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Review

Translating the therapeutic potential of neurotrophic factors to clinical 'proof of concept': A personal saga achieving a career-long quest

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ABSTRACT

While the therapeutic potential of neurotrophic factors has been well-recognized for over two decades, attempts to translate that potential to the clinic have been disappointing, largely due to significant delivery obstacles. Similarly, gene therapy (or gene transfer) emerged as a potentially powerful, new therapeutic approach nearly two decades ago and despite its promise, also suffered serious setbacks when applied to the human clinic. As advances continue to be made in both fields, ironically, they may now be poised to complement each other to produce a translational breakthrough. The accumulated data argue that gene transfer provides the 'enabling technology' that can solve the age-old delivery problems that have plagued the translation of neurotrophic factors as treatments for chronic central nervous system diseases.

A leading translational program applying gene transfer to deliver a neurotrophic factor to rejuvenate and protect degenerating human neurons is CERE-120 (AAV2-NRTN). To date, over two dozen nonclinical studies and three clinical trials have been completed. A fourth (pivotal) clinical trial has completed all dosing and is currently evaluating safety and efficacy. In total, eighty Parkinson's disease (PD) subjects have thus far been dosed with CERE-120 (some 7 years ago), representing over 250 cumulative patient-years of exposure, with no serious safety issues identified. In a completed sham-surgery, double-blinded controlled trial, though the primary endpoint (the Unified Parkinson's Disease Rating Scale (UDPRS) motor off score measured at 12 months) did not show benefit from CERE-120, several important motor and quality of life measurements did, including the same UPDRS-motor-off score, pre-specified to also be measured at a longer, 18-month post-dosing time point. Importantly, not a single measurement favored the sham control group. This study therefore, provided important, well-controlled evidence establishing 'clinical proof of concept' for gene transfer to the CNS and the first controlled evidence for clinical benefit of a neurotrophic factor in a human neurodegenerative disease.

This paper reviews the development of CERE-120, starting historically with the long-standing interest in the therapeutic potential of neurotrophic factors and continuing with selective accounts of past efforts to translate their potential to the clinic, eventually leading to the application of gene transfer and its role as the 'enabling technology'. Because of growing interest in translational R&D, including its practice in industry, the paper is uniquely oriented from the author's personal, quasi-autobiographic perspective and career-long experiences conducting translational research and development, with a focus on various translational neurotrophic factor programs spanning 30+ years in Big Pharma and development-stage biotech companies. It is hoped that by sharing these perspectives, practical insight and information might be provided to others also interested in translational R&D as well as neurotrophic factors and gene therapy, offering readers the opportunity to benefit from some of our successes, while possibly avoiding some of our missteps.

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Introduction

Neurological diseases continue to constitute a huge unmet medical need, contributing to an ever-increasing financial burden on our health care system as well as personal burden on families and society. Neurotrophic factors arguably provide the greatest, current opportunity to significantly improve the treatment of a range of neurological diseases. The more obvious opportunities involve chronic neurodegenerative diseases, like Alzheimer's, Parkinson's, Huntington's and ALS where improvement in symptoms, restoration of function of degenerating neurons and slowing or even reversing disease progression seems feasible. Additionally, less-obvious opportunities for neurotrophic factors include a host of other poorly treated disorders for which loss of neurons and neuronal function have also been linked to their key symptoms (Apkarian et al., 2004; Jeste and Lohr, 1989; LaVail et al., 1998; Sheline et al., 1999), including chronic pain, psychiatric diseases (e.g., schizophrenia and depression) and blindness. In contrast to other potential therapeutic approaches, studies with neurotrophic factors in animal models have consistently supported the idea that they have the capacity to restore the function of dying neurons, improve compromised morphology and phenotype, and protect neurons from further degeneration in human neurodegenerative diseases. My personal interest in the potential of neurotrophic factors as therapeutics for human neurodegenerative disease spans 25 years, long ago captured in an editorial written for Neurobiology of Aging entitled: 'Neurotrophic Factors: Can the Degenerating Brain Be Induced to Heal Itself?' (Bartus, 1989a). The editorial described the therapeutic promise seen in neurotrophic factors and was intended to help stimulate broader interest in their potential use for treating neurodegenerative diseases (Box 1). It seems revealing that more than two decades later, despite the early excitement surrounding their promise, no controlled study has yet produced sufficiently robust clinical benefit to support regulatory (e.g., FDA) approval.

However, clear progress has been made in both basic and translational research, including insight gained from a completed multicenter, sham-surgery controlled study testing CERE-120, or AAV2-NRTN (neurturin) in moderately advanced Parkinson's disease (PD) subjects. While the results of this trial did not meet the goal of generating data sufficient to support FDA approval, they did nonetheless produce multiple lines of evidence demonstrating that NRTN was able to produce some of the desired clinical benefit in Parkinson's patients. Though benefit was not seen on the primary endpoint (improvement on UPDRS-motor-off score, measured at 12 months, post-dosing, compared to sham-surgery), many other pre-specified and clinically important motor and quality of life measurements did show improvement

Box 1
Early comments regarding the translational potential of neurotrophic factors, as well as the delivery challenges posed when translating them as treatments for human neurodegenerative diseases.

- *Editorial written*, 1988. *Neurotrophic Factors: Can the degenerating brain be induced to heal itself*? 'When one considers the history of neurology, the idea that one might be able to treat patients so that their brain cells might either withstand deadly perturbations or regenerate to a healthier, more functional state is truly revolutionary. Never before in the history of medical science could we imagine that means to induce damaged parts of the brain to heal.'
  Excerpted from Bartus, 1989a Neurobiology of Aging, Volume 10, Page 513.
- *Editorial written*, 1988. *Delivery to the Brain: The problem lurking behind the problem*. 'The challenge of delivering new therapeutics for neurodegenerative diseases seems particularly important when one considers that the more novel therapeutic approaches to treat the pathogenic or pathologic variables involve peptides and proteins.'
  Excerpted from Bartus, 1989b Neurobiology of Aging, Volume 10, Page 621.

with CERE-120 at 12 months, with even more measurements showing benefit at 18 months (including the UPDRS-motor-off score at this later, blinded time-point). Equally important, not a single measurement at any time point similarly favored the sham control group. Moreover, the data continued to support the general safety of the approach. Thus, while the program has not yet generated data sufficient to gain regulatory approval, it has provided important 'clinical proof of concept' evidence in a well-controlled trial, demonstrating that a neurotrophic factor can significantly improve the symptoms of a neurodegenerative disease, enabled by the use of gene transfer as a safe and effective delivery method (Marks et al., 2010).

The development of CERE-120 has therefore grown into one of the leading novel therapeutic programs for PD, with three separate clinical trials completed and another multi-center, controlled trial now fully enrolled, with clinical assessments continuing to evaluate safety and possible efficacy over time. The program is supported by over two dozen nonclinical studies and over a dozen peer-reviewed publications. To date, approximately 80 PD subjects have been dosed, some as long ago as 7 years, with no serious, unexpected safety issues noted. The time and effort required to advance the program to its current state have been significant, while the translational lessons learned and insight gained were substantial.

At the time we began working on AAV-NRTN (CERE-120), it is likely that few would have predicted this program would advance to its current position among translational neurotrophic factor and CNS gene therapy programs. Several formidable programs had already achieved a significant head-start well before Ceregene even began their first animal experiments with AAV-NRTN (CERE-120). Amgen (one of the most successful biotech companies, ever) had already launched a directly-competing clinical program in PD using a closely-related and far better-characterized neurotrophic factor, GDNF (glial cell line-derived neurotrophic factor). Similarly, Cephalon (a very successful publically-traded, biopharmaceutical company) had already advanced a small molecule, neurotrophic factor mimetic into PD clinical trials with much promise (Saporito et al., 2002; Wang and Johnson, 2008). Around the time Ceregene was merely opening its doors, another company (Avigen), armed with a war-chest of funds generated from the public sale of their stock, had already completed their nonclinical program and filed an IND to test a gene therapy product for PD. Finally, while we were still in the midst of executing our nonclinical program, yet another company (Neurologix) was launching a clinical trial for yet another gene transfer product for PD. Thus, all of these competing programs were literally years ahead of ours. Yet, each of these programs has since been discontinued by those companies (and some of the companies have since disappeared), while Ceregene's program has continued to advance. What translational lessons might be learned and shared?

Translational R&D is a complicated endeavor, providing as many opportunities for avoiding pitfalls as it does hurdles and unexpected obstacles. Over the past 10–15 years, interest in identifying and developing new therapeutics has grown in academia and government, mirroring a focus traditionally held by industry. In an effort to address some of the knowledge gaps inherent in this growing trend, this manuscript uses the example of the many translational issues and activities that preceded and eventually contributed to the development of CERE-120. To help provide more unique insight into translational R&D, the manuscript offers a more personal account of the author's translational experiences in industry, including the gradual migration to the use of gene transfer as the enabling technology to overcome the significant translational obstacles presented by neurotrophic factors. As such, this is not intended to be a general review of all the advances made in the neurotrophic factor or gene transfer fields during the past 25 years, nor an attempt to acknowledge all of the breakthroughs or major contributions to those fields. Rather, this manuscript is intended as a personal account of a career in translational research and the perspectives gained, as they gradually evolved to the opportunity to lead the effort to apply gene transfer to translate neurotrophic factors and establish clinical proof of concept. My hope is that by sharing this saga, some of the events and challenges I experienced may prove as informative to others as they have been valuable to me, and perhaps in some way, maybe even as interesting. Moreover, it might provide an opportunity for others interested in developing new products to benefit from our successes as well as avoid the inefficiencies inherent in repeating some of our missteps.

Big Pharma: a training ground for translational R&D and personal introduction to neurotrophic factors

Because my early interest in science was focused on a desire to seek new treatments for serious CNS disorders, it was natural for me to adopt the unconventional career path of accepting a position in industry, rather than a more traditional academic appointment. After all, industry is where the vast majority of new, innovative products have been, and continue to be developed (though the genesis of many basic ideas behind those products very often originate in academia or the National Institutes of Health (NIH)).

The world involving new drug discovery and development has changed dramatically in the 30+ years since I became involved with translational R&D. Small molecules, and in particular oral pills, were by far the most widely used type of product. Pharmaceutical science had barely begun to employ then-novel receptor binding and kinetic approaches to help target the discovery and development of novel therapeutics or define mechanisms of action of existing medications. Biologics had not yet emerged as a therapeutic entity and neurotrophic factors as treatments for neurodegenerative diseases had not yet been considered. Gene therapy was still a decade or more away from reality, and even biotechnology had not grown to become a bona fide therapeutic approach. Even relatively simple monoclonal antibodies, which now represent an ever-expanding, multibillion dollar biopharmaceutical business, were still more than a decade away from demonstrating proof of concept for any human disease. Thus, while the initial programs I was involved with had little or nothing to do with neurotrophic factors or gene therapy, in reality, all translational R&D involves many common features and challenges. Moreover, they share a singular common goal of conducting tests to determine whether a potential product possesses sufficient safety and efficacy to ultimately be used to treat a human disease. Thus, as described below, the experiences gained during this time ultimately played an important role in the later development of AAV-NRTN for PD.

More specifically, my initial responsibility primarily involved the challenge of helping to establish and eventually lead the first-ever programs intended to treat the major cognitive deficits of AD and related age-related maladies. Additionally, I also served as a role player for many other programs intended to treat other CNS problems, providing either empirical or conceptual support (see Table 1). My early research on treatment approaches for the memory loss in AD eventually produced a body of work that helped establish the link between the loss of function of basal forebrain cholinergic neurons and the major cognitive symptoms of AD and related disorders; i.e., the so-called 'Cholinergic Hypothesis' (see reviews by Bartus et al., 1982; Bartus and Dean, 2009). Given its 'neurological' nature, it also provided me an early introduction to neurotrophic factors and some of its early pioneers. The "Cholinergic Hypothesis" had gradually gained recognition as a useful heuristic tool as well as translational concept, in that it provided the conceptual framework and empirical foundation that eventually translated to the first four drugs ever approved by the FDA for AD (see Bartus, 2000). It was because of a growing interest in the cholinergic hypothesis that I was exposed to early evidence supporting the radical idea that endogenous proteins, called neurotrophic factors, might possibly reverse and stop the degeneration of neurons in something as complicated and destructive as AD. Nerve growth factor (NGF) had been the first neurotrophic factor

Table 1
Major translational R&D programs in Big Pharma (1973–1988: Parke-Davis & Lederle Labs, both since acquired by Pfizer).

Product	Mechanism	Indication(s)	Status/stage of development
Pramiracetam	Novel nootropic	Alzheimer's disease (AD); mild cognitive impairment (MCI)	Approved and marketed in select EU countries: 1990s
AChE inhibitors	Cholinergic function enhancers	AD; MCI	Concept and independent translational research contributed to 1st four products approved for AD: mid 1990s to early 2000s
Sonata (zaleplon)	1st non-benzodiazepine sedative/hypnotic	Insomnia	Approved and marketed: 1999
CI-275838	Novel-class nootropic	AD	Phase 1 — suspended due to side effects: 2000
Bicifadine	Novel serotonin–norepinephrine reuptake inhibitor (SNRI)	Depression; chronic pain	Phase 3 — program terminated by licensee: 2009
Indiplon	Novel, non-benzodiazepine sedative/hypnotic	Insomnia	Phase 3 — "approvable letter"; development suspended by licensee: 2009
Ocinaplon	1st GABA-complex agonist anxiolytic void of sedative side effects	Anxiety	Phase 2 — liver tox; development suspended by licensee: 2009

discovered and was eventually shown to provide crucial neurotrophic support for the same basal forebrain cholinergic neurons that were degenerating in AD and that formed the focus for research related to the cholinergic hypothesis. While participating on panels and in symposia by virtue of my role in helping to formulate and empirically establish the cholinergic hypothesis (e.g., a 1982 Science paper was soon-after designated a "Citation Classic" by ISI, ranking #4 among neuroscience/neurology papers from the early 1980s to mid-1990s), I came to know Franz Hefti and Fred (Rusty) Gage. They were both independently engaged in pioneering research demonstrating that under conditions of neurodegeneration, increasing NGF levels could restore function and protect these same cholinergic neurons from further degeneration and death. It was soon clear to me and others that the intriguing and powerful characteristics of neurotrophic factors reported by Gage, Hefti and others might possibly provide the potential to literally revolutionize the treatment of neurodegenerative diseases.

While I soon found myself dabbling in the neurotrophic field via collaborations with colleagues in academia (e.g., Azmitia et al., 1988; Kordower et al., 1988), a question that I began to ask was how I might engage in a more formal translational research program intended to develop neurotrophic factors for therapeutic purposes. This hardly was the type of program to be easily embraced at Big Pharma, for in many different ways the concept was simply too far ahead of industry, which still primarily focused on small molecules, mostly orally administered. However, before that question is addressed, it might be useful to provide a more detailed overview of the potential therapeutic advantages of neurotrophic factors, as viewed 25 years ago and continues to be viewed today.

Neurotrophic factors: can the degenerating brain be induced to heal itself?

The editorial I wrote nearly 25 years ago that has the same title as this section (Box 1) reflected the enthusiasm I felt for neurotrophic factors, though it was clearly too early for anyone to know whether they might actually live up to this early promise. However, it would have been even more difficult for anyone to have known that after over two decades of intense animal research and many attempts to show efficacy in human clinical trials, their therapeutic utility would remain unproven. Perhaps even more surprising, despite that extended timeframe and continued uncertainty, enthusiasm for their therapeutic potential has nonetheless remained relatively high.

This raises the obvious question: what was it about neurotrophic factors that stimulated such interest, and why after 25 years of research and development, with no approved product to show, has the promise of this class of molecules persisted? The unique features of neurotrophic factors and the therapeutic possibilities they provoke, when combined with the wealth of mutually-corroborating scientific evidence about them, make neurotrophic factors a compelling target for translational R&D (Box 2). They offer the opportunity for paradigm-shifting, transformational new therapies for serious, unmet medical problems. Yet, they enjoy a deep empirical foundation about their biology, posing only a fraction of the risk normally associated with such new opportunities. Even more enticing, many agree that inherent in the complex but powerful biology of neurotrophic factors lies the likelihood that if one can demonstrate a significant reduction in clinical symptoms, then that neurotrophic factor should likely also provide additional benefit by helping to delay, reverse or possibly even halt disease progression. That is, the same repair genes activated by the neurotrophic factor that restores the function of degenerating neurons to provide symptomatic improvement should also produce healthier neurons that are more able to withstand the pathogenic pressures that drive disease progression. As many have noted in the past, this possibility of reversing and slowing disease progression represents the 'holy grail' for neurological diseases and neurotrophic factors currently seem to provide the best opportunity for accomplishing this.

Specialized Biopharma: the opportunity to formally attempt to translate neurotrophic factors as treatments for neurodegenerative diseases

The 15 years spent at Big Pharma working with teams of scientists that focused on the successful discovery and early development of several novel therapies (Table 1) provided invaluable, practical R&D experience that also served to highlight the significant obstacles that would be faced by any program intending to translate neurotrophic factors to products. It convinced me that if neurotrophic factors were to be successfully developed as therapeutics, it would require a longer-term commitment and higher tolerance for risk than most Big Pharmas at the time likely possessed. There simply were too many obstacles and unknown risks that needed to be confronted to compete for resources with other programs and concepts. Indeed, when one considers that no such product has yet achieved FDA approval, now 25 years later, that perspective would have proven to be correct! Cephalon would later make significant progress developing a small-molecule that mimicked the effects of a neurotrophic factor as a treatment for PD and the program advanced nicely through in vitro and nonclinical testing and into clinical trials with much promise and excitement. However, the disappointing results obtained in a controlled clinical trial in PD and the subsequent termination of the program amply underlie the

Box 2
Neurotrophic factors, their therapeutic promise and translational appeal.

Neurotrophic factors are endogenous proteins that, during ontogeny and early maturation, are responsible for neuronal differentiation, survival and establishing appropriate efferent connectivity of the nervous system (Huang and Reichardt, 2001; Snider and Johnson, 1989; Sofroniew et al., 2001). Each of the many different neurotrophic factors thus far identified serves these roles for only a relatively small number of specific neuronal types or populations. Once the nervous system has developed, the levels of these proteins decline and they assume a more maintenance-like function during adulthood and throughout the aging process (Hefti et al., 1989; Huang and Reichardt, 2001; Snider and Johnson, 1989; Sofroniew et al., 2001). However, research over the past several decades from around the globe has consistently demonstrated that under conditions of neurodegeneration, supra-physiological (i.e., biopharmaceutical) levels of a neurotrophic factor can induce neuronal repair genes in degenerating neurons. Induction of these repair genes routinely produces morphological and functional restoration of the neurons, significant slowing further neurodegeneration and even protecting against death (Hefti et al., 1989). Thus, neurotrophic factors provide the opportunity to substantially rejuvenate degenerating neurons in humans – morphometrically and functionally – potentially improving symptoms, extending the utility of current pharmacotherapies and possibly slowing, halting or even reversing disease progression.

Neurotrophic factors appear to provide functional and morphological benefits to their responsive neurons, no matter how the neurons are damaged or impaired — an extremely important point for translational purposes. They have consistently provided benefit following a wide variety of neuronal perturbations, including cutting and/or crushing axons; exposure to neurotoxins, free radical donors, inflammatory agents and other cytotoxic agents; genetic mutations; protein processing defects and the effects of age, alone and in combination with other perturbations. Neurotrophic factors therefore seem to represent a 'final common therapeutic pathway' to neuronal restoration and protection, likely providing potential benefit independent of many possible pathogenic cascade(s) that are truly responsible for the disease in humans and free from the traditional burdens of theoretical insight and assumptions surrounding those issues. In other words, the potential therapeutic benefits of neurotrophic factors seem to be 'pathogenic neutral', which offers a major translational advantage, given the complexity inherent in most chronic neurodegenerative diseases and the uncertainty and controversy regarding which pathogenic variables might be most important. If one is able to identify a neuronal population whose degeneration and/or loss of function has been linked to the symptoms or pathogenesis of a disease, then the appropriate neurotrophic factor can likely provide restorative effects without any need for a clear understanding of the pathogenic variables involved. This rather unique characteristic of neurotrophic factors provides a significant, perhaps unprecedented opportunity to reduce risks in developing a 'first-in-class' therapeutic for a serious, unmet need. Moreover, it leverages decades of cross-disciplinary research from around the globe that collectively establishes nonclinical proof of mechanism and proof of concept for the potential benefit of neurotrophic factors when degeneration of a specific neuronal population is known to represent a key feature in the manifestation of a human disease.

difficulties inherent in translating novel, paradigm-shifting concepts into clinical reality (Saporito et al., 2002; Wang and Johnson, 2008).

In the mid to late 1980s, the venture capital world and Wall Street became increasingly interested in novel, higher-risk treatments for CNS diseases leading to the formation of several new start-up companies, some of which specifically defined neurotrophic factors as a therapeutic focus area. I was recruited in mid-1988 as VP, R&D and Chief Scientific Officer at Cortex, being the first full time employee to sign-on. Cortex was founded on the basis of the scientific strengths and reputations of three University of California, Irvine professors. The underlying assumption was that they, and inventions from their laboratories, would provide the ideas to fuel the R&D of the company, leading to new, innovative products for neurodegenerative disorders, with neurotrophic factors being the main approach.

Without question, each of the UCI professors had been engaged in seminal research, including neurotrophic factors; however, with a single exception, none of their research had generated any patentable neurotrophic-related inventions — a fact that did not become clear to us until we began to develop R&D strategies for Cortex (lesson learned!). The single exception involved an intriguing observation that systemically-administered n-hexacosanol, a long-chain fatty alcohol, could produce robust neurotrophic-like activity, protecting cholinergic neurons in the medial septum from degeneration and death following transection of their projection to the hippocampus (Borg et al., 1990). The translational implications of achieving robust neurotrophic activity following parenteral administration of a potential therapeutic were obvious; better still, the phenomenon had not yet been reported (and presumably observed) by others and therefore the concept was patentable (and a license could be obtained from the University of California). Indeed, if replicated and developed into a product, the data would have circumvented the serious delivery issues that have continued to plague the translation of neurotrophic factors for over 20 years. The major problem with the idea was that we at Cortex were not able to replicate the intriguing results. Recognizing the importance of gaining as much certainty as possible, I arranged a collaboration between a co-author from the original study who was still working at UCI and an independent investigator (Jeffrey Kordower, University of Illinois Medical Center). Unfortunately, even with the participation of one of the original investigators, we still could not replicate the original hexacosanol observations and apparently no one has done so since. The good news is that we saved significant time and otherwise-wasted resources by first conducting the appropriate tests to assure that the initial results were as robust and promising as they seemed — a somewhat obvious exercise that is too often skipped when new companies find themselves under intense pressure to develop products quickly and thus too often allow themselves to compromise rigorous scientific practice and judgment.

Though we attempted to quickly establish a discovery program to search for and identify more novel neurotrophic factors, de novo at Cortex, that effort was not successful and was eventually abandoned in favor of other (non-neurotrophic factor) programs we had been able to develop that showed promise, including adenosine agonists (Jacobson et al., 1992; Maillard et al., 1994) and calpain inhibitors (Li et al., 1993) (Table 2). Another noteworthy effort initiated just prior to my leaving Cortex was their current AMPAkines program which remains their major focus today. All three of these programs were the brain-child of Gary Lynch (one of the 3 UCI professors associated with Cortex).

The calpain inhibitor program became the most advanced program while I was at Cortex (see, Bartus, 1997, for review) and ironically soon indirectly contributed to a new opportunity to return to efforts to develop neurotrophic factors as useful therapeutics. During

Table 2
Major translational R&D programs in Specialized Neuropharma (1988–1992: Cortex).

Product	Mechanism	Indication(s)	Status/stage of development
Multiple neurotrophic factors	Varied	Neurodegeneration	Program terminated 1990; poor IP and/or biological activity
Hexacosanol	Purported neurotrophic mimick	Neurodegeration	Program terminated 1989, lack of efficacy
Adenosine agonists	Neuronal modulator	Stroke; closed head injury	Program terminated 1991, poor therapeutic index; significant sedation at all dose levels
Calpain inhibitors	Relatively potent and selective inhibitors of the neutral, calcium-dependent protease, calpain	Acute and chronic neurodegenerative diseases	Novel inhibitors developed with preclinical evidence of greater selectivity over existing molecules; licensed to Alkermes, 1992

my third year at Cortex, we formed a joint-development program for our calpain inhibitor program with Alkermes, a more-mature biopharmaceutical company with better financial resources. Within a year after the agreement was initiated, Alkermes elected to take full control of the calpain program and offered me a position to join the company as Sr. VP of preclinical R&D. My responsibilities were to continue leading the effort to develop the calpain inhibitors, while also directing a planned expansion of Alkermes' neuroscience effort, which included a program specifically focused on translating neurotrophic factors to treat neurodegenerative diseases. The opportunities and challenges involved in leading a translational program that focused on neurotrophic factors were a key factor in my decision to join Alkermes.

Transporting NGF across the BBB for treating Alzheimer's disease

At the time, Alkermes was focused on developing novel methods of surpassing the BBB (blood–brain barrier) to enable potentially valuable therapeutics into the brain. The technology underlying their neurotrophic program was based, in large part, on the seminal work and patents of William Pardridge (UCLA), who pioneered the concept of using endogenous BBB transport systems, such as transferrin receptors that normally transport iron from the blood into the brain, as a means to carry therapeutic agents across the BBB (Pardridge et al., 1991). This approach offered a possible solution to the realization that if NGF or other neurotrophic factors were ever to be developed as therapeutics, effective delivery methods would have to be developed. Alkermes' program involved the use of a proprietary chemical construct, consisting of an antibody fragment to the transferrin receptor, chemically conjugated to NGF. The well-known, biologically characterized neurotrophic properties of NGF, coupled to a means of transporting it across the BBB using an endogenous transport system, seemed to represent an interesting, practical and potentially important translational opportunity.

The program had even greater personal appeal to me, in that the intended indication for NGF was Alzheimer's disease, with the therapeutic target being the same degenerating cholinergic neurons that served as the focus for the 'cholinergic hypothesis' I had helped conceive and establish a decade earlier. I was well-aware that significant dose-limiting toxicity (primarily GI-related) blunted the efficacy that might otherwise be achieved with the cholinesterase inhibitors in clinical development at the time (and since approved). The problem was, at least in part, related to the lack of specificity of those drugs for the degenerating cholinergic neurons in the targeted basal forebrain, coupled with the ubiquitous distribution of healthy cholinergic neurons in the peripheral, parasympathetic nervous system (and also other regions of the brain). Thus, the use of parenteral cholinergic pharmaceuticals to correct the central cholinergic deficit linked to the early cognitive deficits of AD was proving to pose serious obstacles that limited the clinical benefit, with no clear solution at hand. This problem has not been solved more than 30 years after the conceptualization of the cholinergic hypothesis, emphasizing how difficult and challenging many translational issues can be.

The idea of enhancing the function of the degenerating cholinergic neurons with a neurotrophic factor like NGF that would selectively target the degenerating neurons responsible for the major early-stage symptoms of AD had clear appeal. When I joined Alkermes, the data that supported the concept involved the demonstration that the NGF/transferrin-receptor-antibody conjugate (named, AK-26), enhanced the survival of neonatal cholinergic neurons transplanted into the ocular chamber of an adult rat's eye (Friden et al., 1993). Since the transplanted tissue developed a surrogate BBB in the vitreal chamber, it served as a model for testing whether agents normally excluded could now cross the BBB due to their conjugation to the carrier. While the results published in that study with NGF supported the potential promise of the concept, it left many large, lingering questions that remained to be addressed before the approach might advance to a genuine 'development' program, let alone translated to clinical testing. Foremost of these was the nagging concern that Alkermes and its collaborators had been unable to demonstrate consistent or robust efficacy of the systemically administered NGF conjugate in the 'gold standard' in vivo model for NGF, the 'fimbria–fornix transection' model (or FFX model) of cholinergic degeneration. Hefti and others had shown that when the axons of septo-hippocampal cholinergic axons are severed, the neurons quickly degenerate and lose their ability to express phenotypic markers, but with elevated concentrations of NGF, the degenerating neurons are restored, regaining their morphology and expression of functional proteins in these neurons. The failure to achieve more reliable and robust results with AK-26 in the FFX model raised serious concerns that defined one of several key issues that required additional focused research to try to resolve before this technological approach might advance to a genuine development program.

Over a period of several years, working with several different collaborators, nonclinical proof-of-concept was gradually established for this approach. It was established that systemically (IV) administered AK-26 (i.e., NGF conjugated to a transferrin receptor antibody) could be transported across the BBB to provide neurotrophic support for degenerating cholinergic neurons in a variety of animal models (Backman et al., 1996, 1997; Bartus, 1999; Bartus et al., 1996a; Charles et al., 1996; Kordower et al., 1994). However, the magnitude of the trophic response was often not as great as that achieved with NGF infusions directly into the brain and we were never able to find clear evidence for neuroprotection by AK-26 in the FFX model. In the end, the relatively modest benefits, as well as the lack of an explanation for the failure to achieve reliable and significant effects in the FFX model gave many of us pause for concern. Eventually, those concerns, coupled with the identification of significant side effects (e.g., inflammation and pain) induced by exposing peripheral tissue to high levels of AK-26 made continued development of this program impractical and it was unceremoniously terminated.

As the focus on neurodegenerative diseases increased during the so-called 'decade of the brain', which coincided with the gradual emergence of biopharmaceuticals (including neurotrophic factors) as potentially important new treatments, appreciation also increased for the obstacles posed by the BBB. Three other innovative technologies at Alkermes offered additional possibilities to address the delivery problems posed by proteins, generally, and neurotrophic factors, specifically.

Table 3
Major translational R&D programs in Specialized Biopharma (1992–2002: Alkermes).

Product	Mechanism	Indication(s)	Stage of development/status
Calpain inhibitors	Potent selective inhibitors of neutral, calcium-dependent protease	Acute and chronic neurodegenerative diseases	Preclinical proof of concept; program discontinued due to lack of sufficient bioavailability into brain; 1995
Nerve growth factor (NGF)	Transferrin receptor antibody conjugated to NGF enabling passage across the BBB	Alzheimer's disease	Non-clinical proof of concept, program terminated; 1995
Sustained hGH	Injectable formulation of essential growth factor; provides sustained blood levels for 1 week following single injection	hGH deficiency; small stature. Technology also tested, but not practical for delivering neurotrophic factors to brain.	Approved by FDA; 1999
Cereport (RMP-7) BBB permeabilizer	B2 bradykinin agonist that modulates opening of tight junctions comprising the BBB	Initially all BBB possibilities; later glioma focus. Technology found not suitable for neurotrophic factors to brain	Phase 2b — discontinued, 1999, due to change in regulatory climate and corporate priorities
Pulmonary triptans	Formulation/route provides far faster and more reliable blood levels, also avoids problems related to gastric slowing during migraine attacks. All features provide potentially greater efficacy	Migraine	Concept conceived and pilot data established feasibility, 2000. Program moth-balled, but nearly identical pulmonary product concept now developed by Vectura Group. Also, MAP Pharmaceuticals, Inc. successfully completed Phase 3 trials in 2011 with similar pulmonary DHE product.
Pulmonary flurbiprophen	Formulation/route provides far faster and more reliable blood levels, potentially providing greater efficacy	Break-through pain	Nonclinical proof of concept established, 2000. Program moth-balled 2002.
Pulmonary epinephrine	Bronchial modulator provides far faster blood levels and is less invasive (compared to Epi injectable pen)	Anaphylaxis	Phase 1 — 'mothballed', 2002; later licensed by Civitas Therapeutics
Pulmonary L-dopa	Formulation of dopamine precursor provides more rapid and reliable blood levels	Rescue therapy for PD	Non-clinical POC established, 2001; program initially mothballed but licensed by Civitas Therapeutics and now their lead program
Pulmonary diazepam	Formulation/route provides far faster and reliable blood levels, potentially providing greater efficacy	Epilepsy	Concept conceived and pilot data established feasibility, 2001. Program moth-balled in 2002 but similar product concept (using nasal route) now being developed by Acorda Therapeutics
Vivitrol	Once/month injectable formulation of naltrexone providing more consistent blood levels, while avoiding daily patient adherence issues	Alcohol and opiate abuse	Approved by FDA; 2006
Pulmonary zolpidem	Formulation/route provides far faster and reliable blood levels, potentially providing greater efficacy	Sleep maintenance insomnia (or 'middle of the night waking')	Concept conceived and pilot data established feasibility, 2001. Program moth-balled 2002. Transcept, Inc. independently received FDA approval for similar product concept (using sublingual route) in 2011
Risperdal Consta	Injectable formulation of atypical antipsychotic, provides sustained release of therapeutic for 2 weeks/injection	Schizophrenia and bipolar disorder	Approved in US and EU; 2003
Bydureon	Injectable formulation of insulin-use modulator, Byetta, provides sustained release of therapeutic for 2 weeks/injection	Diabetes	Approved in EU and US; 2011 and 2012, respectively

While none of these programs ultimately solved the formidable delivery problems posed by neurotrophic factors, as discussed later, they and other programs at Alkermes (see Table 3) nonetheless provided invaluable experience with a range of translational R&D issues, including direct involvement with, and input in, regulatory and clinical activities (an opportunity that more often exists in smaller, development-stage companies versus larger Big Pharma, because team members are often required to wear many 'different hats', work in very intimate multidisciplinary teams and extend their efforts well outside their areas of formal training and past experience).

Opening the BBB (blood brain barrier) by relaxing the tight junctions

One program Alkermes had developed to overcome delivery obstacles posed by the BBB involved the use of a chemically-synthesized, novel bradykinin B2 receptor agonist to transiently relax the tight junctions comprising the BBB, thus temporarily opening it to permit high concentrations of a systemically circulating therapeutic agent to cross into the brain (Bartus, 1999; Bartus et al., 1996b, 1996c). In principle, this technology might provide another means to circumvent the BBB to allow neurotrophic factors to reach their CNS targets. However, as

more was learned about the phenomenon of relaxing the tight junctions by activating the B2 receptors located on the luminal surface of brain capillaries, it became increasingly clear that neurotrophic factor proteins would not benefit; the increased permeability achieved was limited to smaller molecules (i.e., less than 10 kDa) and lasted only 5 to 10 min before the BBB spontaneously reformed via tachyphylaxis (Bartus et al., 1999; 2000; Elliott et al., 1996; Inamura et al., 1994). Thus, while we advanced the RMP-7 program into several clinical studies in glioma patients, including controlled trials in both Europe and the United States (Bartus, 1999; Emerich et al., 2001), it did not provide a solution for the problems of delivering neurotrophic factors to the brain.

Sustained release of protein as alternative delivery method

Another major technological innovation at Alkermes involved the ability to formulate therapeutically beneficial molecules, including proteins (and therefore neurotrophic factors), into injectable, biodegradable polymeric microspheres. Once so-formulated and injected into the body (including the brain), the polymers comprising the microspheres gradually dissolve into innocuous monomers, slowly releasing the therapeutic agent in a reasonably sustained fashion. The ability to encapsulate proteins for sustained release following injection provided yet another possible technological opportunity to overcome some of the delivery issues of neurotrophic factors by providing long-lasting benefit following an injection of the neurotrophic factor (Bartus et al., 1998). We launched a small pilot project intended to explore whether NGF-laden microspheres might be stereotactically injected into the brain to achieve sustained levels of NGF and provide sustained neuroprotection. It soon became clear, however, that even though one could produce sustained release of NGF in the brain, it was not possible to achieve a sufficiently large amount of neurotrophic factor, into a sufficiently small microsphere mass to provide a sufficiently sustained neurotrophic benefit. In short, the duration of effect achieved with a microsphere mass that could be injected into the brain without causing physical stress was too short to achieve a duration of neurotrophic activity sufficiently long to justify the neurosurgical interventions required to maintain neurotrophic activity for chronic neurodegenerative diseases. While this represented another disappointing outcome, in terms of translating neurotrophic factors, this 'sustained release' technology nonetheless, again provided the opportunity to continue to gain valuable insight and translational experience with several other products, including proteins and biopharmaceuticals (see Table 3) that eventually proved helpful when later leading the development of CERE-120.

Pilot program involving AAV2-GDNF

A final program at Alkermes that was much more directly related to CERE-120 (AAV-NRTN) was initiated as a small pilot project, investigating whether AAV might be used to deliver GDNF to the degenerating nigrostriatal neurons in a 6-OHDA rat model of PD. This project was launched by others (Scott Putney, then at Alkermes and Barry Hoffer, a long-time consultant and collaborator of Alkermes and a pioneer in neurotrophic factor research), primarily to help determine whether Alkermes might eventually pursue gene transfer as an alternative means of delivering proteins for therapeutic purposes. An SBIR (Small Business Innovation Research) grant was awarded to help support the work and I was later given responsibility to manage the grant and personnel performing the research for its final 1.5 years. Ironically, my confidence and interest in gene therapy as a viable translational approach were admittedly not great at the time. Not only did the approach seem to me to be relatively complicated, uncontrolled and variable (compared to more traditional biopharmaceutical technologies), but safety issues generated by others in the field (Kohn et al., 2003; Raper et al., 2003), along with relatively poor outcomes in the initial studies at Alkermes, left me unimpressed and generally skeptical. However, the experience did serve to introduce me to the concept of gene transfer, and eventually the project was able to generate results worthy of publication (McGrath et al., 2002).

At this point in time, I had spent nearly 30 years engaged in translational research and development in Big Pharma and specialized biopharmaceutical companies and even though I was privileged to have participated on numerous teams that had successfully discovered and developed many different novel products, none of the neurotrophic factor programs had proven to be sufficiently strong to merit advancing into clinical development (Tables 2 and 3). And while the 15 years in Biotech, especially the 10 years at Alkermes, provided widespread translational experience with nonclinical, clinical and regulatory issues by virtue of the number of concepts we were attempting to develop into programs to advance into human clinical testing (Table 3), this experience made it clear that the delivery issues first described nearly 25 years earlier for biopharmaceuticals (including neurotrophic factors) were presenting even greater and more difficult challenges than I or likely anyone else had imagined (Box 1).

I was now convinced that if neurotrophic factors were ever going to be successfully translated to the clinic, the delivery obstacles needed to be addressed head-on, and this would likely require a truly innovative, possibly revolutionary approach. My experience at Alkermes had convinced me that even the most sophisticated methods currently available at the time for delivering purified proteins into the brain were not likely to solve the multitude of overlapping delivery problems and obstacles that existed for neurotrophic factor proteins. Even though many of us interested in neurotrophic factors had gradually become comfortable with the likely need for stereotactic neurosurgical methods to deliver neurotrophic factors, I was not yet entirely convinced that gene transfer provided the critical 'enabling' technological innovation, especially given its level of complexity (relative to more traditional pharmaceutical approaches) as well as its past safety record. Moreover, I was still in the midst of developing additional novel products and product concepts that I felt were interesting and represented important potential advances, including Vivitrol for alcoholism and opiate abuse (Bartus et al., 2003; Overstreet et al., 1999), pulmonary L-dopa for rescue therapy for PD (Bartus et al., 2004), pulmonary triptans and NSIADs for migraine and breakthrough pain (Salzberg-Brenhouse et al., 2003), pulmonary diazepam for acute epilepsy attacks, pulmonary zaleplon for sleep maintenance insomnia, and several others (see Table 3) and thus initially resisted considering the idea very seriously. However, over time the opportunity to focus exclusively on translating neurotrophic factors as viable clinical products became too compelling and thus looking back, now a decade later, the possibility that gene transfer could make all the difference in the world is exactly the bet that I eventually would make.

'Delivery to the brain: the problem lurking behind the problem'

If neurotrophic factors indeed have such therapeutic potential, why have so many attempts been unsuccessful, to date? Or, more to the point of this section, what is it about neurotrophic factors and their potential use as therapeutics for chronic neurodegenerative diseases that make their delivery to targeted neuronal populations so difficult?

Over the past two decades, numerous clinical trials involving several different neurotrophic factors and neurodegenerative diseases have been conducted (Apfel, 2002; Apfel et al., 1998, 2000; Gill et al., 2003; Jonhagen et al., 1998; Lang et al., 2006; Marks et al., 2008; Miller et al., 1996; Nutt et al., 2003; Penn et al., 1997; Slevin et al., 2005; Tuszynski et al., 2005; Wellmer et al., 2001), and yet the quality and magnitude of any claimed successes do not yet allow one to declare victory from a translational point of view. More specifically, despite multiple clinical tests involving several different neurotrophic factors and neurodegenerative diseases, until gene transfer, no clear evidence of sufficient, robust clinical efficacy has yet been achieved

Box 3
Delivery obstacles posed by neurotrophic factors and deficiencies with traditional pharmaceutical delivery approaches.

Neurotrophic factors pose a number of unique and difficult delivery issues. First, they must target the brain, which is somewhat sequestered from the main circulatory system by the tight junctions between the endothelial cells comprising the cerebral vessels, known as the blood–brain barrier (BBB). A further complication for treating chronic neurodegenerative diseases is that adequate levels of neurotrophic factors must be maintained for very long periods of time (i.e., for years), for once the proteins return to basal levels, their benefit typically reverses and is lost (Fischer et al., 1987; Hefti et al., 1989; Snider and Johnson, 1989; Sofroniew et al., 2001). Similarly, it is important that a sufficient proportion of the degenerating cell population be exposed to the neurotrophic factor in order to produce effective restoration of neuronal function and thus achieve measureable clinical improvement (though the exact proportion required for therapeutic benefit is currently not clear and likely varies between situations). Because serious side effects have been observed when delivery of neurotrophic factors have inadvertently exposed non-targeted brain sites (e.g., periventricular tissue) it is important to accurately predict, control and restrict protein delivery specifically to the intended target (Day-Lollini et al., 1997; Eriksdotter Jonhagen et al., 1998; Kordower et al., 1999; Nauta et al., 1999; Nutt et al., 2003; Penn et al., 1997). Further complicating the translation of neurotrophic factors, they are proteins and chronic protein delivery can be notoriously difficult because of aggregation, misfolding and development of neutralizing antibodies. Moreover, as proteins, they cannot be taken orally, do not cross blood–brain barrier (BBB) naturally and cannot typically be administered systemically, even if linked to a BBB carrier, due to side effects often associated with exposing systemic organs and tissue (McMahon, 1996; Pezet and McMahon, 2006). These issues, individually and collectively, render safe and effective delivery of neurotrophic factors extremely challenging. For this reason, a consensus opinion has emerged that the successful translation of neurotrophic factors to the human clinic will first require that these crucial delivery issues be solved (Bartus et al., 2007a; Kordower et al., 1999; Lang et al., 2006; Nutt et al., 2003; Salvatore et al., 2006; Sherer et al., 2006).

Unfortunately, traditional pharmaceutical formulations and delivery approaches are unable to solve these multiple problems. For this reason, a number of more innovative methods have been attempted to deliver neurotrophic factor proteins to the CNS, but none has proven successful. For example, various means of transporting neurotrophic factors across the BBB following systemic administration have been attempted. One method that showed early promise exploited endogenous transport receptor-mediated systems located on the abluminal surface of cerebral capillaries (e.g., transferrin transport receptors), described earlier in the Specialized Biopharma section, but thus ultimately proved impractical due to serious side effects induced by exposing non-targeted tissue outside the brain to the neurotrophic factor following intravenous injections (e.g., see McMahon, 1996; Pezet and McMahon, 2006), coupled with only-modest benefit. Infusions of neurotrophic factor proteins directly into the ventricles of the brain showed promise in animal studies of neurodegeneration but significant side effects in humans occurred when periventricular tissue was exposed to high concentrations of the neurotrophic factor (Eriksdotter Jonhagen et al., 1998; Kordower et al., 1999; Nauta et al., 1999, 2003; Penn et al., 1997). Subsequent infusions of the proteins directly into the degenerating parenchyma using chronically indwelling pumps and cannula reduced some, but not all safety issues (Hovland et al., 2007; Lang et al., 2006). However, results from these efforts have been mixed, at best (Gill et al., 2003; Lang et al., 2006; Slevin et al., 2005), with the single, controlled study of GDNF in PD showing no evidence of any real benefit (Lang et al., 2006). Many have concluded that the point source used to deliver the protein in those studies produced poor distribution throughout the putamen, at least partly contributing to the negative results (Lang et al., 2006; Morrison et al., 2007; Salvatore et al., 2006; Sherer et al., 2006). Supporting this concern is the argument made in the seminal paper reporting positive, preliminary results with GDNF (Gill et al., 2003), wherein even the authors of that study concluded that a better means of delivering the protein into the brain was required than that provided by the chronically indwelling hardware and cannula that they used (Gill et al., 2003). Moreover, even if that technology could be improved to provide more wide-spread distribution of the protein, documented complications associated with the use of indwelling hardware warn of continuing, serious safety risks. These include the formation of neutralizing antibodies to GDNF (Hovland et al., 2007; Lang et al., 2006) (most likely due to the protein leaking during routine filling of the subdermally implanted infusion pump) and apparent degeneration of distant cerebellar neurons (most likely due to protein leaking along paths of lease resistance, formed by the implanted cannula, from the targeted putamen to the surface of the brain and eventually to the distant cerebellum) (Hovland et al., 2007). In other words, while the scientific foundation for neurotrophic factors extends several decades and is considered well-established, attempts to translate the therapeutic potential to the clinical arena has been largely disappointing because the technology required to deliver these complex proteins in a safe, controlled and sustained fashion to specific, targeted areas of the brain has been grossly inadequate.

in a controlled clinical trial to convincingly establish that neurotrophic factors can clearly improve the clinical status in any neurodegenerative disease, let alone delay its progression.

Fortunately, during the two decades of clinical trials attempting to translate the therapeutic potential of neurotrophic factors, much has been learned about their biology and translational requirements. This cumulative insight provides important details about the obstacles that must be confronted and solved before neurotrophic factors might be developed as successful human therapeutics. Many of these issues and the continuing difficulties posed in delivering neurotrophic factors to the human brain are summarized in Box 3.

Thus, while the editorial cited in Box 1 ("Delivery to the brain: the problem lurking behind the problem") accurately forewarned that a major translational stumbling block for neurotrophic factors might involve the successful delivery to the brain, that concern has proven far more prescient than I or anyone would have had reason to believe at the time. Similarly, even today, while no one can be certain that solving these long-standing delivery issues will necessarily produce clear clinical benefit to patients receiving neurotrophic factors, it had became increasingly clear to me by the time I was leaving Alkermes that unless the delivery problems were solved, reliable and meaningful clinical benefits of neurotrophic factors would likely never be achieved.

The new millennium in Biotech: personally going "all in" to translate neurotrophic factors, using gene transfer as the means to overcome the long-standing delivery issues

Background

Ceregene was founded in 2001, specifically to develop neurotrophic factors for neurodegenerative diseases, using gene transfer as the delivery approach. Shortly after Jeff Ostrove (Ceregene's President and first

employee) joined the company, he and several SAB members contacted me about leading the R&D effort. The modest exposure I gained to gene therapy via the pilot AAV-GDNF project at Alkermes gave me some personal appreciation that the technology might have the potential to solve some of the more difficult delivery challenges that plagued the neurotrophic field. While several published accounts had, importantly, demonstrated that gene therapy could deliver neurotrophic factors to produce efficacy in animal models of neurodegeneration (e.g., Aebischer et al., 1996; Bensadoun et al., 2000; Bjorklund et al., 2000; Bohn, 1999; Choi-Lundberg et al., 1998; Kirik et al., 2000b; Kordower et al., 2000; McGrath et al., 2002; Tuszynski et al., 1996)), no paper yet adequately addressed the many formidable translational issues that persisted. These issues included: (1) whether gene therapy could prove to be sufficiently safe (a concern we at Ceregene learned was shared by many in the venture capital community as we attempted to raise necessary funding for the company a year later), (2) whether gene therapy could accurately express proteins in targeted sites in the CNS in a sufficiently predictable, controlled and effective fashion, and (3) whether protein expression via gene therapy would necessarily eliminate many of the problems that historically plagued efforts to chronically deliver proteins, including protein aggregation, formation of neutralizing antibodies and the need for sustained bioactivity over long periods of time. Thus, while many investigators had already established that gene transfer could provide the means to generate positive neurotrophic responses in animal models of neurodegeneration (e.g., Aebischer et al., 1996; Bensadoun et al., 2000; Bjorklund et al., 2000; Bohn, 1999; Kordower et al., 2000; McGrath et al., 2002; Tuszynski et al., 1996), it seemed apparent that if this approach were ever to advance to human clinical testing it still remained to be determined whether gene transfer was also able to satisfy many fundamentally important translational hurdles that had mostly remained untested by the community. In essence, the litany of concerns I had when considering the opportunity to join Ceregene soon formed the basis of a translational R&D strategy designed to carefully test whether the approach could be responsibly used to deliver neurotrophic factors to the brains of human volunteer patients and beyond.

Though still not entirely convinced that gene transfer could safely and effectively deliver neurotrophic factors to the CNS at the time I joined Ceregene in late 2002, I was now willing to take on the challenge to help design and execute an R&D strategy intended to define the therapeutic opportunities and limitations of the technology. Relatively soon after joining the company, my responsibilities were formally expanded to include all nonclinical, clinical, regulatory and quality control activities. The company was already actively developing AAV2-NGF (CERE-110) for Alzheimer's disease when I joined, and while it had decided to add a Parkinson's disease program involving AAV2-NRTN (CERE-120), the viral construct had not yet been made, nor had any animal models or a product development strategy been successfully established. Thus, I inherited the opportunity to be involved on the ground level in the conceptualization and development of CERE-120 for PD. Within a few years, we had added several more programs to Ceregene's portfolio, all of which used AAV to deliver a neurotrophic factor to a specific neuronal population responsible for the major symptoms involved with a serious medical disease (Table 4). The simplicity of this concept and the economies of scale it provided enabled a small, development-stage company like Ceregene, to construct a true portfolio of several different product candidates.

The focus on Parkinson's disease and potential value of neurturin (NRTN)

Parkinson's disease (PD) is a chronic, debilitating disease whose major symptoms involve loss of motor ability, including bradykinesia, tremors and problems with gait and balance. It is widely recognized that these major symptoms result from the progressive loss of function and eventual death of the nigrostriatal dopamine neurons. While available pharmaceutical agents for Parkinson's disease are generally effective during the earlier stages of the disease, as the disease progresses, the benefit of existing treatments eventually wanes. In time, this leaves patients unable to initiate or control movement during major portions of each day (i.e., 'wearing off' phenomenon), while simultaneously causing disabling, drug-related dyskinesias due to the development of peak-dose sensitivity. While deep brain stimulation (DBS), which involves a neurosurgical procedure and implantation of chronically indwelling hardware, offers temporary relief of certain motor symptoms, it often occurs with significant complications (Deuschl et al., 2006) and does not provide satisfactory benefit to many PD patients. No current treatment, including DBS, is able to slow disease progression. In contrast, the therapeutic potential of neurotrophic factors to improve the symptoms and delay disease progression of neurodegenerative diseases, including Parkinson's, has been appreciated for decades (see earlier sections) (Apfel et al., 2000; Eriksdotter Jonhagen et al., 1998; Mufson et al., 1999; Seiger et al., 1993). Neurturin is a naturally-occurring protein that has potent neurotrophic effects on midbrain dopamine neurons, including those originating in the nigra that degenerate in PD (Kotzbauer et al., 1996). It is a close structural and functional analog of glia cell line derived neurotrophic factor (GDNF), which prior to Ceregene, had been the focus of the vast majority of basic and clinical research related to neurotrophic factor treatment for PD, suggesting potential therapeutic value for treating the degenerating nigrostriatal dopamine neurons (e.g., Gash, et al., 1995; 1996; Hoffer, et al., 1994).

Gene transfer as an 'enabling technology'

Gene transfer (sometimes also called 'gene therapy') involves the use of a harmless viral vector to deliver the gene for the therapeutic protein directly to a targeted site so that the cells in close proximity

Table 4
Major translational R&D programs in biotech (2002–present: Ceregene).

Product	Mechanism	Indication	Stage of development/status
CERE-110 (AAV2-NGF)	Trophic factor for basal forebrain cholinergic neurons	Alzheimer's disease	Controlled multicenter Phase 2 ongoing
CERE-120 (AAV2-NRTN)	Trophic factor for midbrain dopamine neurons	Parkinson's disease	Clinical proof of concept established in Phase 2a; dosing completed in revised multicenter controlled Phase 2b trial — assessments ongoing
CERE-120 (AAV2-NRTN)	Trophic factor for striatal neurons	Huntington disease	Non-clinical proof of concept established
CERE-130/135 (AAV2-IGF1)	Trophic factor for spinal cord motor neurons innervating musculature	ALS	Non-clinical proof of concept established
CERE-140 (AAV2-NT4)	Tropic factor for retinal cells (i.e., rods, cones and ganglion cells)	Multiple serious ocular diseases causing blindness	Non-clinical proof of concept established

to that site will be transduced and programmed to express and secrete the protein in a continuous, long-term fashion. Thus, rather than attempting to exogenously deliver the large, 3-dimensionally-complex protein directly to the targeted site, the *gene* for the protein is delivered to the targeted site, thereby inducing local cells to manufacture and secrete the protein through their endogenous systems.

Gene transfer has emerged as a practical means of potentially overcoming all the obstacles associated with delivering neurotrophic factors to the brain, thus possibly providing the 'enabling' technology required for translating the use of these proteins into viable biotherapeutics for human neurodegenerative diseases. While the need for standard stereotactic surgical techniques does require a 'paradigm shift' from the way human diseases have traditionally been treated, the magnitude of the problems posed by neurodegenerative diseases demands that truly innovative approaches be given serious consideration. The progress made by DBS, both in terms of improved patient status as well as patient and care-giver acceptance, offers compelling evidence that neurosurgical approaches can be both effective and practical. The indwelling hardware and other esoteric aspects of DBS arguably makes it a more complicated and invasive approach than gene transfer, which based on current data will likely involve a single, once-per-lifetime surgical, dosing procedure. Despite the clinical benefits of DBS and its deserved, growing acceptance, it has no impact on the underlying pathogenesis and thus does nothing to slow disease progression, falling well short of the true promise embodied in a neurotrophic factor/gene transfer approach. Thus, the collective characteristics of safe, very long-term, controlled protein expression that can be targeted to specific sites or systems seems to support that idea that gene transfer may have finally solved the delivery problems for neurotrophic factors.

Adeno-associated virus (AAV) has emerged as a favorite vector for translational purposes for several important reasons. One of the most important of these is that AAV2 is naturally replication-deficient and does not easily or normally integrate into the host chromosome, thus greatly reducing the possibility for insertional mutagenesis. Most authorities agree it generally provides potentially life-long expression of the protein in the targeted area of the body following a single administration of the viral vector (Hadaczek et al., 2010; McCown, 2011). Finally, AAV is only weakly immunogenic and has not been associated with any human disease or symptoms (despite the majority of humans having been exposed to it naturally). In sum, with over 80 clinical protocols initiated and more than 1000 patients administered a variety of AAV gene transfer constructs, AAV has gradually gained acceptance as a generally safe and effective means of delivering therapeutic proteins in a sustained, long-lasting fashion (Journal of Gene Therapy: http://www.wiley.com//legacy/wileychi/genmed/clinical).

The design and construction of CERE-120 (AAV-NRTN) as a novel gene therapy product for PD

CERE-120 is a gene delivery construct comprising a genetically-engineered, adeno-associated virus serotype-2 (AAV2) vector that lacks all of the viral protein-coding sequences and thus encodes only the human neurturin (NRTN) cDNA (Gasmi et al., 2007b). From the outset, the design, methods, materials and controls used to create and manufacture CERE-120 were focused on providing a safe and effective product for human use. Similarly, as reviewed in more detail in later sections, an extensive nonclinical program was launched to thoroughly test CERE-120 for expression patterns and kinetics, safety and bioactivity/efficacy and when unusual or unexpected findings were uncovered, every effort was made to understand them so that nothing would be left to chance or bias that might impact human safety or the likely viability of the product. This is the main reason why over two dozen nonclinical studies were conducted with CERE-120, the majority of which were launched well before the first human subject was ever dosed.

The genome encompassed in CERE-120 consists of AAV2 inverted terminal repeats (ITRs) flanking a NRTN expression cassette containing a CAG promoter (consisting of a human cytomegalovirus (CMV) enhancer, chicken B-actin gene and rabbit B-globin gene slice acceptor), the human mature NRTN cDNA fused to the human β-nerve growth factor (βNGF) pre/pro sequence, and a human growth hormone gene (hGH) polyadenylation signal. The substitution of the natural pre–pro sequence with the efficient human NGF pre–pro signal sequence was done to assure efficient secretion of NRTN from the transduced cells, which the natural NRTN pre–pro sequence was not able to provide (Gasmi et al., 2007b; see later section titled 'Key translational issues confronted').

As has been well-established for all AAV2 vectors, the genome does not integrate into the host chromosome, but rather forms a stable episome within the nucleus, enabling the single-stranded NRTN DNA comprised in CERE-120 to pair with its complimentary sequence, producing continuous NRTN protein expression. Studies in cell culture and brain established that CERE-120-mediated NRTN is selectively expressed in neurons, with immunohistochemical staining demonstrating that over 99% of all NRTN-positive cells were also positive for the neuron-specific antibody, Neu-N and negative for all other cell types, including numerous glia markers. However, in clear contrast to the published dogma in the field, our studies demonstrate that *AAV does not selectively transduce neurons*, for when we substituted the typical CAG promoter with the glia-specific GFAP promoter, glia cells now expressed the transgene protein, but not neurons (Ceregene, unpublished results). In other words, by merely changing the promoter (and leaving all elements of the AAV2 vector construct identical), one can completely alter the cell type in which protein expression occurs. Thus, these data indicate that both neurons and glia must be transduced by AAV2 but that the promoter determines whether or not the transgene will be expressed in a specific cell type.

AAV2-NRTN (CERE-120) was intended to overcome all the delivery issues discussed earlier by circumventing the major obstacles associated with delivering sustained quantities of biologically active proteins into selectively-targeted brain tissue. It is designed to deliver the gene for NRTN to targeted neurons, programming these neurons to provide continuous, long-term, controlled protein expression in selective, stereotactically-targeted regions of the brain. An important element that predicted the safety of CERE-120 is that, in contrast to many prior instances where serious side effects have been observed with gene therapy, only relatively small quantities of vector are required to provide relatively widespread coverage of the targeted putamen with NRTN protein. Additionally, significant systemic exposure is avoided, as is non-targeted exposure of other neuronal sites. Finally, because the CNS is relatively immunologically privileged and AAV2 is relatively non-inflammatory, the probability of eliciting an immune or inflammatory reaction is further reduced.

At the same time, the opportunity for robust, long-lasting therapeutic effects to treat chronic neurodegenerative diseases following a single CERE-120 administration seemed plausible with AAV2 in combination with the transcriptionally active CAG promoter, for research from other laboratories had shown this combination provided continuous, persistent expression of the transgene protein over several years (Bankiewicz et al., 2006) (an observation since replicated with CERE-120). Thus, CERE-120 was intended to provide a life-time of NRTN-mediated neurotrophic factor support following a single administration, offering additional safety advantages as well as eliminating numerous inconveniences associated with repeat surgical procedures.

The initial CERE-120 non-clinical program

Introduction

The targeting approach for delivering CERE-120 (and thus NRTN) to the degenerating nigrostriatal neurons adopted the prevailing viewpoint that it was not necessary (and possibly counter-productive) to

deliver neurotrophic factors directly to the degenerating nigral cell bodies located deep in the substantia nigra, for many studies had shown that administering the factor to the terminal fields in the striatum was both necessary and sufficient (e.g., Kirik, et al., 2000a; for a detailed discussion of this issue, see Bartus et al., 2011a, 2011b). Thus, the initial nonclinical program adopted that approach, administering CERE-120 to the striatum to evaluate several translational characteristics essential to justify, support and guide eventual clinical testing in Parkinson's patients using the same striatal-dosing approach.

The first and most obvious characteristic evaluated was whether the NRTN expressed following CERE-120 administration provided robust neurotrophic support for nigrostriatal dopamine neurons, improving their function and protecting against degeneration. In essence, this was the easiest and most straight-forward component of the entire nonclinical program, for it merely required replicating what others had already established for other neurotrophic factors (especially GDNF) using gene therapy (see prior sections and papers cited). Beyond that, a number of other important translational questions remained to be answered for which little data yet existed for any neurotrophic factor, whether delivered via viral vectors or other means. For example, could CERE-120 be delivered safely, for sufficiently long periods of time and with a sufficiently wide therapeutic index (i.e., ratio between dose required for efficacy versus dose when side effects are noted) to justify advancing the program to clinical testing in chronic PD patients who would require years, if not decades of therapeutic benefit? This issue was greatly complicated by the heightened sensitivity to the possible safety risks associated with injecting viral vectors into humans, due to some of the early, unrelated safety issues experienced in the gene therapy field, briefly referred to earlier. This concern was further exacerbated because we intended to inject CERE-120 directly into the brain and once administered, could not suspend protein expression if any serious side effects were to develop (while 'regulatable' vectors were under development at the time, none were considered sufficiently advanced to provide a practical solution to this problem, and indeed none has yet advanced into clinical testing, now almost a decade later; see the following section for more details). Other questions for which very little data existed in the field, but were equally important for translational purposes, involved whether protein expression followed a predictable and orderly dose–response and thus whether the amount of protein, as well as its location and pattern of expression, could be adequately controlled. Finally, it was also important to establish that the NRTN expression would remain reasonably consistent over long periods of time and would not migrate from the targeted site. Of course, in the course of conducting these studies, it was inevitable that unexpected findings or novel observations would be made, most of which required experimental follow-up to assure that no uncertainties or mysteries surrounded the performance or effects of CERE-120 occurred in animals prior to initiating the first-ever tests in human PD subjects.

It was with these issues in mind that the CERE-120 nonclinical program was developed, implemented and executed. Less than two years elapsed from the time of CERE-120's construction to the filing of an IND with the FDA to test CERE-120 in humans. During this time, 20 separate nonclinical studies (Bartus et al., 2007, 2011a; Gasmi et al., 2007a, 2007b; Herzog et al., 2007, 2008, 2009, 2011; Kordower et al., 2006) were initiated (obviously, many in parallel; see Table 5), serving as a lasting testimony to the talent and commitment of the scientists at Ceregene to advance this program forward as efficiently and responsibly as possible (see Acknowledgment section for several of the most outstanding). During this time, we were confronted with a number of unexpected, and at times, difficult translational issues that required resolution in order for the program to advance, thus requiring additional, previously unplanned studies to be conducted (therefore eventually leading to 20 different nonclinical studies initiated prior to the start of testing in humans).

Key translational issues confronted

During the course of testing and developing CERE-120, a number of translational issues and obstacles were encountered, some of them conceptual (or theoretical) and others empirical, requiring a fuller understanding of the viral vector's bioactivity. Some of the more controversial, worrisome or unusual are reviewed, below.

Table 5
Overall summary of CERE-120 non-clinical program.

Striatal targeting (20 + studies)	Dose related effects	Kinetics (change over time)	Conclusions
NRTN expression	Predictable, dose–response	Stable expression, 1 month after dosing; no changes up to nearly 2 years later	Predictable, stable, controlled expression
Safety/toxicity	No toxicity at any dose	No short term or longer term toxicity	No evidence of any toxicity at any dose or time point, even with administration of excessive doses
Bioactivity/'efficacy'	Dose–response effects dependent on spread (volume) of protein and area of target covered	Varies with model but persistent benefit in all instances	Robust efficacy consistently observed on a variety of animal models and assortment of different objective endpoints
Nigral targeting (8 additional studies)	**Dose related effects**	**Kinetics (change over time)**	**Conclusions**
NRTN expression	Predictable dose–response, with far better protein coverage in striatum following nigra targeting, compared to more conventional striatal targeting	Stable expression after approximately 3 weeks	Broad coverage of substantia nigra, limited to target area
Safety/toxicity	No serious side effects at any dose; moderate effects on weight at excessive dose, only — linked to mistargeted protein far from nigra	No significant safety issues at any time point measured; modest effects on weight with mistargeted protein seen within 2–3 weeks.	No evidence of any toxicity with properly targeted NRTN; no serious side effects at any dose
Bioactivity/'efficacy'	Evidence of superior neurotrophic response with degenerating neurons following nigra, versus striatal delivery	No evidence for loss of efficacy	Evidence of superior efficacy with nigra targeting (compared to terminal fields, only) when neurons are undergoing degeneration

Decision to use a 'non-regulatable' vector. One of the first decisions we had to make was whether to try to employ one of the so-called 'regulatable vectors' that were under development versus the use of a vector that would produce steady, unregulated protein expression. The issue here was both simple and clear: ample published data existed that an AAV2 vector, when used in conjunction with a CAG-like promoter, would likely provide relatively permanent expression of the therapeutic protein in the transduced cells. On the one hand, this characteristic clearly offered potential advantages for any product intended to treat a chronic neurodegenerative disease via a neurosurgical procedure. On the other hand that characteristic raises understandable safety concerns, in that if any serious side effect were to emerge from NRTN expression it would be difficult, if not impossible, to halt expression. Thus, this issue was given very serious consideration at Ceregene, with discussions among the development team as well as with Ceregene's SAB. Ceregene also had discussions with a company that was formed specifically to develop and use regulatable vectors for gene transfer products (Rheogene). In the end, we decided that none of the available regulatable vectors at the time were truly ready for clinical development, and collectively concluded that all of them posed more translational issues and/or as many inherent safety issues as did the use of an unregulatable vector.

Notwithstanding the progress that was being made with regulatable vectors, none of the systems available had yet been shown to be sufficiently safe and effective for human use. Indeed, regulatable promoters require the uncontrolled expression of a 'regulator protein', and thus suffer a potential conceptual flaw that while they are intended to provide the means to turn on and off the expression of the therapeutic protein, the regulator protein (required to regulate gene expression), is itself expressed in an unregulated fashion. The issue this raises is that the regulator protein could prove to be as toxic, if not more so, than the therapeutic protein, especially since very little is often known about the long-term consequences, in vivo, of expression of any of the regulator proteins known to us at the time. Since some of them were not mammalian, possible immunogenicity was a very plausible concern. It was relatively easy for us to test extremely high, excessive doses of CERE-120 to see what safety liability might lie latent with chronic high levels of NRTN expression. Fortunately, none could be found (see Safety of CERE-120 section). Finally, the use of regulatable vectors also necessarily carries the additional conceptual liability of requiring inherent 'polypharmacy', for it requires the targeted tissue to be exposed to the therapeutic protein, as well as the regulator protein and the pharmaceutical agent used to turn the regulator protein on and off. These characteristics add significant complexity to a solution for a concern that was, and still is, largely hypothetical.

For all of these reasons, Ceregene and its SAB unanimously elected to move forward without the use of a regulatable feature to our vector. We have since found no safety issues with CERE-120 and neither the FDA nor any of the seven different European regulatory agencies we met with to discuss the CERE-120 program raised any concerns about our use of a 'non-regulated' vector (perhaps given the extremely clean safety profile of CERE-120), we did face extremely stiff resistance when we presented our program and plans to the NIH's DNA Recombinant Advisory Committee (i.e., the so-called "RAC"). Ironically, despite the intense controversy faced many years ago at the RAC when Ceregene presented its decision to not use a regulatable vector, no regulatable vector has yet been successfully developed and used in a single human trial. Rheogene (a company founded specifically to develop and implement regulatable vectors) has long-ago disappeared and, most importantly, CERE-120 has not yet been linked to a single serious side effect in animals or humans. To be clear, when (and probably, not if) regulatable vectors have been shown to be clearly safe and effective, they will and should be used for many translational programs. However, even then, the determination should be data-driven, based on a careful risk–benefit assessment involving the particular transgene, clinical indication and patient population. When clinical trials and patient safety are involved, "one size" never fits all.

Concerns regarding pre-existing antibodies to AAV. A major concern that existed in the gene therapy field prior to launching the CERE-120 program was the impact that preexisting antibodies to the viral vector might have on performance of the vector, expression of the transgene or effect to the injected tissue. It was widely recognized that a majority of people have been exposed to naturally-occurring AAV and that 30 to 60% of humans have neutralizing antibodies to AAV (Calcedo et al., 2009). What was not clear is the extent to which these neutralizing antibodies might negate the viral vector, or possibly even impair the transduced cells, either rendering the treatment ineffective, or worse yet, causing a serious inflammatory reaction in the brain. One possible solution we considered, which if implemented, would have greatly complicated and possibly compromised later recruitment, would be to first screen all potential subjects for neutralizing antibodies and exclude them from participating in clinical trials (and thus later exclude all PD patients presenting neutralizing antibodies from receiving treatment, unless another clinical trial was later conducted to establish safety and effectiveness in this subpopulation). Clearly, obtaining empirical data to guide our decision was preferred and as we considered how to address this concern and held discussions with numerous immunological experts, we gained increasing appreciation for the fact that the route of immunization often significantly impacts the response to subsequent re-exposure to the antigenic stimulus (i.e., AAV). Moreover, the prevailing presumption was that humans were primarily exposed to AAV via the respiratory system and we soon concluded that no adequate, nor validated artificially-immunized models of AAV existed to address this issue adequately. Thus, we were presented with a bit of a quandary. We had learned that many nonhuman primates had been exposed to AAV and many presented neutralizing antibodies to the virus and this provided us with a reasonable approach to empirically address this issue.

Testing for the existence of neutralizing antibodies (Ab) revealed that nearly 50% of the monkeys presented neutralizing Ab to AAV (Ceregene, unpublished results). Monkeys were distributed among treatment groups such that a number of monkeys with and without neutralizing Ab were included in key CERE-120 groups, which allowed the assessment of the potential impact of the presence of prior Ab to AAV on the performance of, or tolerance to, administration of CERE-120. The results of this effort were quite revealing and very comforting. First, when we compared the titer of neutralizing Ab to AAV in serum from monkeys prior to and following CERE-120 administration, we found all possible outcomes: (1) no change in Ab titer; (2) amplification of Ab titer in monkeys previously presenting Ab at baseline; and (3) induction of Ab in monkeys previously negative at baseline. Interestingly, all these outcomes proved to be an epiphenomenon as far as CERE-120 safety and effectiveness was concerned. That is, none of these varied AAV Ab outcomes had any impact on any safety/tox outcome, including inflammatory markers at the site of administration in the brain, the transduction and expression of NRTN, or the bioactivity or 'efficacy' in any model system. Thus, these data argued that prior existing antibodies to AAV have no impact on protein expression or bioactivity following AAV-viral mediated gene transfer *to the brain*, at least following a single administration. Importantly, the clinical data, to date, appear entirely consistent with the predictions made from these nonhuman primate results.

'Apparent' lack of NRTN expression and/or bioactivity. As we barely began our efforts to design and test a novel vector to deliver the NRTN gene for PD, we quickly ran into a major issue; apparently extremely poor NRTN expression, measured in the conditioned medium from cells presumably transduced with the NRTN gene 48 h earlier (Ceregene, unpublished results). We confirmed and expanded these

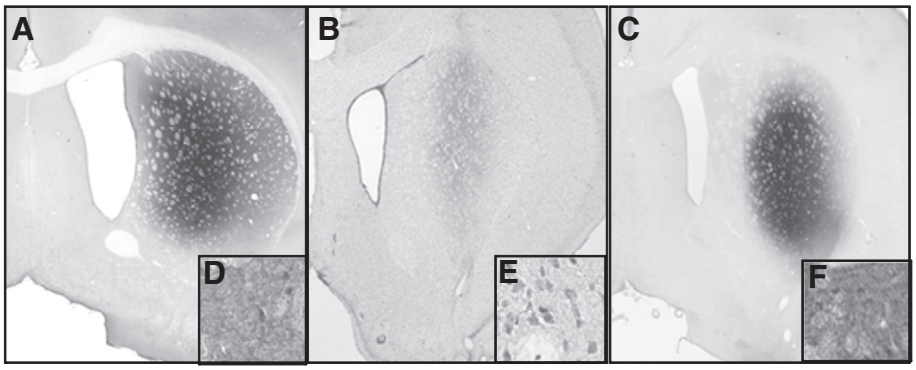

Fig. 1. Immunohistochemical photomicrographs showing transgene protein expression in rat striatum following administration of three different vector constructs: panels A (and D): low and high power magnification views of robust GDNF expression following injection of vector containing GDNF gene with its natural pre–pro sequence; panels B (and E): low and high power magnification views of weak NRTN expression following injection of vector containing NRTN gene with its natural pre–pro sequence (note high power view suggests most protein remains intracellular); panels C (and F): low and high power magnification views of NRTN following injection of CERE-120, which express NRTN gene via substitution of human NGF pre–pro sequence (note substantially more robust immunohistochemical signal, with clear suggestion of ample extracellular protein). See section "Apparent lack of NRTN expression and/or bioactivity" for more details.

results by injecting the same vector into the striatum of rats, noting poor NRTN immunohistochemical signal, whereas a similar vector expressing GDNF (as a positive control) produced an intense signal (see Fig. 1). High-magnification examination of the NRTN-labeled histology slides revealed somewhat better evidence of NRTN expression, but it appeared to be only intracellular, with no clear evidence for protein outside the transduced striatal neurons. To further evaluate the performance of this vector, we tested it in 6-OHDA treated rats, comparing it to a similar vector expressing GDNF, and found no evidence for neuroprotection, while the GDNF vector provided significant neuroprotection (Fig. 2). These data clearly suggested that NRTN was not being secreted effectively from the transduced cells, and while the absolute level of protein also seemed low (at least as measured by immunoreactivity), we did not know to what extent elevated levels of NRTN sequestered in the soma might affect expression efficiency of NRTN within those cells. Recognizing that protein expression is regulated via the pre–pro sequence, we began a search for alternative pre–pro secretion sequences to substitute in the hope that might correct this problem. We eventually constructed, tested and confirmed the effectiveness of substituting the human NGF pre–pro for the natural NRTN pre–pro (Figs. 1 and 2). While we were not certain as to what extent use of a chimeric gene might create safety issues or pose concerns for the FDA, we elected to move forward with an AAV2 vector substituting the human NGF pre–pre sequence, followed by the remaining NRTN genome as our potential neurotrophic factor product for PD and it thereafter enjoyed the full focus of our development efforts. We first confirmed that the NRTN protein expressed from CERE-120 had an amino acid sequence identical to that of endogenous, full length human NRTN. We then addressed possible issues involving the use of a chimeric gene head on by testing extremely high doses (by any standard or traditional definition) and long post dosing safety time points to assure that if any safety problems existed, we would more likely induce them. If this occurred we would have the opportunity to evaluate and better understand them and hopefully establish risk mitigation procedures. In the end, we found no evidence of any safety issue with CERE-120, and it has continued to prove itself to be the cleanest and safest product I have ever tested in any program at any time in my career, including many already approved for use in humans (Tables 1 and 3).

Loss of vector due to binding to dosing hardware. Early in the program we also discovered that our highly purified vector binds to conventional stainless steel injection needles with high affinity, thus significantly altering the concentration of the product delivered to the brain, in a non-linear fashion (a problem we discovered that does not occur as readily when less-pure vector preparations are injected because the impurities block the vector from binding to the stainless steel surface) (Ceregene, unpublished results). Because we had not anticipated that the "vector recovery" studies would really identify a problem, and were primarily treating it as a simple box-checking exercise prior to filing our IND, we had already initiated some of our safety/tox studies by the time we discovered the issue. Thus, we elected to repeat some of

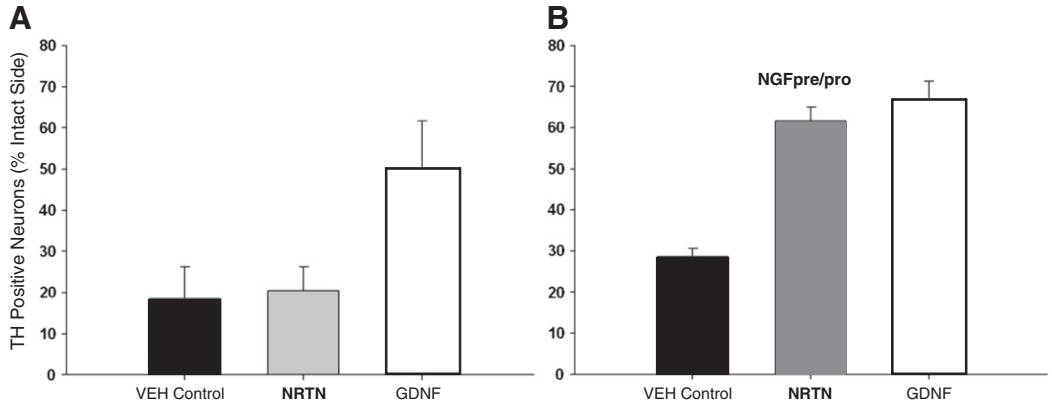

Fig. 2. Protection of nigrostriatal dopamine neurons against 6-OHDA toxicity with vectors described in Fig. 1. (A) Note that vector containing NRTN gene with its natural pre–pro is ineffective (vector containing GDNF gene was included as positive control); (B) vector containing NRTN gene but substituting human NGF pre–pro provides comparable protection to vector containing GDNF gene. See section "Apparent lack of NRTN expression and/or bioactivity" for more details.
Data in panel B were derived from results previously published; Gasmi et al., 2007a).

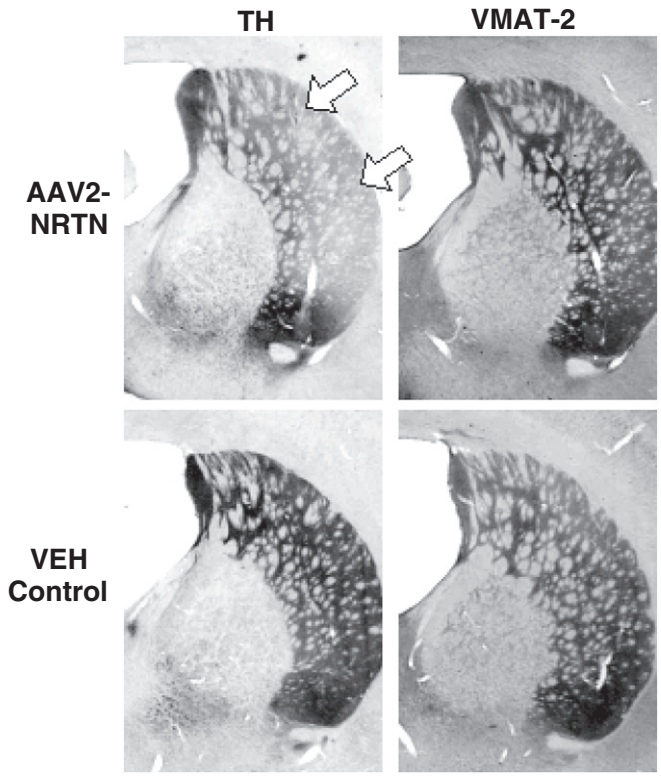

Fig. 3. Photomicrographs showing species-specific, down-regulation of TH immunohistochemical signal following expression of high concentrations of NRTN in rat striatum. Note that while TH-positive staining is decreased following AAV2-NRTN (compared to vehicle-treated rats) no loss of VMAT-2 staining is seen, confirming that dopamine neurons and terminals are intact. Other studies also demonstrated dopamine function remained intact despite reduction in TH-straining. Also, the phenomenon was shown to be species specific and does not occur in primates. See section "Dose related los of TH immunoreactivity" for further details.

the rat safety/tox studies to determine if we could retain the same, wide dose-range and therapeutic index we had initially thought we had established. In addition to causing us to repeat part of our safety/tox program, this discovery also required an intense effort to develop and validate a practical means to inhibit the vector/stainless steel binding prior to filing the IND and initiating the planned CERE-120 clinical trials. Following a series of bench experiments with the vector and dosing hardware, we learned that the binding was saturable and we therefore elected to develop a method to saturate all of the vector binding sites prior to use immediately before each surgery. For purposes of filing an IND, this required assurance and formal validation that this method would indeed work under a range of conditions possibly encountered in the operating room. While the method allowed us to responsibly move forward with our clinical program, we knew this was a temporary solution and we therefore continued working to find a more satisfactory solution that eventually could be used during commercialization of CERE-120. In time, we developed a proprietary non-stainless steel injection needle that was implemented prior to our Phase 2a study and has since been used for all our human studies (Gasmi, 2012).

Dose-related 'loss' of TH immunoreactivity. Another of the more troublesome observations made while in the midst of analyzing our safety/toxicology (high dose) studies, was a dramatic reduction in TH expression in the targeted nigrostriatal neurons (Fig. 3). This dose-related effect initially suggested that high expression levels of NRTN might possibly be neurotoxic (even causing death of many nigrostriatal neurons). Obviously, this provided some cause for concern and we immediately launched an intensive investigation to better understand the phenomenon and thus assess its impact on the potential viability and continued development of CERE-120 as a therapeutic agent.

We first examined VMAT-2 immunoreactivity and observed no changes as a function of CERE-120 dose or level of NRTN expression, despite reduced TH signal (Fig. 3). The preservation of VMAT staining gave us some level of comfort because this suggested that CERE-120 was not overtly toxic to the nigrostriatal terminals, although the reduced TH signal still could reflect impaired function of the targeted dopamine neurons (Ceregene, unpublished results). We launched a series of additional studies to provide more insight into the functional status of the neurons with down-regulated TH. This functional characterization included neurochemical analyses of striatal dopamine and dopamine metabolites as well as behavioral analysis using motor tasks that reflect nigrostriatal function.

Rats treated with a high dose of CERE-120 (2×10^{10} vg/striatum) for 6 months demonstrated the most dramatic reduction in TH and thus we used this condition to assess possible neurochemical changes in their nigrostriatal dopamine neurons, by neurochemical analyses of fresh striatal tissue. Specifically, the ratios of striatal dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured using high performance liquid chromatography (HPLC). Changes in the ratios of these metabolites have been shown, and are generally accepted to reflect changes in dopamine activity and turnover at the synaptic level (e.g., Bartus, et al., 2004). Following a high dose of CERE-120 (that reduced TH-immunoreactivity), we observed a small but significant increase (37%) in the ratio of the dopamine metabolites (HVA and DOPAC) to dopamine levels in the CERE-120-treated rats, compared to formulation buffer control rats, reflecting a modest increase in the rate of dopamine turnover and enhanced dopaminergic activity in CERE-120 treated rats. Additionally, a small, but statistically significant decrease (21%) in absolute levels of dopamine was also observed, which is also consistent with a moderate increase in dopamine turnover. Thus, the neurochemical assessment supported an increase in dopamine function of the high dose CERE-120 rats, and certainly not the decrease in dopamine function that might have been suggested by the decrease in TH-immunoreactivity (Ceregene, unpublished results).

Similarly, performance on a number of behaviors that have been shown to be dependent on an intact nigrostriatal system were examined in rats 6 months after CERE-120 administration to the striatum. These included adjusted forelimb stepping in response to movement, vibrissae-elicited forelimb placing, and plank and rod walk tasks. No differences were observed between CERE-120 treated and formulation buffer control treated rats in any of these behaviors, despite the reduction in TH-immunoreactivity, thus this again confirmed the functional integrity of the nigrostriatal system following delivery of high dose CERE-120 for 6 months (Ceregene, unpublished results).

Importantly, our research in rats not only argued that the TH-down-regulation likely has no impact on the function of dopamine neurons, but further work in nonhuman primates suggested that this most likely was species-specific (restricted to rats). That is, several studies performed in non-human primates clearly demonstrated enhanced intensity of TH staining following CERE-120 administration, both in the nigra as well as in the terminal fields of the striatum. This enhanced TH signal was observed at 3 months to 12 months (i.e., well within the temporal window of when a decreased signal is seen in rats) and occurred consistently at a 60-fold range of CERE-120 doses. In contrast to the observations following high-dose CERE-120 in rats, in no case was a decrease in TH staining ever observed following CERE-120 in nonhuman primates.

Coincidentally, around the time we were completing our studies, two publications from Bjorklund and Kirik using GDNF (Georgievska et al., 2004; Rosenblad et al., 2003) provided independent findings very similar to ours, with even more compelling evidence that the phenomenon was a transient (fully reversible), compensatory response to increased dopamine activity following delivery of the neurotrophic

factor. Thus, these beautifully-conceived studies persuasively argued that delivery of neurotrophic factors, such as GDNF (and by association, NRTN), to young healthy rats causes a transient increase in dopamine activity, requiring a compensatory decrease in TH activity in order to maintain normal levels of dopamine activity. Thus, they argued that the loss of TH staining is merely a normal compensatory mechanism intended to maintain normal dopamine activity in healthy neurons, providing welcome support that the down-regulation we had unexpectedly observed with our high dose CERE-120 rats was not an issue that likely posed any risk to PD patients.

Nonetheless, given the importance of this issue, we wanted to move forward with as much certainty as possible. We reasoned that since age is an important risk factor for Parkinson's disease, tests in aged rats might provide important, additional information for whether this phenomenon poses a risk to PD patients. Moreover, we also imagined that aged rats might provide a unique means to further differentiate the compensatory explanation from the 'toxicity' alternative possibility. We surmised that because aged rats suffer a modest reduction in dopaminergic function spontaneously, one might expect that the phenomenon would be less robust in aged rats if it was truly compensatory in response to enhanced NRTN-induced dopamine function. That is, there should be less need for a compensatory reaction to merely restoring function toward more normal levels, where the levels are already reduced (as they are with age). On the other hand, if the decreased TH signal represents a toxic effect to a high dose NRTN, one might expect to see it exaggerated in aged rats, where the ability to overcome neurodegenerative perturbations is generally less evident than in young rats, and the effect of toxic challenges are therefore often more severe. Consistent with the interpretation that it is a compensatory response, the decrease in TH was less robust in aged rats than in young rats given the same dose (Ceregene, unpublished results).

Thus, all of the available evidence generated was consistent with the concept that the decrease in TH observed in the rat following elevations in either GDNF or NRTN does not reflect a toxic liability but rather a normal, compensatory reaction that maintains normal levels of dopaminergic synaptic activity in the context of continuous neurotrophic factor stimulation. Moreover, this phenomenon did not occur in nonhuman primates and together these two general observations provided the empirical support to move forward with confidence that human subjects would not be exposed to undue risk following continuous NRTN expression, even at high doses.

Risk of cerebellar toxicity, due to reports by others using GDNF. In the midst of preparing our initial pre-IND documentation for the FDA and Appendix M materials for the NIH's DNA Recombinant Advisory Committee (aka the 'RAC'), informal reports began to circulate on toxicity induced in nonhuman primates from a program that roughly parallels the general goals and objectives of CERE-120. This program (sponsored by Amgen) used indwelling cannula and a subdermal pump to infuse GDNF, an analog of NRTN, into the striatum of advanced PD patients. While it therefore differed in significant ways from ours using AAV to express NRTN, the similarities of two closely-related neurotrophic factors delivered to the same brain sites for the same indication and purpose could not be ignored. Specifically, Amgen announced suspension of their program because of Purkinje cell loss and associated astrocytosis in the cerebellum in several monkeys receiving high-dose infusions with GDNF for 6 months; many monkeys in this study also showed dose-related antibodies to GDNF, as did many PD subjects treated in a similar manner. The data from this study were initially presented in poster form and we were able to get a copy of it, prior to its eventual publication (Hovland et al., 2007), both of which offered important details regarding these potentially troubling observations. Because the observations of possible toxicity following chronic infusions of GDNF might predict similar toxicity with chronic expression of NRTN, we evaluated the data reported and expended great effort to learn as much as we could about the phenomenon, including details not included in the poster and publication. It was important to gain a detailed understanding of the toxicity reported with GDNF in order to assess possible risk in our own program and determine whether continuing to develop CERE-120 was appropriate. In time, with careful due diligence, we became comfortable that the toxicity reported following infusions of purified GDNF did not predict that similar effects should be seen with gene-mediated expression of NRTN.

The authors pointed out that the mechanisms of action for the cerebellar pathology and the development of antibodies were unknown, although they did conclude that an important factor was likely leakage of the GDNF protein into the cerebrospinal fluid (CSF) or blood from the infusion pump implanted under the skin and/or the cannula implanted in the striatum. Data supporting that hypothesis include the fact that a clear, dose-related increase in recombinant GDNF was seen in the CSF, while a significant number of monkeys in the high-dose group even had detectable levels of recombinant GDNF in their plasma. Importantly, we also noted that several other toxicology findings were consistent with mistargeted protein delivery to periventricular areas. These included dose-related changes in the classic signs for mistargeted (in this case, more-likely leaking) trophic factor in periventricular sites, including weight loss and meningeal thickening in the area of the medulla oblongata and segments of the spinal cord. Because we recognized from the start of our program that prior attempts to test NGF in AD patients and GDNF in PD patients via infusions in the lateral ventricles produced the same troubling side effects, we were already routinely testing for these possible effects in our animal studies.

We were therefore able to quickly and easily differentiate our results with CERE-120, expressing NRTN via gene transfer, from those infusing purified GDNF via artificial hardware. Importantly, the differences were striking. First, in contrast to the GDNF infusion study, NRTN has never been detected in serum or CSF, even at excessive doses of CERE-120 or after a full year of continuous NRTN expression. Secondly, no antibodies to NRTN have ever been detected with any dose of CERE-120 or time point. Third, none of the classic signs of non-targeted neurotrophic factor exposure to periventricular sites have ever been seen with CERE-120, despite careful, protocol-prescribed attention and formal analyses specifically intended to identify possible changes in weight, pain, meningeal thickening or axon sprouting. Finally, no evidence of any histological changes was noted in the cerebellum, or any other brain region or body system for that matter. The latter finding is particularly cogent, for we routinely provide an extensive series of brain tissue from our safety/toxicology studies to an outside neuropathologist for independent assessment. Coincidentally, the same neuropathologist that examined our CERE-120 tissue participated in the GDNF assessment (and is an author on the GDNF publication). After becoming aware of the GDNF-cerebellar findings, we specifically asked that he review sections of the cerebellum for any signs of pathology or histological stress. Despite clear and intimate knowledge of the changes seen with GDNF and instructions to identify any changes of any kind following CERE-120, no toxicological findings were seen. While it is still not clear what mechanism(s) might be responsible for the constellation of safety issues raised by the high-dose GDNF monkeys, we concluded that each of them could be most parsimoniously linked to problems in protein delivery related to inherent deficiencies with the use of indwelling hardware, leading to leakage of the protein into the cerebral spinal fluid system of the CNS during the chronic infusions and possibly also into the circulatory system during routine refilling of the pumps. The leakage of GNDF, in turn, caused untargeted and undesirable periventricular and possibly systemic exposure, inducing the same unwanted neurotrophic response in the periventricular sites previously reported when neurotrophic factors were intentionally infused into the lateral ventricles (Eriksdotter Jonhagen et al., 1998; Nutt et al., 2003). We felt this conclusion enjoyed further, independent support because we noted

that another laboratory independently reported that infusions of radiolabeled GDNF into the monkey putamen (using a pump/cannula system similar to that used in the Hovland et al. study) produced leakage of GDNF to superficial layers of the occipital cortex and cerebellum (Salvatore et al., 2006).

In conclusion, none of the toxicity seen with infusions of GDNF in monkeys had been, or has been seen following CERE-120 (expression of NRTN via gene transfer), despite even longer CERE-120 time points and delivery of the highest doses feasible. While it is possible that GDNF possesses a much less desirable safety profile than does NRTN, a more likely explanation for all the data is that the major factor causing the neurotoxicity observed in the monkeys of Hovland et al. (2007), was untargeted delivery of the protein due to a necessarily cumbersome and suboptimal delivery approach. Thus, these data, in conjunction with those collected in the course of developing and testing CERE-120, argue for the *relative safety* and effective delivery of proteins *using gene-transfer*, compared to chronic infusions of protein via mechanical hardware. This same assessment also provided a logical and parsimonious explanation for the development of neutralizing antibodies to GDNF in several of the human subjects enrolled in Amgen's controlled Phase 2 trial (Lang et al., 2006) while also providing comfort that this phenomenon was not likely to be seen with CERE-120. Thus, we proceeded with the development of CERE-120, despite Amgen's abandonment of their program and it has now been many years since their reports of toxicity in monkeys and humans and we continue to observe no evidence of toxicity or antibodies to NRTN in any animals or Parkinson's subjects administered CERE-120.

Translational advantages of CERE-120

Despite the sometimes unique and often unexpected challenges confronted when developing CERE-120 for entry into human testing, we nonetheless enjoyed a number of major empirical, translational 'breaks' or advantages related to the performance of CERE-120. These greatly facilitated our ability to move the nonclinical program forward efficiently and responsibly into clinical testing in PD subjects. These included the observations: (1) that CERE-120 produces a predictable, controlled, dose-dependent volume of NRTN expression, (2) that NRTN expression reaches an asymptote approximately 1 month, post-dosing and exhibits no change in amount, location or pattern of protein expression thereafter (for at least one to two years later), and (3) that CERE-120 lacks any detectable toxicity or side effects, even at extremely high, excessive doses, thus simplifying translation of doses in animals to those intended for humans (see Bartus et al., 2011a for a detailed discussion regarding translating doses to humans using inter-species 'scaling'). If any of CERE-120's characteristics were markedly different, it is unlikely this program could have been translated to the human clinic as efficiently or effectively as it was, let alone become the first to establish clinical proof of concept for neurotrophic factors in human neurodegenerative diseases as well as gene transfer for a CNS indication. The more we studied CERE-120 and understood it, the more we appreciated how 'well-behaved' it performed. Greater detail regarding these positive attributes is provided in the following sections.

Kinetics and expression patterns of NRTN following CERE-120 injections

The primary objective of delivering CERE-120 is to achieve expression of NRTN throughout the striatum, while at the same time avoiding NRTN exposure to non-targeted sites within the CNS that could conceivably cause side effects. In particular, we felt it important to avoid exposing periventricular areas to NRTN since direct infusion of other neurotrophic factors (e.g., GDNF, NGF) into the ventricles had been shown to produce adverse effects in animals and humans (Day-Lollini et al., 1997; Eriksdotter Jonhagen et al., 1998; Kordower et al., 1999; Nutt et al., 2003). Thus, it was important that we achieve an understanding for the variables that influence the volumetric spread (location) of protein as well as the amount, so that this insight might later be applied for dose-selection in humans. It was also important to understand the kinetics of expression following CERE-120 administration in order to rationally design efficacy experiments, and to know when maximum expression is achieved in order to interpret longer-term safety date for CERE-120.

A series of studies was conducted to examine the time course of NRTN expression following CERE-120 administration and the relationship between CERE-120 dose, NRTN volume of distribution (via immunohistochemistry) and levels of NRTN (via ELISA). Collectively, these studies established that: (1) NRTN expression is detectable as early as 1–2 days after administration of CERE-120 (Gasmi et al., 2007b); (2) NRTN volume of expression and levels of expression reached an asymptote at approximately 1 month after CERE-120 dosing (Gasmi et al., 2007b); (3) volume of NRTN expression remains stable throughout the life-span of the rat and for at least a full year in monkeys (Herzog et al., 2009, 2011); (4) the volume of distribution of NRTN can be controlled by manipulating the dose of CERE-120 in both rats (Gasmi et al., 2007b) and monkeys (Herzog et al., 2008, 2009); and (5) by distributing several injections of CERE-120 within the putamen at predetermined dose levels, it is possible to express NRTN throughout a significant portion of the putamen, while avoiding exposure to other (non-targeted) brain regions (Gasmi et al., 2007a, 2007b; Herzog et al., 2007, 2008, 2009, 2011). (The latter point was later extended to the substantia nigra (Bartus et al., 2011a).).

In summary, the 'kinetics of expression' of NRTN following CERE-120 administration into the striatum reveals stable and well-controlled protein largely restricted to the targeted region. These data formed the basis of dose selection for nonclinical and clinical studies and the initial foundation for the CERE-120 development program.

Bioactivity and efficacy of NRTN following CERE-120 administration

Recognizing that no animal model can truly capitulate all the important elements of any human neurodegenerative disease (for discussion of these issues, see reviews by Bartus, 1988; Bartus et al., 1983), we elected to test CERE-120 in multiple animal models (Table 6). The presumption made was that because PD is a complex disease that most likely involves many different etiologic and pathogenic variables that ultimately lead to degeneration and death of the nigrostriatal dopamine neurons, no animal model can reliably predict efficacy for restoring function of these neurons. Thus, if one could demonstrate that CERE-120 mediated NRTN expression can overcome a wide variety of neural perturbations to these neurons in animals, then it may more likely help rescue function and status of these same neurons in PD patient's degeneration, no matter what factors are responsible for their degeneration.

A series of experiments in rats and monkeys was conducted to establish that the NRTN protein expressed following CERE-120 administration is capable of producing a neurotrophic response to nigral dopamine neurons and that this translates to positive outcomes using a variety of measurements in a number of different animal models of PD and dopamine deficiency. Studies in young, naive monkeys established that CERE-120, delivered to the striatum, produces sustained protein expression in striatum and nigra, in a controlled, dose-related fashion, leading to an appropriate biological trophic signaling in the nigra (e.g., enhanced tyrosine hydroxylase [TH] and activation of pERK) (Herzog et al., 2008, 2009). Studies in aged rats and monkeys, demonstrated similarly appropriate responses to CERE-120, including hypertrophy of dopamine neurons in aged rats (Herzog et al., 2011), and increased numbers of TH-positive neurons, enhanced pERK and F-Dopa PET signaling in aged monkeys, up to 8 months post-dosing (Herzog et al., 2007). In young rats treated with CERE-120 and then allowed to grow old (i.e., 20 months later), NRTN expression did not change and remained biologically active for 18 to 20 months, post dosing, as indicated by elevated pERK immunoreactivity in nigral neurons (Herzog et al., 2011). In the 6-OHDA rat model of PD (or nigral dopamine depletion), CERE-120 was able to protect nigral neurons in a dose-related fashion, up to 7 months after administration (maximum

Table 6
Variety of preclinical models and end points demonstrating neurotrophic activity with CERE-120.

Model	NRTN expression	Impact on targeted DA neurons	Functional impact
6-OHDA rats	Dose-related and long-term (8 months+)	Increased cell survival	Behavioral benefit (rotation)
Aging rats	Very long-term (20 months+)	Enhanced pERK	nd
Aged rats	Predictable volume of expression confirmed	Classic neurotrophic factor-induced DA cell hypertrophy; increased pERK signal	nd
Young monkeys	Dose-related expression, persisting for 12 months+	Activated pERK; DA cell hypertrophy	nd
Aged monkeys	Predictable volume and duration of expression confirmed (8 months+)	Enhance TH in striatum and nigra; Increased # TH+ neurons; DA cell hypertrophy	Enhanced striatal F-dopa PET signal
MPTP monkeys	Predictable volume and duration of expression confirmed (10 months+)	Preservation/protection neurons; enhanced TH in striatum and nigra; enhanced pERK in nigra	Improvement in battery of motor tasks

nd = not determined.

time point examined). This neuroprotective effect was linked to functional improvement on the amphetamine-induced rotational task (Gasmi et al., 2007a). Finally, in the MPTP nonhuman primate model, CERE-120 produced long-lasting improvement in motor behavior beginning within 1 to 3 months after CERE-120 dosing and persisting for 10+ months, the longest time point examined. CERE-120 also provided protection against nigral neuron degeneration and loss of striatal DA fibers, as well as enhanced TH and pERK in the preserved nigral neurons of these same monkeys (Kordower et al., 2006).

Together, these data demonstrated a consistent pattern of bioactivity and efficacy following CERE-120 administration, establishing that the NRTN protein expressed is robustly and consistently bioactive and can therefore protect and/or restore dopamine neurons and function in each of the model systems tested (Table 6).

Safety of CERE-120

Perhaps the most important component of the nonclinical development program for CERE-120 involved tests of safety and potential toxicity. Constructing and executing an unusually thorough and conservative nonclinical safety program prior to moving into human testing seemed appropriate for several related reasons. First, the history of efforts to translate the promise of gene therapy into clinical trials has been fraught with many unanticipated and sometimes serious side effects. Secondly, intervention in the human brain necessarily raises even greater concerns when considering the possible side effects that might occur with a novel experimental intervention. Finally, CERE-120 was expected to produce relatively permanent (i.e., irreversible) NRTN expression, necessitating that the burden of proof for safety be set higher than normal to establish that no harm could reasonably be expected from administration of CERE-120 or long-term expression of NRTN.

For these reasons, the nonclinical toxicology/safety program for CERE-120 tested large numbers of rats and monkeys, at doses hundreds of times higher than those required for bioactivity and efficacy in the animal models. In order to better prepare for the possibility that serious side effects might occur from entirely unanticipated sources or in unexpected ways, the toxicology evaluations were not limited to the CNS, but rather included tissues throughout the body and the entire brain, using multiple endpoints. In total, the safety/toxicity program, alone, consisted of six studies in rats and four more in monkeys, including tests on aged rats and aged monkeys (since age is a major risk factor in PD). Collectively, these studies established a wide safety margin for CERE-120. In fact, no CERE-120-related toxicity was observed at any dose or time point in any study conducted. Therefore, no 'maximum tolerated dose' could be identified (since even the highest, excessive doses were well-tolerated), including the highest dose that could be practically administered. (Note: vectors will aggregate and fall out of solution at extreme concentrations, and injury to brain tissue will occur if excessive volumes are infused; these factors necessarily limit the highest dose that can be administered to an animal).

In summary, these studies evaluating the safety and toxicity of CERE-120 up to one year in rats and monkeys demonstrated: (1) no adverse effects on general health, diet, etc.; (2) no adverse effects on neurological or behavioral assessments; (3) no histopathology on cerebrum, cerebellum, brain stem, or spinal cord; (4) none of the toxicological changes seen with intraventricular infusion of NGF or GDNF in animals and humans, including weight loss, pain, Schwann cell hyperplasia or axon sprouting; (5) no functional or morphological adverse effects on the nigrostriatal system, nor any CERE-120 related inflammatory reactions; (6) no significant changes in clinical chemistry or hematology; (7) no histopathological effects on peripheral organs; (8) no detectable NRTN in CSF or serum; and (9) no detectable serum antibodies to NRTN. In conclusion, extensive testing in nearly 250 rats and over 30 monkeys, involving doses far in excess of those intended for the clinic, up to a year following CERE-120 administration revealed not a single toxicological finding or safety concern, thus providing further support for advancing this program into clinical testing in humans.

Synopsis: preclinical proof of concept support for CERE-120

In summary, 20 nonclinical studies were conducted with CERE-120 to establish both safety and proof of concept in animal models to support the initial Phase 1 clinical trial in PD patients (Table 5). These studies consistently demonstrate that: (a) a single stereotactic administration of CERE-120 provides NRTN selectively to the targeted areas in the brain, (b) the amount and volume of NRTN expression follow an orderly dose–response relationship, (c) expression levels and rate of expression are predictable, and (d) the volume of brain tissue and region exposed remains stable for many years, once an asymptote is reached at approximately one month. Additionally, NRTN-expressed via CERE-120 is potently bioactive, inducing the conventional surrogate markers established for neurotrophic activity and enhanced dopamine neuronal function, including cellular hypertrophy, pERK activation and enhanced tyrosine hydroxylase expression. CERE-120 is also able to restore target innervation and protect nigrostriatal neurons against degeneration in multiple animal models. Finally, CERE-120 has generated no evidence of toxicity or any side effects, even at dose-multiples hundreds of times higher than required for bioactivity and efficacy in animal models of nigrostriatal neurodegeneration. In sum, the nonclinical program established that CERE-120 is able to produce the desired trophic response in targeted neurons in a safe and effective fashion, therefore supporting the advancement of the program into human testing in PD patients.

Identifying appropriate CERE-120 doses for testing in humans

As the nonclinical program continued to generate safety and efficacy data that supported testing CERE-120 in human Parkinson's patients, it became increasingly important to consider how we might select safe and potentially effective doses to be tested in humans. The issues confronted with a 'first in human' gene therapy vector targeted to a relatively small area of the brain were necessarily far different from those confronted when selecting initial human doses for typical pharmaceuticals, especially systemically-delivered small molecules or biopharmaceuticals. At least three separate and relatively unique translational obstacles complicated this activity. First, contrary to nearly all other 'first in human' tests of novel products, use of gene transfer does not allow the opportunity to withdraw the biopharmaceutical agent once delivered, for the vector will likely continue to express the transgene protein for the life of the transduced cells. Secondly, since the NRTN is specifically expressed in targeted sites within the human brain, it is not possible to monitor dose levels via plasma pharmacokinetic methods, as is commonly done following systemic administration of novel agents; indeed, no surrogate is even available to confirm successful NRTN expression or the distribution of vector when injected into the human brain. Thus, other than the lack of observable safety issues, no hard data is generated to help guide 'dose escalation' in human testing (such as drug levels in plasma or tissue, as is often used for more typical drugs under development). Finally, because administration of CERE-120 requires an invasive procedure (i.e., stereotactic brain surgery), ethical issues (and regulatory guidance) dictate that one cannot initially test intentionally very low(i.e. virtually safe) doses and then gradually increase the dose to approach presumed therapeutically relevant levels, as is often done for small molecules. Rather, the risk of surgery to the subject requires that all doses administered have at least a rational chance of providing meaningful efficacy. These circumstances collectively required that the starting "first-in-human" doses be sufficiently low to likely be safe and also sufficiently high to rationally be efficacious. Fortunately, the nonclinical data were extensive, including the wide safety margins established for CERE-120 and the kinetics of expression established in rats and monkeys (see prior sections). All these data proved to be immensely helpful in addressing this problem.

First, extensive nonclinical testing demonstrated that the dose–response function for CERE-120 was very different from that of conventional small molecules. With the latter, as the dose is gradually increased, one moves from a 'no effect level' through a range of doses until reaching one that provides the desired therapeutic effect. Eventually, even higher doses produce undesirable toxicity (thus defining a maximum tolerated dose). In contrast to this conventional scenario, with CERE-120, an extremely wide range of doses was shown to provide the desired neurotrophic effect, with no dose level showing any sign of toxicity. As long as the level of NRTN expression was sufficient to be detected via standard immunohistochemical methods, we consistently observed clear signs of the desired neurotrophic activity. Thus, a very wide range of doses was seen to produce roughly equivalent neurotrophic effects at the cellular level with no dose showing any sign of cellular stress, or toxicity. Secondly, tests in rats and monkeys established that we could predict and control the volume (and amount) of protein expressed by varying the vector genome dose of CERE-120.

These data collectively argued that we did not need to concern ourselves with the conventional pharmaceutical concepts of a maximum tolerated dose or human 'therapeutic index' for CERE-120, when selecting the doses to be tested in humans. Rather, with CERE-120 it was more important to focus on identifying doses that would adequately cover the putamen (i.e., with enough protein to be detected by immunohistochemistry — which we established produced the desired neurotrophic response), while avoiding NRTN expression outside the borders of the putamen (which we considered the primary source of potential toxicity, since prior studies infusing neurotrophic factors in both humans and primates reported that serious side effects could be induced if high concentrations of the protein were exposed to periventricular tissue).

Using the extensive dose–response data we accumulated in animal studies establishing how volume of NRTN expression changed as a function of CERE-120 dose, and considering differences in the volume of targeted brain structures (both putamen and later substantia nigra), we sought to select human doses that were equivalent to those found to be both effective and safe in our animal studies. When defining the initial human doses, we applied three 3 simple principles: (1) the more broadly the targeted site is covered with elevated NRTN, the better (i.e., cover as many of the degenerating terminals as possible); (2) it was not necessary to worry about NRTN levels being elevated too high in any part of the targeted putamen, but (3) NRTN spreading outside the targeted putamen should be avoided, with particular attention to the possibility of protein (or vector) following paths of least resistance (e.g., large cerebral vessels and white matter tracts) and thus exceeding the intended targets. Of course, given that we had not yet collected any evidence that vector spread and protein expression in human brain would necessarily precisely follow that established for monkeys (i.e., it could spread further), we selected two reasonably conservative doses for our initial Phase 1 trial, estimated (based on animal expression data) to likely cover between 25 and 40% of the putamen. Later quantification of NRTN expression from CERE-120-treated human autopsy cases conservatively estimated putaminal coverage to be ~15% (Bartus et al., 2011b); currently, it is not known whether AAV and/or NRTN diffuse less widely in human PD brain (compared to nonhuman primate

Table 7
Cross-species dose equivalents (based on relative volume of targeted brain sites), enabling rational, species-to-species dose comparisons.

	Species	Vol. of target (mm^3)	Efficacy considerations		Safety considerations	
			Dose/hemisphere ($vg \times 10^9$)	Dose equiv. ($vg \times 10^9/mm^3$)	Dose/hemisphere ($vg \times 10^9$)	Dose equiv. ($vg \times 10^9/mm^3$)
Striatum/putamen	Rat (striatum)	25	0.16–4.0	0.006–0.16	20	0.8
	Monkey (striatum)	1200	150	0.13	1800	1.5
	Human Ph 1: *first in humans* (Putamen)	4000	65–270	0.016–0.07	270	0.07
	Human Ph 2a (Putamen)	4000	270	0.07	270	0.07
	Human Revised Ph 1/2b (Putamen)	4000	1000	0.25	1000	0.25
Substantia nigra	Rat	2.5–3.0	1.6	0.6	60	22.2
	Monkey *Projected*	50–60	32	0.6	n/a	n/a
	Human Ph 1: *first in humans*	n/a	n/a	n/a	n/a	n/a
	Human Ph 2a	n/a	n/a	n/a	n/a	n/a
	Human revised Ph 1/2b	300–500	200	0.5	200	0.5

Note: for all rat SN dosing calculations, a volume of 2.7 mm^3 was used; for monkey SN calculations, a volume of 55 mm^3 was used; and for SN human calculations, a volume of 400 mm^3 was used. Also, n/a = not applicable (for substantia nigra not targeted in initial Ph1 and Phase 2a studies).

brain) or alternatively whether the relatively poor conditions and processing of human post-mortem autopsy tissue produced a significant under estimation of the actual NRTN volume of expression achieved. This is discussed in some detail in Bartus et al. (2011a).

We used the relative volumes of the targeted putamen (for humans) or striatum (for animals) to compute comparable, species-specific "dose equivalents" (Table 7). This allowed us to use the efficacy and safety data collected in animals to project 'human equivalent doses' (scaled on the basis of relative volume of the targeted putamen) and to identify comparable doses for humans that provided significant neurotrophic effects in animal models, while also being well within the range of doses shown to be safe and void of any toxicity in animals. Using this approach, the highest Phase 1 human dose (0.07 vg $\times 10^9$/mm^3) was approximately 11.5 to 21.5 times lower than dose equivalents shown to be safe in rats (0.8 vg $\times 10^9$/mm^3) and monkeys (1.5 vg $\times 10^9$/mm^3), respectively; see Table 7. At the same time, both Phase 1 doses were well within the dose range shown to be efficacious in 6-OHDA rats (0.006 to 0.016 vg $\times 10^9$/mm^3), while the higher of the two Phase 2 doses approached the single dose tested and shown to be effective in MPTP monkeys (i.e., it was 54% of the 0.13 vg $\times 10^9$/mm^3 MPTP monkey dose, by volume of target). However, as discussed below, with the accumulated, long-term safety data generated in monkeys since the initiation of the CERE-120 clinical program, and the additional safety data generated in the initial Phase 1 and Phase 2a clinical trials, we have since further increased the CERE-120 dose to the putamen so that in the current Phase 2b trial, the human dose is nearly twice the comparable MPTP dose and 64% greater than the highest 6-OHDA comparable dose.

Initial clinical experience with CERE-120: establishing clinical proof of concept for neurotrophic factors

Initial Phase 1

On the basis of a successful nonclinical program, a 'first-in-humans' Phase 1 safety trial (CERE-120-01) in moderately-advanced Parkinson's disease patients was initiated in 2005 and completed in 2006. Two dose levels of CERE-120 were tested (6 subjects each), delivered bilaterally into the terminal fields of the degenerating nigrostriatal neurons in the putamen. This study provided initial evidence for the human safety of CERE-120, for no SAEs (serious adverse events) were noted during the entire duration of the trial. Additionally, preliminary data suggested possible clinical improvement at 12 months, post-dosing with this small population of PD subjects, showing statistically significant improvement (compared to their pre-CERE-120 baseline scores) on a number of clinically relevant motor and quality of life endpoints (Marks et al., 2008).

Recognizing the preliminary nature of these open-label data, we moved quickly to initiate a multi-center, sham surgery-controlled, double blind Phase 2 trial.

Controlled Phase 2a trial

Introduction. Ceregene initiated a multicenter, sham-surgery-controlled, double-blinded Phase 2a trial (CERE-120-02) in 2006, intended to further evaluate safety, while also testing for efficacy in advanced Parkinson's disease. This trial, completed in 2008, had three primary goals: (1) generate additional data supporting the safety and feasibility of this therapeutic approach in patients with Parkinson's disease by expanding to multiple sites across the United States; (2) generate data under double-blinded, controlled conditions that would provide initial "proof of concept" evidence for this approach in human disease and; (3) achieve a sufficiently robust benefit in a single, predetermined "primary endpoint" to support product registration with the FDA and international regulatory agencies. The proof of concept data in particular should include improvement in a number of relevant, predetermined motor and health-related quality-of-life assessments, and ideally would eventually be complemented by histological data (through examination of the brains from trial patients who might someday come to autopsy). When the opportunity for histological analyses occurs, the data should demonstrate that CERE-120 produces predictable and controlled NRTN expression in the targeted region of the Parkinson's brain (putamen) and that the expressed NRTN can enhance the status of the degenerating dopaminergic neurons (e.g., increased TH expression).

For purposes of achieving proof of concept, these additional measures were essential, for it is only through an assessment of several objective, relevant and mutually-corroborating measures (including the primary endpoint) that one might ascertain whether CERE-120 is able to exert a clinical benefit on PD and thus establish proof of concept. For registration purposes, these multiple measures were of potential importance to better characterize and confirm benefits across multiple clinical domains and for negotiating the language of the label with the FDA, in the event that a statistically significant effect on the primary endpoint was achieved. Most important for purposes of generating registration data for the FDA, however, was the primary endpoint. This was the motor component (part 3) of the Unified Parkinson's Disease Rating Scale (UPDRS) in the practically-defined motor off state (i.e., no Parkinsonians medications for at least 12 h), measured at 12 months, post treatment. The protocol was also designed to generate blinded data on a subset of patients (based on the sequential timing of enrollment) at 15 to 18 months post-surgery and the results of these longer-term effects were presented as part of the secondary analysis, particularly for "proof of concept" purposes.

Results from controlled Phase 2a trial demonstrate 'clinical proof of concept' for therapeutic benefit of CERE-120. The initial multi-center, double-blind, sham-surgery controlled trial with CERE-120 produced several important findings. First, additional data supporting the safety of the dosing procedure and treatment approach were clearly achieved, with no unexpected serious toxicity or side effect observed. Importantly,

Table 8
"Motor-related" and "Quality of Life" (QOL) endpoints measured in double-blind, controlled Phase 2 trial. Several measures suggested benefit of CERE-120 at 12 months and even more motor and QOL measures showed benefit at 18 months (* designates statistical significance; (*) designates strong trend, bolded). No measure suggested similar benefit of sham. Data adapted from Marks et al., 2010.

	12 month blinded evaluation			15–18 month blinded evaluation		
	Sham surgery: change from baseline	CERE-120: change from baseline	p Value at 12 months	Sham surgery: Δ from baseline	CERE-120: Δ from baseline	p Value at 18 months
UPDRS I "off"	0.95	−0.32	<**0.01** *	1.27	−0.26	**0.02** *
UPDRS II "off"	−2.25	−3.35	0.4	0.82	−3.32	**0.07** (*)
UPDRS II "on"	1.6	−0.89	**0.03** *		*Not tested*	
UPDRS III "off"	−6.95	−7.19	0.9	−5.64	−11.21	**0.02** *
PD diary "off"	−0.23 h	−1.00 h	**0.07** (*)	−0.52 h	−1.48 h	**0.09** (*)
PD diary "on without troubling dyskinesia"	0.80 h	1.00 h	0.3	0.55 h	2.25 h	**0.05** *
Timed walking "off"	−3.00 s	−2.65 s	0.6	−0.55 s	−8.11 s	**0.02** *
PDQ-39	1.20	−2.83	**0.03** *		*Not tested*	

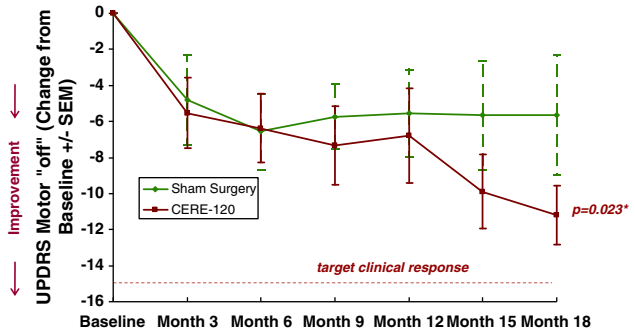

Fig. 4. Changes in UPDRS motor-off scores over time, providing 'Proof of Concept' evidence from well controlled trial that CERE-120 is able to improve the function of advanced Parkinson's disease (PD) patients. Note that while this 'primary endpoint' did not demonstrate a clinical benefit from CERE-120 at 12 months, a protocol-prescribed analysis of longer-term, blinded data showed significant benefit by 18 months. These data corroborate the benefit of CERE-120 which is also seen in several secondary endpoints at 12 months, and the improvement of even more measurements at 18 months, with no measure similarly favoring the sham group (see Table 9).
Data adapted from Marks et al., 2010).

intriguing evidence of a clinical benefit from CERE-120 was obtained when the effects of CERE-120 versus sham treatment were compared using blinded data from motor and quality of life assessments. At the 12 month protocol-specified evaluation period, several endpoints provided evidence for CERE-120 benefit, including findings in well-validated PD scales such as the PDQ-39 quality-of-life scale and patients' self-rated motor diaries, without a single measure favoring the sham-surgery group (Table 8). However, the primary endpoint designated for registration purposes (i.e., UPDRS motor-off part 3) did not discern a difference between improvements observed in the CERE-120 and sham at the 12 month evaluation time point (though both groups did show equivalent improvement compared to their pre-treatment baseline scores; $p<0.05$).

Providing further 'proof of concept' support for the potential benefit of CERE-120, analyses of data prescribed in the formal Statistical Analysis Plan and performed on the subset of patients who remained blinded beyond 12 months, demonstrated an increasing clinical benefit of CERE-120, with several additional secondary measures showing statistical significance, including the protocol-specified primary efficacy measure (UPDRS motor-off) when assessed at 18 months, post-dosing ($p<0.023$) (Marks et al., 2010). Once again, no measure favored the sham-surgery group (see Table 8 and Fig. 4).

In an editorial accompanying the publication, Benabid (2010) independently supported the conclusion that the data established proof of concept, stating that the findings "provide the first clinical evidence of a clinical benefit of gene therapy in Parkinson's disease; these results will serve as a starting reference that it is hoped will be exceeded in future trials". While this independent endorsement was welcomed, it was also tempered in the minds of us developing CERE-120 by the appreciation that the effects achieved, while clearly promising, were likely not yet sufficient to enable successful translation to widespread clinical practice. Many of us felt that the results needed to be optimized and fortunately, observations from post-mortem tissue of CERE-120-treated subjects provided essential insight to help direct that effort.

Further 'proof of concept' support from autopsy tissue. Analyses of post-mortem brains from two subjects treated with AAV2-NRTN who died of causes unrelated to CERE-120 administrated 6 weeks and 3 months earlier provided further proof of concept evidence in PD, as well as unique insight for optimizing dosing of CERE-120 in advanced PD patients (Bartus et al., 2011b). Those data were recently replicated and extended with autopsy tissue from two more patients treated with CERE-120 four-plus years prior to dying of unrelated causes (Ceregene, unpublished data).

Several new and insightful findings from the autopsy tissue were reported by Bartus et al. (2011b). First, NRTN expression in the targeted PD putamen following CERE-120 delivery was confirmed, demonstrating CERE-120 performed as it was designed to do (Fig. 5). Secondly, a clear but modest increase in TH signal (a commonly accepted surrogate for improved integrity of DA neurons) was observed in the PD putamen and linked to targeted NRTN expression, offering the first evidence that enhanced status of dopamine neurons can be achieved in the advanced PD brain (using the accepted surrogate of TH immunohistochemistry; Fig. 5). These data provided further proof of concept support for CERE-120, establishing that CERE-120 can provide targeted expression of NRTN in the human brain and that the NRTN expressed via CERE-120 can then enhance the status of degenerating dopamine terminals. Reinforcing these observations, an independent guest editorial (Lewis and Standaert, 2011) stated that the results of this study provided "proof that gene therapy with AAV2-NRTN (aka CERE-120) results in functional transgene expression in target cells in humans".

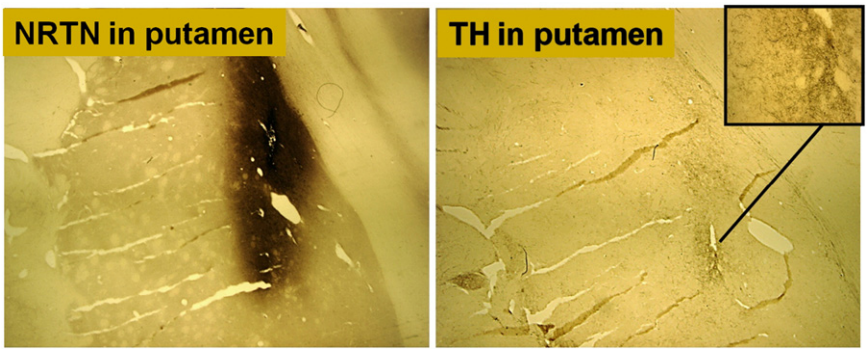

Fig. 5. Examples of neurturin expression (NRTN; left panel) and tyrosine hydroxylase induction (TH; right panel) in the Parkinson's putamen following CERE-120 administration. Similar data exists for patients treated 1 month to 4 years post-dosing, and following death from unrelated causes. These data provided the first evidence that vector-mediated gene transfer can be used to express a neurotrophic factor in human brain (left panel) and this, in turn, enhances degenerating nigrostriatal neurons (right panel). However, the response observed was relatively weak and inconsistent compared to that achieved in animal models. This is most likely due to relatively small levels of NRTN achieved in the soma of degenerating neurons following expression in the terminal fields, adding further support for the idea that a serious deficiency in axonal transport exists in PD and other chronic neurodegenerative diseases, thus blunting the biological response to the NRTN expressed only in putamen.
Data adapted from Bartus et al., 2011.

At the same time, despite the NRTN expression in the targeted putamen, evidence for NRTN accumulation *was sparse in the substantia nigra* pars compacta (SNc — where the degenerating cell bodies originate). This observation was unexpected, based on dogma supported by decades of independent research with neurotrophic factors and animal models around the globe, as well as many, more recent studies directly with CERE-120; (see Bartus, et al., 2011b). The fact that NRTN expression in the targeted terminal fields (in the putamen) is not mirrored by corresponding NRTN in the neuronal cell bodies (in the substantia nigra) reveals a fundamental difference in the function of degenerating nigrostriatal neurons in advanced PD, versus conventional animal models. This difference suggests a serious deficiency in retrograde axonal transport in advanced PD. The hypothesis that NRTN and/or CERE-120 is not adequately transported from the dopamine terminals to the cell bodies is consistent with an emerging literature that long axons die back and suffer deficiencies in axonal transport as an early pathogenic event in a number of neurodegenerative diseases, including PD (Braak et al., 1999; De Vos et al., 2008; Morfini et al., 2009; Raff et al., 2002; Roy et al., 2005) (for a more detailed discussion, see Bartus et al., 2011b).

Based on these observations, we concluded that to gain the maximum benefit of CERE-120 in advanced PD, delivery will have to be expanded to include targeting the degenerating cell bodies, as well as the terminal field. We further expect that this will prove true for any other neurotrophic factor or vector expressing a neurotrophic factor intended to restore neuronal function and status in most human neurodegenerative diseases. Consistent with the general idea of impaired transport of NRTN to the cell bodies, though we observed induction of tyrosine hydroxylase in the terminal fields of the putamen of the PD autopsy cases, it was far less extensive and reliable than that observed in prior primate studies with CERE-120 following equivalent NRTN expression in the putamen (Bartus et al., 2011b). Moreover, no clear evidence for enhanced TH was seen in the nigra cell bodies of the PD autopsy cases, arguing that higher levels of NRTN are required in the substantia nigra cell bodies to achieve the desired and expected neurotrophic responses, and the low levels achieved were likely insufficient to produce a satisfactory clinical response. Thus, further dose-optimization, specifically involving direct targeting of nigra cell bodies with CERE-120 is required and was implemented as part of a new Phase 1/2b protocol.

Performing due diligence on the concept of targeting the substantia nigra with CERE-120 to enhance the bioactivity on NRTN in PD subjects

Prior to implementing the novel dosing paradigm of targeting the substantia nigra in patients, we initiated a 'risk:benefit assessment' to help identify and evaluate potential risks, relative to expected benefit to patients. Three general areas of potential risk were evaluated: (1) stereotactic surgery; (2) exposure of SN neurons (and their afferents and efferents) to NRTN; and (3) exposure of neurons far outside the SN due to mistargeted NRTN. This analysis was performed by senior medical and scientific staff at Ceregene, in close collaboration with a number of key opinion leaders and consultants, involving several neurosurgeons, noted neurologists, several neuroscientists and psychiatrists familiar with mid-brain dopamine function as well as Ceregene's SAB. The overwhelming consensus was that the plan to target the substantia nigra was logical and scientifically justified, that any of the risks identified were merely hypothetical, had a low probability of occurring and could likely be mitigated if they did occur. Thus, an overwhelming, near-unanimous consensus existed that the potential benefits far outweighed any hypothetical risks. This position was later also widely endorsed by the Parkinson's disease neurology and neurosurgical community, as evidenced by the number and quality of excellent sites that enthusiastically agreed to participate in the clinical trials. Additionally, a competitive LEAPS (Linked Efforts to Accelerate Parkinson's Solutions) award was granted by the MJFF (Michael J Fox Foundation for Parkinson's Research) to help defray some of the costs of an eventual Phase 2b trial, providing further independent support for the approach and proposed strategy.

Additional nonclinical studies prior to new dosing approach

Prior to implementing this novel dosing paradigm in humans, we conducted eight additional nonclinical experiments to evaluate the feasibility, safety and effectiveness of targeting the substantia nigra (SN) with AAV2-NRTN (Bartus et al., 2011a) and to better understand and appraise recent warnings of serious weight loss that might occur with targeting the SN with neurotrophic factors (Manfredsson et al., 2009a, 2009b; Su et al., 2009). Finally, we also sought to define an appropriate dose of AAV2-NRTN that should safely and effectively cover the SN in PD patients.

As an initial experiment, we determined SN-volume for rats, monkeys and humans. We then employed these values to calculate comparable dose-equivalents for each species by scaling each dose, based on relative SN volume. Using this information, we then injected AAV2-GFP to monkey SN to quantify AAV2-vector distribution and confirm our calculations that it provided reasonable SN coverage. Building on these data, we then selected a ~200-fold-range of AAV2-NRTN doses (and a single AAV2-GDNF dose) and administered them to rat SN. This produced an intentionally wide range of protein expression to evaluate the consequence of NRTN protein in, around and distant from the targeted SN.

In contrast to recent warnings regarding nigra targeting of neurotrophic factors (Manfredsson et al., 2009a, 2009b; Su et al., 2009), no dose produced any serious side effects or toxicity, though we replicated the modest reduction-in-weight-gain reported by others with AAV-GDNF, via our highest AAV2-NRTN and the AAV2-GDNF dose, only. A dose-related increase in NRTN expression was seen, with the lower doses limiting NRTN to the peri-SN and the highest dose producing mistargeted-NRTN well-outside the SN. Using differences in location of NRTN expression throughout the midbrain and diencephalon across the range of doses, and correlating with either the presence or absence of changes in weight, we were able to demonstrate that the reduction-in-weight-gain following excessive-doses can clearly be dissociated from NRTN in the targeted SN, and is linked to mistargeted NRTN in the diencephalon. Moreover, we further demonstrated that prior destruction of the dopaminergic SN neurons via 6-OHDA had no impact on the weight loss phenomenon, further dissociating neurotrophic exposure to SN dopamine neurons as the culprit for weight changes. We also showed that more than 50% of the effect on weight can be easily mitigated with simple dietary supplementation of preferred foods to rats, providing further confidence that the low risk of an effect on weight in PD patients could likely be mitigated with appropriate instructions and dietary intervention. Finally, we demonstrated that relatively low AAV2-NRTN doses provided significant neuroprotection against 6-OHDA toxicity (compared to the very high doses that had no toxicity), establishing a wide therapeutic-index for nigral targeting of CERE-120.

Thus, despite the warnings that serious safety issues were likely to occur if the SN is targeted with GFLs, and the intense controversy that followed, a careful empirical evaluation of the concept established that the concerns are largely unfounded for: (1) massive doses would be required, far in excess of anything that one might imagine giving to humans, once properly scaled doses are calculated and considered; (2) even with significant mistargeted protein in mid-brain, not a single side effect of any type could be detected, unless the mistargeted protein spreads to the distant diencephalon; and (3) even with massive doses leading to wildly mistargeted protein in diencephalon, the only side effect observed was modest reduction in food intake, which correlated with similarly modest reduction in weight, both of which could be largely mitigated by dietary supplementation of preferred foods.

Collectively, these data provided direct empirical support for the idea of targeting the SN with AAV2-NRTN in PD patients, demonstrating that properly targeted and scaled AAV2-NRTN provides safe and effective NRTN expressions. They also provided the means to define an

Table 9
Clinical history and status of CERE-120.

Study (protocol no.)	Year started–completed	# Subjects	# Treated: CERE-120	Other details	Information gained
Phase 1 (CERE-120-01)	2005–06	12	12	2 Sites; open-label	Initial safety
Phase 2a (CERE-120-02)	2006–08	58	40	9 Sites; controlled, double-blinded	Additional safety; initial info re: efficacy (mixed); initial proof of concept established
Revised Phase 1 (CERE-120-09)	2009–10	6	6	3 Sites; open-label	Safety for nigra targeting; larger dose
Revised Phase 2b (CERE-120-09)	2010–13[a]	51	~25	11 Sites; controlled, double-blinded	Additional safety data; additional efficacy information
Long-term follow-up (CERE-120-03)	2006–ongoing	100+	~85	Follow-up for 5 years, post-surgery (controls and CERE-120)	Long-term safety and general PD well-being

[a] Dosing completed; assessments continuing until early 2013.

appropriate human-equivalent dose for proceeding into the planned clinical trial, using empirically-based scaling to account for marked differences in SN volume between species.

Dose-target optimized Phase 1/2b protocol: maximizing the effects of CERE-120

A new 'dose-target' optimized Phase 1/2b protocol (CERE-120-09) was developed in 2009, based on the insight gained from the initial Phase 2a proof of concept data, the related autopsy evaluation, and the results of the nonclinical studies evaluating the effects of CERE-120 targeted to the SN. Also invaluable were the feedback and enthusiastic support received from a host of advisors and consultants, the principle investigators and neurosurgeons for the clinical sites we contacted and others less directly involved with the program, such as the MJFF.

Several significant protocol improvements were implemented in an attempt to increase the rate and magnitude of a neurotrophic response to CERE-120, while also improving our ability to measure clinical benefit in a controlled trial. These changes include adding a dose of CERE-120 directly into the substantia nigra (as briefly described; see Fig. 6), increasing the dose 4-fold to the putamen (justified on the basis of the additional clinical and nonclinical safety data generated with CERE-120 since the program was launched), employing a longer evaluation period (from 12 months to at least 15 and up to 24 months, depending on the amount of time in the protocol since the subject was dosed), and instituting several protocol changes intended to reduce the placebo response as well as increase our sensitivity and power to detect a treatment effect.

The Phase 1 safety component of this study was initiated in late 2009 and successfully completed in mid-2010. It involved two dose cohorts (the first using the same putaminal dose as in the prior Phase 2a trial and adding the SN dose; the second retaining that SN dose but increasing the putaminal dose by 4-fold). No serious or unexpected safety issues were noted (including no change in weight of any subject), providing the evidence to justify advancing to the Phase 2b double-blind, efficacy/safety component.

The sham-surgery, double-blind, Phase 2b efficacy/safety component of the protocol was powered at greater than 90% to see a difference between sham and CERE-120 on the motor-off (part 3) UPDRS (Unified Parkinson's Disease Rating Scale), with half of the 50 subjects receiving treatment and the other half receiving a sham surgical procedure. In addition to the efforts at Ceregene, the trial was conducted at eleven (11) of the leading movement disorder medical centers in the United States (University of California — San Francisco, Emory University, Baylor University, Duke University, University of Alabama — Birmingham, Stanford University, Columbia University, University of Pennsylvania, Rush University, Beth Israel — NYC and Mt Sinai Medical Center — NYC). It was launched near the end of 2010, with enrollment and dosing completed in November 2011. Top-line data is anticipated in early 2013.

Synopsis of program

Over 20 nonclinical studies and 3 clinical protocols have now been successfully completed with CERE-120 (Tables 5 and 9). To date, no safety issues have been identified, despite dosing over 80 Parkinson's patients up to 5+ years ago and literally hundreds of animals, at dose multiple many times higher than required for efficacy. Indeed, when

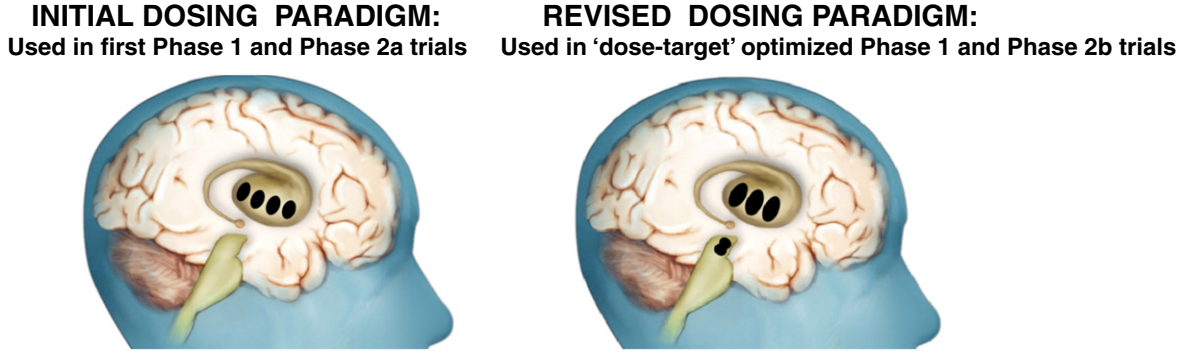

Fig. 6. Comparison of two different dosing schemes used in CERE-120 clinical trials. Left: 4 separate needle tracts were used to distribute CERE-120 into the putamen (only) of each hemisphere in the initial Phase 1 and Phase 2a studies. Right: 3 separate needle tracts were used to distribute a 4-times higher dose into the putamen of each hemisphere, as well as an additional needle tract delivering CERE-120 directly to the substantia nigra of each hemisphere in the revised, 'dose-target' optimized Phase 1 and Phase 2b trials. These and other protocol changes were implemented based on insight gained in the initial 'first-in-human' controlled clinical trial of gene transfer combined with a neurotrophic factor. All were intended to maximize the probability of eliciting and detecting a meaningful difference between the treated and control subjects in the current CERE-120 Phase 2b multi-center, sham-surgery controlled trial.

one compares the safety record for CERE-120, relative to the collective safety record for similar surgically-mediated interventional PD trials, such as fetal and other tissue transplantations, as well as infusions of GDNF using indwelling hardware, it is clear that the incidence of probable, treatment-related SAEs is far higher in the latter efforts. Thus, ironically, the use of *gene transfer* to improve the function of degenerating nigrostriatal neurons (e.g., by delivering neurotrophic factors) can now be considered a *far safer approach* than the alternative methods used previously or currently. CERE-120 not only continues to provide safe, long-term, persistent expressions of NRTN restricted to the targeted brain regions, but also provides evidence of long-term, persistent neurotrophic activity.

Data from a past controlled trial in Parkinson's patients provided proof of concept support for CERE-120's clinical benefit, with several relevant motor and quality of life endpoints showing statistically significant and clinically meaningful improvements in blinded comparisons to sham treatment. Analysis of autopsy tissue qualitatively demonstrated the desired biological effects of CERE-120 in Parkinson's disease brain, further supporting the clinical proof of concept. However, an analysis of the clinical and autopsy data suggested that the effect was not yet optimized and required an adjustment of the dosing paradigm (directly targeting the substantia nigra). Thus, we sought to build upon what we had learned from the initial 'first-in-humans' Phase 1 and Phase 2 trials and better optimize the potential therapeutic effects of NRTN while also improving the ability of the protocol to detect a potential treatment effect. As stated in an independent editorial in Movement Disorders: "As one of the few therapies immediately available that may not only slow PD progression, but also improve outcomes, we feel that the potential benefits of a clinically successful CERE-120 treatment cannot be ignored" (Lewis and Standaert, 2011). While the outcome of any single clinical trial is uncertain and history warns us that disappointing results can occur for a wide variety of reasons (including some having nothing to do with whether a treatment actually works), it is hoped that, minimally, the insight gained and information shared with the scientific/medical community during the course of developing CERE-120 will eventually prove valuable to others. Thus, in the worse case, the 'clinical proof of concept' data generated for CERE-120 and the data, anecdotes and lessons accumulated along the way that are shared herein, as well as in other publications, may prove helpful to others as they continue the quest to develop neurotrophic factor treatments for PD and other neurodegenerative diseases, as well as integrate gene transfer into more common translational activities.

Disclosure statement

The author is an officer of Ceregene, Inc. (San Diego, CA), the company developing AAV-NRTN (CERE-120) as a treatment for Parkinson's disease. He is paid a salary and has been awarded stock options by the company.

Acknowledgments

Many people contributed to the body of work outlined in this perspective review and the author recognizes that far less would have been achieved without the help, support and advice they provided. First, the author gratefully acknowledges the role of Jeffrey Ostrove (President and CEO of Ceregene), as we developed gene transfer as an enabling technology to deliver neurotrophic factors to the brain for Parkinson's disease (via CERE-120) and other serious, poorly treated neurodegenerative diseases. Jeff contributed invaluable talent and experience to the development of Ceregene's gene transfer programs, having studied AAV as part of his PhD and later spending years leading the manufacturing and process development activities of a commercial facility. Thus, he brought immediate experience and sophistication in cGMP manufacturing of viral vectors to Ceregene, which positively influenced CERE-120's initial design, process and the establishment of many controls, which in turn contributed directly to the excellent safety record CERE-120 would eventually achieve.

Special recognition is also owed to Dr. Stephen Sherwin, who had previously co-founded and served as CEO of Cell Genesys for years (before it merged with BioSante Pharmaceuticals) and who truly launched Ceregene as a corporate entity. While Mark Tuszynski and Jeffrey Kordower are generally recognized as the early 'scientific founders' of Ceregene, given their appreciation that gene transfer might provide the means for safe and effective delivery of neurotrophic factors to the brain, it was Dr. Sherwin who took the nascent 'corporate concept' and contributed badly needed translational sophistication and business savvy to give the company the initial credibility required to recruit quality people while also securing the bulk of the company's initial funding. Just as importantly, he recognized the need to substantially increase the IP (intellectual property or patent portfolio) far beyond that established by the scientific founders. In keeping with this requirement, Ceregene licensed multiple patents from Cell Genesys and other parties, covering a wide array of essential technology, including everything needed to manufacture viral vectors for therapeutic purposes. Thus, Dr. Sherwin enabled Ceregene to acquire the crucial tools as well as the necessary resources to make a serious run at using gene transfer to deliver neurotrophic factors as therapeutics, and for this reason, many consider him to be the 'corporate founder' of Ceregene.

Similarly, the contributions and advice from Ceregene's entire SAB (Scientific Advisory Board) are gratefully acknowledged, including those of Inder Verma (a pioneer in the gene therapy, oncology and other fields), Rusty Gage (an early pioneer in neurotrophic factors, gene therapy, stem cells and general neurodegeneration-related research), Gene Johnson (co-discoverer of neurturin and other neurotrophic factors and scholar on issues related to novel treatments for PD), Jude Samulski (an early and continuing pioneer on the use AAV as an important vector for gene therapy), Warren Olanow (an internationally-recognized movement disorder specialist), Leon Thal (a world-renowned Alzheimer's neurologist), Jeffrey Kordower (a widely-recognized leader in animal models and neurotrophic factors and long-time friend and collaborator) and Mark Tuszynski (a leader in use of NGF and gene therapy for the cholinergic system). In particular, the advice and support of Jeffrey Kordower and Warren Olanow for PD-specific issues early in the program, and Gene Johnson, Rusty Gage and Inder Verma for numerous other gene transfer and neuroscience-related issues deserve special acknowledgement related to the development of CERE-120. Additionally, other colleagues who have been particularly helpful include Anthony Lang, Nicholas Boulis, Ron Alterman, Joao Siffert, David Barba, Mehdi Gasmi, Dominick Vacante, Kathy Bishop and Chris Herzog. Also, special recognition is given to all the scientific and clinical personnel at Ceregene who made substantial contributions to the CERE-120 program, as often evidenced by their role as co-authors in the numerous publications emanating from the program. In this regard, Tiffany Baumann deserves special recognition for major contributions to clinical operations and strategic decisions since the initial Phase 1 CERE-120 trial, while also providing invaluable regulatory support to the author over the past several years. Sincere thanks and appreciation are also extended to the excellent neurology and neurosurgical personnel at the sites participating in the CERE-120 trials. Without the help and involvement of all these friends and colleagues, CERE-120 would not have reached its current stage of development.

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their continued interest, support (financial and otherwise) during the course of the several clinical trials conducted with CERE-120, as well as the many courageous people with Parkinson's disease who volunteer to participate in experimental drug trials as well as agree to donate their brains so that much more might be learned and therefore others might benefit from their efforts.

References

Aebischer, P., et al., 1996. Intrathecal delivery of CNTF using encapsulated genetically modified xenogeneic cells in amyotrophic lateral sclerosis patients. Nat. Med. 2, 696–699.

Apfel, S.C., 2002. Nerve growth factor for the treatment of diabetic neuropathy: what went wrong, what went right, and what does the future hold? Int. Rev. Neurobiol. 50, 393–413.

Apfel, S.C., et al., 1998. Recombinant human nerve growth factor in the treatment of diabetic polyneuropathy NGF Study Group Neurology 51, 695–702.

Apfel, S.C., et al., 2000. Efficacy and safety of recombinant human nerve growth factor in patients with diabetic polyneuropathy: a randomized controlled trial. rhNGF Clinical Investigator Group JAMA 284, 2215–2221.

Apkarian, A.V., et al., 2004. Chronic back pain is associated with decreased prefrontal and thalamic gray matter density. J. Neurosci. 24, 10410–10415.

Azmitia, E.C., et al., 1988. Use of tissue culture models to study neuronal regulatory trophic and toxic factors in the aged brain. Neurobiol. Aging 9, 743–758.

Backman, C., et al., 1996. Systemic administration of a nerve growth factor conjugate reverses age-related cognitive dysfunction and prevents cholinergic neuron atrophy. J. Neurosci. 16, 5437–5442.

Backman, C., et al., 1997. Carrier mediated delivery of NGF: alterations in basal forebrain neurons in aged rats revealed using antibodies against low and high affinity NGF receptors. J. Comp. Neurol. 387, 1–11.

Bankiewicz, K.S., et al., 2006. Long-term clinical improvement in MPTP-lesioned primates after gene therapy with AAV-hAADC. Mol. Ther. 14, 564–570.

Bartus, R.T., 1988. The need for common perspectives in the development and use of animal models for age-related cognitive and neurodegenerative disorders. Neurobiol. Aging. 9, 445–451.

Bartus, R.T., 1989a. Neurotrophic factors: can the degenerating brain be induced to heal itself? Neurobiol. Aging. 10, 513.

Bartus, R.T., 1989b. Drug delivery to the brain: the problem lurking behind the problem? Neurobiol. Aging. 10, 621.

Bartus, R.T., 1997. The Calpain hypothesis of neurodegeneration: evidence for a common cytotoxic pathway. Neuroscientist 314–327.

Bartus, R.T., 1999. The blood–brain barrier as a target for pharmacological modulation. Curr. Opin. Drug Discov. Devel. 2, 152–167.

Bartus, R.T., 2000. On neurodegenerative diseases, models, and treatment strategies: lessons learned and lessons forgotten a generation following the cholinergic hypothesis. Exp. Neurol. 163, 495–529.

Bartus, R.T., Dean III, R.L., 2009. Pharmaceutical treatment for cognitive deficits in Alzheimer's disease and other neurodegenerative conditions: exploring new territory using traditional tools and established maps. Psychopharmacology (Berl) 202, 15–36.

Bartus, R.T., et al., 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science 217, 408–414.

Bartus, R.T., et al., 1983. Behavioral and pharmacological studies using animal models of aging: implications for studying and treating dementia of Alzheimer's type. Banbury Report 1515: Biological Aspects of Alzheimer's Disease. Cold Spring Harbor Laboratory, pp. 207–218.

Bartus, R.T., et al., 1996a. Dissociation of p75 receptors and nerve growth factor neurotrophic effects: lack of p75 immunoreactivity in striatum following physical trauma, excitotoxicity and NGF administration. Restor. Neurol. Neurosci. 10, 49–59.

Bartus, R.T., et al., 1996b. Permeability of the blood brain barrier by the bradykinin agonist, RMP-7: evidence for a sensitive, auto-regulated, receptor-mediated system. Immunopharmacology 33, 270–278.

Bartus, R.T., et al., 1996c. Controlled modulation of BBB permeability using the bradykinin agonist, RMP-7. Exp. Neurol. 142, 14–28.

Bartus, R.T., et al., 1998. Sustained delivery of proteins for novel therapeutic agents. Science 281, 1161–1162.

Bartus, R., et al., 1999. Use of Cereport (RMP-7) to increase delivery of carboplatin to gliomas: insight and parameters for intracarotid infusion via a single-lumen cannula. Drug Del. 6, 15–21.

Bartus, R.T., et al., 2000. Evidence that Cereport's ability to increase permeability of rat gliomas is dependent upon extent of tumor growth: implications for treating newly emerging tumor colonies. Exp. Neurol. 161, 234–244.

Bartus, R.T., et al., 2003. Vivitrex, an injectable, extended-release formulation of naltrexone, provides pharmacokinetic and pharmacodynamic evidence of efficacy for 1 month in rats. Neuropsychopharmacology 28, 1973–1982.

Bartus, R.T., et al., 2004. A pulmonary formulation of L-dopa enhances its effectiveness in a rat model of Parkinson's disease. J. Pharmacol. Exp. Ther. 310, 828–835.

Bartus, R.T., et al., 2007. Issues regarding gene therapy products for Parkinson's disease: the development of CERE-120 (AAV-NTN) as one reference point. Parkinsonism Relat. Disord. 13 (Suppl. 3), S469–S477.

Bartus, R.T., et al., 2011a. Properly scaled and targeted AAV-NRTN (neurturin) to the substantia nigra is safe, effective and causes no weight loss: support for nigral targeting in Parkinson's disease. Neurobiol. Dis. 44, 38–52.

Bartus, R.T., et al., 2011b. Bioactivity of AAV2-neurturin gene therapy (CERE-120): differences between Parkinson's disease and nonhuman primate brains. Mov. Disord. 26, 27–36.

Benabid, A.L., 2010. Reflection and reaction: gene therapy for Parkinson's disease: do we have the cure? Lancet Neurol. 9, 1142–1143.

Bensadoun, J.C., et al., 2000. Lentiviral vectors as a gene delivery system in the mouse midbrain: cellular and behavioral improvements in a 6-OHDA model of Parkinson's disease using GDNF. Exp. Neurol. 164, 15–24.

Bjorklund, A., et al., 2000. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res. 886, 82–98.

Bohn, M.C., 1999. A commentary on glial cell line-derived neurotrophic factor (GDNF). From a glial secreted molecule to gene therapy. Biochem. Pharmacol. 57, 135–142.

Borg, J., et al., 1990. Peripheral administration of a long-chain fatty alcohol promotes septal cholinergic neurons survival after fimbria–fornix transection. Brain Res. 518, 295–298.

Braak, H., et al., 1999. Extensive axonal Lewy neurites in Parkinson's disease: a novel pathological feature revealed by alpha-synuclein immunocytochemistry. Neurosci. Lett. 265, 67–69.

Calcedo, R., et al., 2009. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J. Infect. Dis. 199, 381–390.

Charles, V., et al., 1996. Atrophy of cholinergic basal forebrain neurons following excitotoxic cortical lesions is reversed by intravenous administration of an NGF conjugate. Brain Res. 728, 193–203.

Choi-Lundberg, D.L., et al., 1998. Behavioral and cellular protection of rat dopaminergic neurons by an adenoviral vector encoding glial cell line-derived neurotrophic factor. Exp. Neurol. 154, 261–275.

Day-Lollini, P.A., et al., 1997. Hyperplastic changes within the leptomeninges of the rat and monkey in response to chronic intracerebroventricular infusion of nerve growth factor. Exp. Neurol. 145, 24–37.

De Vos, K.J., et al., 2008. Role of axonal transport in neurodegenerative diseases. Annu. Rev. Neurosci. 31, 151–173.

Deuschl, G., et al., 2006. Deep brain stimulation: postoperative issues. Mov. Disord. 21 (Suppl. 14), S219–S237.

Elliott, P.J., et al., 1996. Intravenous RMP-7 selectively increases uptake of carboplatin into rat brain tumors. Cancer Res. 56, 3998–4005.

Emerich, D.F., et al., 2001. The development of the bradykinin agonist labradimil as a means to increase the permeability of the blood–brain barrier: from concept to clinical evaluation. Clin. Pharmacokinet. 40, 105–123.

Eriksdotter Jonhagen, M., et al., 1998. Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. Dement. Geriatr. Cogn. Disord. 9, 246–257.

Fischer, W., et al., 1987. Amelioration of cholinergic neuron atrophy and spatial memory impairment in aged rats by nerve growth factor. Nature 329, 65–68.

Friden, P.M., et al., 1993. Blood–brain barrier penetration and in vivo activity of an NGF conjugate. Science 259, 373–377.

Gash, D.M., et al., 1995. Morphological and functional effects of intranigrally administered GDNF in normal rhesus monkeys. J. Comp. Neurol. 363, 345–358.

Gash, D.M., et al., 1996. Functional recovery in parkinsonian monkeys treated with GDNF. Nature 380, 252–255.

Gasmi, M., et al., 2012. Needle assembly for use in delivering precise dosages of proteinaceous pharmaceutical compositions and methods for use of same. US Patent # 8092429 B2 Jan 10, 2012.

Gasmi, M., et al., 2007a. AAV2-mediated delivery of human neurturin to the rat nigrostriatal system: long-term efficacy and tolerability of CERE-120 for Parkinson's disease. Neurobiol. Dis. 27, 67–76.

Gasmi, M., et al., 2007b. Striatal delivery of neurturin by CERE-120, an AAV2 vector for the treatment of dopaminergic neuron degeneration in Parkinson's disease. Mol. Ther. 15, 62–68.

Georgievska, B., et al., 2004. Overexpression of glial cell line-derived neurotrophic factor using a lentiviral vector induces time- and dose-dependent downregulation of tyrosine hydroxylase in the intact nigrostriatal dopamine system. J. Neurosci. 24, 6437–6445.

Gill, S.S., et al., 2003. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. Nat. Med. 9, 589–595.

Hadaczek, P., et al., 2010. Eight years of clinical improvement in MPTP-lesioned primates after gene therapy with AAV2-hAADC. Mol. Ther. 18, 1458–1461.

Hefti, F., et al., 1989. Function of neurotrophic factors in the adult and aging brain and their possible use in the treatment of neurodegenerative diseases. Neurobiol. Aging. 10, 515–533.

Herzog, C.D., et al., 2007. Striatal delivery of CERE-120, an AAV2 vector encoding human neurturin, enhances activity of the dopaminergic nigrostriatal system in aged monkeys. Mov. Disord. 22, 1124–1132.

Herzog, C.D., et al., 2008. Transgene expression, bioactivity, and safety of CERE-120 (AAV2-neurturin) following delivery to the monkey striatum. Mol. Ther. 16, 1737–1744.

Herzog, C.D., et al., 2009. Expression, bioactivity, and safety 1 year after adeno-associated viral vector type 2-mediated delivery of neurturin to the monkey nigrostriatal system support CERE-120 for Parkinson's disease. Neurosurgery 64, 602–612.

Herzog, C.D., et al., 2011. Gene transfer provides a practical means for safe, long-term, targeted delivery of biologically-active neurotrophic factor proteins for neurodegenerative diseases. Drug Deliv. Transl. Res. 1, 361–382.

Hoffer, B., et al., 1994. Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. Neurosci. Lett. 182, 107–111.

Hovland Jr., D.N., et al., 2007. Six-month continuous intraputamenal infusion toxicity study of recombinant methionyl human glial cell line-derived neurotrophic factor (r-metHuGDNF in rhesus monkeys). Toxicol. Pathol. 35, 676–692.

Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677–736.

Inamura, T., et al., 1994. Intracarotid infusion of RMP-7, a bradykinin analog: a method for selective drug delivery to brain tumors. J. Neurosurg. 81, 752–758.
Jacobson, K.A., et al., 1992. Synthesis and biological activity of N6-(p-sulfophenyl)alkyl and N6-sulfoalkyl derivatives of adenosine: water-soluble and peripherally selective adenosine agonists. J. Med. Chem. 35, 4143–4149.
Jeste, D.V., Lohr, J.B., 1989. Hippocampal pathologic findings in schizophrenia. A morphometric study. Arch. Gen. Psychiatry 46, 1019–1024.
Jonhagen, M.E., et al., 1998. Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. Dement. Geriatr. Cogn. Disord. 9, 246–257.
Kirik, D., et al., 2000a. Preservation of a functional nigrostriatal dopamine pathway by GDNF in the intrastriatal 6-OHDA lesion model depends on the site of administration of the trophic factor. Eur. J. Neurosci. 12, 3871–3882.
Kirik, D., et al., 2000b. Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J. Neurosci. 20, 4686–4700.
Kohn, D.B., et al., 2003. Occurrence of leukaemia following gene therapy of X-linked SCID. Nat. Rev. Cancer 3, 477–488.
Kordower, J.H., et al., 1988. Nerve growth factor receptor immunoreactivity in the non-human primate (*Cebus apella*): distribution, morphology, and colocalization with cholinergic enzymes. J. Comp. Neurol. 277, 465–486.
Kordower, J.H., et al., 1994. Intravenous administration of a transferrin receptor antibody-nerve growth factor conjugate prevents the degeneration of cholinergic striatal neurons in a model of Huntington disease. Proc. Natl. Acad. Sci. U. S. A. 91, 9077–9080.
Kordower, J.H., et al., 1999. Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. Ann. Neurol. 46, 419–424.
Kordower, J.H., et al., 2000. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290, 767–773.
Kordower, J.H., et al., 2006. Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. Ann. Neurol. 60, 706–715.
Kotzbauer, P.T., et al., 1996. Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Lett. Nat. 384, 467–470.
Lang, A.E., et al., 2006. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann. Neurol. 59, 459–466.
LaVail, M.M., et al., 1998. Protection of mouse photoreceptors by survival factors in retinal degenerations. Invest. Ophthalmol. Vis. Sci. 39, 592–602.
Lewis, T.B., Standaert, D.G., 2011. Parkinson's disease, primates, and gene therapy: vive la difference? Mov. Disord. 26, 2–3.
Li, Z., et al., 1993. Peptide alpha-keto ester, alpha-keto amide, and alpha-keto acid inhibitors of calpains and other cysteine proteases. J. Med. Chem. 36, 3472–3480.
Maillard, M.C., et al., 1994. Adenosine receptor prodrugs: synthesis and biological activity of derivatives of potent, A1-selective agonists. J. Pharm. Sci. 83, 46–53.
Manfredsson, F.P., et al., 2009a. Tight Long-term dynamic doxycycline responsive nigrostriatal GDNF using a single rAAV vector. Mol. Ther. 17, 1857–1867.
Manfredsson, F.P., et al., 2009b. Nigrostriatal rAAV-mediated GDNF overexpression induces robust weight loss in a rat model of age-related obesity. Mol. Ther. 17, 980–991.
Marks Jr., W.J., et al., 2008. Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. Lancet Neurol. 7, 400–408.
Marks Jr., W.J., et al., 2010. Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomised, controlled trial. Lancet Neurol. 9, 1164–1172.
McCown, T.J., 2011. Adeno-associated virus (AAV) vectors in the CNS. Curr. Gene Ther. 11, 181–188.
McGrath, J., et al., 2002. Adeno-associated viral delivery of GDNF promotes recovery of dopaminergic phenotype following a unilateral 6-hydroxydopamine lesion. Cell Transplant. 11, 215–227.
McMahon, S.B., 1996. NGF as a mediator of inflammatory pain. Philos. Trans. R. Soc. Lond. B, Biol. Sci. 351, 431–440.
Miller, R.G., et al., 1996. A placebo-controlled trial of recombinant human ciliary neurotrophic (rhCNTF) factor in amyotrophic lateral sclerosis rhCNTF ALS Study Group Ann. Neurol. 39, 256–260.
Morfini, G.A., et al., 2009. Axonal transport defects in neurodegenerative diseases. J. Neurosci. 29, 12776–12786.
Morrison, P.F., et al., 2007. Convective delivery of glial cell line-derived neurotrophic factor in the human putamen. J. Neurosurg. 107, 74–83.
Mufson, E.J., et al., 1999. Distribution and retrograde transport of trophic factors in the central nervous system: functional implications for the treatment of neurodegenerative diseases. Prog. Neurobiol. 57, 451–484.
Nauta, H.J., et al., 1999. Intraventricular infusion of nerve growth factor as the cause of sympathetic fiber sprouting in sensory ganglia. J. Neurosurg. 91, 447–453.
Nutt, J.G., et al., 2003. Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. Neurology 60, 69–73.
Overstreet, D.H., et al., 1999. Crews FT. Suppression of alcohol intake by chronic Naloxone treatment in P rats: tolerance development and elevation of opiate receptor binding. Alcohol. Clin. Exp. Res. 23 (11), 1761–1771.
Pardridge, W.M., et al., 1991. Selective transport of an anti-transferrin receptor antibody through the blood–brain barrier in vivo. J. Pharmacol. Exp. Ther. 259, 66–70.
Penn, R.D., et al., 1997. Intrathecal ciliary neurotrophic factor delivery for treatment of amyotrophic lateral sclerosis (phase I trial). Neurosurgery 40, 94–99 discussion 99–100.
Pezet, S., McMahon, S.B., 2006. Neurotrophins: mediators and modulators of pain. Annu. Rev. Neurosci. 29, 507–538.
Raff, M.C., et al., 2002. Axonal self-destruction and neurodegeneration. Science 296, 868–871.
Raper, S.E., et al., 2003. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab. 80, 148–158.
Rosenblad, C., et al., 2003. Long-term striatal overexpression of GDNF selectively downregulates tyrosine hydroxylase in the intact nigrostriatal dopamine system. Eur. J. Neurosci. 17, 260–270.
Roy, S., et al., 2005. Axonal transport defects: a common theme in neurodegenerative diseases. Acta Neuropathol. 109, 5–13.
Salvatore, M.F., et al., 2006. Point source concentration of GDNF may explain failure of phase II clinical trial. Exp. Neurol. 202, 497–505.
Salzberg-Brenhouse, H.C., et al., 2003. Inhibitors of cyclooxygenase-2, but not cyclooxygenase-1 provide structural and functional protection against quinolinic-acid induced neurodegeneration. J. Pharmacol. Exp. Ther. 306 (1), 218–228.
Saporito, M.S., et al., 2002. Discovery of CEP-1347/KT-7515, an inhibitor of the JNK/SAPK pathway for the treatment of neurodegenerative diseases. Prog. Med. Chem. 40, 23–62.
Seiger, A., et al., 1993. Intracranial infusion of purified nerve growth factor to an Alzheimer patient: the first attempt of a possible future treatment strategy. Behav. Brain Res. 57, 255–261.
Sheline, Y.I., et al., 1999. Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. J. Neurosci. 19, 5034–5043.
Sherer, T.B., et al., 2006. Crossroads in GDNF therapy for Parkinson's disease. Mov. Disord. 21, 136–141.
Slevin, J.T., et al., 2005. Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. J. Neurosurg. 102, 216–222.
Snider, W.D., Johnson Jr., E.M., 1989. Neurotrophic molecules. Ann. Neurol. 26, 489–506.
Sofroniew, M.V., et al., 2001. Nerve growth factor signaling, neuroprotection, and neural repair. Annu. Rev. Neurosci. 24, 1217–1281.
Su, X., et al., 2009. Safety evaluation of AAV2-GDNF gene transfer into the dopaminergic nigrostriatal pathway in aged and parkinsonian rhesus monkeys. Hum. Gene Ther. 20, 1627–1640.
Tuszynski, M.H., et al., 1996. Gene therapy in the adult primate brain: intraparenchymal grafts of cells genetically modified to produce nerve growth factor prevent cholinergic neuronal degeneration. Gene Ther. 3, 305–314.
Tuszynski, M.H., et al., 2005. A phase I clinical trial of nerve growth factor gene therapy for Alzheimer disease. Nat. Med. 1–5.
Wang, L.H., Johnson Jr., E.M., 2008. Mixed lineage kinase inhibitor CEP-1347 fails to delay disability in early Parkinson disease. Neurology 71, 462 author reply 462–3.
Wellmer, A., et al., 2001. A double-blind placebo-controlled clinical trial of recombinant human brain-derived neurotrophic factor (rhBDNF) in diabetic polyneuropathy. J. Peripher. Nerv. Syst. 6, 204–210.