

Therapeutic potential of CERE-110 (AAV2-NGF): Targeted, stable, and sustained NGF delivery and trophic activity on rodent basal forebrain cholinergic neurons

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ABSTRACT

Treatment of degenerating basal forebrain cholinergic neurons with nerve growth factor (NGF) in Alzheimer's disease has long been contemplated, but an effective and safe delivery method has been lacking. Towards achieving this goal, we are currently developing CERE-110, an adeno-associated virus-based gene delivery vector that encodes for human NGF, for stereotactic surgical delivery to the human nucleus basalis of Meynert. Results indicate that NGF transgene delivery to the targeted brain region via CERE-110 is reliable and accurate, that NGF transgene distribution can be controlled by altering CERE-110 dose, and that it is possible to achieve restricted NGF expression limited to but covering the target brain region. Results from animals examined at longer time periods of 3, 6, 9 and 12 months after CERE-110 delivery indicate that NGF transgene expression is stable and sustained at all time points, with no loss or build-up of protein over the long-term. In addition, results from a series of experiments indicate that CERE-110 is neuroprotective and neurorestorative to basal forebrain cholinergic neurons in the rat fimbria–fornix lesion and aged rat models, and has bioactive effects on young rat basal forebrain cholinergic neurons. These findings, as well as those from several additional non-clinical experiments conducted in both rats and monkeys, led to the initiation of a Phase I clinical study to evaluate the safety and efficacy of CERE-110 in Alzheimer's disease subjects, which is currently ongoing.

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Introduction

Protecting and restoring the basal forebrain cholinergic neurons (BFCNs) of the nucleus basalis of Meynert (NBM) is a logical approach to treating mild to moderate Alzheimer's disease. Although the etiology of Alzheimer's disease is largely unknown, three primary neuro-anatomical features characterize the Alzheimer's diseased brain: amyloid plaques, neurofibrillary tangles, and the loss of neurons. Severe cholinergic neuronal death occurs in the NBM of the basal forebrain in Alzheimer's disease (Whitehouse et al., 1981, 1982; Coyle et al., 1983; Lyness et al., 2003), and cholinergic loss in Alzheimer's disease correlates significantly with severity of dementia and synapse loss (Perry et al., 1978a,b; Bierer et al., 1995). Cholinergic blockade in humans and monkeys impairs cognition in ways that are qualitatively similar to cognitive dysfunction associated with mild to moderate Alzheimer's disease (Bartus and Johnson, 1976; Robbins et al., 1997; Taffe et al., 1999, 2002), and modest pharmacological augmentation of cholinergic function improves Alzheimer's disease symptoms (Birks, 2006; Burns and O'Brien, 2006; Hansen et al., 2007). In addition, cholinergic axonal projections of BFCNs regulate neuronal activity in

the cortex and hippocampus, which places BFCNs in a unique position to influence a diverse array of executive functions (Bucci et al., 1998; Kilgard and Merzenich, 1998; Baxter and Chiba, 1999; Furey et al., 2000).

Treatment of basal forebrain cholinergic degeneration in Alzheimer's disease with NGF was proposed many years ago (e.g. Chen et al., 1989; Phelps et al., 1989; Hefti and Schneider, 1991; Hefti et al., 1996). In animal models, NGF prevents the death of BFCNs after axonal injury (Williams et al., 1986; Hefti, 1986; Rosenberg et al., 1988; Koliatsos et al., 1990, 1991a,b; Tuszynski et al., 1990; Tuszynski and Gage, 1995), reverses their spontaneous age-related atrophy (Chen and Gage, 1995; Lindner et al., 1996; Smith et al., 1999; Conner et al., 2001), and improves learning and memory in lesioned and aged rats (Fischer et al., 1987, 1991; Williams et al., 1991; Markowska et al., 1994; Tuszynski and Gage, 1995; Chen and Gage 1995; Martinez-Serrano et al., 1996). In Alzheimer's disease, NGF levels are reduced in the BFCNs of the NBM (Mufson et al., 1995; Scott et al., 1995). At the same time, NGF levels in the natural target of BFCN axons, the cortex, have generally been found to be elevated in Alzheimer's disease brains (Crutcher et al., 1993; Mufson et al., 1995; Scott et al., 1995; Hellweg et al., 1998; Fahnstock et al., 2001; Peng et al., 2004). This suggests retrograde axonal transport of NGF from the cortex to the BFCN somata is defective in Alzheimer's disease (Mufson et al., 1995, 1999; Scott et al., 1995). Supporting this concept, defective retrograde

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transport of NGF by BFCNs has been demonstrated in a mouse model of Alzheimer's pathology (Cooper et al., 2001; Salehi et al., 2003, 2006). Therefore, it is possible that circumventing this putative retrograde transport defect by administering NGF directly to BFCNs could both prevent loss of BFCNs and augment function of remaining BFCNs in Alzheimer's disease (i.e. Hellweg et al., 1990).

While administration of NGF to BFCNs could be an effective treatment for Alzheimer's disease, a safe and effective means of accurately delivering NGF to BFCNs has been lacking. NGF needs to be continuously administered since non clinical studies have found that when NGF is withdrawn its effects are not maintained (Montero and Hefti, 1988; Niewiadomska et al., 2002). NGF protein does not readily cross the blood-brain barrier when administered systemically (Lapchak et al., 1993) and therefore must be administered directly to the brain in order to be most effective. Because NGF administration needs to bypass the blood-brain barrier, and be continuous, an initial clinical trial of NGF for Alzheimer's disease tested continuous infusion of NGF into the cerebral ventricles. Unfortunately, adverse side effects arose in subjects in that trial, and the trial was halted without observation of substantial benefits (Olson et al., 1992; Eriksson et al., 1998). Subsequent non clinical research has revealed that neuroanatomical changes occur in response to the broad distribution of NGF in the cerebrospinal fluid (CSF) following intracerebroventricular administration, and that these changes are related to the physiological side effects induced by the action of NGF on non-target cells. Importantly, it has also been established that both the neuroanatomical changes and the physiological side effects can be avoided by direct administration of NGF into the brain parenchyma (Olson et al., 1991; Day-Lollini et al., 1997; Winkler et al., 1997; Pizzo et al., 2002). Thus, effective delivery of NGF requires both consistent exposure of the protein to the targeted NBM, while avoiding non-targeted exposure to other brain regions.

To this end, gene transfer may be the most effective and practical delivery method available, in that it can provide controlled and sustained delivery of a therapeutic protein such as NGF to a targeted brain region following a single surgical procedure, without the complications of indwelling hardware. In support of this concept, a Phase I clinical study of *ex vivo* gene transfer (autologous fibroblasts transfected with a retroviral vector to express NGF) has shown none of the adverse effects associated with off-target NGF delivery, and suggests potential effects on brain metabolism and, possibly, cognition (Tuszynski et al., 2005). However, the *ex vivo* gene transfer approach is limited by a decline in NGF protein expression from the cells over 18 months post-implantation, while manufacturing complexities and costs make this approach impractical for application to larger numbers of patients. Therefore, CERE-110, a genetically engineered, replication defective adeno-associated virus serotype 2 (AAV2) vector that contains the full-length human β -nerve growth factor (NGF) cDNA, is currently being developed for the delivery of NGF for Alzheimer's disease. The AAV2 vector was chosen because it preferentially transduces neurons, delivers the transgene predominantly as non-integrated DNA (thereby reducing the possibility of insertional mutagenesis), and results in long-lasting gene expression following a single administration to the brain parenchyma. A series of non clinical experiments were performed to: (1) examine NGF transgene kinetics and pattern of expression, (2) determine the dose-response relationship between CERE-110 and resulting NGF expression, and (3) test the hypothesis that NGF delivered via CERE-110 provides the expected trophic activity to NBM basal forebrain cholinergic neurons. Findings from these studies, described herein, support accurate, reliable targeting and control of NGF transgene expression, sustained and stable transgene expression over long time periods, bioactivity of NGF expressed from the viral vector, and efficacy in the rodent models of BFCN degeneration relevant to AD. Results from these studies provide support for a Phase I clinical study of CERE-110 in subjects with mild to moderate AD, which is currently ongoing (Arvanitakis et al., 2007).

Materials and methods

Vector plasmid constructs

A prototype vector, AAV2-NGF-wPRE, was used for the fimbria-fornix lesion experiment. The wPRE element was subsequently removed from the vector due to potential safety concerns (for example, see Kingsman et al., 2005) and this resulting vector, called CERE-110, was used in all other experiments. The AAV2-NGF-wPRE vector genome contains the AAV2 inverted terminal repeats (ITRs) flanking a transgene expression cassette containing the CAG promoter (Niwa et al., 1991), the human NGF cDNA, the woodchuck hepatitis post-transcriptional regulatory element (wPRE) (Donello et al., 1998) and the human growth hormone gene (hGH) polyadenylation signal (polyA) (Stratagene). To generate the AAV2-NGF-wPRE plasmid, the CAG-NGF-wPRE cassette (Blesch et al., 2005) was isolated by restriction digest and cloned into the pAAV-MCS (Stratagene) plasmid. The CERE-110 expression cassette was created by removing the wPRE from AAV2-NGF-wPRE. In addition, the ampicillin selection cassette was replaced by the kanamycin selection cassette. Restriction digestions and nucleotide sequence determination confirmed plasmid integrity.

Cell culture and vector production

Unless otherwise specified, cells were cultured in phenol red and antibiotic-free Iscove's Modified Dulbecco's Medium (IMDM)-5% FBS supplemented with 4 mM L-Glutamine (Irvine Scientific) at 37 °C in 5% CO₂. Both vectors were produced by overnight triple plasmid calcium phosphate transfection of subconfluent 293 cells using an equimolar cocktail of the following plasmids: a vector genome plasmid, an AAV2 *rep/cap* plasmid, and an adenovirus helper plasmid, which encodes adenoviral genes necessary for AAV2 particle production. Medium was replaced the following morning, and 2 to 3 days post-transfection cells were harvested and lysed by mechanical disruption in a deoxycholate containing buffer to release vector particles. Cellular DNA, RNA and residual plasmid DNA were then digested with 100 U/mL of Benzonase (Merck) for 3 h at 37 °C. AAV2-NGF-wPRE vector was purified by filtration and affinity (heparin) chromatography followed by dialysis in an isotonic saline formulation buffer (FB; 2 mM MgCl₂ in PBS) using a Slide-A-Lyzer 10,000 MWCO (Pierce). CERE-110 vector was purified by filtration, affinity (heparin) and ion exchange chromatography. CERE-110 vector particles were then subjected to centrifugal filtration on 50 KDa filter units (Millipore) and concentrated into FB. Upon concentration the bulk product was sterilized-filtered on a 0.2 μ m filter unit and then filled into 0.5 mL polypropylene cryovials. Vector titers were determined by dot blot or quantitative PCR using specific primers and are expressed in vector genomes (vg).

Experimental subjects

One hundred and thirty young male Sprague Dawley rats and twenty-one 21-month old male Fischer 344 rats (Harlan) were used in these studies. Rats were provided food and water ad libitum and maintained on a 12-hour light/dark cycle. All experiments were conducted in accordance with the guidelines of the Office of Laboratory Animal Welfare and the Ceregene, Inc. Institutional Animal Care and Use Committee.

Experimental details

For all surgeries, rats were anesthetized with a mixture consisting of xylazine (3.25 mg/kg), acepromazine (0.62 mg/kg) and ketamine (62.5 mg/kg), and placed in a stereotaxic frame (Stoelting). The skull was exposed and burr holes made with a Dremel drill above the injection sites. Vector or formulation buffer (FB) control was delivered via a 10 μ L Hamilton syringe attached to a 26 gauge beveled stainless steel needle. Injections were made at a rate of 0.5 μ L/min, via an

injection pump (Stoelting). Following each injection, the needle was left at the injection site for 1 min, retracted 1–3 mm, and then held in place an additional minute, followed by removal from the brain. Details of the injection parameters for each experiment (i.e. target brain structure, stereotaxic injection coordinates, injection volume, injection dose) are summarized in Table 1.

Fimbria–fornix lesion model experiment

In the fimbria–fornix lesion model experiment, 4 male Sprague Dawley rats were injected with 2 μ L of AAV-NGF-wPRE (5.2×10^9 vg) in the right hemisphere medial septum. Nine days later, an ipsilateral aspirative lesion of the fimbria–fornix was performed on vector injected animals as well as 4 control (lesion-only) animals. At 2 weeks post-lesion, all animals were sacrificed for histological analysis.

CERE-110 dose–range testing experiment

In the dose–range testing experiment, 38 male Sprague Dawley rats were injected with a range of doses of CERE-110 or FB control in the NBM. Animals for NGF immunohistochemical analysis were injected in the right hemisphere NBM with one of the following CERE-110 doses: 1.8×10^8 vg, 2.7×10^8 vg, 5.3×10^8 vg, 1.1×10^9 vg, 1.8×10^9 vg, or 5.3×10^9 vg ($n=3$ animals/dose). Animals for NGF ELISA analysis were injected in the NBM bilaterally with one of the following CERE-110 doses: 8.8×10^7 vg, 1.8×10^8 vg, 2.7×10^8 vg, 3.5×10^8 vg, 5.3×10^8 vg, 8.8×10^8 vg, 1.1×10^9 vg, 1.8×10^9 vg, 2.7×10^9 vg, 3.5×10^9 vg, 5.3×10^9 vg, or 1.1×10^{10} vg ($n=3$ NBM/dose). Two additional animals were injected bilaterally with 1 μ L of FB control. All animals were sacrificed at 2 weeks post-injection for NGF analysis by immunohistochemistry or ELISA.

Aged rat model experiment

Twenty-one 21-month old male Fischer 344 rats were injected with 1×10^8 vg CERE-110 (11 animals) or FB control (10 animals) in the right hemisphere NBM. During the course of the study, 4 animals were found dead and 1 animal was euthanized 2 days prematurely due to his moribund condition. Four of the 5 animals had evidence of neoplasia and 1 animal had evidence of chronic renal failure. Both of these are common causes of spontaneous death in aged rats. Of the 5 animals that died or were sacrificed prematurely, 2 were in the formulation buffer control group and 3 were in the CERE-110 treated group. Mortality rates were not significantly different between treatment and control groups (2/10 vs. 3/11, X^2 test, $p>0.05$). After a 3-month post-injection period, all remaining animals were sacrificed for histological analysis.

Long-term experiment

A total of 84 young male Sprague Dawley rats were injected with FB control or one of 2 different doses of CERE-110 (1×10^8 or 2×10^9 vg

per NBM) bilaterally in the NBM. Six animals per group were sacrificed at 3 and 6 months post-injection and 8 animals per group were sacrificed at 9 and 12 months post-injection for histological analysis. One animal in the 12-month FB control group died prematurely, due to spontaneously occurring pulmonary carcinoma.

Histology

Perfusion and tissue processing for immunohistochemistry

Animals were overdosed with an anesthetic cocktail and transcardially perfused with ice-cold 0.9% saline followed by 2% paraformaldehyde (PFA) with 0.2% parabenzoquinone (PBQ). Brains were removed, post-fixed for 2 h in 2% PFA with 0.2% PBQ, and cryoprotected in 30% sucrose at 4 °C. Brains were coronally sectioned on a sliding microtome at 40 μ m and sections stored in cryoprotectant at –20 °C.

Immunohistochemistry

Immunohistochemistry was performed on separate 1-in-6 series of sections using antibodies raised against NGF (rabbit anti-NGF, used at 1:1000, a gift from Dr. J. Conner, UCSD, La Jolla, CA) or ChAT (goat polyclonal anti-ChAT, used at 1:500 Chemicon). Free-floating sections through the forebrain were blocked with 5% horse serum in TBS/0.25% Triton X-100 for 1–2 h and then incubated overnight at 4 °C with the primary antibody. This was followed by incubation with biotinylated secondary antibody (donkey anti-rabbit, used at 1:500, Jackson ImmunoResearch, for NGF and horse anti-goat, used at 1:333, Vector Laboratories, for ChAT) for 3 h at room temperature. Sections were visualized with avidin-biotinylated peroxidase complex procedure (Vector Laboratories) using 3,3-diaminobenzidine (DAB) as the chromogen. After sections were mounted onto glass slides they were dehydrated and coverslipped with DPX mounting media.

Nissl staining and AChE histochemistry

Successful lesion of the fimbria–fornix pathway was confirmed by examination of cresyl violet stained sections through the lesioned area and by loss of acetylcholinesterase (AChE) staining in the medial septum cholinergic neuron target region, the hippocampus. Cresyl violet stain was performed by immersing sections in 0.2% cresyl violet solution followed by dehydration with ethanol and xylene and coverslipping with DPX. For AChE histochemistry, sections were incubated in a solution containing 24% sodium sulfate (Na_2SO_4), 0.15% glycine, 0.002% copper sulfate (CuSO_4), 0.12% acetylthiocholine iodide and 0.00037% tetraisopropylpyrophosphoramidate (Iso-OMPA; Sigma) in a 0.05 M maleate buffer overnight at 37 °C. After rinsing with 20% Na_2SO_4 followed by 10% Na_2SO_4 , staining was visualized using 4% $(\text{NH}_4)_2\text{S}$ for 1 min. Sections were then rinsed with distilled water and incubated in 10% formalin for 20 min. Slides were dehydrated and coverslipped with DPX.

Quantitation of NGF by ELISA

At scheduled sacrifice, brains were collected fresh and flash-frozen on dry ice. Three 1-mm thick hemi-coronal slices centered on the injection site were collected from each hemisphere. Each slice was homogenized in a buffer (phosphate buffer, pH 7.0, 400 mM NaCl, 0.1% Triton X-100, 5 mM EDTA and 0.5% BSA) at a tissue concentration of 10 μ L of buffer per 1 mg tissue. A protease inhibitor cocktail (Sigma) was added at 1 μ L per 20 mg tissue. Quantification of NGF was performed using the NGF Emax Immunoassay System kit (Promega), which is specific for NGF, exhibiting typically less than 3% cross-reactivity with other neurotrophic factors (Promega, 2007). Each sample was analyzed in duplicate at two dilutions (1/20 and 1/50) and optical density was measured at 450 nm using an optical plate reader (Versamax, Molecular Devices). All groups were compared in the same assay to reduce the influence of interassay variances; measured NGF levels were uncorrected for recovery of NGF-spiked samples. Data

Table 1
Summary of injection parameters used in different experiments

Experiment	Target BFCN structure	Injection site coordinates			Injection volume (μ L)	AAV2-NGF dose per hemisphere (vg)
		AP ¹ (mm)	ML ¹ (mm)	DV (mm)		
Fimbria–fornix lesion model	MS	0.3	–0.7	–8.0 ¹	2	5.2×10^9
Dose range testing	NBM	–1.5	± 2.5	–8.0 ¹	0.5, 1 or 2	8.8×10^7 to 1.1×10^{10}
Aged rat model	NBM	–1.5	–2.5	–7.0 ²	1	1×10^8
Long-term young rat model	NBM	–2.5	–3.8	–6.8 ²	1	1×10^8 ; 2×10^9
		–1.5	± 2.5	–8.0 ¹	1	
		–2.5	± 3.8	–7.2 ¹	1	

BFCN = basal forebrain cholinergic neuron; MS = medial septum; NBM = nucleus basalis of Meynert; AP = anterior–posterior; ML = medial–lateral; DV = dorsal–ventral; vg = vector genomes.

¹ Measured from bregma.

² Relative to dura.

were analyzed using SOFTmax PRO 4.0 and the highest value of the three hemi-coronal sections from each hemisphere was reported.

Morphometric analysis

Using unbiased stereological techniques (optical fractionator, StereoInvestigator software v5.0, MicroBrightField Inc.) an estimation of the total number and size of ChAT-positive cells was determined on a 1-in-6 series of sections through the basal forebrain medial septum (fimbria–fornix lesion model experiment) or NBM (aged rat model and long-term experiments). Quantitative analyses were performed by an individual blind with respect to treatment group.

Statistical analysis

Statistical analyses were performed using SigmaStat v2.03 (SPSS). Preliminary analyses were performed to verify that the data were

normally distributed. Differences between groups were assessed using Student's *t*-test or analysis of variance (ANOVA) followed by Tukey multiple comparison post-hoc testing, when appropriate. A significant difference between groups was established if $p < 0.05$.

Results

AAV2-NGF prevents degeneration of BFCNs in the rat fimbria–fornix lesion model

Complete lesioning of the fimbria–fornix pathway was confirmed in all animals by examination of cresyl violet stained sections covering the lesion area and by loss of acetylcholinesterase staining in the axonal target region of the medial septal cholinergic neurons, the hippocampus (data not shown). Robust expression of NGF protein was observed in the medial septum of all AAV2-NGF injected animals, as determined by NGF immunohistochemistry (Fig. 1A). As shown in

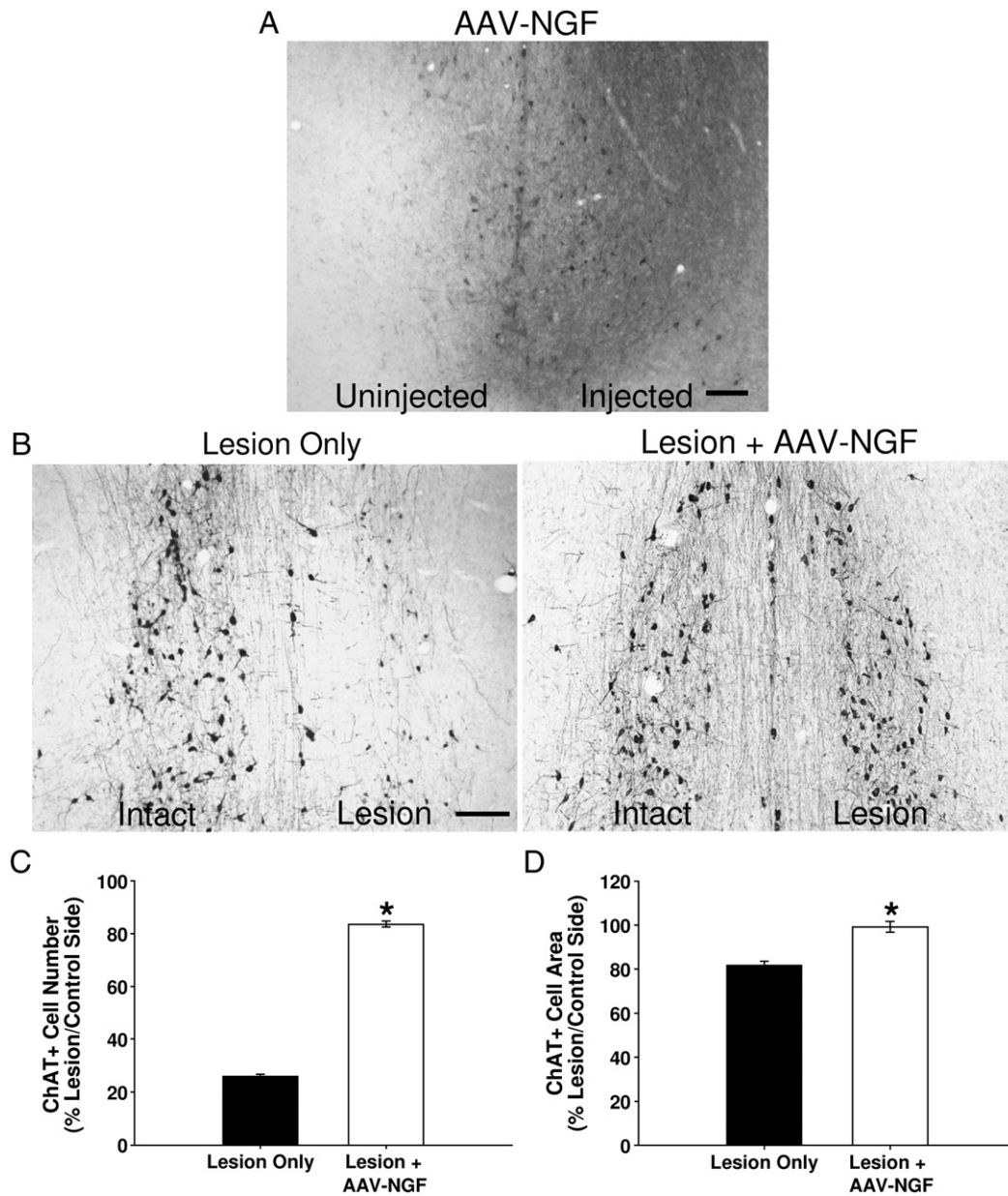


Fig. 1. AAV2-NGF is neuroprotective in the rat fimbria–fornix lesion model of basal forebrain cholinergic neuronal degeneration. (A) Robust NGF protein expression, as detected by NGF immunohistochemistry, is evident in the AAV2-NGF injected side of the medial septum. (B) Following unilateral lesion of the fimbria–fornix pathway of the rat brain, ChAT immunostained cells in the medial septum (MS) are shown in uninjected control and AAV2-NGF injected brains. Lesion and vector injection were performed on the right side. (C) The number of ChAT-positive medial septum neurons is significantly increased in AAV2-NGF injected brains compared to controls. (D) The cell area of ChAT-positive medial septum neurons is also significantly increased in AAV2-NGF injected brains compared to controls. Error bars represent standard error of the mean (SEM). * $p < 0.05$. Scale bars = 100 μ m.

Figs. 1B and C, in uninjected rats the lesion reduced the number of ChAT-positive cells in the medial septum to $26.1 \pm 0.5\%$ of the control (unlesioned) side, and diminished the size of remaining ChAT-positive cells to $81.9 \pm 1.6\%$ of control side (Fig. 1D). AAV2-NGF significantly prevented the loss of ChAT-positive BFCNs ($83.7 \pm 1.0\%$ of control side; $p < 0.05$ compared with lesion-only animals; Figs. 1B and C) and completely prevented the reduction in cell size ($99.2 \pm 2.4\%$ of control side; $p < 0.05$ compared with lesion-only animals; Fig. 1D). These results demonstrate that AAV2-mediated delivery is effective in providing biologically active NGF protein to BFCNs and yielded the characteristic trophic response, in a standard well-characterized model of cholinergic neuronal degeneration.

CERE-110 results in targeted and controlled NGF delivery to the NBM

Fig. 2A shows representative images of NGF immunohistochemistry, illustrating the resulting NGF protein expression in the NBM

from a range of CERE-110 doses. A noticeable increase in resulting NGF protein distribution was evident when the dose of CERE-110 was increased. In addition, a qualitative difference in the intensity of NGF immunohistochemical staining between CERE-110 dose groups was evident such that the intensity of staining increased, both intracellularly (closed arrows) and extracellularly (open arrows), as the dose of CERE-110 delivered to the NBM increased. Analysis of NGF immunohistochemistry over the range of doses tested also indicated that it was possible to achieve targeted and localized delivery of NGF to the rat NBM. Review of the NGF immunohistochemical data and the anatomy of the rat NBM indicated that a dose of 1×10^8 vg/NBM was optimal for providing as much exposure of the rat NBM to vector-derived NGF as possible without having significant diffusion of protein to brain regions distal to the NBM. This dose was operationally defined as the 'optimal' dose of CERE-110 for the rat NBM. Quantification of NGF protein levels by ELISA confirmed that the amount of NGF produced following CERE-110 administration could be controlled by

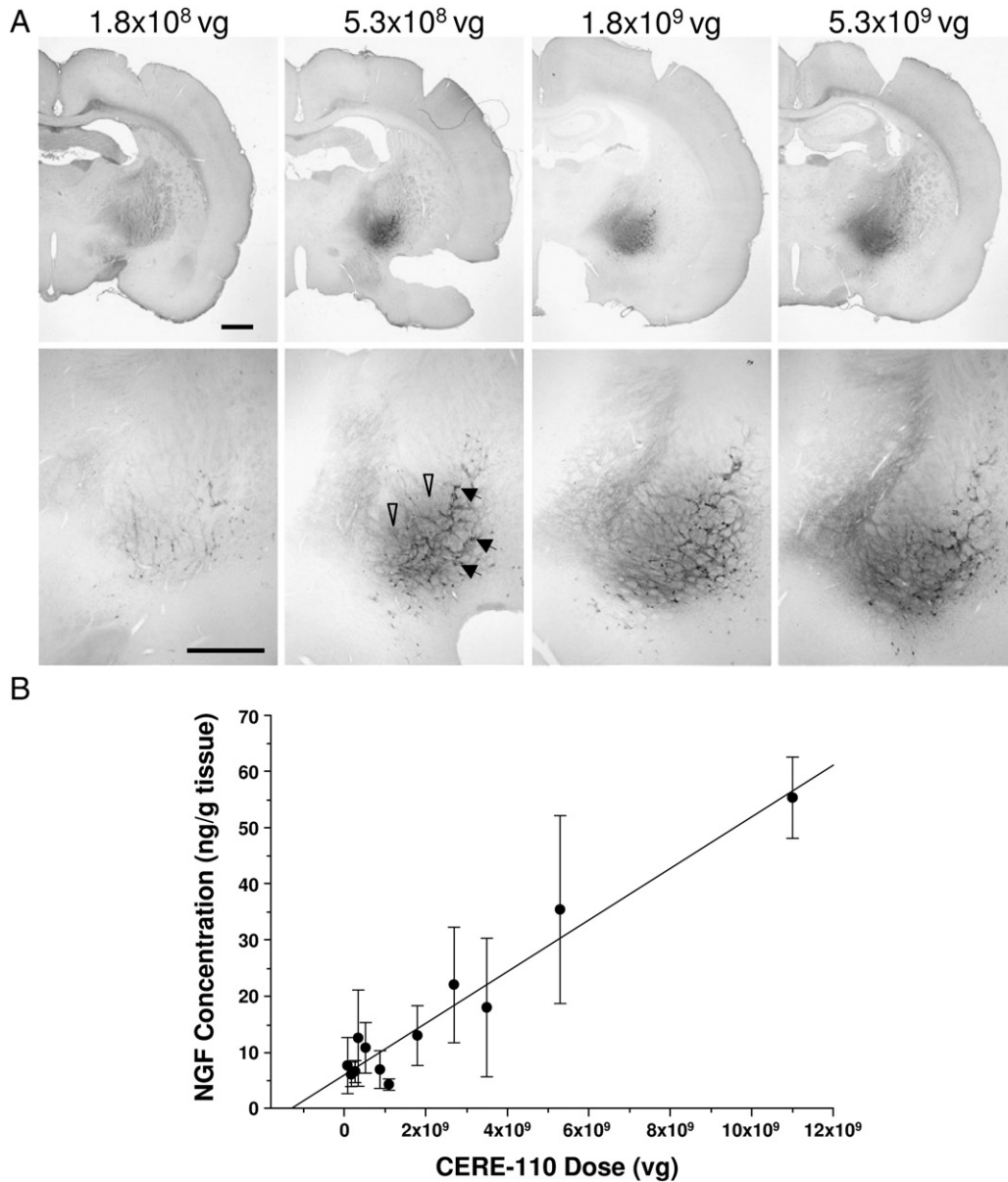


Fig. 2. Dose-related expression of NGF protein following CERE-110 delivery to the NBM of the young rat. (A) Immunohistochemical staining for NGF shows increased protein expression both intracellularly (closed arrows) and extracellularly (open arrows) with escalating doses of CERE-110 delivered to the NBM of the rat brain. Injection volume was held constant at $1 \mu\text{L}$ for all injections and CERE-110 vector dose was manipulated by varying vector concentration. Scale bar = 1 mm . As shown by ELISA (B), brain tissue NGF concentration is a function of the number of vector genomes of CERE-110 delivered to the NBM ($R^2 = 0.60$, $p < 0.001$). Data are expressed as ng NGF protein/gram wet brain weight; Error bars represent standard error of the mean (SEM). vg = vector genomes.

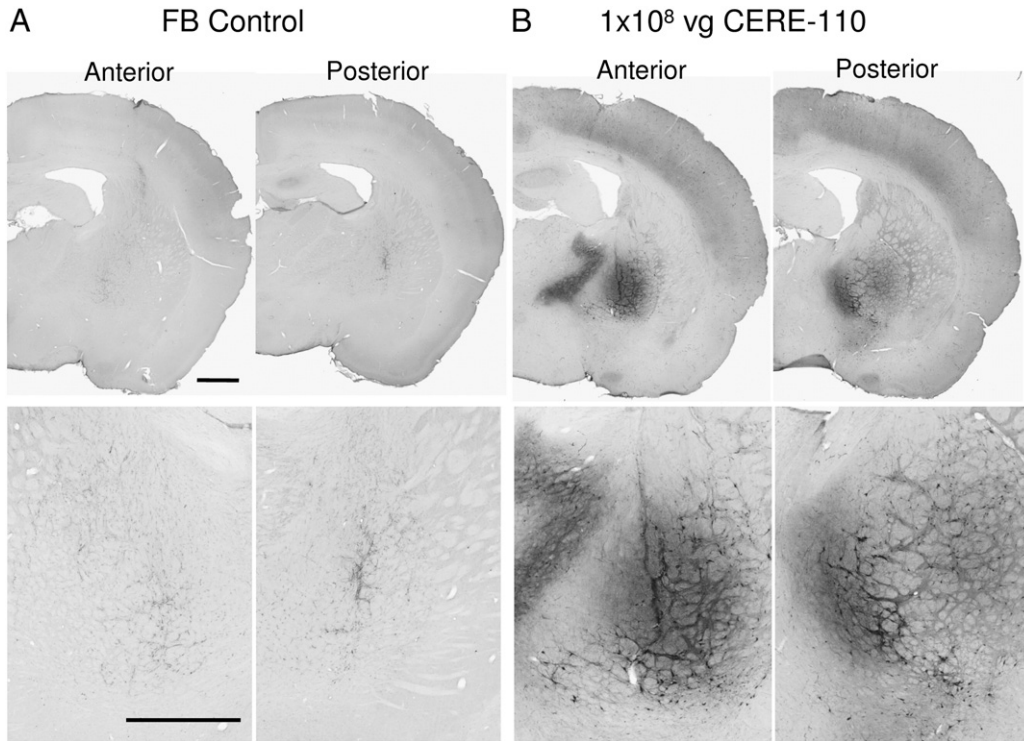


Fig. 3. NGF expression at 3 months following CERE-110 delivery to the NBM of the aged rat brain. NGF immunohistochemical signal in the FB control injected NBM (A) is similar in pattern and intensity to endogenous NGF expression normally seen in uninjected brains. In the CERE-110 injected aged rat brain (B), robust expression of NGF protein above endogenous levels is observed, providing NGF coverage of the target NBM and diffuse expression in the cortex, in the region of terminal fields of NBM neurons. Scale bar=1 mm. vg=vector genomes.

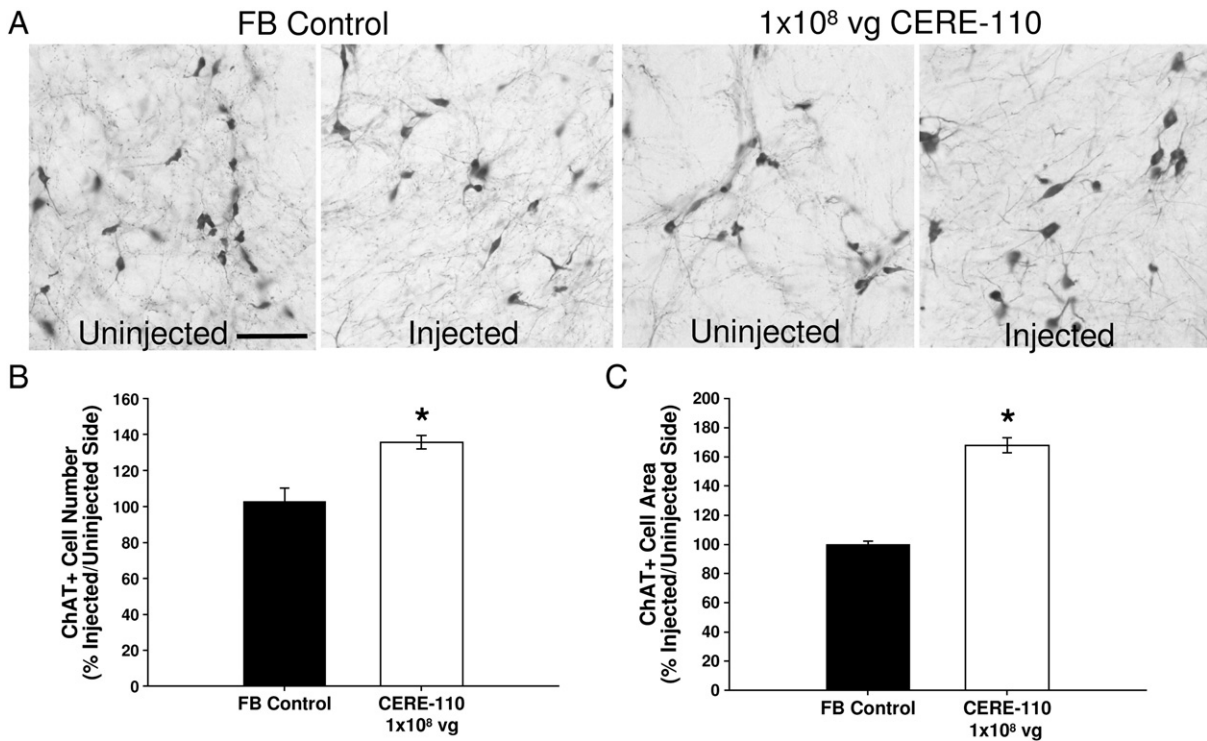


Fig. 4. Bioactivity of CERE-110 on NBM basal forebrain cholinergic neurons in the aged rat. (A) ChAT-positive immunostained cells in the NBM in control FB injected and CERE-110 injected aged rat brains. Injection of FB or CERE-110 was performed on the right side, while the left side remained uninjected for comparison purposes. (B) The number of aged rat NBM neurons positively stained for ChAT is significantly increased in CERE-110 injected brains compared to FB injected controls. (C) The cell area of aged rat NBM ChAT-immunopositive neurons is significantly increased in CERE-110 injected brains compared to FB injected controls. Error bars represent standard error of the mean (SEM). **p*<0.05. Scale bar=100 μ m. vg=vector genomes.

altering delivered vector dose (Fig. 2B). Regression analysis indicated that parenchymal brain NGF concentration was significantly positively correlated with the dose of CERE-110 injected ($R^2=0.60$, $p<0.001$). The defined optimal dose of CERE-110 (1×10^8 vg/NBM) corresponds to an NGF dose of approximately 6 ng/mg tissue, as measured by ELISA. Taken together, these results demonstrate that the amount and distribution of NGF protein delivered via CERE-110 is dependent upon the vector dose injected and that it is possible to achieve targeted NGF expression restricted to but covering the target NBM.

CERE-110 restores the cholinergic phenotype of BFCNs in the aged rat

The efficacy of CERE-110 was also tested in the aged (21-month old Fischer 344) rat. As determined from the rat dose–range testing study described above, the ‘optimal’ dose of CERE-110 (1×10^8 vg/NBM) was used. Injections of 1×10^8 vg CERE-110 into the NBM resulted in robust expression of NGF protein over the NBM target brain region, as assessed by NGF immunohistochemistry (Fig. 3B). As intended with this dose, NGF immunohistochemistry was generally confined to the target NBM and did not spread to the ventricular boundary, where NGF could potentially leak into the CSF. As shown in Fig. 4, in control rats, FB injection did not have a significant effect on the number or size of ChAT-positive cells in the NBM. In FB injected animals, ChAT-positive cell number was $102.7 \pm 7.7\%$ of the number on the uninjected (contralateral) side NBM, and cell size was $99.9 \pm 2.2\%$ of the uninjected side (Figs. 4B and C). In contrast, injection of CERE-110 resulted in trophic effects on NBM neurons, as measured by ChAT-positive cell number ($135.9 \pm 3.7\%$ of uninjected side; $p<0.001$ compared with FB injected; Figs. 4A and B) and ChAT-positive cell size ($167.9 \pm 5.1\%$ of uninjected side; $p<0.005$ compared with FB injected; Figs. 4A and C). These results demonstrate that CERE-110 is efficacious in providing sustained production of biologically active NGF and imparting significant trophic support to cholinergic neurons in the aged brain.

CERE-110 produces the characteristic hypertrophic response of BFCNs in the young rat

It has previously been shown that NGF protein delivery to the NBM results in hypertrophy of cholinergic neurons in young rodents (Pizzolo

et al., 2002). In order to test whether CERE-110 has similar effects and whether these effects persist over long time periods, an experiment was conducted in which CERE-110 was injected to the NBM in young, healthy rats and resulting transgene expression and potential cholinergic neuron hypertrophy assessed at 3, 6, 9 and 12 months post-administration. In FB control animals at 3, 6, 9 and 12 months, endogenous NGF expression was detected in the NBM and was not greater than NGF levels normally seen in uninjected rat NBM using this assay (data not shown). Substantial NGF expression above endogenous levels was detected in the NBMs of all CERE-110 injected animals at 3, 6, 9 and 12 months following CERE-110 administration (Fig. 5). The distribution and degree of immunohistochemical signal in high dose (2×10^9 vg/NBM) animals (Fig. 5, bottom panels) far exceeded that in ‘optimal’ dose (1×10^8 vg/NBM) animals (Fig. 5, top panels), and was evident in many of the natural targets of NBM cells (i.e., cortex, amygdala, thalamus). The extent of NGF immunohistochemical signal in optimal dose animals was largely confined to the NBM, with a small amount observed in the cortex, a natural target of NBM neurons (Fig. 5, top panels). NGF immunohistochemical signals were similar across all time points at both dose levels (Fig. 5), indicating that transgene expression was sustained and stable up to 1 year after CERE-110 delivery.

A consistent significant difference in cholinergic NBM cell size between FB control injected and CERE-110 injected groups was observed at all time points examined (Fig. 6B). A significant difference in cell size was observed at 3 months (means \pm SEM: FB control= $174 \pm 8 \mu\text{m}^2$; optimal dose CERE-110= $298 \pm 9 \mu\text{m}^2$; high dose CERE-110= $321 \pm 6 \mu\text{m}^2$; one-way ANOVA, $p<0.001$), 6 months (FB control= $178 \pm 8 \mu\text{m}^2$; optimal dose CERE-110= $307 \pm 8 \mu\text{m}^2$; high dose CERE-110= $320 \pm 13 \mu\text{m}^2$; one-way ANOVA, $p<0.001$), 9 months (FB control= $168 \pm 6 \mu\text{m}^2$; optimal dose CERE-110= $279 \pm 10 \mu\text{m}^2$; high dose CERE-110= $307 \pm 10 \mu\text{m}^2$; one-way ANOVA, $p<0.001$), and 12 months (FB control= $159 \pm 3 \mu\text{m}^2$; optimal dose CERE-110= $288 \pm 9 \mu\text{m}^2$; high dose CERE-110= $287 \pm 11 \mu\text{m}^2$; one-way ANOVA, $p<0.001$). Post-hoc analyses indicated a significant difference in cholinergic NBM cell size between FB control and CERE-110 high dose groups and FB control and CERE-110 optimal dose groups (Tukey least significant difference, $ps<0.001$) and no difference between CERE-110 optimal and high dose groups at all time points. At each of 3, 6, 9 or 12 months, no significant differences in cholinergic NBM cell

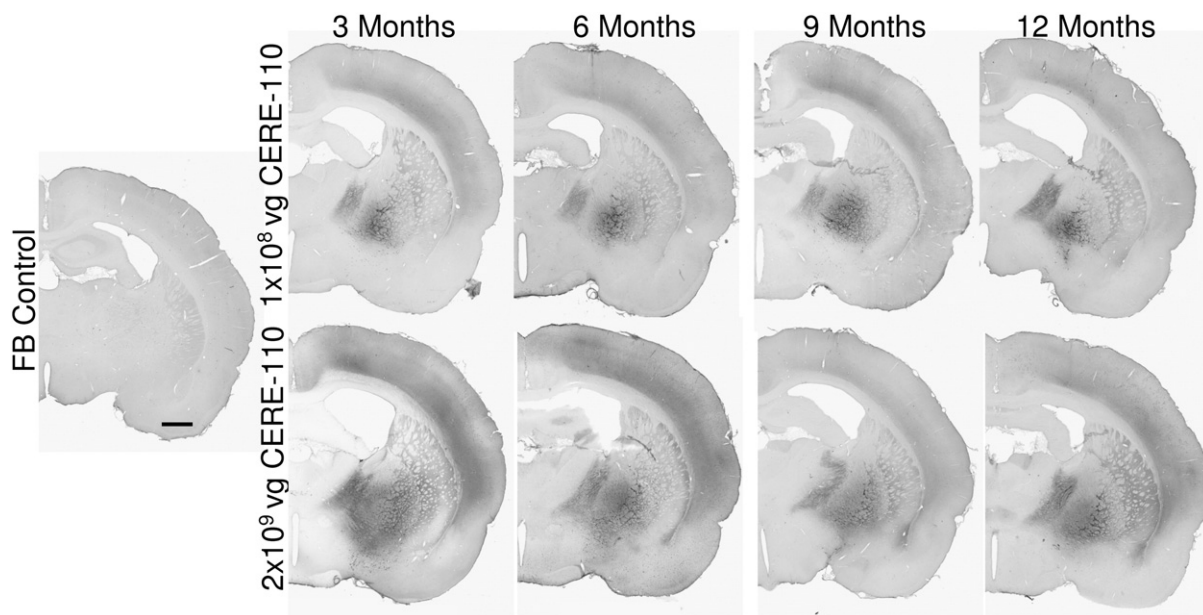


Fig. 5. NGF expression at 3, 6, 9 and 12 months following CERE-110 delivery to the NBM of the young rat. NGF immunostained cells and secreted protein is shown in (A) control formulation buffer (FB) injected rats at 3 months, (B) optimal dose (1×10^8 vg/NBM) CERE-110 injected rats at 3, 6, 9 and 12 months, and (C) high dose (2×10^9 vg/NBM) CERE-110 injected rats at 3, 6, 9 and 12 months (C). Scale bar = 1 mm. vg = vector genomes.

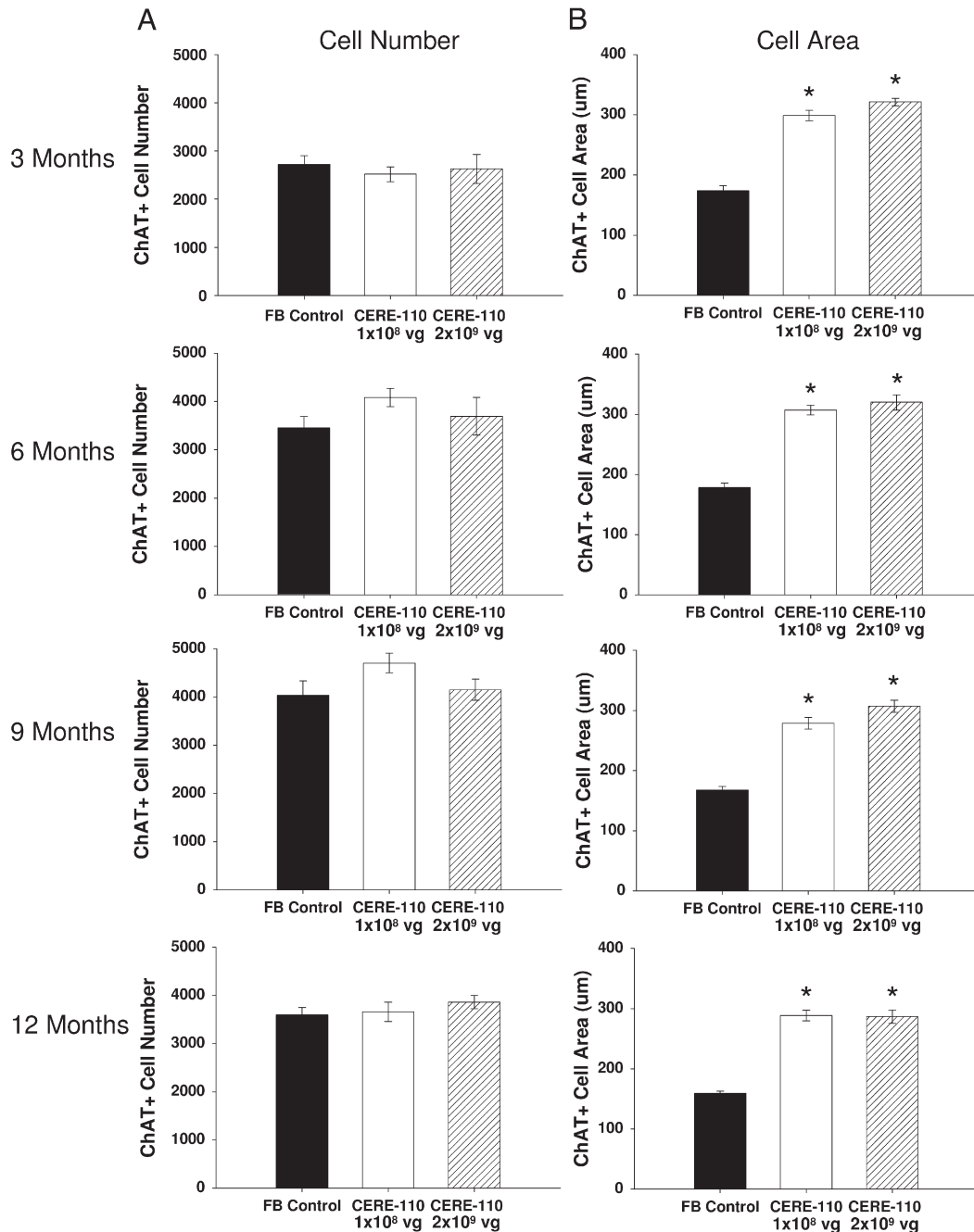


Fig. 6. Increased ChAT-positive cell area but no change in cell number following CERE-110 delivery to the NBM of the young rat. (A) NBM ChAT-positive cell number at 3 months, 6 months, 9 months, and 12 months following delivery of formulation buffer control and optimal and high dose CERE-110 to the NBM. (B) NBM ChAT-positive cross-sectional cell area at 3 months, 6 months, 9 months and 12 months following FB control and optimal and high dose CERE-110 delivery to the NBM. Error bars represent standard error of the mean (SEM). *Significantly different compared to FB control ($p < 0.05$).

number were observed between groups ($p > 0.05$; Fig. 6A). NBM cholinergic cell number was equivalent between groups at 3 months (means \pm SEM: FB control = 2721 ± 177 ; optimal dose CERE-110 = 2515 ± 152 ; high dose CERE-110 = 2624 ± 301 ; one-way ANOVA, $p > 0.05$), 6 months, (FB control = 3455 ± 237 ; optimal dose CERE-110 = 4081 ± 189 ; high dose CERE-110 = 3696 ± 390 ; one-way ANOVA, $p > 0.05$), 9 months (FB control = 4037 ± 292 ; optimal dose CERE-110 = 4704 ± 201 ; high dose CERE-110 = 4148 ± 218 ; one-way ANOVA, $p > 0.05$), and 12 months (FB control = 3596 ± 168 ; optimal dose CERE-110 = 3663 ± 215 ; high dose CERE-110 = 3863 ± 176 ; one-way ANOVA, $p > 0.05$). Thus, CERE-110 delivery resulted in long-term biologically active NGF transgene product that had a consistent, stable trophic effect on basal

forebrain cholinergic neurons of the NBM for up to 1 year, with no cell loss.

Discussion

Gene delivery of the neurotrophic factor NGF to degenerating and dying basal forebrain cholinergic neurons is being developed as a potential treatment for mild to moderate Alzheimer's disease. In support of this concept, research over the past three decades has supported the importance of basal forebrain cholinergic neurons in the cognitive symptoms that characterize the earlier stages of this disease (Bartus, 2000). Similarly, research published over the past two

decades has shown that BFCNs respond positively when they are supplied with exogenous NGF, often returning function to aged or injured neurons and enabling these neurons to withstand significant neurodegenerative perturbations. Studies reported herein clearly indicate that AAV2 vector-mediated delivery is effective in providing NGF protein to BFCNs, with *in vivo* biological consequences, in the fimbria–fornix lesion model, the aged rat model of cholinergic neuronal degeneration, and bioactive neuronal responses in young animals.

In the clinic, the goal is to expose as much of the human NBM as possible to vector-derived NGF, without significant diffusion of NGF to distal brain regions, especially CSF-containing ventricles and meninges. Studies were performed in which various doses of CERE-110 were injected into the rat NBM, and results indicate that NGF transgene delivery to the targeted brain region is reliable and accurate, and that NGF transgene distribution can be controlled by altering CERE-110 dose. We demonstrate that it is possible to achieve restricted NGF expression limited to but covering the target brain region, and that a dose of 1×10^8 vg/NBM is optimal for providing as much exposure of the rat NBM to vector-derived NGF as possible without having significant diffusion of protein to brain regions distal to the NBM or the CSF. Results from animals examined at longer time periods of 3, 6, 9 and 12 months after CERE-110 delivery indicate that NGF transgene expression is stable and sustained at all time points, with no loss or build-up of protein over the long-term.

In addition, we provide evidence that CERE-110 mediated delivery of NGF protein provides neuroprotective or neurorestorative effects in several rodent models of BFCN degeneration directly relevant to Alzheimer's disease. Multiple studies have shown that fimbria–fornix lesion-induced degeneration of BFCNs can be prevented by treatment of the neuronal cell somata with NGF (Hefti, 1986; Williams et al., 1986; Kromer, 1987; Rosenberg et al., 1988; Tuszyński et al., 1990; Koliatsos et al., 1991a,b). Results in the fimbria–fornix lesion model of cholinergic neuronal degeneration demonstrate that the use of an AAV2-based vector is an effective means of delivering NGF protein to prevent degeneration of BFCNs. Results from this experiment are consistent with similar published reports of *in vivo* neuroprotection of BFCNs after AAV vector-mediated delivery of NGF in this model (Mandel et al., 1999; Wu et al., 2003, 2005). In aged rats, BFCNs are not lost, but they both shrink in size and lose expression of phenotypic markers such as p75, TrkA, and ChAT (Smith et al., 1993; Greferath et al., 2000; Stemmelin et al., 2000; Saragovi, 2005). These pathologic changes, also observed in the Alzheimer's disease brain, can be reversed in aged animals by administration of NGF. This response has been linked to improved cognitive function in aged rats (Fischer et al., 1987, 1991; Chen and Gage, 1995; Martinez-Serrano et al., 1996), as well as enhanced cortical reinnervation in aged monkeys (Conner et al., 2001). Results in the aged rat demonstrate that CERE-110, delivered at the operationally defined optimal dose of 1×10^8 vg/NBM, is an effective means of delivering NGF to provide significant trophic support to aging cholinergic neurons, as assessed by enhanced cholinergic cell size and cell number in the NBM of 24-month old rats. This dose was also effective in producing a biological response (cell hypertrophy) in NBM neurons in young rats without adversely affecting NBM cholinergic cell numbers at each of 3, 6, 9 and 12 months following vector injection. The biological response provided by the optimal dose of CERE-110 (1×10^8 vg/NBM) was not significantly less than the response produced by a 20× greater dose of CERE-110 (2×10^9 vg/NBM). This result is consistent with the concept that a maximal response occurs at the dose that exposes a preponderance of the cholinergic neurons of the NBM to NGF protein, as assessed by immunohistochemistry, and that increasing the CERE-110 dose above the operationally defined optimal dose results in no greater biological effect. Notably, in this study, NBM cellular hypertrophy was not associated with adverse behavioral changes in young rats as assayed by daily cage side observations or the functional observation battery (Moser, 2000; Bishop et al., manuscript in preparation).

Based on these proof-of-concept data in animal models of cholinergic neuronal degeneration, one can test the hypothesis that CERE-110 delivered to the basal forebrain region containing the NBM in subjects with Alzheimer's disease may have a positive effect on BFCN function and cognitive functions regulated by the cholinergic system. BFCN loss has been linked to cognitive dysfunction in Alzheimer's disease, and further, is presumed to contribute to the progression of Alzheimer's disease symptoms. This hypothesis has been validated by the many controlled clinical trials that have demonstrated the efficacy of drugs that marginally augment CNS cholinergic function in Alzheimer's disease (Hake, 2001). The first four drugs approved by the Food and Drug Administration for Alzheimer's disease, the cholinesterase inhibitors (ChEIs), act through the common mechanism of prolonging cholinergic synaptic signaling. CERE-110-mediated NGF delivery might show cognitive benefit similar to the ChEIs, since NGF is predicted to effectively increase availability of acetylcholine (ACh) in the cerebral cortex. However, CERE-110 may provide significant additional cognitive benefit by: 1) preventing the death of BFCNs, 2) increasing the vitality of remaining BFCNs, and 3) generating more ACh in the cortex than do ChEIs (especially since the systemically administered ChEIs have significant dose-limiting toxicity associated with peripheral cholinesterase inhibition). Neither of the first two of these benefits of NGF has been clearly associated with the use of ChEIs. Moreover, preserving and revitalizing the BFCNs themselves would be expected to preserve and restore the natural spatial and temporal patterns of ACh transmission in the cortex, which ChEIs do not.

In addition to the loss of BFCNs, other neuroanatomical changes occur in Alzheimer's disease. These include the accumulation of amyloid plaques and neurofibrillary tangles, as well as loss of other neuronal populations. Currently it is unclear how the various factors related to those anatomical changes interact, and to what extent those changes might also contribute to the cognitive dysfunction in Alzheimer's disease (Mesulam, 1999; Bartus, 2000; Blusztajn and Berse, 2000; Hardy and Selkoe, 2002; Isacson et al., 2002). Nor is it known how bolstering cholinergic function might affect those pathogenic processes, although use of ChEIs does not appear to significantly alter the course of the underlying disease. It is anticipated that preventing the loss of, and/or augmenting the function of, remaining BFCNs will improve symptoms of Alzheimer's disease and reduce cognitive decline. This could occur directly if the loss of BFCNs significantly contributes to the progression of Alzheimer's disease, and/or indirectly if the loss of BFCNs contributes significantly to other aspects of Alzheimer's disease, such as accumulation of β -amyloid, that may be relevant to disease progression. Towards testing these hypotheses, clinical testing of CERE-110 is currently underway. A small Phase I clinical study to examine the safety of CERE-110 delivered to the NBM in Alzheimer's disease patients is ongoing (Arvanitakis et al., 2007), and a double-blind, sham surgery-controlled, Phase II study to further examine the efficacy and safety of CERE-110 is planned.

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