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Adeno-associated virus-mediated delivery of glial cell line-derived neurotrophic factor protects motor neuron-like cells from apoptosis

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Motor neuron disorders including amyotrophic lateral sclerosis may benefit from the induction of neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) that are known to be trophic and protective for motor neurons. However, the application of such factors is limited by an inability to successfully target their expression in the nervous system. In this study we investigate the potential of using adeno-associated virus (AAV) as a vector for gene delivery into motor neuron-like cells. In initial experiments on the motor neuron cell line NSC-19 using a recombinant AAV vector expressing the reporter gene β-galactosidase (AAV-LacZ), we successfully demonstrate the utility of AAV for gene transfer. In addition, a recombinant AAV vector expressing GDNF was shown to express and secrete high levels of the neurotrophic factor into the surrounding media of NSC-19 infected cells. Finally, the AAV-GDNF vector is demonstrated to act in a neuroprotective fashion. Withdrawal of trophic support from NSC-19 cells through serum deprivation results in a subsequent increase in the number of cells entering apoptosis. However, the percentage of apoptotic cells are significantly reduced in cells infected with the AAV-GDNF vector, as compared to AAV-LacZ or uninfected controls. This work demonstrates the potential of using AAV as a vector in motor neuron-like cells and should prove important in devising future gene therapy strategies for the treatment of *in vivo* motor neuron disorders. *Journal of NeuroVirology* (2001) **7,** 437–446.

Keywords: amyotrophic lateral sclerosis; adeno-associated virus; glial cell line-derived neurotrophic factor; gene therapy

Introduction

Amyotrophic lateral sclerosis (ALS) is a chronic neurodegenerative disorder characterised by progressive degeneration of motor neurons in the spinal cord and brain stem with associated muscular denervation and atrophy leading ultimately to paralysis and death. The disease is sporadic in approximately 90% of cases whereas the remaining 10% are familial in origin. Mutations in the gene encoding the enzyme copper/zinc superoxide dismutase (Cu/Zn-SOD) have been identified in 20% of familial and in 2% of sporadic cases. The pathogenic mechanisms underlying the disease process remain poorly understood, although glutamate-mediated neurotoxicity and/or oxidative stress prior to cell death have been implicated (Shaw, 1999). At this point, there is no effective therapy available for ALS.

Recent evidence, however, suggests that ALS and associated neurodegenerative disorders may benefit from the induction of specific neurotrophic factors that have been shown to be important in the maintenance, survival, and regeneration of neurons. Ciliary

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neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and glial cell line-derived neurotrophic factor (GDNF) have been demonstrated as trophic factors for motor neurons (Aebischer and Kato, 1995; Yuen and Mobley, 1996). Of these, perhaps the most promising for motor neurons is GDNF. GDNF has been shown to increase the choline acetyltransferase (ChAT) activity of embryonic motor neurons (Zurn *et al*, 1994), to rescue developing motor neurons from natural and axotomy-induced cell death (Oppenheim *et al*, 1995; Yan *et al*, 1995) and to protect adult motor neurons from avulsion-induced cell death (Li *et al*, 1992). More recently GDNF and neurturin, a homologue of GDNF, have been shown to increase ChAT activity in postnatal motor neurons and to protect from chronic glutamate-mediated toxicity (Bilak *et al*, 1999). In addition, in a comparative study on postnatal motor neurons, GDNF and insulin-like growth factor-1 were shown to increase ChAT activity and to be protective against glutamate-mediated neuronal cell death whereas BDNF, NT3, and CNTF showed no such effects (Corse *et al*, 1999). Because glutamate-mediated toxicity is implicated in the pathogenesis of ALS, this observation may indicate that GDNF may be of greater relevance as a neuroprotective factor in the treatment of ALS than BDNF, NT3, and CNTF.

Important in any therapeutic strategy will be the ability to deliver and express the appropriate trophic factor(s) at the target site in the CNS. Due to their polypeptidergic chemical structure and short serum half-life trophic factors cannot be efficiently administered to CNS tissue. Thus far, clinical trials with neurotrophic factors delivered via repeated subcutaneous administration of recombinant proteins have failed to yield clinical improvement and have been complicated by severe side effects evidenced with the high doses administered. Thus, alternative strategies to increase neurotrophic factor levels within or surrounding the target cell population are actively being explored.

One method by which this may be achieved is to employ a gene transfer approach to achieve targeted expression. We have shown previously that a peripheral intramuscular inoculation with a herpes simplex virus (HSV) vector can result in retrograde transport within the sciatic nerve, and subsequent transduction of the innervating spinal cord motor neurons (Keir *et al*, 1995). Although this study demonstrated the feasibility of using a viral vector to express a foreign gene within the spinal cord, significant cytotoxicity is associated with the use of HSV vectors (Wood *et al*, 1994; Keir *et al*, 1995), pointing out the need to develop less cytotoxic vectors. Replication-deficient adenovirus vectors have also been successfully used to transduce neuronal cells *in vivo* (Le Gal La Salle *et al*, 1993) and, importantly, an adenovirus vector expressing the neurotrophic factor NT-3 was recently shown to be of therapeutic benefit in a mouse model of motor neuron disease, the *pmn* mutant mouse (Haase *et al*, 1997). However, viral toxicity and the host immune response to viral proteins and/or contaminating helper virus may also limit the application of adenoviral vectors (Byrnes *et al*, 1995; O'Leary and Charlton, 1999).

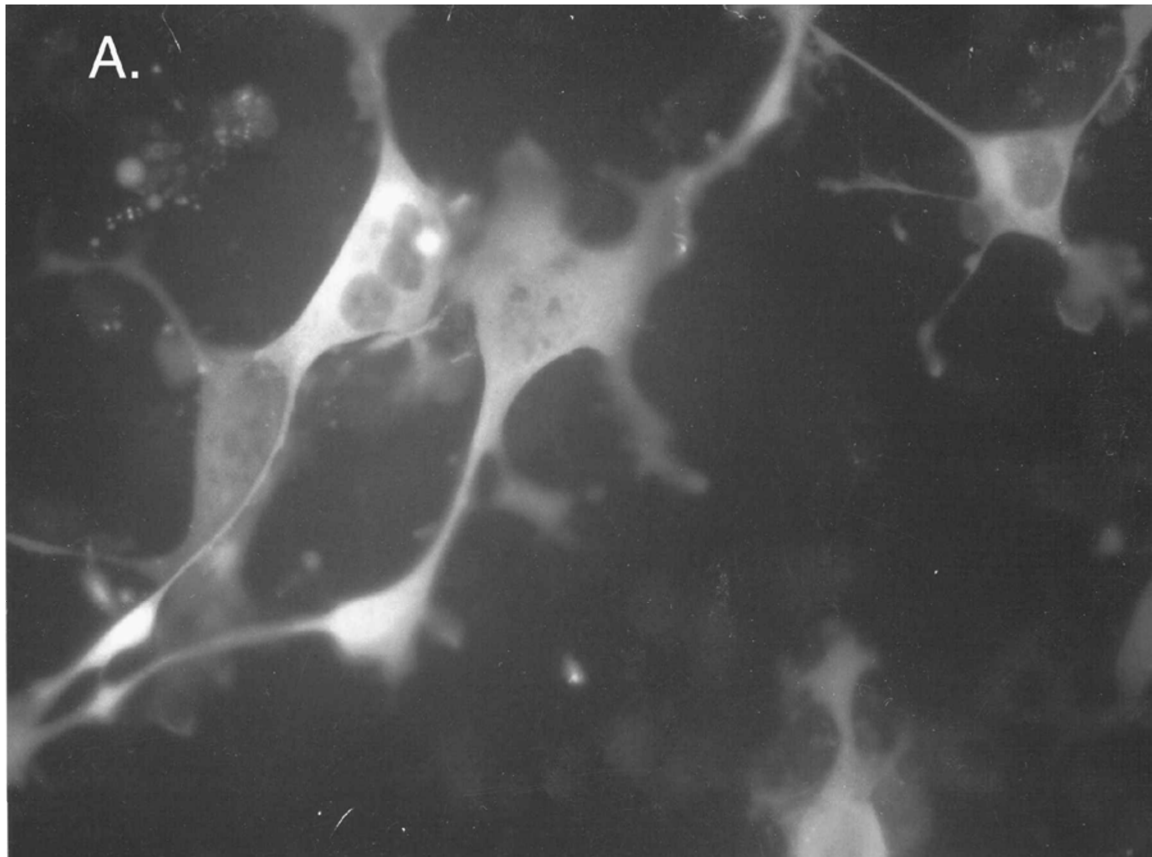
More recently, vectors based on the defective parvovirus adeno-associated virus (AAV) have been proposed for gene transfer to the CNS and have a number of features which may make them particularly suitable for this purpose. Wild-type AAV has no known associated pathology, and recombinant vectors typically have over 95% of the viral genome removed and can be produced to a high titer free of contaminating helper virus. In addition, AAV is capable of producing efficient transduction and stable integration, with accompanying long-term gene expression in both dividing and nondividing cell populations (Monahan and Samulski, 2000). In the nervous system the efficacy of AAV vectors for gene transfer has been demonstrated in a variety of studies including dissociated cell cultures of primary human glial cells (Keir *et al*, 1997) and human NT neurons (Du *et al*, 1996) and also in hypothalamic organotypic explant cultures (Keir *et al*, 1999). Long-term *in vivo* gene expression has been described in neurons of the normal and 6-OH-dopamine lesioned rodent brain (Kaplitt *et al*, 1994; McCown *et al*, 1996) and in normal and injured rodent spinal cord (Peel *et al*, 1997).

In this study, we examine the potential of using AAV as a vector for gene transfer to motor neuron-like cells using the NSC-19 motor neuron cell line. The NSC-19 cell line was generated through the fusion of mouse neuroblastoma N18TG2 cells and a motor neuron-enriched 12–14-day embryonic mouse spinal cord cell culture (Cashman *et al*, 1992). NSC-19 cells exhibit characteristic features of motor neurons, including a multipolar neuronal morphology, expression of ChAT and neurofilament proteins, and the ability to synthesise, store, and release acetylcholine and to generate action potentials. They have been widely used as an *in vitro* model for functional studies in motor neurons and also in examining the effects of toxicity and oxidative stress on motor neurons (Keller *et al*, 1999; Pedersen *et al*, 1999) and as such are a good *in vitro* model for studies on motor neurons. We demonstrate that AAV can be used for gene transfer in this model and for effective delivery of the neurotrophic factor GDNF. Furthermore, infection with an AAV-GDNF vector is also demonstrated to have a neuroprotective effect on NCS-19 cells subjected to serum deprivation

Results

AAV-LacZ transduction of the NSC-19 motor neuron cell line

The ability and efficiency of utilising AAV as a vector for gene transfer into motor neurons was first investigated using the recombinant AAV-LacZ vector pdx-31



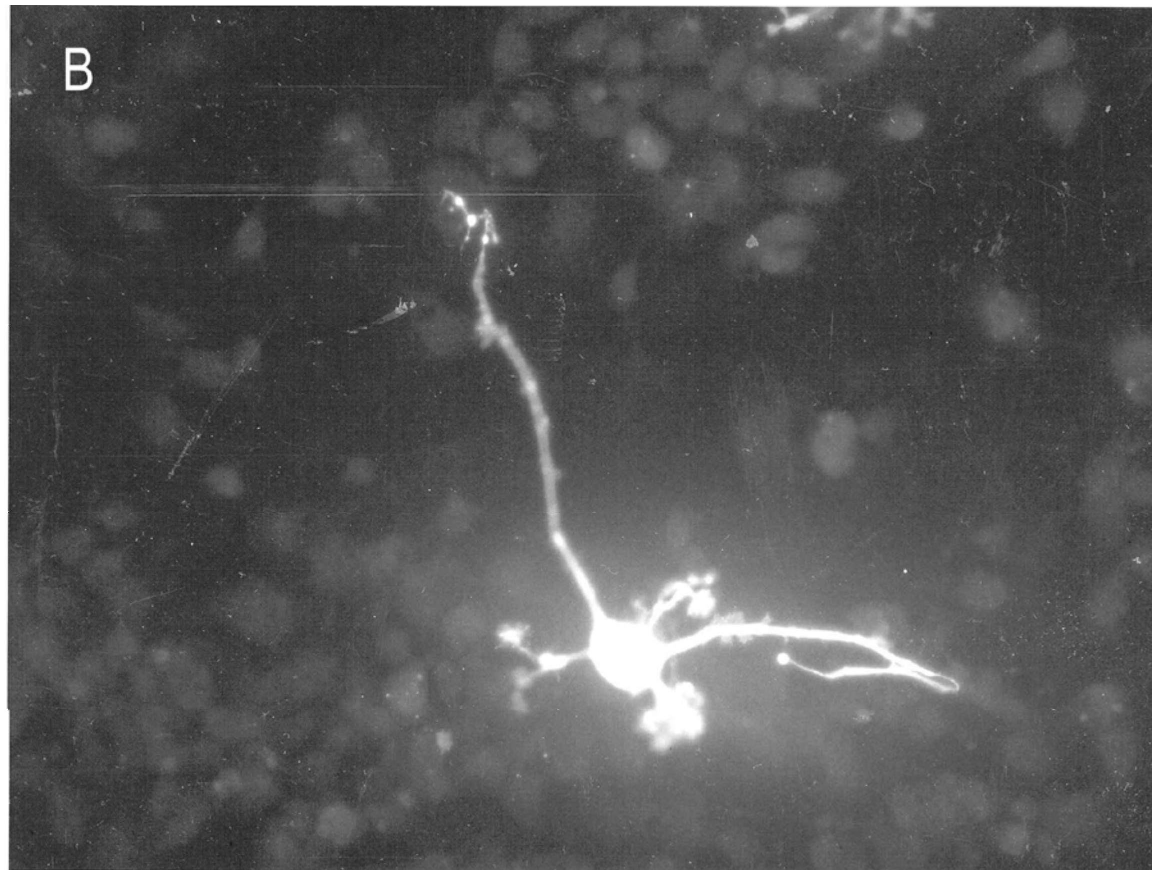
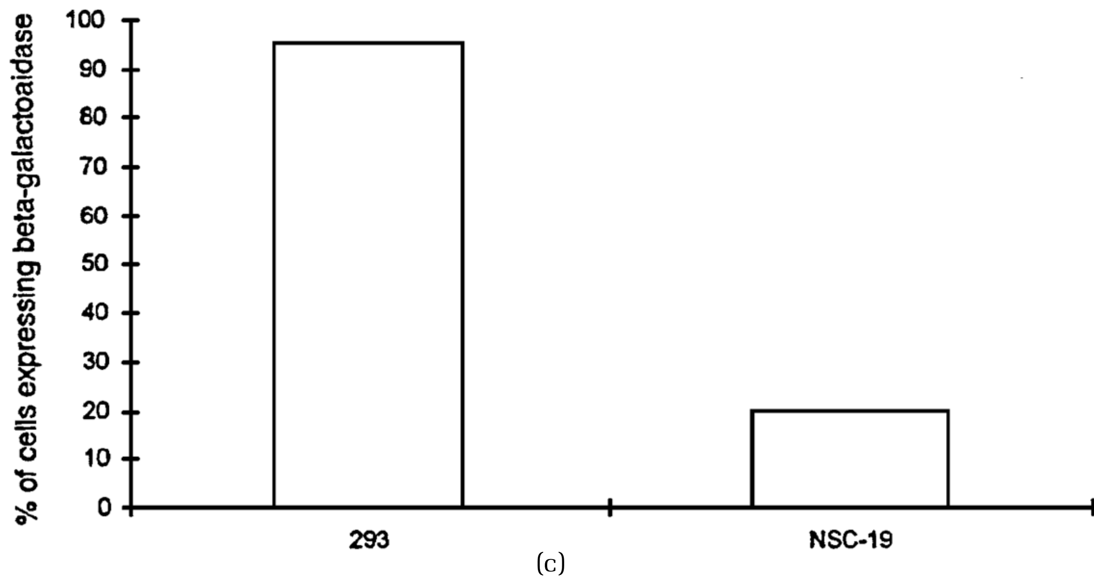


Figure 1 β-Galactosidase expression from the AAV-LacZ vector pdx-31. (A) Human embryonic kidney 293 cells and (B) NSC-19 motor neuron cell line showing expression of β-galactosidase 24 h p.i. as evidenced by immunohistochemical detection. (C) Percentage of 293 and NSC-19 cells expressing β-galactosidase 24 h p.i. with the AAV-LacZ vector at a titre of 1×10^7 virus particles/well. Infections were set up in triplicate. *(Continued)*



(c)

Figure 1 (Continued)

which expresses a cytoplasmic form of the reporter gene β-galactosidase. The NSC-19 motor neuron cell line was infected with serial dilutions of virus with titres ranging from 1×10^7 down to 1×10^3 virus particles per well. The human embryonic kidney 293 cell line which is known to be highly sensitive to infection by AAV was treated as above, providing a positive control for infection. Examination for β-galactosidase expression was at 24 h p.i. At this point, expression of β-galactosidase was detected in both the NSC-19 and 293 cells as evidenced by immunocytochemistry (Figure 1A, B). The transduction efficiency, with equivalent titre of virus, was typically 5–10-fold lower in NSC-19 cells than in the control 293 cells. At the highest titre of virus used over 95% of 293 cells and approximately 20% of NSC-19 cells were positive for β-galactosidase expression (Figure 1C). Increasing the titre of virus did not increase the rate of transduction in NSC-19 cells above approximately 20–25% of the population. As expected serial dilutions of the vector stock lowered the transduction rate in both lines and no expression of β-galactosidase was detected in uninfected control cultures.

Production of GDNF in 293 and NSC-19 cells

The ability of AAV to deliver and express GDNF was next confirmed in cells infected with the rAAV-GDNF vector. NSC-19 and 293 cells were infected with 1×10^8 virus particles of the AAV-GDNF vector and production of GDNF was measured by assaying levels of the trophic factor secreted into the medium by using an ELISA technique. AAV-LacZ infected cultures served as a negative control for GDNF production and the addition of 1000 pg of recombinant standard protein to one culture well served as a positive control for GDNF. In cells infected with the rAAV-GDNF vector, GDNF production was evident by 24 h p.i. and the expression was maintained up to 72 h p.i. (Figure 2A, B). Levels of GDNF detected were higher in the media of 293 transduced cells, which is

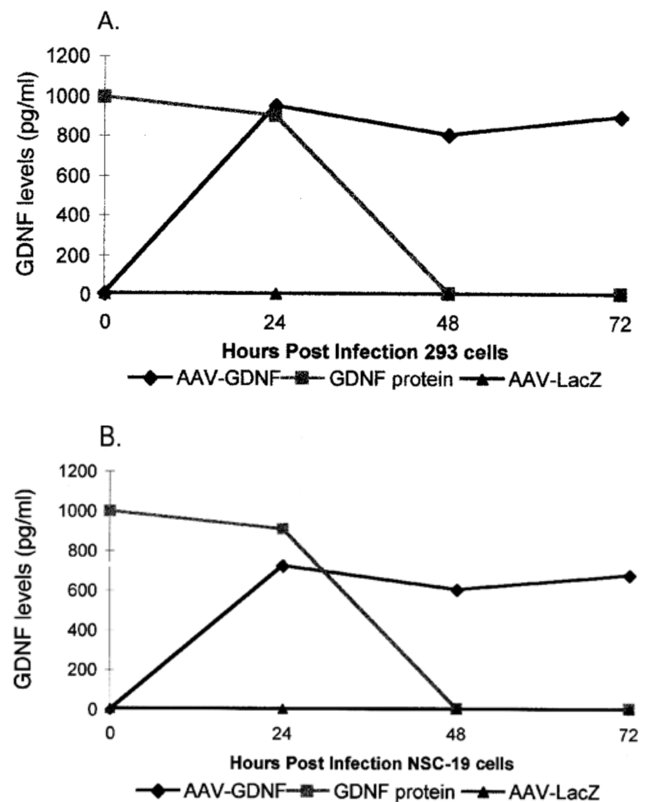


Figure 2 ELISA Detection of GDNF protein. (A) GDNF levels detected in the media of 293 cells after treatment with the AAV GDNF vector, recombinant GDNF protein, or the AAV-LacZ. (B) GDNF levels detected in the media of NSC-19 cells after treatment with the AAV-GDNF vector, recombinant GDNF protein, or the AAV-LacZ.

consistent with the greater transduction efficiency observed in this cell line using the LacZ vector. There were no detectable levels of GDNF measured in cells infected with the AAV-LacZ vector. In cells that received recombinant protein, GDNF was detected at the 0- and 24-h time points but was absent subsequently after cells were washed and replaced with fresh media.

AAV-mediated expression of GDNF attenuates apoptotic cell death in motor neurons resulting from serum deprivation
Withdrawal of trophic support through serum deprivation has been shown to induce a variety of cells to enter apoptotic cell death. NSC-19 cells were subjected to serum deprivation and the degree of apoptosis was determined by calculating the number of cells with condensed and fragmented nuclei. By 24 h post serum deprivation, a significant increase in the number of apoptotic cells was observed as compared to NSC-19 cells maintained in 10% FCS ($P < 0.003$). The number of apoptotic cells increased over time such that by 48 h a 5-fold increase was evident ($P < 0.001$) (Figure 3A, B).

To examine whether the AAV-GDNF vector would have a neuroprotective effect on NSC-19 cells entering apoptosis, cells were infected prior to, simultaneously with, and post serum deprivation. NSC-19 infected with AAV-GDNF 24 hours prior to serum deprivation showed a significant reduction in the number of apoptotic cells ($P < 0.01$) as indicated by nuclear DNA condensation and fragmentation (Figure 4A). If cells were infected with AAV-GDNF simultaneously with serum deprivation, a similar neuroprotective effect was observed ($P < 0.015$, Figure 4B). In NSC-19 cells infected 24 h after serum deprivation had been initiated, no such protective effect was observed (data not shown). There was no significant difference in the level of apoptosis between cells infected prior to or simultaneously with serum deprivation. No neuroprotective effect was observed in cells that were infected with the AAV-LacZ vector regardless of the time of delivery.

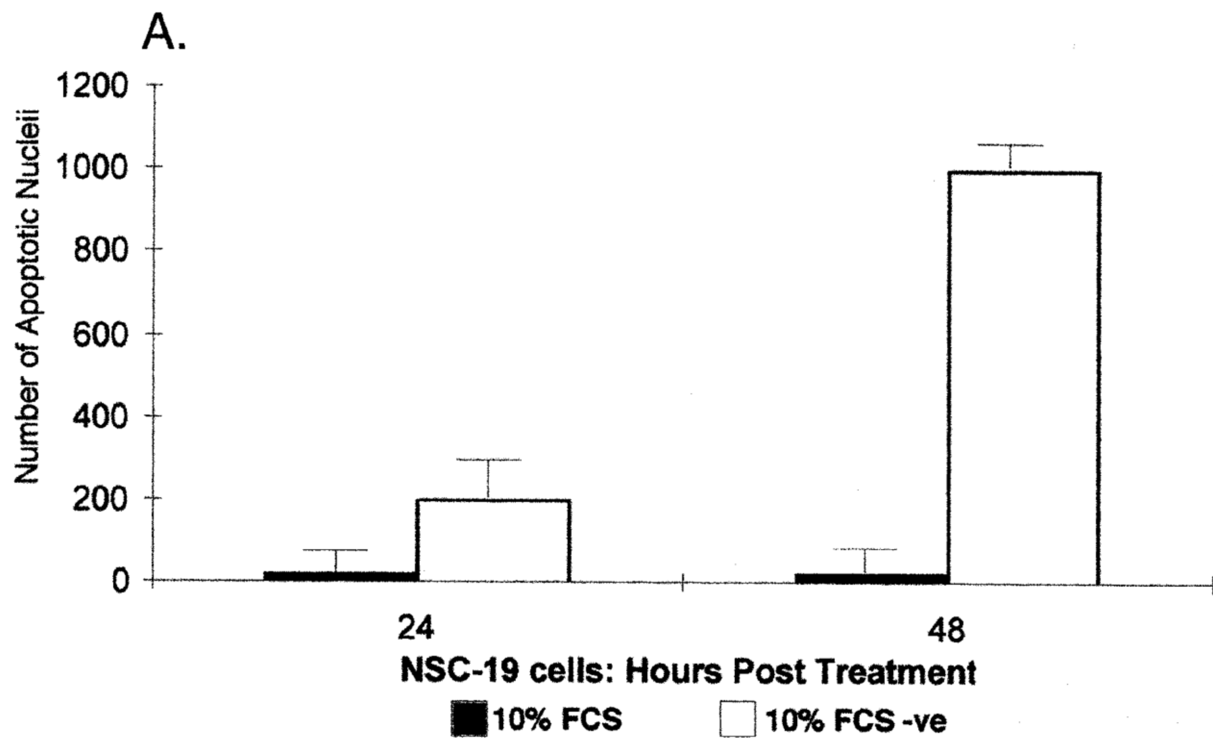
Discussion

The inability to deliver effectively neurotrophic factors to target areas within the central nervous system limits the potential therapeutic application of such factors for the treatment of neurodegenerative disorders such as ALS. In this study, we demonstrate that adeno-associated virus can be used as a vector for gene transfer into motor neuron-like cells and furthermore, that an AAV vector expressing the neurotrophic factor GDNF can be neuroprotective for motor neurons.

Using an AAV vector expressing β-galactosidase gene transfer was successfully demonstrated in the NSC-19 motor neuron cell line. NSC-19 cells proliferate upon culture and express many characteristics of motor neurons. Mature NSC-19 cells extend neurites, can generate action potentials, and express the neurofilament triplet proteins NF 68, NF 150, and NF 200. In addition, NSC-19 cells express choline acetyltransferase and synthesize and store acetylcholine. No expression of the astrocyte specific intermediate filament GFAP was detected in NSC-19 cultures (Cashman *et al*, 1992). As such, NSC-19 cells are a good *in vitro* model of motor neurons in which there is no evidence of glial-like cells that could interfere with the results presented.

The transduction efficiency observed in NSC-19 cells after infection with AAV was relatively low, even at the highest concentrations of vector used. This is somewhat surprising as AAV has been shown previously to transduce a variety of dissociated neural cultures with high efficiencies at comparable concentration (Du *et al*, 1996; Keir *et al*, 1997). Reasons for this may include poor receptor availability on NSC-19 cells as compared to other cell types examined. AAV attachment and infection is mediated through membrane-associated heparan sulfate proteoglycans (Summerford *et al*, 1999) with $\alpha V\beta 5$ integrin and human fibroblast growth factor receptor 1 acting as co-receptors for infection (Summerford and Samulski, 1998; Qing *et al*, 1999). It remains to be established whether all these receptor types are present on NSC-19 cells. Interestingly, studies using HSV vectors have shown a similar pattern of low transduction efficiency in this cell line (authors' observations). As the primary attachment of HSV is believed to occur through an interaction of HSV-specific glycoproteins with heparan sulfate moieties located on cell surface proteoglycans, low transduction rates may indicate a reduced availability of heparan sulfate on NSC-19 cells. Although detailed studies using AAV to specifically target motor neurons *in vivo* remain to be carried out available evidence would appear to indicate that receptor availability should not be a limiting factor *in vivo*. AAV appears to efficiently and preferentially transduce neuronal rather than glial cells (Peel *et al*, 1997) when injected into the spinal cord, and HSV has been shown to effectively bind to and target spinal cord motor neurons (Keir *et al*, 1995).

Upon infection with the AAV-GDNF vector, high levels of GDNF were secreted into the media of NSC-19 and 293 cells. As expected from the results obtained with the AAV-LacZ vector, the levels of GDNF detected in the media of NSC-19 cells were lower than in 293 cells, a finding consistent with the lower transduction efficiency detected there. The levels were not, however, as low as would be expected from the 5–10-fold reduction observed with the LacZ vector as evidenced by immunohistochemical detection of β-galactosidase. This perhaps reflects a greater sensitivity in the detection method of GDNF as opposed to



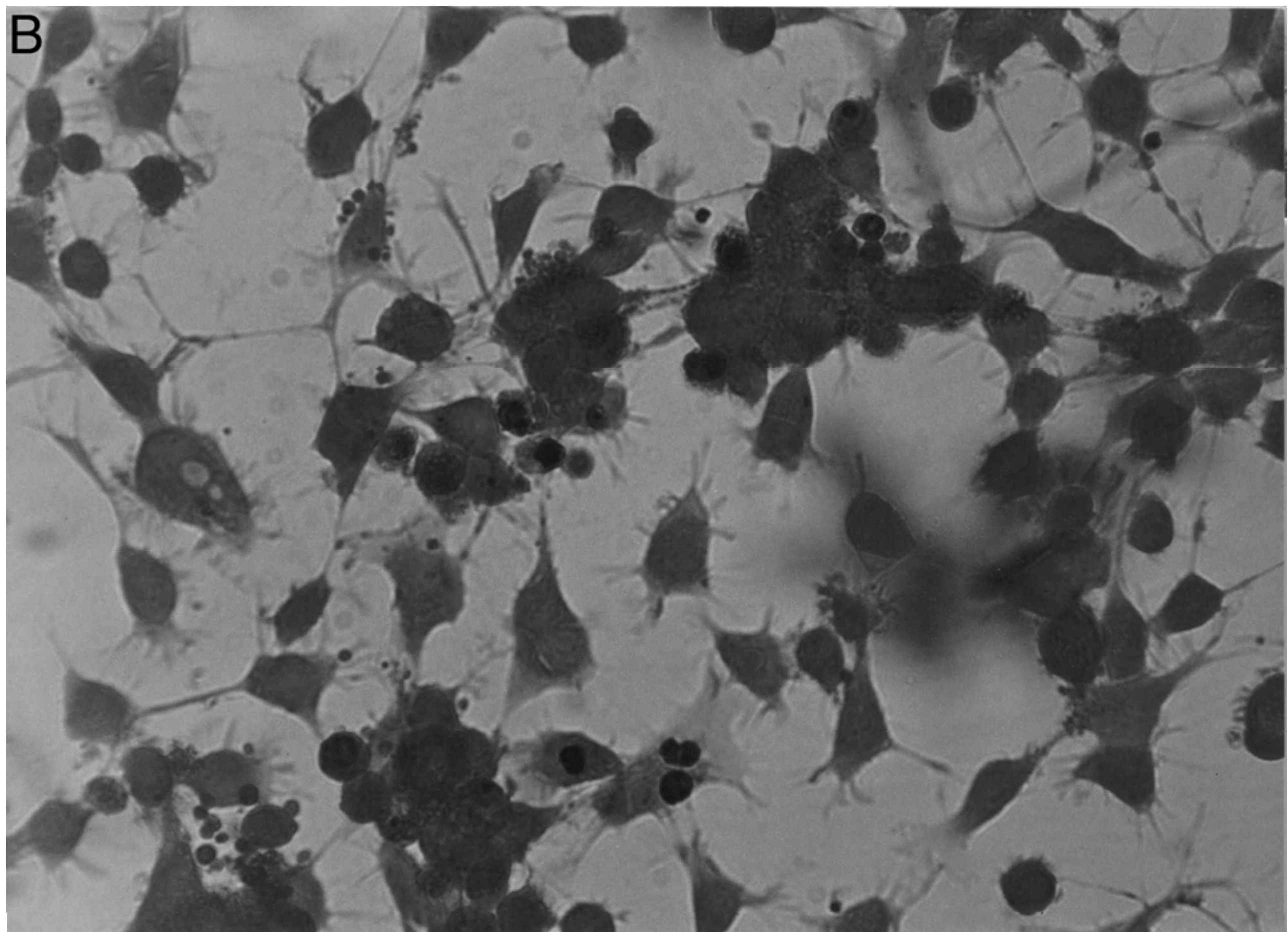


Figure 3 Detection of apoptosis in NSC-19 cells following serum deprivation. (A) Number of apoptotic NSC-19 cells after 24 and 48 h detected/well following washing of cells and replacement with either DMEM plus 10% FCS or DMEM. Significant increases in the number of apoptotic nuclei are seen at both 24 ($P < 003$) and 48 ($P < 0.001$) h. The numbers represent the means of 3 separate samples for each treatment group. (B) Apoptotic nuclear DNA fragmentation in NSC-19 cells as evidenced by the incorporation of biotinylated nucleotides using a TUNEL assay for apoptosis (Promega). Cultures are counter stained with Luxol Fast Blue and apoptotic nuclei appear black.

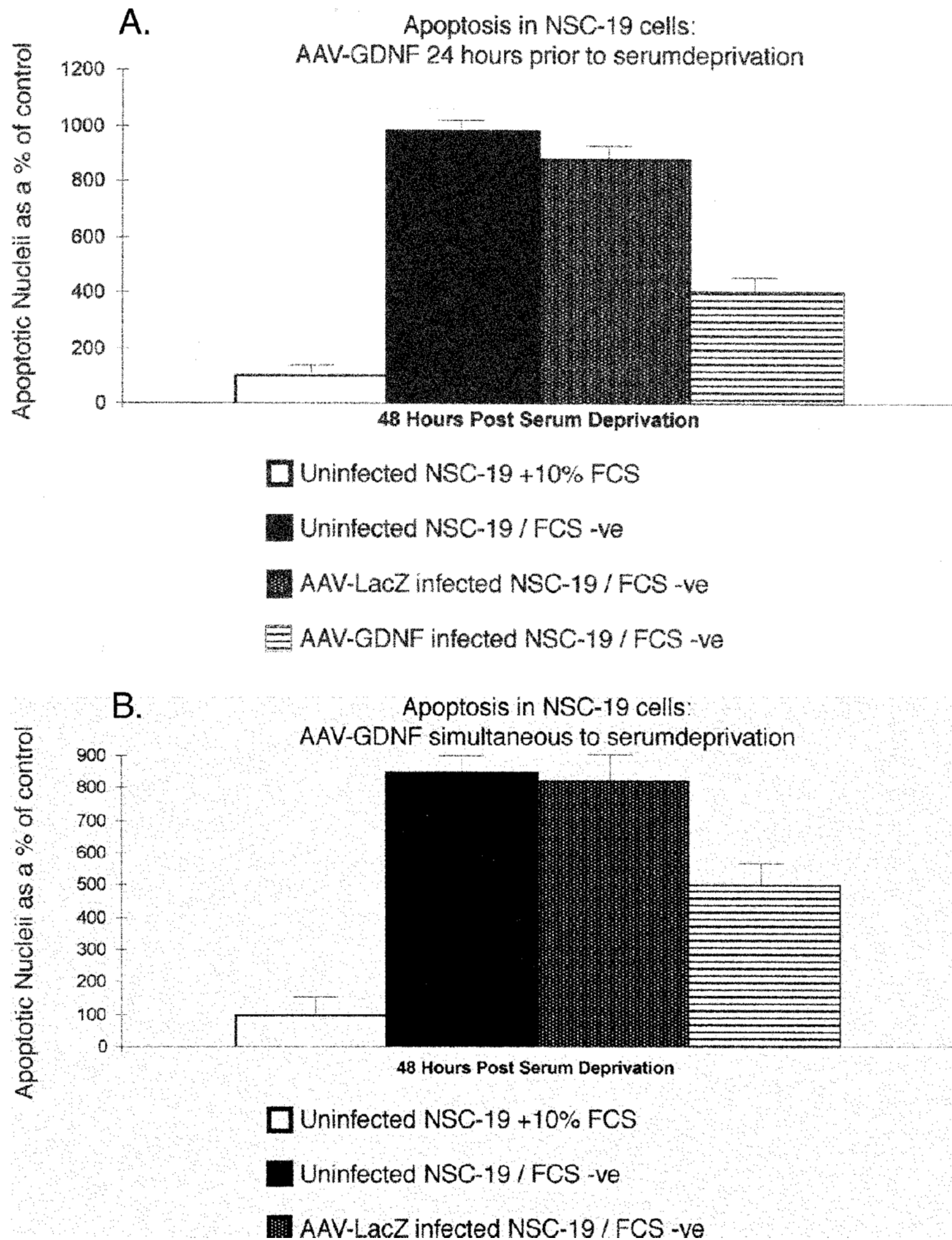


Figure 4 Effects of AAV-GDNF on apoptosis in NSC-19 cells. The number of apoptotic nuclei are expressed as a percentage of uninfected NSC-19 cells maintained in DMEM plus 10% FCS. (A) AAV-GDNF given 24 h prior to the commencement of serum deprivation significantly reduces the number of apoptotic nuclei detected 48 h post serum deprivation ($P < 0.01$; $n = 3$ by one-way analysis of variance), as compared to serum deprived uninfected or AAV-LacZ infected cells. (B) AAV-GDNF given simultaneously with the commencement of serum deprivation results in a similar significant reduction in the number of apoptotic nuclei detected 48 h post serum deprivation ($P < 0.015$; $n = 3$ by one-way analysis of variance), as compared to serum deprived uninfected or AAV-LacZ-infected cells. The numbers represent the means of 3 separate samples for each treatment group.

that of β-galactosidase. The expression of GDNF was maintained throughout the time course of the study in cells infected with the AAV-GDNF vector. Because the cells were washed and the media was replaced after each time point collected this represents fresh production of GDNF in these cells. In contrast, in cells that received GDNF standard as a positive control no GDNF could be detected after the initial media containing the recombinant protein had been replaced with fresh media after the 0- and 24-h time points.

A number of potent neuroprotective properties of GDNF on motor neurons have been reported. These include rescuing motor neurons from programmed and axotomy-induced cell death and rescuing postnatal motor neurons from chronic glutamate-mediated degeneration. Here, we report that an AAV-GDNF vector can offer significant neuroprotection for NSC-19 cells subjected to serum deprivation. When NSC-19 cells were subjected to serum deprivation, a marked increase in the number of apoptotic cells over time was demonstrated. However, in cultures infected with the AAV-GDNF vector a significant reduction in the number of apoptotic cells was observed in cultures that were infected prior to, or simultaneously with, serum withdrawal from the cultures. It is not possible to distinguish whether the neuroprotective effect occurred only in those cells transduced with the AAV-GDNF vector or whether GDNF secreted into the media is able to act in a paracrine way on surrounding cells. The latter hypothesis, though, seems reasonable as high levels of GDNF are detected in the conditioned media of the cell population and would be available to act on cellular receptors. GDNF is believed to act on cells through a two-component cellular receptor complex consisting of a glycosylphosphatidylinositol-linked α subunit and a transmembrane tyrosine kinase receptor (Durbec *et al*, 1996; Jing *et al*, 1996; Treanor *et al*, 1996). The secretion of GDNF from a subset of transduced cells which could then act in a paracrine fashion on surrounding cells may offer a significant advantage over the subcutaneous administration of protein. There was no significant protection observed in cultures that were infected post serum deprivation presumably because the programmed cell death was already initiated in these cultures and is not reversible through the expression of GDNF. No significant difference was observed in the rate of cell death between cultures infected prior, or simultaneously, to serum deprivation. The reason for this is likely due to the rapid uptake, transport, and expression of AAV. Infusion of AAV into the rodent brain has shown binding to neuronal cell surfaces to occur within 6 min and to be transported to the nucleus within 30 min (Bartlett *et al*, 1998), and reporter gene expression is observed as early as 6 h p.i. in NSC-19 cells (authors' observations). Importantly, transduction with the AAV-LacZ vector had no effect on the level of apoptosis, indicating that infection with AAV does not influence the rate of cell death.

In summary, we have shown that AAV can be used as a vector for gene transfer into motor neuron-like cells and that an AAV vector expressing the motor neuron trophic factor GDNF can be neuroprotective for motor neurons. This work has importance for the development of gene therapy strategies for motor neuron disorders. Future work should focus on the ability of AAV to specifically target motor neurons *in vivo* and for the treatment of models of motor neuron-like disease.

Materials and methods

Tissue culture
The NSC-19 motor neuron cell line was generated by the fusion of murine neuroblastoma N18TG2 cells and a motor neuron-enriched 12–14-day-old embryonic mouse spinal cord cell culture as described previously (Cashman *et al*, 1992). The human embryonic kidney 293 cell line was purchased from ATCC. Both NSC-19 cells and the human embryonic kidney 293 cell line were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum and appropriate antibiotic.

Virus production
The recombinant adeno-associated virus (rAAV) vector pdx31-LacZ has been described previously (McCown *et al*, 1996) and produces a cytoplasmic β-galactosidase signal. For the construction of the rAAV-GDNF vector, two primers were used to PCR amplify the rat GDNF cDNA (forward primer 5′ gatccgaggtgccgaagcc 3′ and reverse primer 5′ ccagggtcagatacatccac 3′). The PCR product was cloned into the Not I sites of the plasmid pTR-UF1 after Klenow enzyme fill in. The orientation of the insert was confirmed by DNA sequencing. The vectors are replication incompetent with 96% of the viral genome having been removed and replaced with a cytomegalovirus promoter-transgene cassette. The production of adenovirus-free rAAV viral particles was carried out as described elsewhere (Xiao and Samulski, 1998), yielding vector stocks of 1×10^9 virus particles/ml for the AAV-pdx31-LacZ vector and 1×10^{13} virus particles/ml for the AAV-GDNF vector.

Viral infection
Cells were grown to 75% confluence in 6-well plates on sterile glass coverslips. The media was removed and replaced with 200 μl of fresh media containing the appropriate AAV vector at varying multiplicities of infection. Virus was allowed to bind and absorb for 30 min at 37°C, whereupon cells were washed and replaced with appropriate fresh media with or without 10% FCS and maintained at 37°C in 5% CO_2.

Immunohistochemistry for β-galactosidase
Cultures were fixed in 4% paraformaldehyde and washed twice in PBS. β-Galactosidase expression was detected by incubating cultures in rabbit

anti-β-galactosidase (5 prime/3 prime, 1 : 100) for 1 h at room temperature, rinsed in PBS 3 times and then incubated in swine anti-rabbit immunoglobulins directly conjugated to FITC (Dako, 1 : 50) for 1 h.

Detection of GDNF
Following infection with the rAAV-GDNF vector 200-μl aliquots of supernatant were removed from the cultures at predetermined time points. The first sample was collected at zero hours immediately after AAV binding had occurred and a 200-μl aliquot of fresh media was replaced. Subsequent time points were collected at 24, 48, and 72 h p.i. where, following collection of the 200-μl aliquot, the remaining media was removed and replaced with fresh media. The samples of supernate were stored at -20°C until assayed for GDNF. Levels of GDNF were detected by an ELISA technique using the Promega GDNF E_{max} Immunoassay system (Promega), according to the manufacturers instructions. Control cultures received either the AAV-LacZ vector to act as a negative control or 1000 pg of recombinant standard protein to serve as a positive control.

TUNEL assay for apoptosis
Following treatment of serum deprivation and/or virus infection cultures were fixed in 4% paraformaldehyde and apoptotic cells were detected by measuring nuclear DNA fragmentation with the Promega "Dead-end" Colorimetric Apoptosis Detection system (Promega), which end labels the fragmented DNA of apoptotic cells using a modified TUNEL (TdT mediated dUTP Nick End Labelling) assay.

Statistical analysis
Statistical analysis of the increase in apoptotic nuclei after serum deprivation was performed by a one-tailed Student's *t*-test. Analysis of the neuroprotective effect of the AAV-GDNF vector was performed by one-way analysis of variance.

Acknowledgements

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