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Huntington's disease: pre-clinical models to therapy using genetically-modified immunoisolated cells and lentiviral vectors

Review Article

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Abbreviations: 3-nitropropionic acid (3-NP); Alzheimer's disease, (AD); amyotrophic lateral sclerosis, (ALS); baby hamster kidney, (BHK); blood-brain barrier, (BBB); choline acetyltransferase- (ChAT); ciliary neurotrophic factor, (CNTF); cytomegalovirus, (CMV); fibroblast growth factor-1, (FGF-1); glial cell-line derived neurotrophic factor, (GDNF); Glial fibrillary acid protein, (GFAP); glutamic acid decarboxylase, (GAD); Huntington's disease, (HD); leukemia inhibitory factor, (LIF); nerve growth factor, (NGF); neuronal intranuclear inclusions, (NII); neurotrophin 3, (NT3); Parkinson's disease, (PD); phosphoglycerate kinase 1, (PGK); Poly(acrylonitrile-co-vinyl chloride), (PAN/VC); poly(ethylene oxide), (PEO); positron emission tomography, (PET); quinolinic acid (QA); tetracycline, (Tet); Tet-response element, (TRE); transactivator, (tTA); tyrosine hydroxylase, (TH); wild-type, (WT)

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Summary

Huntington's disease (HD) is a devastating genetic disorder. Despite the absence of effective therapy, there has been a explosion in interest for developing treatment strategies aimed at lessening or preventing the neuronal death that occu in this disease. In large part, the renewed interest in neuroprotective strategies has been spurred by our increasin understanding of the genetic and molecular events that drive the underlying neuropathology of HD. This escalatin understanding of the biological underpinnings of HD is derived from several convergent sources represented b investigators with clinical, genetic, molecular, physiological and neurobehavioral backgrounds. The diversity of dat being generated has, in turn, produced a unique time in HD research where an impressive number of potentia therapeutics are coming to the forefront. This review outlines the use of intracerebrally delivered CNTF usin immunoisolated, genetically-modified cells and lentiviral vectors. Each approach has advantages and disadvantages bu both have produced impressive pre-clinical demonstrations of neuronal preservation in models of HD. These benefits ar highlighted in the context that HD is a neurodegenerative disorder in which genetic detection provides a clear an unequivocal opportunity for neuroprotection.

I. Introduction

Neurodegenerative diseases are characterized by a progressively debilitating cognitive and motor deterioration. Among the most problematic and prevalent neurological disorders are those associated with the loss of specific populations of neurons. Approximately 12 million people in the United States suffer from such neurological disorders resulting in public expenditures and secondary medical expenses that exceed $400 billion annually (Rubin and Gold, 1992). Advances in molecular biology, genetic engineering, proteomics, and genomics are identifying an increasing number of proteins, peptides and other compounds with enormous treatment potential. Most of these compounds are, however, not biologically active in the brain following systemic administration, largely because of the blood-brain barrier (BBB). The BBB regulates the internal chemical environment of the CNS using mechanisms of low passive permeability combined with a highly selective transport system between the blood and the brain (Rubin and Staddon, 1999). A number of strategies are being pursued to circumvent the BBB. Some of the techniques available for delivering therapeutic molecules directly into the brain have fairly long histories and include: 1) carrier-, or receptor-mediated transcytosis (Friden et al, 1993; Friden 1994); 2) osmotic opening (Jiao et al, 1996; Kroll and Neuwelt, 1998); 3) direct infusion with stereotactic guidance (Kordower at al., 1993; Riddle

et al, 1997; Mufson et al, 1999); 4) osmotic pumps (Vahlsing et al, 1989; Olson et al, 1991); 5) sustained-release polymer systems (Winn et al, 1989; Hoffman et al, 1990; Langer and Moses, 1991); 6) cell replacement / cell therapy (Freed et al, 1990; Yurek and Sladek, 1990; Dunnett and Bjorklund, 1994) and, 7) direct gene therapy (Bowers et al, 1997; Zlokovic and Apuzzo, 1997; Barkats et al, 1998; Kaplitt et al, 1998). In this review, we discuss 2 promising techniques for direct delivery of molecules to the brain parenchyma. Specifically, we review laboratory studies demonstrating the use of genetically-modified immunoisolated cells and lentiviral vectors to deliver ciliary neurotrophic factor (CNTF) to the brain as a means of potentially treating Huntington's disease (HD). The pre-clinical evidence supporting the therapeutic potential of these approaches is highlighted with discussions of the advantages of each approach. The current state of initial clinical trials using encapsulated cells therapy for HD is also discussed.

II. Neuropathology of Huntington's disease

Huntington's disease (HD) is a devastating autosomal dominant neurodegenerative disorder resulting from a genetic defect at the IT15 gene locus of the short arm of chromosome 4. HD is found in all regions of the world with an overall prevalence of 5-10 per 100,000 individuals (Conneally 1984). While the age of onset is usually between the mid-thirties to late fifties, juvenile (<20 years old) and late onset (>65 years old) cases have been reported. From the time of diagnosis, an intractable course of mental deterioration and progressive motor abnormalities occurs that invariably results in death. On average patients suffer 17 years of symptomatic illness.

Clinically, HD is characterized by involuntary choreiform movements, psychiatric and behavioral changes, and frequently dementia (Martin and Gusella, 1986; Greenamyre and Shoulson, 1994). The emotional disturbances may occur as early as a decade before the onset of the movement disorder. There are no effective treatments (Penney et al, 1990). Although medications may reduce the severity of chorea or diminish the behavioral symptoms, they do not increase patient survival or substantially improve quality of life (Shoulson, 1981). Pathogenetically, HD is characterized by a programmed, premature death of specific populations of neurons. The genetic defect on chromosome 4 results in an increase in the number of polyglutamine-encoding CAG repeats at a site encoding for the protein Huntingtin (The Huntington's Disease Collaborative Research Group, 1993; Ashizawa et al, 1994). The expanded CAG repeats produces a "gain of function" that mediates the clinical and pathological sequelae of HD, although the mechanism by which this occurs remains unknown. In HD, the most striking pathological changes occur in the caudate nucleus and putamen with the medium-sized spiny neurons being particularly vulnerable. In contrast, the large aspiny interneurons and medium aspiny projection neurons are less affected and degenerate later in the disease process (Ferrante et al, 1985; Vonsattel et al, 1985; Ferrante et al, 1987). Neurochemically, the major defect in HD involves the loss of GABA-containing neurons that provide inhibitory innervation to the globus pallidus and substantia nigra pars reticulata. Early in the disease, the GABA-ergic/enkephalinergic projection to the external globus pallidus appears to be preferentially affected (Albin et al, 1990, 1992). Levels of striatal ChAT, substance P, CCK, and angiotensin-converting enzyme are also decreased. Interestingly, neurons containing NADPH-diaphorase such as somatostatin and neuropeptide Y actually increase expression in HD (DiFiglia et al, 1997). While other subcortical and cortical brain regions may also show degenerative changes, the degree of degeneration varies, does not correlate with the severity of the disease (Albin et al, 1990, 1992; Greenamyre and Shoulson, 1994) and is commonly overshadowed by the striatal changes.

Ultrastructurally, neuronal intranuclear inclusions (NII) are observed in numerous brain regions including the striatum, amygdala, hippocampus, red nucleus, and cerebellum. Interestingly, these ultrastructural abnormalities have been reported to occur at a higher frequency in juvenile-onset cases of HD, while dystrophic neurites (axonal inclusions) may be more prominent in later-onset cases. Both NII and dystrophic neurites are immunoreactive for N-terminal huntingtin and ubiquitin antibodies (DiFiglia et al, 1997; Becher et al, 1998).

III. Genetic detection provides an opportunity for neuroprotection in Huntington's disease

Unlike many other neurodegenerative diseases, the polyglutamine expression in HD permits an unequivocal diagnosis of HD early in life, even in utero (The Huntington's Disease Collaborative Research Group, 1993). The ability to identify early on individuals with HD provides the opportunity to design interventions far before the development of substantial neurodegeneration and the expression of the behavioral changes. This is in contrast to other neurodegenerative diseases including Parkinson's disease (PD) and Alzheimer's disease (AD), where the lack of a sufficiently early and reliable diagnostic test make it difficult to design neuroprotective approaches with the same temporal benefit as might be possible in HD (Calne and Langston, 1983). In PD, a diagnosis is not possible prior to an extensive loss of striatal dopamine and severe degeneration of dopaminergic neurons in the substantia nigra. Although PD can be diagnosed, using PET, before the onset of symptoms, even this diagnosis relies on the substantial loss of striatal dopamine function. In AD, patients exhibit marked decreases in cortical cholinergic function and considerable accumulation of neuritic plaques and neurofibrillary tangles within the first year of diagnosis. In both instances, a pathological cascade has been initiated and is extensively developed, making it difficult to slow the process and likely impossible to reverse the already considerable neuronal death. Unfortunately, most neurodegenerative diseases are characterized by this same pattern (i.e. a substantial damage must occur within the central nervous system

prior to onset of clinical symptoms). HD is a clear exception to this pattern. If the appropriate interventions were available, they could be used within a wide window of therapeutic opportunity that occurs well before the onset of neural degeneration. Accordingly, the preservation of the neuronal cytoarchitecture and physiology of the striatum could be maintained, while forestalling the debilitating consequences of the disease.

IV. Criteria for developing a neuroprotective strategy for Huntington's disease

Establishing the criteria for a neuroprotective therapy in HD requires that certain pre-clinical goals be met. The most logical goal is the prevention of the loss of GABA-ergic medium spiny neurons within the striatum (Emerich, 2001). This is the most prevalent and consistent pathological feature in HD. Preservation of the cell body should also be accompanied by preservation of the normal projections of these neurons. It seems intuitively obvious that preserving the GABAergic cell body without sparing the inputs to the globus pallidus and substantia nigra pars reticulata will be met with limited success. Clinically, it is imperative to target both the motor and higher-order cognitive deficits seen in HD. Obviating or reducing the motor changes without changing the personality changes, dementia, and psychosis associated with HD would be of limited value. While striatal degeneration is clearly linked to the motor changes in HD, the CNS changes underlying the cognitive deficits are more obscure and may be the result of basal ganglia and/or cortical dysfunction. Nonetheless, to maximize the potential of any neuroprotective strategy, the striatal neurons and their afferent and efferent projections, including the cortical projections, should be protected to attempt to prevent both the motor and non-motor sequelae of HD.

V. Role of animal models in developing neuroprotective strategies for Huntington's disease

The ability to devise novel therapeutic strategies in HD is tightly linked to the characterization of highly relevant animal models (Emerich, 2001). Animal models help to elucidate the biological and behavioral manifestations of clinical disorders, and suggest novel therapeutic strategies for their prevention and/or treatment. Although animal models may not reproduce the complex etiologies, pathophysiology, or behavioral abnormalities associated with diseases of the nervous system, they do provide a practical way to explore questions concerning structure and function. Animal models of HD have typically relied on chemically-induced loss of striatal neurons or, more recently, the development of transgenic animals with excessive CAG repeats. These models are mentioned below.

A. Chemical lesions of the striatum

It has been suggested that the neuronal death in HD is related to an underlying excitotoxic process (Olney 1989). Based on this premise, two in vivo systems have been used extensively as animal models for HD. First, directly injecting excitatory amino acids into the striatum recapitulates many of the anatomical and behavioral changes characteristic of HD. While initial studies employed kainic acid (Coyle and Schwarcz, 1976) the excitotoxic model was refined when it was shown that quinolinic acid (QA) more accurately modeled the pathology of HD (Beal et al, 1986, 1989, 1991). QA lesions of the striatum spare both medium spiny neurons that stain for NADPH-diaphorase and large aspiny cholinergic neurons; both of which are spared in HD. Excitotoxic lesions of the striatum in experimental animals also mimic motor and cognitive changes observed in HD (Sanberg et al, 1989; Block et al, 1993). A second model of striatal neurodegeneration uses injections of the mitochondrial toxin 3-nitropropionic acid (3-NP). Both acute and chronic 3-NP produce widespread disturbances in brain succinate dehydrogenase activity together with a selective degeneration of striatal neurons in both rats and monkeys. Recent non-human primate studies have provided compelling evidence that chronic administration of 3-NP can produce progressive striatal degeneration with considerable homology to HD (Brouillet et al, 1999; Hantraye et al, 1999). This homology has been confirmed using anatomical, behavioral (both motor and cognitive testing), and neuroimaging techniques.

Recently, Roitberg and colleagues (2003) directly compared the anatomical and behavioral consequences of intrastriatal injections of QA versus daily systemic intramuscular administration of 3-NP for up to 8 weeks in Cebus apella monkeys. Magnetic resonance imaging scans of the brain were obtained before and after treatment and a detailed immunocytochemical analysis was conducted on the striatum. The animals' behavior was evaluated before, during, and 3 months after administration of the neurotoxin. The QA-treated group displayed large areas of increased signal on T2-weighted images in the caudate and putamen bilaterally. A large area of neuronal loss with glial sparing was observed in the QA-treated group, including the caudate and putamen. Immunohistochemistry and morphometric analyses revealed that both groups had lesions in the striatum, but the 3-NP-treated group displayed smaller lesions restricted to the dorsolateral putamen. Frontal cognitive function as evaluated by object retrieval-detour task test demonstrated a marked deterioration in successful responses, with an increase in barrier reaches in both groups. No significant change in performance of fine motor tasks was observed. QA-treated animals displayed hyperactivity at night. Animals in both groups demonstrated abnormal posture, and the 3-NP-treated group showed spontaneous and apomorphine-induced dystonia and dyskinesia. These results suggest that both QA and 3-NP induce behavioral and morphological features that resemble the juvenile and akinetic-rigid variants of HD, with the group with 3-NP-induced lesions displaying smaller lesions and spontaneous dyskinesia. The further refinement of monkey

models that faithfully reproduce these characteristics will undoubtedly prove valuable and has already played an important role in the development of therapies aimed at local delivery of neurotrophic compounds.

B. Transgenic models of Huntington's disease

Even though lesions of the striatum produced by excitotoxins and mitochondrial inhibitors mimic to a great extent the pathology that occurs in HD, these models do not incorporate the genetically driven onset and time course of striatal pathology. Various transgenic mice that carry a mutant version of the HD gene have been created using either full-length cDNA CAG repeats or the exon 1 of a mutated HD gene carrying a variable number of CAG copies. The most developed and characterized knockout mouse model is the R6/2 line that contains approximately 150 CAG repeats (Mangiarini et al, 1996; Bates et al, 2000; Gutenkunst et al, 2000). These animals show a dramatic and progressive phenotype resulting in death within 16-18 weeks. The cause for the abrupt death is unknown, but prior to that time significant learning and memory deficits appear (approximately 1 month after birth) together with motor changes such as an irregular gait and dystonia. Atrophic medium spiny striatal neurons and neuronal intranuclear inclusions are also evident. A variety of other transgenic models are under study, each with various CAG repeat numbers (see 27 Gutenkunst et al, 2000 for a review). Full characterization of the neuropathology and behavioral changes are not yet available in all cases. Continued efforts will provide new insight into the function and relationship of CAG repeats, HD neuropathology, and the underlying neurobehavioral symptoms. Finally, knock-in or conditional models are not discussed here, but even though they have met with limited success, they hold the promise of creating mouse lines with great homology to HD with the possibility of helping to more fully understand some of the early molecular changes leading to cell degeneration. Even though transgenic model development is only beginning, it is a clear example of how animal models can lead to a rapid and substantial revision of our understanding of the molecular events in HD. Transgenic animal models will play a pivotal role in the development of potential therapeutics. Given the vast libraries of potential compounds that already exist, in vitro systems and invertebrate models including C. elegans and Drosophila will be invaluable high throughput screens for rapid determinations of promising compounds. These drugs can then be screened and tested more thoroughly in the available transgenic models. Further refinements along these lines will also provide continued insight into the cellular and molecular events that underlie the initiation and progression of HD.

IV. Intracerebral delivery of CNTF to protrect striatal neurons

CNTF is a member of the alpha-helical cytokine superfamily, with well-documented functions in the peripheral nervous system. CNTF also exerts a wide range of biological and neuroprotective effects in the CNS (see Apfel 1997 for a revew). In vitro and in vivo, CNTF acts as a survival factor for motor neurons. CNTF has also been reported to protect basal forebrain cholinergic neurons and substantia nigra dopaminergic neurons from lesion-induced degeneration and reduce cell loss and promote functional recovery following cerebral ischemia. Additional neurons that are responsive to CNTF include ciliary ganglion neurons, hippocampal neurons, neurons of the spinal nucleus of the bulbocavernosus, retinal photoreceptors, corticospinal neurons, and progenitor cells that differentiate into oligodendrocytes and astrocytes.

CNTF was one of the first neurotrophic factors to be used in large-scale clinical trials for the treatment of amyotrophic lateral sclerosis (ALS) (The ALS CNTF Treatment Study (ACTS) Phase I-II Study Group, 1995a,b). These trials, that relied on subcutaneous administration of CNTF, were ultimately unsuccessful. Although the exact reason for failure remains unclear possible factors include a poor pharmacokinetic profile of CNTF after systemic delivery or ineffective doses of CNTF attained at the site of degenerating motorneurons. It is also conceivable that pre-clinical studies in large animals would have revealed that CNTF delivery is simply ineffective in ALS. Nonetheless, the potent and diverse effects of CNTF in the CNS have provided the impetus for continued exploration of its therapeutic potential. The in vivo data supporting the ability of CNTF to protect striatal neurons in animal models of HD is a logical extension of these previous studies and is discussed in detail below.

VII. Cell immunoisolation for delivery of neurotrophic molecules

One of the major difficulties in applying CNTF or any neurotrophic factor to the CNS is accessibility. Virtually all neurotrophic factors are large proteins that do not cross the BBB. Therefore, systemic administration will not suffice and techniques must be developed to circumvent the BBB. Local delivery using polymer encapsulated genetically-modified cells is one iteration of cell-based gene therapy with demonstrated pre-clinical and clinical success delivering CNTF to the CNS. Immunoisolation was originally described in 1933 when Bisceglie demonstrated that encapsulated xenograft cells could survive beyond the limit for humoral rejection. The continued refinement of immunoisolation for the CNS owes much of its foundation to investigators focused on peripheral diseases, particularly diabetes (Chick et al, 1977; Lim and Sun, 1980) and Parkinson's disease, using dopamine-secreting cells (Shoichet et al, 1995). Immunoisolation is based on the observation that xenogeneic cells can be protected from host rejection by encapsulating, or surrounding them within an immunoisolatory, semipermeable membrane. Single cells or small clusters of cells can be enclosed within a selective, semipermeable membrane barrier which admits oxygen and required nutrients and releases bioactive cell secretions, but restricts passage of larger cytotoxic agents from the host immune defense system. The selective

membrane eliminates the need for chronic immunosuppression of the host and allows the implanted cells to be obtained from non-human sources, thus avoiding the constraints associated with cell sourcing, which have limited the clinical application of unencapsulated xenogeneic cell transplantation.

There are generally two categories for cell immunoisolation by encapsulation, namely micro- and macro-, each with some benefits and limitations (see Emerich and Winn, 2001 for a review). The majority of CNS transplant studies have employed macroencapsulation. Macroencapsulation involves filling a hollow, usually cylindrical, selectively permeable membrane with cells, generally suspended in a matrix, and then sealing the ends to form a capsule. Polymers used for macroencapsulation are biodurable, with a thicker wall than that found in microencapsulation. While thicker wall and larger implant diameters can enhance long-term implant stability, these features may also impair diffusion, compromise the viability of the tissue and slow the release kinetics of desired factors. Macrocapsules can also be retrieved from the recipient and replaced if necessary.

Using polymer-encapsulated cells to deliver molecules to the CNS requires that several fundamental principles be met including in vitro and in vivo demonstrations of immunoisolation, biocompatibility of the encapsulating material, and long-term product secretion because of the chronic nature of diseases like HD. These are discussed below.

VIII. Validation of the concept of immunoisolation

A. *In vitro* studies

The maintenance of immunoisolation, i.e., capsule integrity, can easily be confirmed in vitro. For instance, in one study, a polydisperse (10^3 - 10^6 g/mol) dextran solution was encapsulated into hollow fibers and the flux of the molecules across the semi-permeable membrane was measured to produce a dextran rejection curve (Dionne et al, 1996). With intentionally damaged control devices, the large molecular weight dextran species easily escaped while intact capsules retained the encapsulated dextran. Capsule integrity can also be confirmed using standard immunological assays such as measuring the protection of encapsulated cells against the cytotoxic killing of antibody (IgG)-mediated complement lysis (Winn and Tresco, 1994). With integral PC12 cell-loaded capsules, in the presence of antibody and complement, the capsular membrane prevented antibody-mediated complement lysis (<10% cell death), while complete killing (100%) was observed in cases of damaged capsules or with PC12 cells not encapsulated.

B. *In vivo* The importance of polymer capsule integrity for xenografted cell survival is illustrated in studies where unencapsulated cells, or cells encapsulated in intentionally damaged membranes, have been implanted into the brain (Aebischer et al, 1991). Intact PC12 cell-loaded capsules implanted into the guinea pig striatum showed no lymphocytic infiltration and a minimal astrocytic reaction by GFAP staining whereas the same cells were rejected when placed into intentionally damaged capsules. Parallel studies confirmed that unencapsulated PC12 cells do not survive following implantation into either the guinea pig or the monkey striatum while encapsulated PC12 cells have demonstrated viability for 6 months in monkeys (Kordower et al, 1995). Similar results have been obtained in rats that received intraventricular implants of bovine adrenal chromaffin cells when implanted in the ventricular space (Lindner et al, 1997). There was no evidence of elevated levels of rat anti-bovine adrenal chromaffin cell IgG or IgM levels in serum from rats implanted with encapsulated xenogeneic adrenal chromaffin cells for nearly 1.5 years in vivo. In contrast, a robust host immune response was induced in animals after implantation of unencapsulated bovine adrenal chromaffin cells.

IX. Biocompatibility of encapsulated cells

Transplant survival, with and without an encapsulating membrane, is mediated by many factors. The cellular / tissue reaction generated by a host in response to a foreign body, typically referred to as biocompatibility, impacts the success of the transplant. Factors affecting biocompatibility include the method of implantation, the implant site, and implant properties, such as composition of the polymer, potential residual processing agents, surface integrity and microgeometry, and the size and shape of the implant. The lack of a significant host tissue reaction to the implant is crucial for the initial viability of the encapsulated cells as well as diffusion from the capsule. While capillary invasion into the capsule walls helps provide nutrients and oxygen in proximity to the encapsulated cells the process of angiogenesis for neovascularization typically evolves in a 4 – 7 day period (Clark, 1996). Therefore, the encapsulated cells must endure an initial period of nutrient and oxygen deprivation obtaining these essential factors only by diffusion. Moreover, since the only means of delivery of the desired cellular products from an encapsulated cell implant is by diffusion, any reaction around the capsule might diminish the diffusion of therapeutic products from the encapsulated cells.

Early investigations utilized electron microscopic techniques to characterize the brain tissue reaction to plastic-embedded metal electrodes and polymer implants (Stensass and Stensass, 1978; Rauch et al, 1986; Winn et al, 1989). Necrosis of the tissue surrounding the polymer capsules implanted into the striatum of rodents was minimal with small Nissl-positive cells and capillaries invading the open trabeculae in the wall of the macrocapsules (Winn et al, 1989). Glial fibrillary acid protein (GFAP) immunocytochemistry revealed local reactive astrocytes one to two weeks after implantation. The intensity of the GFAP reaction diminished rapidly such that by four weeks after implantation the gliotic reaction surrounding the polymer implant was minimal. No significant changes in myelin basic protein reactive oligodendroglia were observed, and neuron specific

enolase-reactive neurons were readily identifiable adjacent to the implant. Subsequent studies with an immunospecific antisera against rat microglia, OX-42, revealed a reaction in magnitude and time course similar to that seen for the astrocytes. These studies clearly demonstrated biocompatibility within the host nervous system, which suggests that the bi-directional transport of low molecular weight solutes across the permselective membrane can be maintained in vivo. While many biocompatibility studies have been conducted with empty polymer macrocapsules (Winn et al, 1989) the inclusion of cells does not appear to significantly impact the host reaction to the polymer device.

It should be noted that in an effort to maintain or even further enhance the biocompatibility for cell line-containing implants, or reduce protein adsorption that may negatively impact the ability to maintain adequate long-term diffusive characteristics, several post-synthesis modifications are possible. Poly(acrylonitrile-co-vinyl chloride) (PAN/VC) hollow fiber membranes which were surface modified by grafting poly(ethylene oxide) (PEO) groups exhibited improved biocompatibility in brain tissue over the unmodified PAN/VC controls (Shoichet et al. 1994). Similar observations were made with PEO-modified poly(hydroxyethyl methacrylate-co-methyl methacrylate) membranes utilized extensively in cellular microencapsulation (Crooks et al, 1995).

X. Long-term product secretion and delivery from encapsulated cells

Because HD entails progressive neurodegeneration, it is likely that a neurotrophic factor therapy such as CNTF delivery will only be beneficial if sustained release of the therapeutic molecule is achieved. Accordingly, before patients suffering from chronic CNS diseases can be routinely implanted with encapsulated cells, long-term survival of the encapsulated cells and continued release of the therapeutic molecule must be demonstrated. Although effective immunoisolation should result in long-term survival of encapsulated cells, few studies have examined implant viability for more than a few months. A few notable exceptions exist and provide compelling evidence about the potential for long-term survival and release of molecules from the cells. Following encapsulation, PC12 cells have been maintained in vitro and in vivo for at least 6 months, while maintaining a typical morphology and clustered arrangement along the lumen of the device (Aebicher et al, 1991; Kordower et al, 1995; Tresco et al, 1992). The cells remain tyrosine hydroxylase (TH)-immunoreactive and mitotically active, with necrosis primarily in regions of high cell density. Electron microscopy confirms the presence of numerous mitochondria, polysomes, and electron-dense secretory vesicles distributed within the cytoplasm. Spontaneous and evoked release of dopamine can be detected from capsules maintained both in vitro, and following explantation from the CNS. Both rodent microdialysis and positron emission tomography (PET) studies in primates have confirmed that encapsulated PC12 cells continue to produce L-dopa / dopamine in situ (Tresco et al, 1992; Subramanian et al, 1997).

As noted above other cell types, including encapsulated bovine adrenal chromaffin cells, also survive for prolonged periods of time (Sagen et al, 1993; Lindner et al, 1997). Intraventricular implants of encapsulated bovine chromaffin cell implants survived for nearly 1.5 years and continued to produce catecholamines and met-enkephalin (Lindner et al, 1997). Polymer-encapsulated, genetically-modified cells also survived and continued to secrete trophic factors such as nerve growth factor (NGF) for 12- 13.5 months in rats (Date et al, 1996; Winn et al, 1996). The cells remained viable and the NGF secreted from the encapsulated cells was 64% higher following removal from the rat lateral ventricles (Winn et al, 1996). NGF transgene copy number was equivalent to pre-implant levels, indicating NGF gene stability. No deleterious effects from long-term NGF were detected using an extensive battery of tests.

XI. Neuroprotection based on delivery of CNTF to the striatum

A. Rodent studies

Studies using the rodent QA model of HD have been conducted to determine the extent of striatal protection produced by encapsulated cells genetically-modified to secrete CNTF (Emerich et al, 1996; 1997). In these studies, a DHFR-based expression vector containing the CNTF gene was transfected into a baby hamster kidney fibroblast cell line (BHK). Using a polymeric device, encapsulated BHK-control cells and those secreting CNTF were transplanted unilaterally into the ipsilateral rat lateral ventricle. One week later, the same animals received unilateral injections of QA (225 nmol) or the saline vehicle into the ipsilateral striatum. As assessed by quantitative behavioral and histological analysis, CNTF cells attenuated the extent of host neural damage produced by QA. Nissl-stained sections demonstrated that CNTF cells significantly reduced the volume of striatal damage produced by QA. Quantitative analysis of striatal neurons further demonstrated that both choline acetyltransferase-(ChAT) and glutamic acid decarboxylase (GAD)-immunoreactive neurons were protected by CNTF implants. After surgery, animals were behaviorally tested for apomorphine-induced rotation behavior. Rats receiving CNTF cells rotated significantly less than animals receiving BHK-control cells.

These studies offered additional evidence of neuronal protection produced by CNTF (Emerich et al, 1997). Bilateral intrastriatal injections of QA produced a marked hyperactivity, an inability to use the forelimbs to retrieve food pellets in a staircase test, increased the latency of the rats to remove adhesive stimuli from their paws, and decreased the number of steps taken in a bracing test that assessed motor rigidity. These animals were also impaired in tests of cognitive function-the Morris water maze spatial learning task, and the delayed nonmatching-to-position operant test of working memory. Prior implantation with CNTF-secreting cells prevented the onset of all the above deficits such that implanted animals were nondistinguishable from sham-lesioned controls.

Neurochemical determination of ChAT and GAD levels revealed that QA decreased striatal ChAT levels by 35% and striatal GAD levels by 45%. In contrast, CNTF-treated animals did not exhibit any decrease in ChAT levels and only a 10% decrease in GAD levels.

These rodent studies initially defined the extent of both the quantitative and qualitative aspects of CNTF's behavioral protective effects. The observation that CNTF prevents the occurrence of both motor and non-motor deficits following QA has particular relevance or HD, which is characterized by a wide range of behavioral alterations. Huntington's chorea, as the name implies, is a disorder most often associated with pronounced motor changes. However, neurological and psychiatric changes frequently occur as much as a decade prior to the onset of motor symptoms. Indeed, given the severity and persistence of the cognitive changes in HD, any viable neuroprotective strategy would necessarily need to exert beneficial effects on cognitive and psychiatric symptoms. Because the rodent HD models are limited to characterizing the cognitive deficits associated with the disease, the non-human primate HD model has become a much-needed platform for investigating the efficacy of neurotrophic factor therapy for treating HD cognitive dysfunctions.

B. Primate studies

The ability of cellularly delivered trophic factors to preserve neurons within the striatum in a rodent model of HD led to similar studies in nonhuman primates, a step that is crucial to the initiation of clinical trials. A paradigm similar to the one employed in the rodent studies was used in nonhuman primates (Emerich et al, 1997). Polymer capsules containing CNTF-producing cells were grafted into the striatum of Rhesus monkeys. One week later, QA was injected into the putamen and caudate proximal to the capsule implants. As seen in the rodent studies, the volume of striatal damage was decreased and both GABAergic and cholinergic neurons destined to degenerate were spared in CNTF grafted animals. Although all animals had significant lesions, there was a 3-fold and 7-fold increase in GABAergic neurons in the caudate and putamen, respectively in CNTF grafted animals relative to controls. Similarly, there was a 2.5-fold and 4-fold increase in cholinergic neurons in the caudate and putamen, respectively in CNTF-grafted animals.

The ability to preserve GABAergic neurons in animals models of HD is an important, though not entirely sufficient, step to develop a useful therapeutic. If the perikarya are preserved without sustaining their innervation, then the experimental therapeutic strategy under investigation is not likely to yield significant value. The striatum is a central station in series of loop circuits that receive inputs from all of the neocortex, projecting to a number of subcortical sites, and then return information flow to the cerebral cortex. One critical part of this circuitry is the GABA-ergic projections to the globus pallidus and substantia nigra pars reticulata, the parts of the direct and indirect basal ganglia loop circuits. One approach to examining the integrity of this circuit is to use an antibody that recognizes GABA-ergic terminals (DARPP-32) to determine if the preservation of GABA-ergic somata within the striatum also results in the preservation of the axons of these neurons to critical extrastriatal sites. Using quantitative morphological assessment of DARPP-32 optical density, it has been shown that monkeys receiving QA lesions have significant reductions in DARPP-32 immunoreactivity within the globus pallidus and substantia nigra. The lesion-induced decrease in GABA-ergic innervation for both of these regions was prevented in CNTF-grafted monkeys demonstrating that this treatment strategy protected GABA-ergic neurons destined to die following excitotoxic lesion, as well as sustained the normal projection systems from this critical population of neurons (Emerich et al, 1997).

The intrinsic striatal cytoarchitecture can be preserved in monkeys by CNTF grafts, and once exposed to these grafts, the cells apparently maintain their projections. The afferents to the striatum, specifically from the cerebral cortex, were also influenced by these grafts. This may be particularly critical if some of the more devastating nonmotor symptoms seen in HD result from cortical changes secondary to striatal degeneration. Since layer V neurons from motor cortex send a dense projection to the post-commissural putamen, a region that was severely impacted by the QA lesion, the effects of QA lesions and CNTF implants on the number and size of cortical neurons in this region were examined. Although the QA lesion did not affect the number of neurons in this cortical area, layer V neurons were significantly reduced in cross-sectional area on the side ipsilateral to the lesion in control-grafted monkeys. This atrophy of cortical neurons was virtually completely reversed by CNTF grafts (Emerich et al, 1997).

A recent set of studies using CNTF producing cells in 3-NP-treated monkeys have replicated and extended these results (Mittoux et al, 2000). Following 10 weeks of 3-NP treatment, monkeys displayed pronounced chorea and severe deficits in frontal lobe cognitive performance as assessed by the object retrieval detour test. Following implantation of CNTF-producing cells, a progressive and significant recovery of motor and cognitive recovery occurred. Histological analysis demonstrated that CNTF was neuroprotective and spared NeuN and calbindin-positive cells in the caudate and putamen. To date, this collection of data provides the only demonstration that a therapeutic intervention can influence the degeneration of striatal neurons and disruption of basal ganglia circuitry in primate models of HD while preventing the motor and cognitive changes that make HD such a devastating disorder. Accordingly, the use of CNTF appears to satisfy the essential preclinical criteria for further investigation as a neuroprotective agent for HD.

C. Human trials

Recently, clinical trials were initiated to determine the safety and tolerability of CNTF-producing cells implanted into the lateral ventricle of HD patients (Bachoud-Levi et al, 2000). Although the results of this trial are not yet available, the trial is designed using a device, containing as many as 10^6 CNTF-producing BHK

cells producing 0.15-0.50 μg CNTF/day implanted into the right lateral ventricle of 6 patients. In this phase I study, the principal goal is to evaluate the safety and tolerability of the procedure. HD symptoms will also be analyzed using neuropsychological, motor, neurological, and neurophysiological tests and the striatal pathology monitored using MRI and PET-scan imaging.

While the CNTF-induced sparing of striatal neurons and maintanence of intrinsic circuitry in animal models of HD is impressive, the magnitude of the effect is less than that seen in rodents. In primates, robust protection is limited to the area of the capsules. However, the area of the lesion remains extensive and it is likely that diffusion of CNTF from the capsule may not be sufficient to protect more distant striatal regions undergoing degeneration. This concept is supported by an experiment that examined the effects of intraventricular grafts of encapsulated CNTF grafts in the nonhuman primate model of HD (Kordower et al, 1999). In contrast to when the capsules were placed directly within brain parenchyma, intraventricular placements failed to engender neuroprotection for any striatal cell types; again suggesting that diffusion is a key factor in the efficacy of this experimental therapeutic strategy. The complete lack of neuroprotection provided by intraventricular implants in primates should be considered more carefully in the current clinical trials being conducted in which encapsulated cells are being placed into the lateral ventricles of HD patients. If human trials are to yield clinically relevant positive effects, the means of CNTF delivery utilized in these studies needs to be improved. Whether this entails grafting more capsules, enhancing the CNTF delivery from the cells by changing the vector system or cell type employed, or changing the characteristics of the polymer membrane remains to be determined.

XII. Summary of cell encapsulation

There is considerable promise of encapsulated cell therapy for treating a wide range of CNS disorders. Still, a number of research avenues exist which are incompletely explored and deserve attention prior to wide-scale clinical use of this technology. These include:

A. Limited diffusion:

In some pre-clinical studies, the extent of diffusion from the implants appears to limit the therapeutic effectiveness of the encapsulated cells (Tresco et al, 1992; Kordower et al, 1996). Given the size of the human brain relative to the rodent and non-human primate brain, the potential problems related to limited tissue diffusion should be examined empirically. Studies in larger animals should be conducted to assess the optimal spacing and distribution of multiple implants and provide information regarding the relative risks of repeated tissue penetrations, tissue damage and the potential for infection.

B. Regulation of dosage

In its most basic iteration, varying the numbers of cells within an implant, the size of the implant, or the use of multiple implants, may permit a range of doses to be delivered. While some long-term cell survival studies have been conducted (Aebischer et al, 1991; Tresco et al, 1992; Kordower et al, 1995; Winn et al, 1996; Lindner et al, 1997), they tend to utilize only CSF-filled spaces and have not systematically examined cell survival and output of the desired molecule over long periods of time. Rather, studies have provided a "snapshot" of survival and output at a single time-point. Large, long-term, well-controlled studies need to be conducted to examine the relationship between variables that include time, cell survival, gene expression, neurochemical output, the initial numbers of cell encapsulated, and the type of semi-permeable membrane and extracellular matrix used for encapsulation. Obviously, such studies are time consuming and expensive. But without them, the conditions optimal for successful cell encapsulation will remain speculative. Some efforts are ongoing in this area and a recent study raised the interesting possibility that dose control for dividing cells could be accomplished with the use of cell-containing microcarriers in nonmitotic hydrogels (Li et al, 1999).

C. Host effects on transplanted cells

Another area that has attracted very little attention, concerns the variability in the in vivo performance of encapsulated cells and the possible role that the host tissue environment plays in this variability (Kordower et al, 1995; Lindner et al, 1996; Lindner and Emerich, 1998). At least some of the variability in device performance is attributable to differences between hosts. While the mechanism(s) underlying these individual differences remain undetermined, several potential candidates exist including the variations in the general health of the animals, between animal differences in immune function and undetected micro-breaches in the polymer membrane prior to or during implantation. The notion that the viability of grafted cells may depend in part on host-related variability in the CNS environment has only been suggested for encapsulated cells to date. However, this emerging concept might also prove to be relevant for all CNS transplantation approaches that are cellular based.

D. Additional clinical studies are needed

Finally, very few clinical studies have been conducted to date. While several small safety studies have been completed, only one large, controlled clinical study has been performed using encapsulation technology. This study evaluated the use of encapsulated adrenal chromaffin cells for the treatment of pain but failed to reveal analgesia sufficient enough to continue the trials (CytoTherapeutics press release, 1999). Importantly, the failure to demonstrate efficacy in chronic pain using encapsulated chromaffin cells should not be interpreted as indicating that the technology itself was unsuccessful. Indeed, several recent reports indicate that adrenal chromaffin cell implants may not produce efficacy as originally suggested (Lindner et al. 2000a; Lindner et al. 2000b; Lindner et al. 2000c; Lindner and Saydoff 2002; Gulwadi et al. 2002; Lindner et al. 2003). Extensive

studies in acute and chronic rodent pain models have failed to find any evidence of analgesia. This lack of effect occurred under conditions that were apparently designed to exactly reproduce previous testing procedures that did demonstrate efficacy. Among the variables examined were the location of implant (intrathecal vs intraventricular), a wide range in cell preparation techniques, and an exhaustive battery of acute and chronic pain tests with and without nicotine stimulation. The authors reported that systemic administration of morphine produced significant analgesia when tested in parallel in the same models. While subtle testing differences cannot be ruled out as contributing factors in the differences between these recent and previous studies, together with the only well controlled clinical trial conducted to date, it appears that, at the least, adrenal chromaffin cells do not produce analgesic effects as consistently as previous reported (Lindner et al. 2000a; Lindner et al. 2000b; Lindner et al. 2000c; Lindner and Saydoff 2002; Gulwadi et al. 2002; Lindner et al. 2003). The other clinical targets under investigation each use CNTF for Retinitis Pigmentosa (initiated by NeuroTech, Inc.), ALS (Aebischer et al, 1996; Zurn et al, 2000) and HD (Bachoud-Levi et al, 2000) and these are apparently modest efforts. Until, larger, controlled clinical trials are conducted, the potential of this technology will not be fully realized. However, caution must be applied when considering any novel therapy for treating brain disorders and the wide scale use of polymer neural implants should be considered only after rigorous scientific experimentation in animal models and their demonstrated efficacy and safety in human clinical trials.

XIII. Lentiviral vectors for delivery of cntf to the brain

Direct intracerebral injections of viral vectors represents another potential means of delivering neurotrophic factors such as CNTF to the brain. While a variety of viral vectors hold promise for CNS delivery lentiviruses possess certain properties that make them well-suited for neurological diseases including high and selective transduction efficiency, long-term gene expression and the absence of sequences coding for viral proteins that evoke potent immune responses. It is beyond the scope of this review to thoroughly discuss the biology of lentiviruses so we will briefly touch on each of these issues and discuss specific experiments related to delivery of CNTF in animal models of HD.

A. High and selective transduction efficiency

In vitro studies have demonstrated that lentiviral vectors expressing B-gal and GFP reporter genes infect cultured rodent neurons, human neuronal progenitor cells, the RTBM1 and SH-SY5Y human neuronal cell lines, human NTN2, and rodent motoneurons (Mochizuki et al, 1998; Poeschla et al, 1998; Chang et al, 1999; Mitrophanous et al, 1999; Cisterni et al, 2000; Frimpong and Spector, 2000; Nakajima et al, 2000). While the majority of in vitro studies have been qualitative in nature they clearly demonstrate that neuronal cells derived from rodent and human sources are permissive to infection by lentiviral vectors. In vivo studies have confirmed the effectiveness of lentiviral infection (Zufferey et al, 1997; Mitrophanous et al, 1999; Pereira de Almeida et al, 2001). Injections of LacZ expressing lentiviral vectors into the striatum of mice and rats infect cells located several millimeters from the injection tract. Quantitative analyses have demonstrated that hundreds of thousands of cells are infected under these conditions. Kordower and colleagues (1999) further injected lentiviral vectors along multiple tracts within the caudate and putamen of nonhuman primates. At 1 and 3 months later detailed stereological counts revealed between 930,000 and 1,500,000 LacZ-positive cells within and surrounding the injection tracts. Not only are high levels of cellular infection observed in the brain following injections of lentiviral vectors, but the majority of the infected cells have a neuronal phenotype. Following intrastriatal injections in mice and rats more than 90% of transduced cells are neurons (Blomer et al, 1997,1998; Rosenblad et al, 2000) and 80-88% of infected cells in primates are neurons (Kordower et al, 1999).

B. Long-term transgene expression

As mentioned above, the chronic and progressive nature of neurodegenerative diseases such as HD will require a long lasting expression of therapeutic molecules. Few studies have systematically examined the duration of lentiviral infection of CNS cells but those that have been conducted are encouraging. LacZ expressing vectors were injected into the striatum of mice and rats and the injection sites were examined 3-12 months later (Naldini et al, 1996; Blomer et al, 1997; Naldini et al, 1998; Pereira de Almeida et al, 2001). Infection rates were high and stable over time with no apparent reduction in the number of infected cells in the striatum, hippocampus, or substantia nigra. Sustained expression (6 months) of GFP has also been reported following injection into rat striatum (Bjorklund et al, 2000) and B-gal expression has been observed for 3 months in primate striatum (Kordower et al, 1999).

C Lack of inflammation and immunological reaction following injection

Although formal pathological and toxicological evaluations of the brain have not been performed, the available evidence suggests an absence of adverse inflammatory reactions following injections of lentiviral vectors. Tissue damage and perivascular cuffing is limited to the region immediately adjacent to the injection site across species (i.e. mouse to primate) (Bensadoun et al, 2000; Deglon et al, 2000; Kordower et al, 1999). Infiltrating lymphocytes and macrophages are reported 2 weeks after injection but the extent of this reaction was equivalent between saline and virus treated animals (Blomer et al, 1997). Mild numbers of CD45 and CD8 cells were found in 2 of 18 primates following intrastriatal injection (Kordower et al, 2000).

The lack of a significant immunological response to lentiviral injections has been suggested using a paradigm in which a lentiviral vector containing the tyrosine hydroxylase gene was injected into the striatum and then 6 months later a second virus (using the GFP reporter gene) was injected into the hind leg of the same animals. In these studies, GFP expression was still detected after 2 weeks (Kafri et al, 1997). Deglon and colleagues (2002) achieved similar results when they demonstrated that striatal neurons could still be infected by LacZ-expressing virus 6 weeks after an injection of GFP-expressing virus into the same site.

XIV. Neuroprotection based on lentiviral delivery of Cntf to the striatum

Several studies have evaluated the utility of lentiviral vectors to deliver CNTF to the CNS in animal models of HD. Although these studies are limited to only rodents so far, the results are compelling and certainly warrant further study. As a first step, de Almeida et al. (2001), evaluated the ability of LacZ-expressing lentiviral vectors with two different internal promoters, the mouse phosphoglycerate kinase 1 (PGK) and cytomegalovirus (CMV), to infect striatal cells. The intrastriatal injection of lenti-beta-Gal vectors resulted in 207, 400 +/- 11,500 and 303,100 +/- 4,300 infected cells, respectively with beta-galactosidase activity higher in striatal extracts from PGK-LacZ-injected animals as compared to CMV-LacZ animals. The efficacy of the system was further examined by injecting either PGK-LacZ- or PGK-CNTF-expressing viruses into the striatum of rats followed by an intrastriatal injection of QA 3 weeks later. The extent of the striatal damage was significantly diminished in the CNTF-treated rats as indicated by the 52 +/- 9.7% decrease of the lesion volume and the sparing of DARPP-32, ChAT and NADPH-d neurons. The anatomical sparing was associated with a significant preservation of motor ability. Control animals displayed 148 +/- 43 apomorphine-induced rotations compared to 26 +/- 22 rotations in the CNTF-treated group.

Subsequent studies in the same model confirmed and extended these effects (Regulier et al, 2002). A tetracycline (Tet)-regulated lentiviral vector was split in two lentiviruses, the first one containing the CNTF or GFP cDNAs under the control of the Tet-response element (TRE) and a second vector encoding the transactivator (tTA). Rats received an intrastriatal injection with the CNTF- and GFP-expressing viral vectors followed 3 weeks later by QA. A significant reduction of apomorphine-induced rotations was observed in the CNTF-on group. In contrast, GFP-treated animals or CNTF-off rats displayed an ipsilateral turning behavior in response to apomorphine. A selective sparing of DARPP-32-, ChAT-, and NADPH-d-positive neurons was observed in the striatum of CNTF-on rats compared to GFP animals and CNTF-off group. Enzyme-linked immunosorbent assay performed on striatal samples of rats sacrificed at the same time point indicated that this neuroprotective effect was associated with the production of 15.5 +/- 4.7 ng CNTF per milligram of protein whereas the residual CNTF expression in the off state (0.54 +/- 0.02 ng/mg of protein) was not sufficient to protect against QA toxicity.

Most recently, lentiviral vectors expressing the human CNTF or LacZ reporter gene were injected in the striatum of wild-type (WT) and transgenic mice expressing full-length huntingtin with 72 CAG repeats (YAC72) (Zala et al, 2004). Behavioral analysis showed increased locomotor activity in 5- to 6-month-old YAC72-LacZ mice compared to WT-LacZ animals. Interestingly, CNTF expression reduced the activity levels of YAC72 mice compared to control animals. In both WT and YAC72 mice, CNTF expression was demonstrated, up to a year after lentiviral injection. Stereological histological analysis revealed that the number of LacZ and DARPP-32-positive neurons were decreased in YAC72-LacZ mice compared to WT-LacZ animals. Assessment of the benefit of CNTF expression in the YAC72 mice was, however, complicated by a down-regulation of DARPP-32 and to a lesser extent of NeuN in all mice treated with CNTF. The expression of the neuronal marker NADPH-d was unaffected by CNTF, but expression of GFAP was increased. Finally, a reduction of the number of striatal dark cells was observed in YAC mice treated with CNTF compared to LacZ. These data indicate that sustained striatal expression of CNTF can be achieved with lentiviruses. Further studies are, however, needed to investigate the intracellular signaling pathways mediating the long-term effects of CNTF expression on dopamine signaling, glial cell activation and how these changes may affect HD pathology.

XV. Summary of lentiviral vector delivery of CNTF for Huntington's disease

The use of lentiviral vectors to deliver neurotrophic factors to the brain is in its infancy. Still, over the last several years considerable data has been obtained that supports the use of this approach to deliver CNTF (and potentially other compounds) to the striatum in models of HD. Several obstacles remain before lentiviral vectors can be considered safe enough for clinical evaluation. Very little is known in general about the most effective timing of delivery or optimal doses of viral particles that should be administered to slow any disease process much less one with a time course as protracted as in HD. While these issues are not necessarily unique to lentiviral vectors, long-term pre-clinical studies are still needed. With specific regard to lentiviral delivery, long-term studies will be needed to demonstrate the absence of an immunological response against viral proteins or transgenes. Moreover, significant safety studies will be required to ensure against issues that are inherent with viral delivery including biodistribution, germline transmission, insertional mutagenesis, and recombination with endogenous retroviruses, development of manufacturing and monitoring suitable for producing repeatable clinical material, and assay development for sensitive detection of retroviral particles. Finally, studies in animal models are needed to optimize vector design, transduction of neurons, and development of an inducible system to shut off transgene production if needed or desired.

XVI. Conclusions and future directions

Intracerebral delivery of CNTF appears to have considerable promise as a means of slowing the degeneration of striatal neurons that die in HD. Indeed, clinical trials are already underway to begin to determine how faithfully the pre-clinical data translates to the clinical situation. It will be critically important to maintain and strengthen the relationship between clinic and basic research to allow rapid incorporation of new developments as they become available using new tools including improved animal models to aid in our understanding of the disease process and the biological effects of long-term CNTF delivery.

This review has focused on a core problem in neurotrophic factor therapy for CNS diseases. Early trials using systemic or intraventricular delivery quickly uncovered the fundamental pharmacokinetic limitations of these modes of delivery. These proteins do not cross the BBB effectively and their diffusion in the brain parenchyma is very limited (Mufson et al, 1999). Intraparenchymal delivery, on the other hand, is effective in animals and allows precise spatial control (Tuszynski, 2000). We propose that cell encapsulation and lentiviral vector technologies will make significant contributions to overcoming these issues.

However, fundamental issues still remain, particularly surrounding our knowledge of the effects of growth factors. For a long time, neurotrophic growth factors were considered primarily in the context of their potential to protect cells from death. However, neurotrophic factors have numerous other biological effects. For instance, they exert profound effects on neurotransmitter systems by regulating enzymatic activity, receptor expression and synaptic generation, elimination and modification (Brodski et al, 2002). They also regulate the subcellular processes underlying neuronal plasticity and maintenance of structural neuronal integrity. These survival-independent effects on morphology are clearly visible in transgenic animals in which NGF is deleted and cell death is inhibited by deleting the proapoptotic protein Bax (Patel et al, 2000). In these double mutant animals, neurons that die in NGF single mutants do survive. However, these rescued cells are dysfunctional and show signs of degeneration, including shrinkage of cell bodies and loss of axons. These results emphasize that keeping neurons alive is not synonymous with preserving their physiological roles, which depend on an intact neuronal morphology.

The biological effects of neurotrophic factors are also so intertwined across and within cellular systems that it may be simplistic to suggest manipulating this sophisticated cellular machinery by treatment with a single factor. While from a pharmacological point of view this approach is the most favorable method, it appears nonphysiological from a cell biological perspective. In the light of numerous findings that have accumulated, mostly after the initiation of the first clinical trials, it must be assumed that all neurons in the vertebrate body have the potential to respond to multiple factors in a broadly overlapping fashion. Cholinergic forebrain neurons respond strongly to NGF and weakly to BDNF, but also to neurotrophin 3 (NT3), leukemia inhibitory factor (LIF), glial cell-line derived neurotrophic factor (GDNF), fibroblast growth factor-1 (FGF-1) and potentially to many other growth factors, cytokines and peptides that remain to be tested (see Apfel, 1997, Brodski et al, 2002 for reviews). As discussed earlier, CNTF also exerts its effects across a broad spectrum of cell populations. Hence, by treating neurons with growth factors in vivo, we might interfere with a network of multiple protein components that is governed by biochemical equilibria, redundancy, pleiotropy and integrative processes that we are only beginning to understand. For the future, efforts should be made to develop finely-tuned treatment regimens, paying particular attention to growth factor cocktails applied in a sequential fashion and to the appropriate subcellular compartments. Treatment regimes should be developed in animal models based on several growth factors, each at specific concentrations and in specific temporal sequences. Indeed, synergistic effects of growth factor treatments in experimental paradigms of neurodegeneration have already been observed (Haase et al, 1997).

Finally, growth factors can actively kill cells. One of the best examples of this is cell death in vitro and in vivo via overexpression of the intracellular domain of p75 (Majdan et al, 1997). Oligodendrocytes in culture become increasingly sensitive to NGF-mediated killing in parallel with upregulation of p75 and embryonic hippocampal neurons are killed by neurotrophins in culture (Casaccia et al, 1996; Friedman, 2000). The analysis of knockout mice supports the notion of p75-mediated cell death. Increased survival of neuronal precursors in the retina and spinal cord is observed in animals with a targeted mutation of p75 (Frade and Barde, 1999). Taken together, these results indicate that cell numbers are regulated during development in a highly balanced fashion by both prosurvival and antisurvival signals originating from neurotrophic factor receptors. The consequences of the proapoptotic effects of neurotrophic factors for their use as therapeutics need to be addressed in future studies.

These examples indicate that the level of pre-clinical preparations for clinical trials with neurotrophic factors is still not sufficient. Successful therapies will likely derive from developing an integrated view of the cellular signaling machinery employed by neurotrophic factors. This knowledge base will then allow integrative approaches based on the application of multiple neurotrophic factors in useful sequence and combinations.

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