From: Mark H. Tuszynski[1,2], M.D., Ph.D.
[1]Department of Neurosciences-0626, University of California, San Diego, La Jolla, CA 92093-0626
[2]Veterans Administration Medical Center, San Diego, CA 92165

Prospects for Gene Therapy for CNS Disease

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*Correspondence and Proofs to:
Mark H. Tuszynski, M.D., Ph.D.
Department of Neurosciences-0626, University of California, San Diego, La Jolla, CA 92093
Telephone: 858-534-8857 , FAX: 858-534-5220
E-mail: mtuszynski@ucsd.edu

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I. Introduction: Why Deliver Genes for Neurological Disease?

Substantial progress has been made over the last 20 years in understanding the basic biology and function of the *normal* nervous system, and in elucidating molecular and cellular mechanisms that underlie neurological disease. Together with these advances in understanding have come discoveries of novel genes and proteins, which collectively present an unprecedented opportunity to intervene in and treat a number of neurological disorders that heretofore have been untreatable.

But these opportunities for treatment are matched by new practical challenges: how can we deliver novel therapeutics to the nervous system? For example, nervous system growth factors offer the potential to prevent cell death and stimulate cell function, but they are large protein molecules that do not penetrate the CNS. Further, growth factors cause adverse effects from stimulation of non-targeted systems if delivered into the CNS widely and without region-specific targeting. Gene delivery offers the potential to provide growth factors to specific regions of the CNS that contain degenerating neurons, thereby *bypassing* diffusion limitations of the blood brain barrier while *restricting* delivery of growth factors to their intended targets. In a second example, effective treatment of developmental abnormalities of the nervous system caused by single gene mutations, such as the inherited mucopolysaccharidoses, would require insertion of a correct copy of a gene into many cells of the nervous system at a developmental or early post-natal time point. This too can theoretically be achieved by gene therapy by introducing gene delivery "vectors" into multiple and broad sites of the CNS, thereby replacing defective gene copies with copies of natural, working genes.

Thus, the potential of gene therapy lies in practically targeting therapeutic substances to precise or broad CNS regions at effective concentrations for sustained time periods. This offers the possibility, depending on the nature of a given disease, to prevent cell death, augment cell function, or replace a developmentally defective gene. The field of gene therapy and its relevance to the treatment of neurological disease has come a long way in the past 20 years, and is likely to become a mainstay of neurological therapy in the next 20 years as we enter the era of "molecular medicine." This chapter will describe the development of gene therapy for neurological disease, and will present examples of its implementation to treat neurological disease together with some of the challenges that remain to be addressed.

II. The Development of Gene Therapy in the Nervous System

The beginnings of gene therapy for diseases of the nervous system can be traced to any one of several potential time points: the identification by early biologists of the critical importance of the nucleus of the cell to the survival and function of each cell; the identification of the double helical structure of DNA in 1962; the initial production and then routine use of "recombinant" DNA in the 1970's; the first use of gene therapy to treat an animal model of neurological disease in 1988 (Rosenberg, et al., 1988); or the first human trial of gene therapy in 1990 (Culver, 1991). See also (Osterman, 1971, Friedmann and Roblin, 1972, Anderson, 1980). As noted above, the real potential of gene therapy for nervous system disease lies in the ability to correct disorders resulting from genetic mutations at even early time points of development, or to deliver therapeutic genes to the nervous system that can in some way compensate, ameliorate or prevent cell loss or dysfunction resulting from disease. A third and more recently emerging use of gene therapy is to *block* the expression of deleterious genes that cause disease, such as Huntingtin or the amyloid precursor protein. Thus, in inborn errors of metabolism, the goal of gene therapy is to replace a mutated or deleted gene. In disorders such as Alzheimer's disease, Parkinson's disease, or spinal cord injury, gene therapy can be used as a tool to deliver therapeutic substances such as growth factors to the nervous system to prevent cell death and stimulate cell function. Lastly, in diseases such as Huntington's disease or Alzheimer's disease, expression of deleterious disease-causing genes could be blocked (by expressing small-interfering RNAs or antisense mRNAs via gene therapy).

To implement gene therapy, a gene of interest must be identified, sequenced, and, ideally, have a well-defined function. The replacement gene is then introduced into the nervous system and expressed for a sufficient time period (possibly indefinitely) to treat the disease.

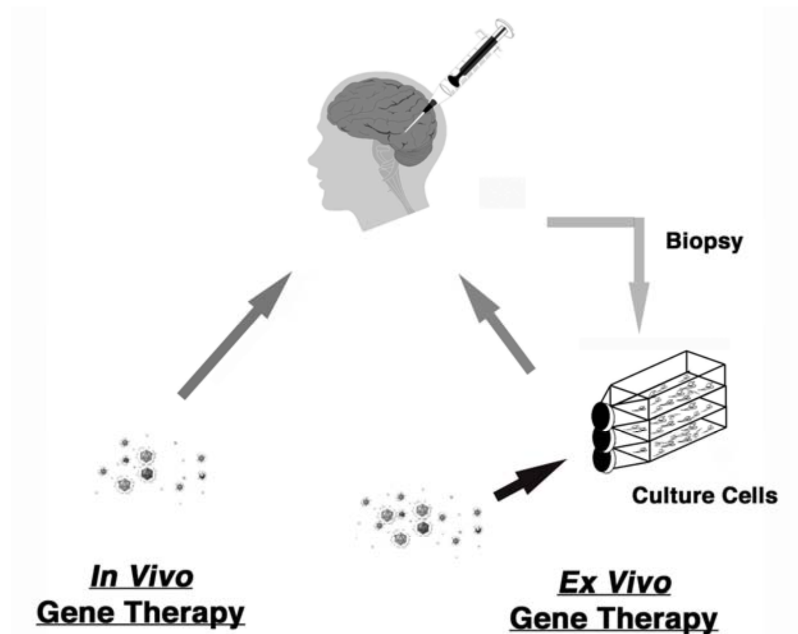
Putting Genes Into Cells: *Ex Vivo* vs. *In Vivo* Gene Therapy

Gene therapy can be divided into two basic approaches: *ex vivo* and *in vivo* gene delivery (**Fig. 1**). In *ex vivo* gene delivery, host cells are obtained from a biopsy and established as *in vitro* (or "*ex vivo*") cultures, and are genetically modified using one of several potential methods described below. Production of the desired gene product by the modified cells can be quantified. Cells are then grafted into the nervous system where they act as localized biological pumps to deliver the gene product. Autologous cells that can act as vehicles of *ex vivo* gene delivery include fibroblasts (obtained from skin biopsies), bone marrow stromal cells, stem cells (e.g., from the bone marrow), clonal lymphocytes, or Schwann cells. Outside the nervous system, tumor cells have also frequently been targets of *ex vivo* genetic modification by enhancing their

production of immune signaling molecules such as cytokines; once re-introduced into the body, the cells may more vigorously stimulate a response of the immune system to tumor antigens.

In vivo gene therapy, on the other hand, circumvents all *ex vivo* cell preparation by instead injecting genes, usually carried within viral "vectors", directly into host cells *in vivo*. This results in the direct genetic modification of cells of the nervous system (Fig. 1).

Schematic of Ex Vivo and In Vivo Gene Therapy



Use of *ex vivo* vs. *in vivo* gene therapy

In the early days of testing the potential of gene therapy for animal models of nervous system disease, *ex vivo* gene therapy vectors generally resulted in better levels of gene product delivery in the nervous system than *in vivo* methods. More recently, however, *in vivo* methods of gene delivery have advanced substantially and now appear to exceed the efficiency of *ex vivo* gene delivery. For this reason, *in vivo* gene delivery methods are likely to become the mainstay of gene therapy for the nervous system, except in cases where cells must also be introduced to treat disease. Examples of the latter could include spinal cord injury or multiple sclerosis, where not only a therapeutic gene would be beneficial (e.g., a growth factor to stimulate axon growth or myelin synthesis), but a replacement cell is also needed to provide a physical bridge in a lesion cavity (spinal cord injury) or to replace myelinating cells (e.g., a Schwann cell or neural stem

cell, in multiple sclerosis). However, with few exceptions, most gene therapy applications will now utilize *in vivo* gene delivery.

Techniques of gene delivery into cells:

New genes can be introduced into cells using several methods. The process begins by constructing a "plasmid" that is comprised of nucleic acids coding for the gene of interest. This gene of interest is expressed by a promoter that should be continuously active. The plasmid can also contain other nucleic acid sequences to regulate, stabilize or augment gene expression or messenger RNAs produced by transcription. Next, the plasmid is introduced into a host cell using either mechanical techniques or using modified viruses that are non-virulent. Mechanical techniques of plasmid introduction include direct injection into cells ("gene gun"), mixture of the plasmid with calcium phosphate (opening pores or channels in the cell membrane for plasmid entry), liposomes (fat particles that surround the plasmid and directly fuse with the cell membrane due to their hydrophobicity), or electrical current ("electroporation", which opens pores in the membrane). Plasmid DNA can also be stabilized and placed directly in the extracellular environment (so called "naked" DNA), allowing direct cell uptake of the DNA.

But the most commonly used and efficient methods for plasmid introduction into cells use viruses, which, through natural selection, have adopted highly efficient means of delivering their genetic material into the nuclei of host cells. In viruses that have been modified for use in gene therapy applications, genes for viral replication and virulence are removed and replaced by therapeutic genes and constitutively active promoters (**Fig. 2**). For *ex vivo* gene delivery, modified retroviral vectors have been used most commonly. For *in vivo* gene delivery, adenovirus, herpes virus, adeno-associated virus (AAV), and retroviruses (e.g., human immunodeficiency virus) have generated the most interest (Muzyczka, 1992, Naldini, et al., 1996b, Jacobs, et al., 1999, Rabinowitz and Samulski, 2000).

Vector Schematic

In vivo gene therapy vectors:

Retroviruses consist of single strands of RNA which, upon entry into a cell, utilize a viral reverse transcriptase enzyme to synthesize viral DNA from RNA (hence the term "retro" virus). This viral DNA is then transcribed to synthesize proteins for viral replication, production of viral envelope or capsid proteins, packaging of viral particles into viral coats, and other virulence-mediating functions. Retroviruses can also code for integrase proteins that direct integration of viral nucleic acid into the host genome. Viral integration into the host genome can result in stable, long-term viral gene expression. Thus, retroviruses have naturally evolved into potent biological mechanisms for expressing genetic material; the challenge of therapeutic gene delivery using viral vectors is to utilize viral mechanisms for expressing therapeutic genes while removing viral genes for virulence. This is generally accomplished by producing gene therapy vectors that are devoid of as many wild-type genes as possible, including all genes for viral replication, envelope production, and capsid production. When coupled with promoters that turn therapeutic gene expression "on" potently and persistently, a mechanism is created for expressing therapeutic mammalian genes at high levels over prolonged time periods in host cells.

One of the earliest retroviral vectors to be tested for use in therapeutic gene delivery was a modified form of the Moloney murine leukemia virus (MLV), in which wild-type genes for viral replication (*rep*), envelope (*env*) production and capsid (*cap*) proteins were removed and replaced by a therapeutic gene of interest. To express the therapeutic gene, in many cases, the wild-type viral promoter contained in the 5'-long terminal repeat (5'-LTR) region of the virus was retained; in other instances, the wild-type MLV promoter was replaced by a promoter from cytomegalovirus (CMV) or other viruses. This vector construct could only infect dividing cells because it entered cells exclusively during the "S" phase of cell division. For gene therapy application to the nervous system, this meant that MLV-based vectors could only be used for *ex vivo* therapeutic gene delivery, since there are very few dividing cells in the adult nervous system. Thus, host cells such as fibroblasts, Schwann cells or bone marrow stromal cells or stem cells would be obtained from a host, cultured *in vitro*, exposed to the MLV-based vector, and then injected back into the host animal to achieve gene delivery. For non-nervous system applications, the retroviral vector itself could be injected *in vivo* and get taken up by actively dividing host cells (e.g., in the bone marrow) to express the gene of interest. In addition, the gene for neomycin resistance (*neo*) was often added to the vector construct to make cells that incorporated the transgene resistant to the antibiotic neomycin; in this manner, cells *in vitro* that successfully incorporated the therapeutic vector could be selected from non-modified cells by adding antibiotics to the cell culture medium. Hence, a pure population of modified cells could be returned to the host. Using MLV-based retroviral vectors and *ex vivo* gene delivery to the nervous system, production of the protein product from the gene of interest, such as a growth factor, could be sustained for time periods of at least one year in the host brain.

Most recently, other RNA-based retroviral vectors have been used in gene therapy applications that do not require host cell division for viral entry into the cell. These include human immunodeficiency virus (HIV)-based vectors, feline immunodeficiency virus (FIV)-based vectors, and lentiviral vectors (Naldini, et al., 1996a). These viruses presumably bind to cell surface receptors to gain entry into cells. Because these vectors are capable of entering non-dividing cells, they can be used for direct gene delivery into non-dividing cells of the adult brain.

As described above for MLV-based gene delivery systems, these vectors are rendered safe by removing wild-type genes responsible for viral replication and particle production. The very few remaining wild-type genes in the vector express no secreted proteins, and hence are not known to elicit an immune response from the host. When used for *in vivo* gene delivery to the nervous system, these vectors predominantly enter neurons (greater than 90% of modified cells in the brain are neuronal) and continue to express their gene product for at least one in the primate brain, the longest time point examined to date. Like MLV vectors, HIV-based vectors integrate into the host genome.

A number of DNA-based viruses have also been adapted for use in gene therapy (Miller, 1990, Muzyczka, 1992, Glorioso, et al., 1995, Naldini, et al., 1996b, Rabinowitz and Samulski, 1998). One of the earliest viruses to be studied for therapeutic gene delivery was the common cold virus, adenovirus. Binding to cell surface-based receptors, this virus enters non-dividing cells and can therefore be used in *in vivo* gene therapy paradigms inside and outside the nervous system. Indeed, this vector has been the most broadly tested of all *in vivo* gene therapy vectors in human clinical trials. However, the most commonly used version of the adenoviral vector expresses a number of wild-type viral proteins and, not surprisingly, has been plagued by resulting inflammatory and immune problems. These problems occur due to both the existence of circulating neutralizing antibodies to adenoviral epitopes, and from the continued expression of wild-type adenoviral proteins from the vector after entry into host cells. Indeed, an immune response to adenovirus led to a patient death in a systemic (non-nervous system) gene therapy trial in 1999, representing a setback to the field of gene therapy. However, the availability of later-generation adeno-associated virus (AAV) and lentivirus vectors substantially reduced this risk of immune response because the vectors do not express wild-type viral genes, and, in the case of lentivirus, humans do not have pre-exsiting circulating antibodies to the virus. The safety profile of the AAV and lentiviral vectors for use in the nervous system is further reinforced by the fact that the absolute quantity of vector delivered for nervous system applications will in general be far lower than quantities used for systemic (non-nervous system) gene therapy. When adenovirus vectors are used for gene delivery in the nervous system, they do elicit immune responses (cite sanchez ramos). More recently, newer generation “gutless” adenovirus vectors have been developed that do not express wild-type viral genes, which may improve the safety profile of adenovirus-based vectors substantially. But for the present, AAV and HIV-based vectors appear to offer safety from the standpoint of an sbsence of significant host immune response to the vector, and duration of in vivo gene expression. As mentioned above, lentiviral vectors continue to express their therapeutic gene for at least one year after delivery to the primate brain, the longest time point examined to date (our unpublished data). AAV vectors also appear to express their gene products for extended time periods in vivo, with persistent expression for at least three and a half years in vivo (K. Bankiewicz, personal communication).

However, a potential drawback of some in vivo gene therapy vectors is the following: depending on the specific site of viral integration into a host genome, host gene expression may become disrupted. Hypothetically, if viral integration occurs in a host genomic sequence that codes for a tumor suppression gene, then tumor genes could become de-repressed, leading to malignant transformation. Alternatively, the integration of viral promoters into the host genome could hypothetically activate the expression of certain host genes, which, if adjacent to coding sequences for oncogenes, could also lead to tumorigenesis. This is a greater hypothetical

problem with in vivo gene delivery vectors that integrate into the host genome, such as retroviruses. AAV, which primarily remains intranuclear but extrachromosomal, is less likely to cause insertional mutagenesis in a host. Indeed, in a retroviral gene therapy trial for severe combined immunodeficiency (SCIDS) in France, most patients showed a clear benefit from gene therapy to replace their defective immune system gene (ref). However, 3 of 11 children in the trial subsequently developed a form of leukemia resulting from insertional mutagenesis. Work is underway to modify the retrovirus and alter its sites of integration into the host genome. Other retroviral vectors, such as HIV-based and lentiviral vectors may not have the same integration site and may therefore have less risk. And again, other efficient in vivo gene therapy vectors such as AAV show little integration into the host genome, and several clinical trials in humans using AAV to date have not reported subsequent development of cancers.

Comparison of *in vivo* vs. *ex vivo* gene therapy

For use in treating neurological disorders, *ex vivo* and *in vivo* gene therapy approaches each have distinct advantages and disadvantages, depending on the potential disease application (Table 1). Advantages of *in vivo* gene delivery include: 1) Simplicity. Gene delivery can be accomplished in a single step of direct vector injection into the desired brain target region, in contrast to the considerable cell processing that is required when using *ex vivo* gene delivery. 2) Minimal invasiveness. *In vivo* gene delivery is achieved by injection of several microliters of vector particles in a fluid solution to the brain and could performed repeated if necessary; *ex vivo* gene therapy delivers cell suspensions that occupy some space in the brain and would be more difficult to repeat.

Potential disadvantages of *in vivo* gene therapy relative to *ex vivo* gene therapy include the following: 1) Non-specificity of target cell infection. Neurons, glia and vascular cells can become genetically modified when *in vivo* vectors are injected into the brain. Whether there might be deleterious effects of expressing certain genes, such as growth factor or neurotransmitter genes, in glia and vascular cells is unknown (although empirically, adverse effects have not been noted in prolonged primate expression studies to date). *Ex vivo* gene therapy allows specific selection of the cell type that expresses the gene(s) of interest prior to delivery to the brain. 2) Toxicity. As noted above, some *in vivo* gene therapy vectors can be directly toxic to host cells, including herpes virus and rabies virus. Early adenovirus vectors elicited immune responses. HIV-based and AAV vector systems are generally safe and non-toxic, and more recently have emerged as potential lead candidates for gene delivery in the nervous system. Newer generation herpes “amplicon” vectors (expressing no wild-type viral genes) and herpes virus / AAV hybrid “amplicon” vectors under development also offer potentially safe gene delivery to the nervous system (Maguir-Zeis, et al., 2001, Heister, et al., 2002 , Wang, et al., 2002). « Gutless” adenoviruses that do not express wild-type viral genes could also allow the re-mergence of adenovirus based systems for nervous system application (Yant, et al., 2002). 3) Risk of malignant transformation. As mentioned above, integrating vectors could cause cancer by interrupting a tumor suppressor gene or mutating a oncogene. This risk also exists for *ex vivo* gene delivery that uses vectors which integrate into the host genome. This risk is far less in non-integrating in vivo vectors such as AAV. 4) Spontaneous recombination with wild-type viruses. It is hypothetically possible that an *in vivo* gene therapy vector could recombine with a wild-type virus, resulting in replication-competent vectors or other mutated viruses with potential for virulence. This risk can be minimized to a range

approaching near impossibility by removing most wild-type coding sequences in the therapeutic vector, by screening recipients of gene therapy for existing infection with wild-type virus such as HIV, and by the creation of hybrid vectors with little if any overlap in wild-type coding sequences.

Table 1: Comparison of *In Vivo* and *Ex Vivo* Gene Therapy

	In Vivo Gene Therapy	***Ex Vivo Gene Therapy***
Advantages	**Simple, Effective** **Minimally Invasive**	**Targets specific cell types** **Safe (little risk of wild-type viral recombination)**
Disadvantages	**Modifies neurons, glia, vessels** **Risk of cancer induction (retroviruses)** **Potential wild-type recombination**	**Cumbersome** **Risk of cancer induction**
	Absence of regulation	**Absence of regulation**

Advantages of *ex vivo* gene delivery relative to *in vivo* gene delivery include the following: 1) Targeting: *Ex vivo* gene therapy can target selected cell types *in vitro* for subsequent grafting to the brain. 2) Safety. *Ex vivo* gene delivery does not introduce infectious viral particles into the brain, hence there is little risk of recombination with wild-type viruses. *Ex vivo* gene delivery utilizes cells from the host, thus there is no risk of immune rejection. Emperically, in a clinical trial of *ex vivo* nerve growth factor (NGF) gene delivery for Alzheimer's disease, there have been no complications to date of the *ex vivo* gene delivery system.

Disadvantages of *ex vivo* gene therapy relative to *in vivo* gene therapy include: 1) Requirement for target cells to divide. As noted above, for host cells to be maintained and genetically modified *in vitro*, they must be capable of dividing. Thus, most post-mitotic cell populations such as neurons cannot act as vehicles of *ex vivo* gene therapy in nervous system. 2) Tumor formation. It is possible that the dividing cells delivered to the brain could form tumors. However, this has not been observed when implanting primary (non-immortalized) cells into the brain, although grafts of immortalized cell lines have formed tumors. "Conditionally immortalized" cell lines which shut off immortalizing genes at 37°C have been successfully implanted into the body without forming tumors (McKay, 1992, Whittemore, et al., 1997). To date, tumor formation has not been observed in a human clinical trial of NGF gene delivery for

Alzheimer's disease. Insertional mutagenesis is also a risk when using viral vectors that integrate into the host genome in *ex vivo* gene therapy, as described above under "disadvantages of *in vivo* gene delivery." 3) Complexity. The *ex vivo* gene therapy process of cell culture, gene modification, and preparation for implantation is cumbersome and time consuming, requiring 2 – 3 months. *In vivo* gene delivery can be performed immediately.

In vivo and *ex vivo* gene therapy both suffer from the drawback that they must be introduced into the brain by direct injection to be effectively administered to the central nervous system (CNS). The blood brain barrier blocks passage of most vectors into the brain. Even were peripheral delivery routes for the CNS available, they likely could not be used because most nervous system applications of gene delivery would require highly specific *and restricted* delivery of genes such as growth factors to specific regions of the brain. Production of the gene of interest in non-targeted regions of the brain could be deleterious. On the other hand, gene delivery to the CNS can be accomplished relatively simply and safely through a small burr hole.

Finally, whereas many gene therapy vectors now efficiently express their gene products for prolonged time periods (years in the case of AAV and lentivirus), the ability to turn "off" gene expression has not yet been perfected. The future development of a practical and effective regulatable gene delivery system will be discussed in more detail below.

Growth Factor Gene Therapy for Neurological Disease: Alzheimer's disease

Neurodegenerative diseases are attractive candidates for gene therapy. Several of these disorders, including Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis, lack truly effective therapies. Other disorders, such as Parkinson's disease, have effective symptomatic therapies but no means to prevent disease progression. The discovery of a class of neuroprotective substances called nervous system growth factors offers the potential for the first time to reduce cell loss in neurological disease and to stimulate the function of remaining neurons. But growth factors are large and polar molecules, and they do not cross the blood brain barrier. Further, they exhibit toxicity if delivered to non-affected brain regions. Thus, effective harnessing of the potential of growth factors to treat neurological disease requires site-specific, intracranial delivery. Gene therapy has emerged as a leading method for achieving long-term, highly localized and regionally-restricted delivery of growth factors to the brain. Clinical trials of growth factor gene delivery are underway in the most common age-related neurodegenerative disorder, Alzheimer's disease (Tuszynski, 2002a), and will soon include Parkinson's disease, and possibly ALS and Huntington's disease.

Growth Factors

There are now known to exist several different families of growth factors, each of which affects the survival and function of various neurons throughout the nervous system. The first nervous system growth factor was identified over 50 years ago by Rita Levi-Montalcini and Viktor Hamburger, and was aptly named "nerve growth factor" (NGF). Levi-Montalcini and Hamburger discovered that mouse sarcoma extracts contained a substance that promoted sensory and sympathetic neuron survival in the embryonic chick (Levi-Montalcini and Hamburger, 1951, Levi-Montalcini, 1987). Over the subsequent decades, several important features surrounding the role of NGF during development were defined. Its structure was elucidated in the early

1970's (Angeletti, et al., 1973), and the gene was cloned in 1983 (Ullrich, et al., 1983). However, the startling discovery that NGF could also influence neuronal survival in the *adult* nervous system came nearly 40 years after its initial discovery.

In 1986 and 1987, three research groups independently reported that injections of NGF protein into the adult brain prevented the death of forebrain cholinergic neurons (Hefti, 1986, Williams, et al., 1986, Kromer, 1987). In 1987, it was also reported that NGF reversed *spontaneous* age-related morphological and behavioral decline in rats (Fischer, et al., 1987). These findings ushered in an era of intense interest in both basic mechanisms of growth factor action in the nervous system, and an exploration of their therapeutic potential to prevent cell death in neurological disease. The ability of NGF to prevent the death of cholinergic neurons was extended to primate systems, where it promoted the survival of 80 - 100% of cholinergic neurons after lesions (Koliatsos, et al., 1990, Tuszynski, et al., 1990, Tuszynski, et al., 1991).

NGF for Alzheimer's Disease

The potential relevance of NGF actions to neuronal loss in Alzheimer's disease (AD) was evident immediately (Hefti and Weiner, 1986, Phelps, et al., 1989). Cholinergic neurons of the basal forebrain undergo profound atrophy and death during the course of Alzheimer's disease (Perry, et al., 1977, Whitehouse, et al., 1981), contributing to cognitive decline (Perry, et al., 1978, Bartus, et al., 1982, Whitehouse, et al., 1982, Candy, et al., 1983). NGF is normally produced throughout life in the brain, and is believed to support the integrity of cholinergic systems (((citation, e.g., Sofroniew))). Notably, NGF transport and availability to support the function and survival of cholinergic neurons is defective in Alzheimer's disease (Mufson, et al., 1995b, Scott, et al., 1995). Thus, NGF could reduce or prevent the cholinergic component of cell loss in Alzheimer's disease.

Delivery of NGF protein to the brain is difficult, however, because NGF is a medium-sized, charged protein that does not cross the blood brain barrier. To reach the brain effectively in animal studies, NGF was pumped into the lateral ventricles where it diffused short distances to reach cholinergic cell bodies (Hefti, 1986, Williams, et al., 1986, Kromer, 1987, Emmett, et al., 1996). Intracerebroventricular infusion of NGF was the only available delivery route for a clinical trial in Alzheimer's disease, and plans were accordingly formulated (Phelps, et al., 1989). However, preclinical distribution and toxicology studies of intracerebroventricular (ICV) infusions revealed several adverse effects of this route of NGF administration were discovered. All of these affects in fact reflected the potency of NGF in stimulating the function of neurons of the adult brain, and all adverse affects were attributable to the broad, non-targeted distribution of NGF that resulted from intraventricular infusions. For example, Isaacson and Crutcher reported that NGF-sensitive sympathetic axons sprouted around the cerebral vasculature after ICV infusion (Isaacson, et al., 1990). Williams reported that ICV infusions of NGF caused weight loss in rats due to a reduction in food intake (Williams, 1991). Perhaps most worrisome, Winkler and colleagues (Winkler, et al., 1997) as well as Emmett and colleagues (Emmett, et al., 1996) reported that Schwann cells, which bear receptors for NGF, migrated into an expanding cell layer in the subpial space surrounding the brainstem and spinal cord after ICV NGF infusions. This Schwann cell response reversed following discontinuation of NGF infusion. NGF infusions also led to sensory axon sprouting and a pain syndrome. These adverse effects all occurred in neuronal systems with known patterns of sensitivity to NGF, and all reflected

stimulation of the relevant neuronal systems by NGF. Thus, it was evident that central intraventricular infusions of NGF protein were an impractical delivery method for a clinical trial in Alzheimer's disease. Indeed, three Alzheimer's disease patients in Sweden received ICV infusions of NGF, and not surprisingly the trial was discontinued due to the development of weight loss and pain (Eriksdotter Jonhagen, et al., 1998).

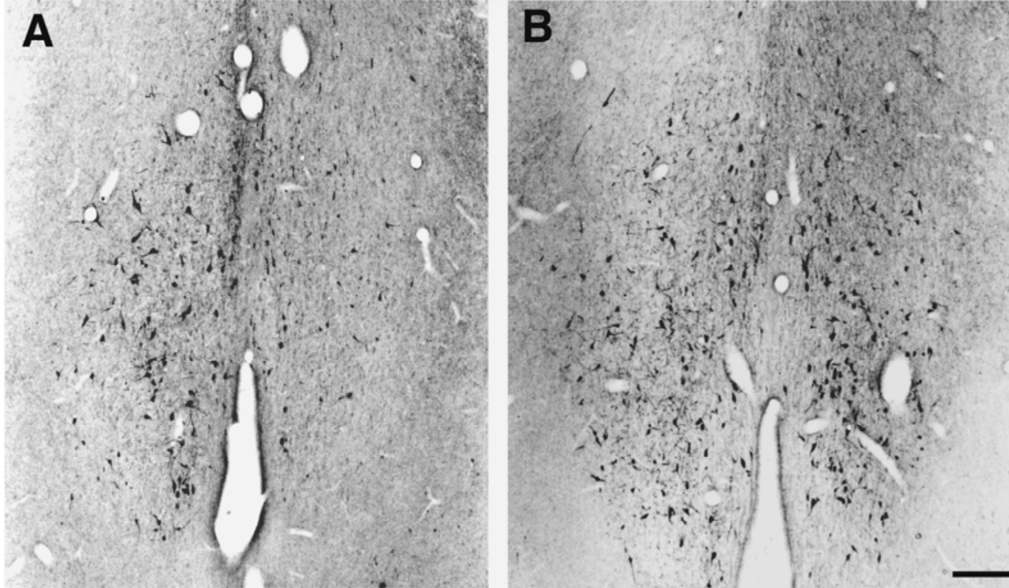
NGF Gene Delivery

An alternative method was required that could deliver NGF across the blood brain barrier, yet *restrict* its delivery to cholinergic cell targets to avoid adverse events. Gene therapy was already emerging as a potential means of achieving sustained and localized protein delivery to the brain. In 1988, the first successful use of *ex vivo* gene delivery to the brain was reported, utilizing NGF gene delivery to rescue cholinergic neurons after injury (Rosenberg, et al., 1988). These early studies of gene therapy in the nervous system used *ex vivo* rather than *in vivo* gene delivery paradigms because the vectors for *ex vivo* gene delivery were superior at the time, both in terms of long-term gene expression in the brain and in safety (absence of immune responses). In the first study of gene delivery to the brain, rat fibroblasts were obtained from skin biopsies and genetically modified to produce and secrete human NGF using retroviral vectors. These cells were implanted into the septal nucleus of rats that had undergone fimbria-fornix lesions. Typically, fimbria-fornix lesions result in the degeneration and death of cholinergic neurons by depriving them these cells from contact with their hippocampal targets. Rats that received *ex vivo* grafts of NGF-secreting fibroblasts exhibited highly significant rescue of cholinergic neurons compared to control subjects that received implants of cells expressing a control reporter gene, ß-galactosidase. Subsequently, Chen and Gage reported that *ex vivo* gene delivery of NGF also reversed cholinergic neuronal atrophy and improved cognition in aged rats (Chen and Gage, 1995).

We then examined whether *ex vivo* NGF gene delivery could also prevent cholinergic neuronal degeneration in the brains of adult primates. Rhesus monkeys underwent fornix lesions followed by implants of primary autologous fibroblasts that were genetically modified to produce and secrete human NGF. Genetically modified cells were placed adjacent to degenerating cholinergic neuronal cell bodies in the basal forebrain. One month later, animals that received NGF-secreting cell implants exhibited a significant reduction in cholinergic degeneration compared to control monkeys that received implants of primary fibroblasts that did not secrete growth factors (Fig. 3; (Tuszynski, et al., 1996)). 68% of neurons were rescued in monkeys that underwent *ex vivo* NGF gene delivery, whereas control monkeys showed survival of only 25% of cholinergic neurons ($p < 0.05$). Further, as we became more experienced in accurately placing *ex vivo* NGF-secreting cells adjacent to degenerating cholinergic neurons, the extent of neuronal protection increased to 92%. This neuronal protection was achieved using NGF doses roughly 500-fold lower than quantities of NGF protein used in previous ICV infusion studies (Koliatsos, et al., 1990, Tuszynski, et al., 1990). The requirement for accurate placement of genetically modified cells in the brain reflected potential improvements in the safety of the gene delivery approach for NGF administration to the brain: NGF diffused only 2 mm from sites of genetically modified cell implantats. This limited diffusion substantially improved the potential safety profile of NGF, reducing the risk that adverse effects would result from protein spread beyond the targeted region. Emerich, Kordower and colleagues also reported cholinergic neuronal rescue in monkeys after fornix lesions, using an alternative form of gene therapy in

which encapsulated xenogenic cells were implanted into the brains of either young or aged monkeys (Emerich, et al., 1994, Kordower, et al., 1994).

NGF Cell Rescue



At this point, it was clear that *ex vivo* NGF gene delivery could prevent lesion-induced degeneration of cholinergic neurons in the primate brain. Cholinergic neurons die in Alzheimer's disease, although the precise mechanism whereby cholinergic neurons (or any neuron, for that matter) die in Alzheimer's disease is unknown. Thus, we thought that it was important to demonstrate that cholinergic neurons degenerating resulting from another mechanism of neural damage could also respond to *ex vivo* NGF gene delivery. Aging is another form of damage to the nervous system, resulting in spontaneous atrophy of cholinergic neurons in rats. Thus, we examined the brains of aged rhesus monkeys for sensitivity to *ex vivo* NGF gene therapy. Examination of the brains of unoperated, aged monkeys revealed that 40% of basal forebrain cholinergic neurons are atrophic (but not dead) compared to young monkeys (Smith, 1999). Notably, three months after undergoing *ex vivo* NGF gene delivery, aged rhesus monkeys exhibited a restoration of functional cholinergic neuronal markers to levels equivalent

to young monkeys (Smith, 1999). Cell size was also restored to levels indistinguishable from young monkeys. Control, aged monkeys that underwent *ex vivo* gene therapy containing a reporter gene but not NGF evidenced no neuroprotection. Further, aged monkeys undergoing *ex vivo* NGF gene delivery demonstrated a return to levels of young monkeys of cholinergic axon projections to the cortex (Smith, 1999).

Dose-escalation/toxicity studies of NGF *ex vivo* gene transfer in primates

The preceding studies demonstrated that *ex vivo* NGF gene delivery could prevent cholinergic neuronal degeneration and ameliorate age-related cholinergic neuronal atrophy in the brains of primates, suggesting that various mechanisms of cholinergic damage in primates were indeed sensitive to *ex vivo* NGF gene delivery. Rodent studies indicated that two additional mechanisms of cholinergic degeneration were also sensitive to NGF delivery: excitotoxic injury (Dekker, et al., 1991) and amyloid-induced degeneration of cholinergic neurons in trisomy-16 mutant mice (((mobley))). Further, NGF diffused no more than 2 mm from brain sites of *ex vivo* gene delivery, in contrast to diffusion distances of several centimeters resulting from intraventricular or intraparenchymal NGF protein infusions (Conner and Tuszynski, unpublished observations). Thus, the prospect that gene therapy could achieve localized and effective delivery of NGF to the brain was emerging.

To further test this possibility before embarking on a clinical trial, a dose escalation/toxicity study of *ex vivo* NGF gene delivery was performed in adult monkeys. Subjects received escalating volumes of autologous NGF-producing cells (autologous fibroblasts) into the nucleus basalis. Volumes ranged 5 ul of cells per injection site to a maximum of 100 ul of cells per injection site. Extent and duration of gene expression were measured together with NGF levels in the cerebrospinal fluid, implanted graft size, weight, and general comfort over a one-year period. At the highest cell injection volumes, fibroblasts refluxed from injections sites yet no resulting toxicity was observed. Weights of all grafted subjects remained stable, and there was no evidence of pain or discomfort as reflected by general activity level. Implanted cells did not migrate and tumors did not form. NGF was not detectable by ELISA in spinal fluid samples taken 6 months and one year post-therapy, and Schwann cells did not migrate into the subpial space of the brainstem or spinal cord. Importantly, gene expression was present for at least one year in the primate brain, assessed from brain biopsies taken at graft sites; NGF levels exceeded physiological levels 5-fold after one year in vivo. Thus, *ex vivo* NGF gene delivery in the primate brain could prevent cholinergic neuronal degeneration, reverse spontaneous age-related cholinergic atrophy, and safely sustain gene expression for one year with no evidence of toxicity.

A phase I clinical trial of NGF gene therapy in Alzheimer's disease

Based upon the preceding safety and efficacy data, a rational basis was established for proceeding to a clinical trial to determine whether *ex vivo* NGF gene therapy could reduce cholinergic neuronal degeneration in AD. NGF could hypothetically benefit AD via two distinct mechanisms: by reducing cholinergic neuronal death, or by *augmenting* cholinergic neuronal function by stimulating NGF-mediated release of acetylcholine in the cortex. Two important questions remained to be tested in the clinical trial, however: 1) Could cholinergic cell loss in AD be prevented by NGF delivery? The underlying cause of neuronal death in AD remains unknown. Whereas extensive neurofibrillary degeneration occurs in cholinergic neurons in AD,

there is little depostion of amyloid in the cholinergic nucleus basalis. As noted above, NGF levels in the cholinergic basal forebrain in AD are significantly reduced compared to age-matched controls (Mufson, et al., 1995a, Scott, et al., 1995), although NGF production in the cortex is not reduced in the AD brain, Thus, defects in NGF retrograde transport appear to exist in AD and could result in cholinergic neuronal degeneration. Delivery of NGF to the cholinergic neuronal cell body using *ex vivo* gene therapy could bypass a transport defect and sustain the survival and function of cholinergic neurons. Even if a primary deficiency of NGF itself does not cause cholinergic neuronal loss in AD, NGF gene delivery could potentially benefit AD by augmenting cholinergic transmission. 2) Would amelioration of the cholinergic component of cell degeneration in AD be sufficient to impact cognitive decline? Multiple neuronal systems decline in AD in addition to cholinergic neurons. However, of the neuronal systems that decline in AD, loss of cholinergic neurons correlates best with synapse loss (Terry, et al., 1991) and dementia severity (Perry, et al., 1978). Further, the cholinergic system plays a critical role in modulating cortical plasticity in the intact brain (Conner, 2003). Indeed, several clinical trials have shown a modest effect of cholinergic system augmentation by cholinesterase inhibitors, even though these oral agents only slightly augment central levels of acetycholine (Davis, et al., 1992, Mayeux, 1999). Thus, preventing cholinergic degeneration in AD, especially early in the disease, could have a signficaint impact on disease progression and quality of life.

A Phase I safety trial of *ex vivo* NGF gene delivery in humans with early stage AD began at UCSD in 2001. Early stage patients were selected for two reasons. First, patients must understand the nature of the procedure that they are undergoing and its potential risks, since this is the first human clinical trial of gene therapy in Alzheimer's disease. Patients with early AD are capable of such insight. The consent of the primary caregiver is also obtained. Second, the hypothetical benefit of NGF in preventing cholinergic degeneration will be of most benefit earliest in the disease, when the greatest population of cholinergic neurons remain to be rescued.

Using the same methods described above for our pre-clinical primate studies, primary autologous patient fibroblasts were genetically modified to produce and secrete human NGF in vitro. After verifying adequate production of human NGF, cells were placed into the human nucleus basalis of Meynert in a dose-escalation trial design. The lowest dose of cells delivered in this clinical trial was effective in rescuing cholinergic neurons in non-human primate studies, and the highest dose used in this trial was well below maximum tolerated cell volumes in monkeys. Eight subjects have undergone the gene delivery procedure, and endpoints that will be examined in the trial include general safety measures as well as cognitive function and glucose metabolism on PET scans.

Potential risks of the study include those that are associated with inadvertent, widespread distribution of NGF protein, including pain, weight loss, or Schwann cell migration into the subpial space. Other risks include hemorrhage from needle passage into the brain, tumor formation from the grafted cells, or migration of the grafted cells. These adverse effects were not encountered in more than 200 NGF-secreting cell implants in pre-clinical primate studies, representing an extensive base of empirical safety data that mitigates the potential risk of a clinical trial. Of potentially greatest concern in this clinical trial would be the development of adverse effects resulting from unintended widespread expression of NGF. Although pain could be treated with analgesics or weight loss controlled with behavioral measures, optimally a means

would be available to turn expression of the therapeutic gene "off". Such means are not currently available, and are under development (see below). Given the impressive efficacy of NGF in preventing loss of cholinergic systems, the extensive empirical safety data in five primate studies (Emerich, et al., 1994, Kordower, et al., 1994, Tuszynski, et al., 1994, Tuszynski, et al., 1996, Smith, 1999, Conner, 2001), the lack of other broadly effective therapies for AD, and the disease's relentlessly progressive course, the clinical trial has proceeded.

In vivo gene therapy for Alzheimer's disease

The current *ex vivo* trial of NGF gene therapy in AD is likely to be followed by a trial if *in vivo* gene therapy for AD. As noted above, the available vectors for *ex vivo* gene therapy were superior to the vectors for *in vivo* gene therapy until recently, hence our clinical program advanced on an *ex vivo* design. The emergence of AAV and lentiviral vectors for *in vivo* gene delivery to the nervous system will likely replace *ex vivo* trials in nervous system disease, unless *in vivo* vectors have unexpected toxicities that have not been revealed to date by primate studies, a possibility that seems unlikely. For applications to Alzheimer's disease, *in vivo* NGF gene delivery has been shown to prevent cholinergic neuronal death in rat models using AAV vectors (Mandel, 1999, Blesch, 2002) and, preliminarily, to reverse age-related cholinergic neuronal atrophy (Klein, et al., 2000). Studies are ongoing with both AAV and lentivirus systems in primates, providing a potential basis for human clinical trials.

Other Genes for AD:

Over 50 nervous system growth factors have been identified to date (Tuszynski, 1999), raising the possibility that growth factors might also be used to target other degenerating neuronal populations in AD. Two other growth factors are of particular interest in this regard: brain-derived neurotrophic factor (BDNF), and basic fibroblast growth factor-2 (FGF-2). BDNF prevents the death of cortical neurons(Giehl and Tetzlaff, 1996, Lu, et al., 2001), and levels of BDNF are diminished in the hippocampus in AD (Phillips, et al., 1991). FGF-2 prevents injury-induced degeneration of entorhinal cortical neurons (Gomez-Pinilla, et al., 1992, Peterson, et al., 1996). Whereas the entire cortex would not be a practical target for growth factor gene delivery in a disorder such as AD, critical areas such as the entorhinal cortex or hippocampus might be candidates for growth factor gene delivery to prevent cell loss. This possibility is the subject of ongoing preclinical studies. In addition, cells have been genetically modified to secrete the neurotransmitter acetylcholine have been shown to significantly ameliorate age-related cognitive decline in rats (Winkler, et al., 1995). However, a practical limitation in the application of a transmitter replacement approach for gene therapy of AD is the large size of the human cortex that would need to be targeted. In contrast, the size of the basal forebrain cholinergic system that is the target of our ongoing trial of *ex vivo* NGF gene delivery in AD is only 1 cm in rostral-caudal extent.

Gene Therapy for Parkinson's disease

Growth Factor Gene Delivery for PD:

Three growth factors are potential therapeutic candidates for gene delivery in the second most common neurodegenerative disorder, Parkinson's disease (PD): glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), and brain-derived neurotrophic factor (BDNF). Progressive loss of dopaminergic neurons occurs in the substantia nigra in PD, and several *in vitro* studies demonstrate that GDNF, NTN or BDNF support the survival of developing nigral

neurons. More importantly, *in vivo* studies in both rodent and primate models of PD demonstrate that these growth factors prevent nigral cell loss and promote functional recovery (Spina, et al., 1992, Frim, et al., 1993, Lin, et al., 1993, Hyman, et al., 1994, Gash, et al., 1996, Kotzbauer, et al., 1996, Winkler, et al., 1996, Connor, et al., 1999, Bjorklund, et al., 2000, Kordower, et al., 2000).

Based on these types of findings, a clinical trial of ICV GDNF protein infusion was performed in PD (Nutt, 2003). However, the non-targeted nature of ICV GDNF infusions induced adverse effects including nausea, weight loss and Lhermitte's signs. Not only did this broad, non-targeted delivery route cause toxicity, but GDNF failed to reach nigral neurons in significant concentrations (J. Kordower, Rush-Presbyterian Medical Center, personal communication). Once again, this outcome reinforces the concept that the adequate clinical assessment of the potential of growth factors to treat human disease will require that effective concentrations of the agents be delivered to neural structures in adequate doses and in a manner that is highly targeted and regionally-restricted, to avoid adverse effects of these potent agents.

Evidence from the pre-clinical literature suggested that the optimal site for delivery of growth factors to degenerating nigrostriatal circuitry might in fact be the region of the caudate/putamen, where a growth factor could induce sprouting of host dopaminergic terminals into their natural striatal targets (Kirik, 2000). GDNF, NTN, or BDNF delivered to the striatum in this manner could promote the survival of nigral neurons because the growth factors are retrogradely transported from the striatum to neurons of the substantia nigra (Mufson, et al., 1996). Rodent studies using *in vivo* gene delivery of GDNF in fact demonstrated significant neuroprotection and functional recovery in rats with experimentally-induced PD (Connor, et al., 1999, Bjorklund, et al., 2000). Subsequently, Kordower and colleagues demonstrated in a primate model of PD, MPT-induced Parkinsonism, that *in vivo* lentiviral GDNF gene delivery induced nearly complete anatomical recovery and functional recovery (Kordower, et al., 2000). In addition, lentiviral gene delivery to aged monkeys also reversed age-related biochemical and anatomcial degeneration of nigrostriatal circuitry (Kordower, et al., 2000). Hence, a clear rationale has been established for pursuing gene delivery in PD, with the potential to both prevent neuronal degeneration and to augment the function of remaining circuitry. However, in the absence of clinically practical regulatable vectors that would allow shut-off of the gene *in vivo*, additional primate studies should be done to demonstrate convindingly the safety of this approach.

Providing additional rationale and proof-of-principle for delivery of growth factors to treat PD, a phase I trial of intraparenchymal infusion of GDNF protein into the stritaum in PD was recently reported (Gill, et al., 2003). 5 patients received continuous infusions of GDNF into a single site in the putamen via an implanted Medtronics pump, and showed signs of both clinical improvement and increased regional fluoro-dopa uptake on PET scan. However, hardware problems occurred in 2 patients and all subjects showed an increase in MRI signal at the infusion site. Hypothetically, gene delivery could be a safer and more effective means of long-term delivery of growth factors to the nervous system compared to chronic infusions and implanted hardware. However, such conclusions cannot be made in the absence of high quality clinical trial data demonstrating both the safety and efficacy in *n vivo* gene delivery.

Neurotransmitter Gene Therapy for PD

Another potential means of using gene therapy in the nervous system in PD is the replacement of neurotransmitters. Neurotransmitter replacement could provide more physiological levels of transmitter replacement in diseases such as PD to smooth out debilitating motor fluctuations that occur in later stages of oral dopamine agonist therapy. Nearly 15 years, Wolff and colleagues used an *ex vivo* gene delivery approach to provide the dopamine synthesizing enzyme tyrosine hydroxylase (TH) to rat's with experimentally induced Parkinson's disease (Wolff, et al., 1989). They demonstrated elevated striatal levels of dopamine and functional recovery. Subsequent studies using TH replacement, or replacement of another enzyme in the dopamine synthesis pathway, dopamine decarboxylase (DDC), have continued to support the feasibility of transmitter-synthesizing gene delivery to the striatum (Kang, et al., 1993, Bankiewicz, et al., 1998, Azzouz, 2002). Clinical trials using this approach are actively being considered. The primary shortcoming of this approach is an uncertainty that the efficacy and safety of non-regulated gene delivery for transmitter synthesis enzymes will exceed that of simple oral agents that are currently available. And unlike growth factor gene delivery, neurotransmitter replacement would not be neuroprotective.

Surgical lesions have recently gained (or regained) substantial popularity in PD treatment, together with a more recent shift to the implantation of stimulating electrodes (deep brain stimulation) to block aberrant excitability in neural systems that contribute to PD symptoms. Taking the approach of a "chemical"lesion of the subthalamic nucleus by gene transfer of glutamic acid decarboxylase (GAD, the synthetic enzyme for the inhibitory neurotransmitter GABA), a clinical trial of *in vivo* AAV GAD gene delivery is ongoing for PD (Luo, et al., 2002). In rodent studies, GAD gene delivery was reported to be effective in reducing the activity of subthalamic circuitry and improving PD-like symptoms. What is lacking in this program is a clear rationale for proceeding with the clinical trial based on compelling data from primate studies. Thus, it is difficult to objectively assess the feasibility of this clinical program. Whereas some risk is justifiable in utilizing a growth factor gene therapy program that offers the potential of fundamentally altering the primary pathogenesis of a neurodegenerative disease such as PD by preventing cell loss, the rationale for transmitter replacement is less clear and therefore the preclinical data should be at least equally strong to that of a growth factor approach. At present, it is not.

Growth factor gene therapy in ALS and Huntington's disease

Growth factors have been identified that are neuroprotective and functionally beneficial in models of Huntington's disease and ALS, offering again the potential to improve disease treatment by reducing cell death. *Ex vivo* gene delivery of ciliary neurotrophic factor (CNTF) in rodent and primate models of Huntington's disease ameliorate cell loss and promotes functional recovery (Emerich, et al., 1996, Emerich, et al., 1997). More recently, AAV-*in vivo* gene delivery of GDNF was reported to exhibit beneficial functional effects in a rat models of Huntington's disease (McBride, et al., 2003). Given the lack of alternative therapies for this neurodegenerative disorder, clinical trials of growth factor gene delivery are being contemplated.

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive and untreatable neurodegenerative disorder. BDNF, GDNF, CNTF and insulin-like growth factor I (IGF-1) have separately been reported to improve cell survival and functional outcomes in animal models of

the disease. Clinical trials with protein infusions of these growth factors have been conducted and failed (Group, 1996, Group, 1999). Yet again, it is likely the case that the routes of growth factor administration in these trials (peripheral or ICV) failed to reach motor neurons in sufficient concentrations to elicit biological stimulation of cell bodies. It is however the case that targeted delivery to motor neurons distributed along the neuraxis in ALS will be practically difficult to achieve. Two gene therapy studies, the first using adenovirus to deliver GDNF (Mohajeri, et al., 1999) and the second using AAV to deliver IGF-1 (Kaspar, et al., 2003), reported that localized delivery of growth factors to functionally critical motor neuronal groups, such as diaphragmatic motor neurons, can prolong survival in animal models of ALS. Interestingly, adenovirus and AAV are retrogradely transported to motor neurons of the spinal cord when injected into muscle. Thus, central neuronal rescue in this disease might be achievable following peripheral gene. These possibilities are also the subject of ongoing deliberation with regard to initiation of clinical trials.

Gene Therapy for Inborn Errors of Metabolism

A significant potential for the field of gene delivery is to replace absent or mutant enzymes that result in disease in the inborn errors of metabolism. Beneficial effects of *in vivo* gene transfer in several animal models of inborn errors of metabolism affecting the nervous system have been reported, including lysosomal storage disease (mucopolysaccharidosis) (Li and Davidson, 1995, Daly, et al., 1999, Stein, et al., 1999), metachromatic leukodystrophy (Consiglio, et al., 2001), alpha-sarcoglycan-deficient mice (Allamand, et al., 2000), arylsulfatase A deificiency (Learish, et al., 1996), beta-hexosaminidase deficiency (the gene affected in Tay-Sachs disease) (Lacorazza, et al., 1996), galactosialidosis (Hahn, et al., 1998), and Fabry disease (Takenaka, et al., 2000). Gene replacement to the bone marrow early in life, or to the neuraxis either early or at slightly later time points, could provide biochemically and symptomatically beneficial therapies for many of these diseases that are otherwise untreatable. Some active work is ongoing in attempting to bring these potential therapies to clinical trials.

The first human gene therapy trial in the nervous system in fact attempted to deliver the enzyme aspartoacylase, which is deficient throughout the neuraxis, to children with Canavan's disease (Leone, et al., 2000). AAV vectors coding for aspartoacylase were injected intraventricularly in two children. This route of ICV injection rendered remote the probability that the desired gene product would be widely accessible to the brain parenchyma. Another clinical trial in Canavan's disease is underway in the U.S., using multiple intraparenchymal injections of *in vivo* AAV expressing aspartoacylase.

Gene therapy for nervous system trauma

Another potential application of gene therapy is to locally deliver growth-promoting substances, such as growth factors, to focal sites of CNS injury to promote cell survival and axon growth. For example, growth factor gene delivery in models of spinal cord injury (SCI) has stimulated the growth of injured axons and generated modest functional recovery in some cases (Grill, et al., 1997, Liu, et al., 1999, Tuszynski, 2002b). Growth factor effects are sustained in the larger spinal cords of primates (Tuszynski, 2002b). Nonetheless, restoring neural circuitry after injury remains a great challenge. An important challenge lying before the practical implementation of growth factor gene delivery for the spinal cord injury is the need for regulated gene delivery: continuous activation of growth factor production in sites of spinal cord injury

leads to failure of axons to regenerate beyond the lesion site because they become "trapped" within the growth factor source. There is a need to turn growth factor gene expression sequentially "on" and then "off" at an injury site to promote axonal growth into, through, and beyond the lesion site. Such studies are ongoing. The prospect of gene delivery to prevent neuronal loss after brain trauma is also a possibility (Lu, et al., 2001). Most applications for growth factor delivery for trauma will require brief bursts of gene expression, or, perhaps more likely, short-term growth factor infusion.

Gene therapy for nervous system cancer

Gene therapy has been investigated in models of intracranial glioma for some time (Short, et al., 1990). In one set of studies, death-inducing genes such as thymidine kinase (TK) can be introduced into rapidly dividing tumor cells in vivo, or passively transferred by injecting cells that express TK into the tumor. Upon addition of the drug gancyclovir, cells that have incorporated thymidine kinase are killed, together with neighboring cells (Barba, et al., 1994, Alavi and Eck, 2001). Early clinical trials are underway. In another approach, genetically modified herpes viruses that express genes to augment presentation of tumor antigens to the host immune system are injected into tumors (Ram, et al., 1997, Shand, et al., 1999, Cohen, et al., 2000, Rainov, 2000, Alavi and Eck, 2001, Markert, et al., 2001, Nanda, et al., 2001a, Todo, et al., 2001). Clinical trials with herpes virus-based approaches to gliomas are in phase III clinical trials.

Optimizing gene delivery for the future:

Regulatable Vectors: An important safety feature that will optimize the future of gene therapy will be the ability to control gene expression in vivo, turning gene therapy expression systems "on" and "off" at will. Such regulatable expression systems have been under development for some time (Gossen, et al., 1994). Ultimately, it is hoped that regulatable vectors will be able not only to stop gene delivery should an adverse event occur, but allow the physician to adjust the therapeutic dose of a delivered gene product *in vivo* much in the way that pharmacological drug doses can be adjusted.

The most thoroughly studied regulatable expression system is probably the tetracycline-sensitive system, developed by Bujard and colleagues (Gossen, et al., 1994). In this controlled expression system, an operon is normally bound to a promoter and allows gene expression to occur. However, when tetracycline is present it binds to the operon, causing it to dissociate from the promoter and turning gene expression off ("tet-off" system). The inverse of this regulatable scheme has also been constructed, wherein the operon binds to the promoter only in the presence of tetracycline, turning gene expression on ("tet-on" system). Using the tet-off system, we have shown that the ability of NGF gene delivery to rescue cholinergic neurons can be completely eliminated by administering an oral tetracycline analog, doxycycline (Blesch, 2001). However, when tested in primates, the tetracycline-regulated system lost efficacy over time due to the formation of host antibodies to its regulatory components (Favre, et al., 2002). Hence, this system requires further development before becoming practical for clinical use. Other systems for regulating gene expression in vivo have been developed, including systems that are regulated in the presence of the steroid ecdysone (Suhr, et al., 1998), and "suicide" systems that can kill genetically modified cells if an "escape" from gene therapy is needed (Nanda, et al., 2001b). Regulatable systems are the subject of ongoing studies.

Convection-Enhanced Gene Delivery: While localized delivery of a therapeutic gene is a clear strength of gene therapy in the nervous system, the practical distance over which genes of interest can de delivered to the brain can sometimes be limiting. For example, in Parkinson's disease, the entire striatum may need to be targeted to effectively deliver growth factor or neurotransmitter genes. "Convection-enhanced" delivery methods have been developed for the delivery of cytotoxic agents to brain tumors, and the same techniques have been adopted for use in delivering genes to broader areas of the brain such as the striatum (Bankiewicz, et al., 1998). Similar approaches could be useful in inborn errors of metabolism, where broad distribution of a therapeutic gene might be needed to achieve phenotypic correction.

Conclusions

The development of molecularly-based therapies in medicine moves inevitably forward. As knowledge regarding the human genome, mechanisms of gene and cell function, and the development of practical gene delivery systems move rapidly forward, so too does the potential for developing more effective therapies for neurological disease. Together with this remarkable potential for altering the landscape of medical therapy comes a need for responsibility on the part of investigators to implement their clinical trials in a rationale and evidence-based manner. Upon this strong foundation of science and responsibility, it is not unlikely that the fate of people facing untreatable diseases will be markedly improved within our lifetimes.

Gene therapy is part of the new era of molecular medicine. Merging the potential of growth factors to rescue neurons with gene therapy technology which can deliver therapeutic substances across the blood brain barrier into specific and restricted brain regions, the possibility exists that neuronal degeneration could be significantly reduced in neurological disorders. Gene therapy's potential is broader still, with the possibility of replacing deficient or mutant genes in inborn errors of metabolism or replacing deficient neurotransmitters in the brain. The newest generation of *in vivo* gene delivery vectors have significantly improved the safety profile of gene therapy, although the need for regulatable expression vectors remains high.

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TABLES:

Table 1: Comparison of *In Vivo* and *Ex Vivo* Gene Therapy

	In Vivo Gene Therapy	***Ex Vivo Gene Therapy***
Advantages	**Simple, Effective** **Minimally Invasive**	**Targets specific cell types** **Safe (little risk of wild-type viral recombination)**
Disadvantages	**Modifies neurons, glia, vessels** **Risk of cancer induction (retroviruses)** **Potential wild-type recombination**	**Cumbersome** **Risk of cancer induction**
	Absence of regulation	**Absence of regulation**

FIGURES:

<u>Figure 1</u>: **Schematic illustration of *in vivo* vs. *ex vivo* gene therapy**.
In vivo gene therapy simply injects a therapeutic vector in the CNS directly to modify native cells of the host brain. *Ex vivo* gene therapy obtains host cells from a biopsy, genetically modifies them *in vitro*, then implants the host's own cells into the CNS to act as localized delivery agents for the therapeutic gene of interest.

<u>Figure 2</u>: **General structure of *ex vivo* gene delivery vectors**. A potent promoter drives the expression of a therapeutic gene of interest, such as a neurotrophic factor (NTF) gene. A second « internal » promoter drives the expression of a gene imparting neomycin resistance to cells. The 5' – 3' construct is terminated by the wild-type 3'-LTR region. *In vivo* gene delivery vectors express only a single novel gene because cells are not « selected » after in vivo transduction.

<u>Figure 3:</u> NGF prevents the death of adult primate cholinergic neurons. (**A**) Following right-sded fornix lesions in adult primates, cholinergic neurons undergo degeneration, reflected by a reduction in the number of choline acetyltransferase-labeled neurons on the right side of the brain. Intact cholinergic neurons are seen on left half of the brain. (**B**) NGF delivery prevents the lesion-induced loss of cholinergic neurons on the right side of the brain. Scale bar = 200 um.