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Increased motoneuron survival and improved neuromuscular function in transgenic ALS mice after intraspinal injection of an adeno-associated virus encoding Bcl-2

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Mutations in the gene encoding Cu/Zn superoxide dismutase (*SOD1*) underlie some familial cases of amyotrophic lateral sclerosis (ALS), a neurodegenerative disorder characterized by loss of cortical, brainstem and spinal motoneurons. Transgenic mice overexpressing a mutated form of human *SOD1* containing a Gly→Ala substitution at position 93 (*SOD1*^{G93A}) develop a severe, progressive motoneuron disease. We investigated the potential of recombinant adeno-associated virus (rAAV) to transfer neuroprotective molecules in this animal ALS model. Initial experiments showed that injection of an rAAV vector encoding green fluorescent protein unilaterally into the lumbar spinal cord of wild-type mice leads to expression of the reporter gene in 34.7 ± 5.2% of the motoneurons surrounding the injection site. Intraspinal injection of an rAAV encoding the anti-apoptotic protein bcl-2 in *SOD1*^{G93A} mice resulted in sustained bcl-2 expression in motoneurons and significantly increased the number of surviving motoneurons at the end-stage of disease. Moreover, the compound muscle action potential amplitude elicited by nerve stimulation and recorded by electromyographic measurements was higher in the rAAV–bcl-2-treated group than in controls. Local bcl-2 expression in spinal motoneurons delayed the appearance of signs of motor deficiency but was not sufficient to prolong the survival of *SOD1*^{G93A} mice. To our knowledge, this study describes the first successful transduction and protection of spinal motoneurons by direct gene transfer in a model of progressive motoneuron disease. Our results support the use of AAVs for the delivery of protective genes to spinal cord motoneurons as a possible way to enhance motoneuron survival and repair.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that mainly affects motoneurons in the cortex, brainstem and spinal cord. It typically occurs in middle adult life, leading to paralysis and death within 3–5 years (1). Approximately 10% of the cases are familial, and 20% of those are associated with dominantly inherited mutations in *SOD1*, the gene encoding Cu/Zn-superoxide dismutase (2). The molecular basis for the selective vulnerability of motoneurons remains unknown. However, the presence of a similar pathology in both sporadic and familial ALS cases, including those without *SOD1* mutations, suggests that common pathogenetic mechanisms may be at the origin of this neurodegenerative disease.

Several lines of transgenic mice overexpressing mutated forms of human SOD1 develop a severe and progressive motoneuron disease closely resembling the human disorder [familial ALS (FALS) transgenic mice] (3–5). Deleterious effects of the mutant SOD1 protein arise through a novel and yet unknown function. Several mechanisms by which mutant SOD1 may lead to motoneuron degeneration have been suggested. These include enhanced peroxidase activity, nitration of tyrosines via formation of peroxynitrite, copper-, zinc- or aggregation-dependent toxicity (for review see refs 6,7), and inhibition of glial glutamate uptake (8). Furthermore, although wild-type SOD1 exerts anti-apoptotic properties (9), mutant SOD1 promotes apoptosis in neuronal cells and caspase-1 is activated in cultured motoneurons expressing mutant SOD1 (9–11). Recently, overexpression of bcl-2 in the progeny from crosses between FALS transgenic and bcl-2 transgenic mice was shown to prolong the survival of FALS mice (12). Similarly, expression of a dominant-negative inhibitor of the caspase interleukin-1β-converting enzyme in FALS transgenic mice was reported to slow the progression of the disease and delay mortality (13).

Adeno-associated virus (AAV) is a non-pathogenic human parvovirus. Recombinant adeno-associated viruses (rAAVs) have been developed in the last few years as vectors for gene transfer and therapy (14,15). rAAVs are not immunogenic (16), lack all viral genes and confer long-term transgene expression in several tissues

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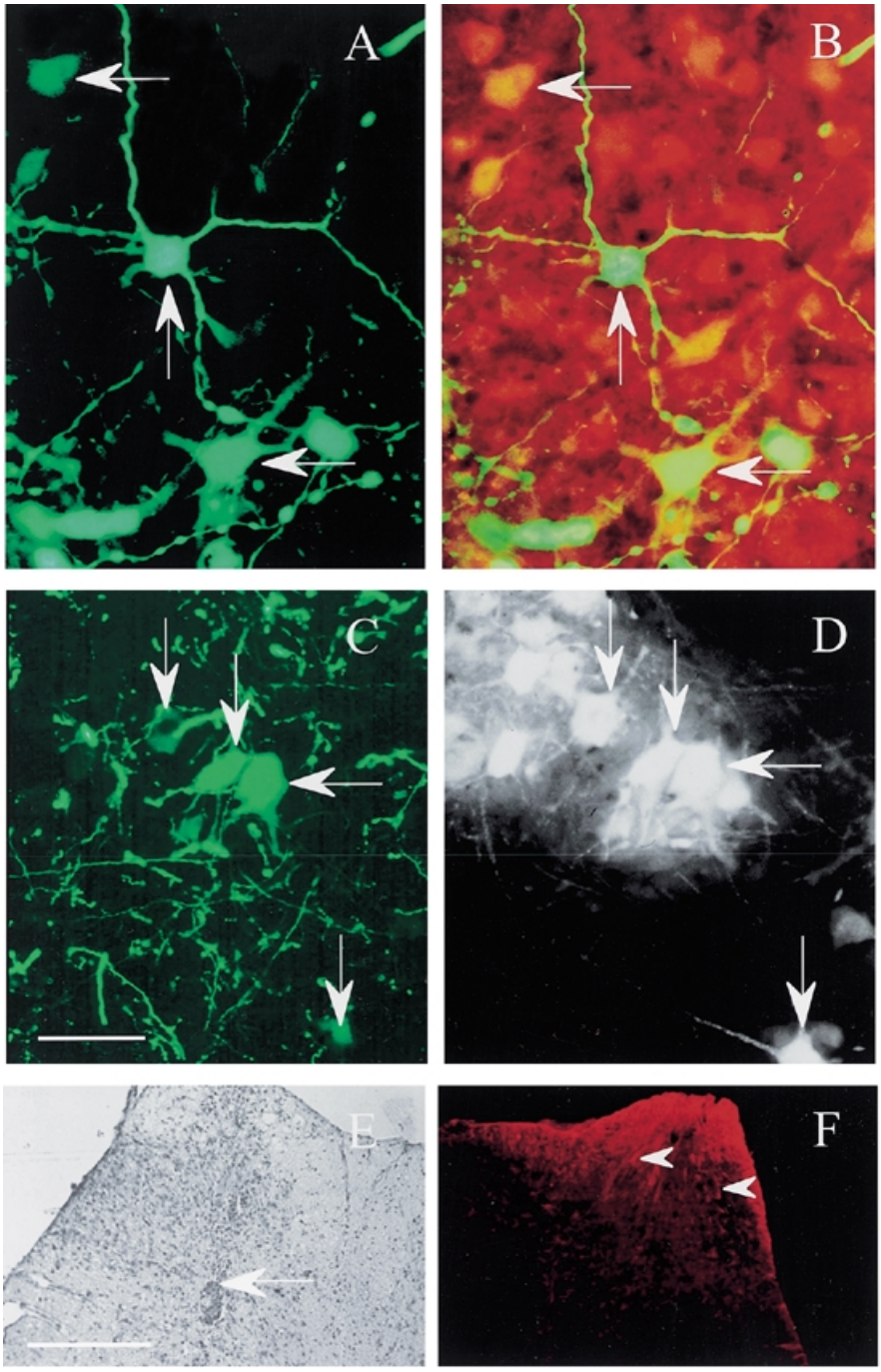

Figure 1. EGFP expression, NeuN immunostaining and FG-retrograde labeling in spinal motoneurons. Wild-type mice (n = 5; 8 weeks old) were injected unilaterally at one site into the spinal cord with 0.5 µl of rAAV–EGFP. Three weeks later, a small cup containing a 2% FG solution in saline was placed on the proximal segment of the transected sciatic nerve for 1 week. (**A** and **C**) EGFP expression in lumbar spinal cord 4 weeks after intraspinal injection. (**B**) EGFP-NeuN double-labeled neurons of the same section as A. (**D**) EGFP-positive motoneurons backed-labeled with FG. Arrows indicate FG/EGFP double-labeled motoneurons and NeuN/EGFP double-labeled neurons. Sections were examined with both rhodamine and fluorescein filters on an Olympus microscope. The EGFP fluorescence is specific and not seen when viewed under rhodamine optics. Nissl-staining (**E**) and immunostaining with antibody J1-31 (**F**) of spinal cord sections of rAAV–EGFP injected mice. Arrow in (E) indicates the needle tract and arrowheads in (F) show mild gliosis.

of immunocompetent animals, including the brain where they preferentially transduce neurons (17,18). In the present work, we have employed an rAAV vector to express human bcl-2 in spinal cord motoneurons of FALS transgenic mice by direct intraspinal injection of the recombinant virus. The effects of bcl-2 expression on motoneuron survival, neuromuscular function and onset of disease were assessed. We demonstrate that rAAV-mediated expression of bcl-2 protects infected spinal motoneurons from

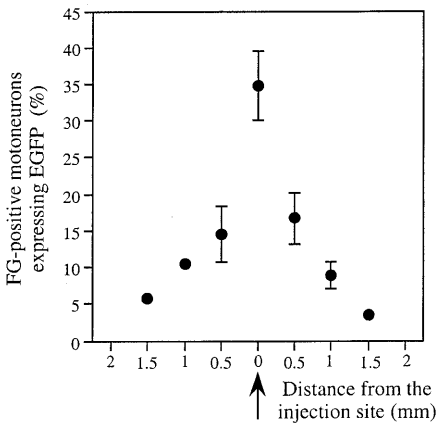

Figure 2. Percentage of FG-positive motoneurons expressing the reporter gene *EGFP* 4 weeks after rAAV–EGFP injection. Twenty sections per point (0, 0.5, 1, 1.5 and 2 mm) and per animal were analyzed over a distance of 4 mm (2 mm on either side of the injection). rAAV was injected at 0 mm (arrow). Values refer to means ± SEM; $n = 5$ animals.

degeneration, slows the decrease in compound muscle action potential (CMAP) and delays the appearance of motor deficiency in FALS transgenic mice.

RESULTS

Analysis of transduction efficiency in the spinal cord of normal mice

To determine the transduction efficiency of rAAV vectors, we performed intraspinal injections of an rAAV virus expressing the marker protein enhanced green fluorescent protein (EGFP) in wild-type C57BL/6 mice. Numerous fluorescent cells were detected in the spinal cord (Fig. 1A and C). To identify the phenotype of these cells, sections were double-immunostained with antibodies to EGFP and NeuN. Most of the EGFP-labeled cells were double-labeled with NeuN (Fig. 1B), whereas only scarce cells were double-stained with GFAP (data not shown). Intraspinal injection of the rAAV vector was associated with a mild degree of gliosis, with no significant cell damage. This observation was made with Nissl staining and immunohistochemistry for astrocyte-specific antigen recognized by monoclonal antibody J1-31, a marker of reactive astrocytes (Fig. 1E and F).

To assess the percentage of motoneurons expressing the reporter gene, motoneurons back-labeled with fluorogold (FG) were analyzed (Fig. 1D). The number of FG-positive motoneurons expressing EGFP was counted in sections of the lumbar spinal cord extending 2 mm in either direction from the needle tract. Double-labeled cells were detected as far as 1.5 mm from the injection site (Fig. 2). In sections surrounding the injection site, 34.7 ± 5.2% ($n = 5$) of the FG-labeled motoneurons expressed EGFP. The percentage of labeled cells decreased with increasing distance from the injection site (Fig. 2).

Onset of disease and survival in rAAV-treated *SOD1*^{G93A} transgenic mice

In *SOD1*^{G93A} mice, with a Gly→Ala substitution at position 93, the first consistent sign of disease is tremor that occurs in one or more limbs at 90–100 days of age. As the disease progresses, the

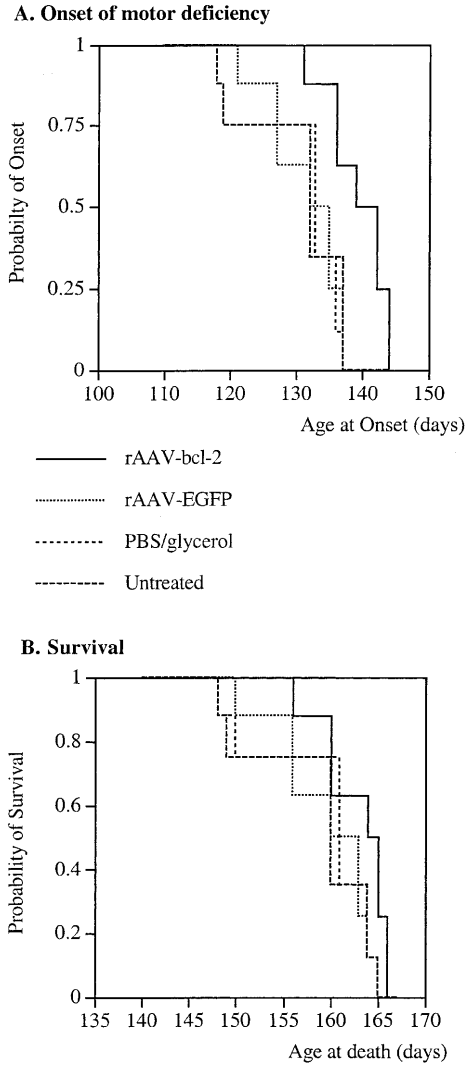

Figure 3. Onset of disease and survival in FALS transgenic mice. (**A**) Cumulative probability of disease onset in rAAV–bcl-2-treated mice and control groups ($n = 7$–8 mice/group). Onset of disease is defined by the appearance of SFPs in gastrocnemius muscle. SFP measurements were made at 60, 70, 80, 90, 100, 110, 120, 130 and 140 days of age. Bcl-2 delays onset of disease by 10.4–12.9 days compared with rAAV–EGFP, PBS/glycerol and untreated animals (rAAV–bcl-2 versus rAAV–EGFP: $P = 0.02$; rAAV–bcl-2 versus PBS: $P = 0.018$; rAAV–bcl-2 versus untreated: $P = 0.014$, Fisher's test). (**B**) rAAV–bcl-2 has no effect on the survival of the FALS transgenic mice.

mice develop proximal muscle weakness and atrophy. At the end-stage of disease (135–140 days of age), the animals are severely paralysed and have lost up to 10% of their body weight (3).

Five-week-old *SOD1*^{G93A} transgenic mice were injected bilaterally at two sites into the lumbar spinal cord with rAAV–bcl-2 ($n = 8$), rAAV–EGFP ($n = 8$) or phosphate-buffered saline (PBS)/glycerol ($n = 7$). An additional group of mice ($n = 7$) remained untreated. To determine the effect of bcl-2 on the onset of motor deficiency, spontaneous fibrillation potentials (SFPs) were measured in the gastrocnemius muscle between 60 and 140 days of age. SFPs are an indication of muscle fiber denervation. The onset of clinical disease, as defined by the first appearance of SFPs in the gastrocnemius muscle, was delayed by 10.4–12.9 days in rAAV–

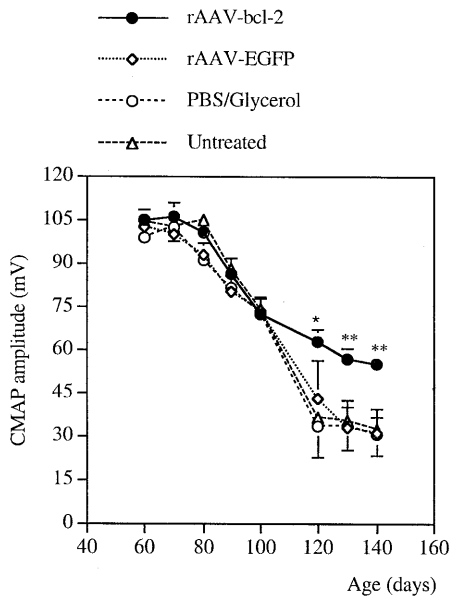

Figure 4. Evoked compound muscle action potential (CMAP) amplitude in the gastrocnemius muscle after stimulation of the sciatic nerve in FALS transgenic mice injected with rAAV–bcl-2 (n = 8), rAAV–EGFP (n = 8), PBS/glycerol (n = 8) and untreated animals (n = 7). Measurments were made at 60, 70, 80, 90, 100, 120, 130 and 140 days of age. Data are expressed in mV ± SEM. *Significant with Scheffé test at $P < 0.01$; **significant with Scheffé test at $P < 0.001$.

bcl-2-treated mice compared with control animals (rAAV–bcl-2: 138.4 ± 2.3 days; rAAV–EGFP: 128 ± 2.6 days; PBS: 126.0 ± 4.3 days; untreated: 125.5 ± 4.0 days; rAAV–bcl-2 versus rAAV–EGFP: $P = 0.02$; rAAV–bcl-2 versus PBS: $P = 0.018$; rAAV–bcl-2 versus untreated: $P = 0.014$, Fisher's test) (Fig. 3A). However, local bcl-2 expression in the lumbar spinal cord was insufficient to prolong the survival of FALS transgenic mice (Fig. 3B). This is consistent with the lack of an effect on the appearance of SFPs in the diaphragm muscle (rAAV–bcl-2: 134.2 ± 3.5 days; rAAV–EGFP: 132.6 ± 4.2 days; PBS: 129.8 ± 2.8 days; untreated: 131.4 ± 5.2 days).

Neuromuscular function in FALS transgenic mice

CMAP amplitudes in gastrocnemius muscle were measured between 60 and 140 days of age as an indication of the number of functional neuromuscular units (19,20). CMAP values in the gastrocnemius muscle were normal and not significantly different between 60 and 80 days of age in both rAAV–bcl-2-treated and control *SOD1*^{G93A} animals [at 80 days of age, rAAV–bcl-2: 100.5 ± 2.6 mV; rAAV–EGFP: 92.7 ± 4.1 mV (n = 8); PBS/glycerol: 90.7 ± 2.1 mV; untreated: 105.0 ± 3.1 mV (n = 7)] (Fig. 4). Thereafter, the CMAP amplitude decreased progressively with time in all groups up to 100 days of age. After 100 days, the decrease in the CMAP amplitude was significantly faster in the controls than in rAAV–bcl-2-treated animals. The slope obtained by regression analysis was significantly lower in the rAAV–bcl-2-treated group (–2.01 ± 0.1 mV/day) compared with the control groups (rAAV–EGFP: –2.62 ± 0.15 mV/day; PBS/glycerol: –2.59 ± 0.21 mV/day; untreated: –2.58 ± 0.12 mV/day). As a consequence, the CMAP amplitude was significantly higher in rAAV–bcl-2-treated mice than in control groups at 140 days of age ($P < 0.001$). The mice were sacrificed 20.2 ± 3 days after the last measurement when they were unable to right themselves.

Table 1. Number of motoneurons at the end-stage of disease

Groups	Location of sections between injection sites (mm)				
	0	0.5	1	1.5	2
Bcl-2	78.5 ± 4.3[a]	108.0 ± 2.7[b]	94.7 ± 4.6[a]	104.8 ± 1.9[b]	85.0 ± 2.0[a]
GFP	57.0 ± 2.6	54.0 ± 4.7	54.8 ± 5.3	59.5 ± 2.8	60.3 ± 1.8
PBS/glycerol	51.0 ± 3.7	55.0 ± 4.8	53.0 ± 2.9	56.3 ± 2.8	55.8 ± 3.4
Untreated	58.5 ± 4.9	57.5 ± 1.8	55.8 ± 3.4	62.8 ± 2.5	67.8 ± 0.9

Cell counts were performed in serial Nissl-stained sections between the two injection sites (injection site no. 1 at 0 mm; no. 2 at 2 mm). Electrophysiological and histological evaluations were performed in the same side of the spinal cord. Only large ventral horn motoneurons with distinct nuclei and nucleoli were included. Spinal injection of rAAV–bcl-2 resulted in a significantly greater number of surviving motoneurons compared with control groups ([a]$P < 0.002$; [b]$P < 0.0001$). Data (means ± SEM) refer to the total numbers of motoneurons on 20 sections at 0, 0.5, 1, 1.5 and 2 mm (n = 5 mice/group). Normal non-transgenic littermates had 151 ± 12 motoneurons at the 0.5 mm spinal cord level.

Motoneuron survival

To determine whether bcl-2 expression protects motoneurons from degeneration, Nissl-stained motoneurons in spinal cord sections of rAAV–bcl-2-injected and control *SOD1*^{G93A} mice were counted 15 weeks following the injection of rAAV vectors (end-stage of disease). Only large cell profiles containing a distinct nucleus with a nucleolus were included. As shown in Table 1 and Figure 5, the number of large motoneurons counted in Nissl-stained sections (Fig. 5A and B) and the number of choline acetyltransferase (ChAT)-positive cells (Fig. 5C and D) was significantly higher in rAAV–bcl-2-treated animals compared with the control groups. At 0.5 mm from the injection site (where bcl-2 protection is maximal) (Table 1), rAAV–bcl-2-treated *SOD1*^{G93A} mice retained 71.5% of the motoneurons of normal non-transgenic littermates (number of motoneurons in non-transgenic mice at the same level of the lumbar spinal cord: 151 ± 12). In contrast, in rAAV–EGFP-treated and other control *SOD1*^{G93A} transgenic animals, only ~40% of the motoneurons remained at the same level of the spinal cord (Table 1). Thus, rAAV-mediated expression of bcl-2 rescued ~50% of the motoneurons that normally degenerate in *SOD1*^{G93A} mice (rAAV–bcl-2 versus rAAV–EGFP at 0.5 mm).

Bcl-2 transgene expression

To assess expression of human bcl-2 at the end-stage of the disease, immunodetection was performed at the time of death, i.e. 15 weeks following the injection of rAAV–bcl-2. Bcl-2-immunolabeled motoneurons are shown in Figure 6A and B. Bcl-2-positive neurons were found over a 5 mm distance along the rostro-caudal axis (Fig. 7). No immunoreactivity for human bcl-2 was observed in rAAV–EGFP-injected mice (Fig. 6C).

DISCUSSION

rAAVs are promising vectors for gene transfer in the nervous system, since they allow long-term expression of transgenes in postmitotic neurons (14,15). These vectors have been used to explore the neuroprotective potential of certain proteins in a

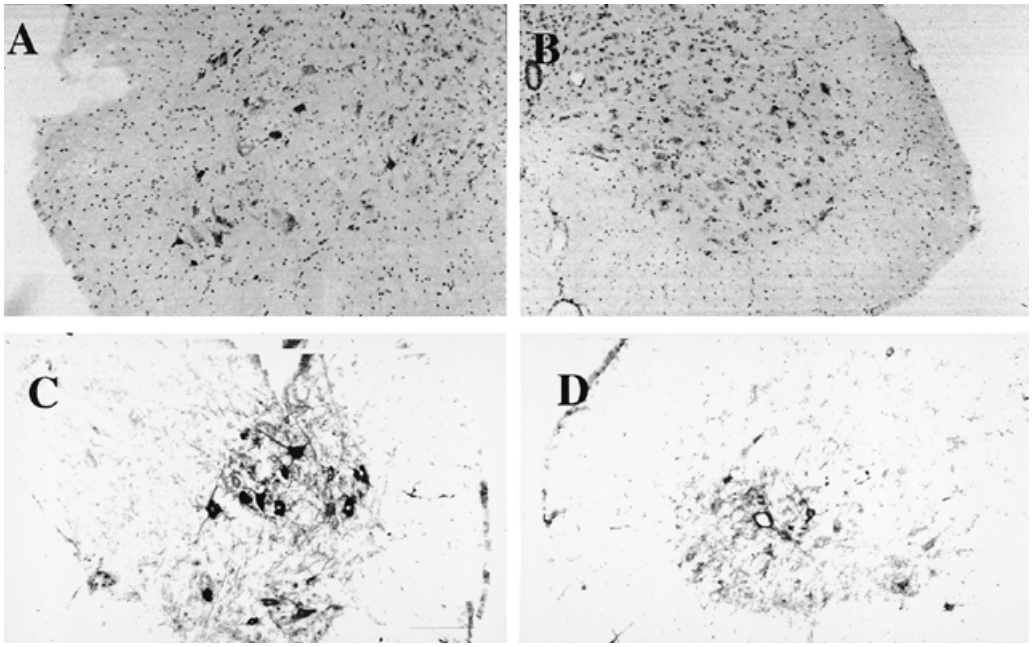

Figure 5. Nissl staining (**A** and **B**) and choline acetyltransferase (ChAT) immunochemistry (**C** and **D**) in rAAV–bcl-2 (A and C) and rAAV–EGFP (B and D) injected mice at the end-stage of disease. Note the larger number of Nissl-stained and ChAT-positive motoneurons in rAAV–bcl-2- compared with rAAV–EGFP-injected animals.

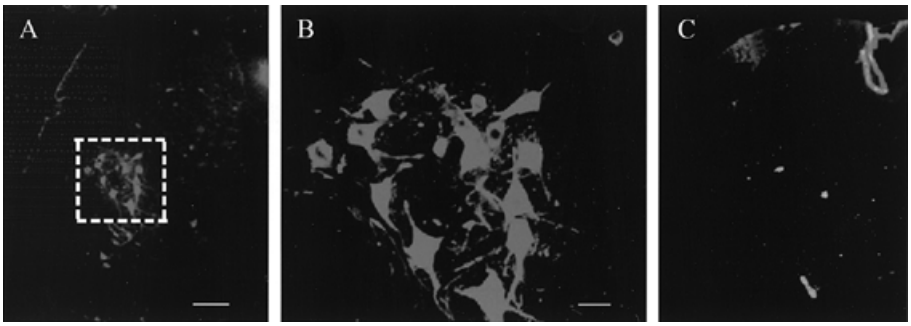

Figure 6. Photomicrographs of spinal cord sections from rAAV–bcl-2- and rAAV–EGFP-injected FALS mice at the end-stage of disease. Expression of human bcl-2 in spinal motoneurons is confirmed by immunohistochemistry using monoclonal antibodies against human bcl-2 (**A** and **B**). No bcl-2-positive motoneurons were observed in the rAAV–EGFP-injected animals (**C**).

number of animal models. Intranigral injection of rAAV–GDNF, for instance, induces significant protection of tyrosine hydroxylase-positive neurons in a rat model of Parkinson's disease (17). We have developed a direct *in vivo* gene transfer method using rAAVs to express genes in murine spinal motoneurons. An *ad hoc* developed system composed of a stereotactic frame and an automatic micropump allows the localized injection of hundreds of nanoliters of viral stock solution into the mouse spinal cord without inducing any significant damage. This is consistent with previous reports that demonstrated the absence of toxicity and inflammatory responses after injection of rAAV vectors into the central nervous system and other tissues (21,22). Moreover, in non-symptomatic *SOD1* mice, CMAP amplitudes were similar before and after rAAV delivery (data not shown), showing that intraspinal rAAV injection does not cause a functional deterioration. Using this method, the reporter gene *EGFP* can be expressed in spinal motoneurons of adult mice. Most of the transduced cells were neurons based on their staining for the neuronal protein NeuN. It was previously reported that neuronal cells are preferentially transduced by rAAV in the rat spinal cord (21). In our experiments, we were able to detect EGFP-transduced motoneurons as far as 1.5 mm from the injection site in the murine spinal cord.

In a second step, we investigated whether rAAV-mediated intraspinal delivery of a neuroprotective gene can interfere with the degeneration of motoneurons in a transgenic mouse model of human FALS. In this model, motoneuron degeneration is caused by overexpression of a mutated form of human *SOD1* (*SOD1*^{G93A}). Although the mechanism by which mutated *SOD1* is toxic to motoneurons is unknown, evidence suggests that apoptosis may be involved. For instance, DNA fragmentation and changes in the localization and levels of bcl-2, bax and bak have been observed in post-mortem spinal cord samples from

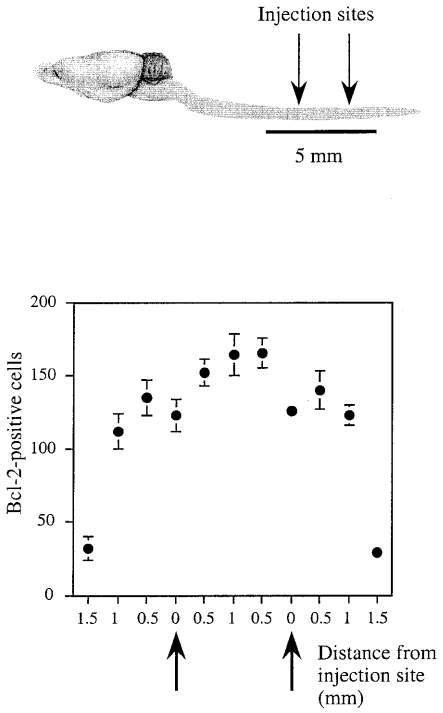

Figure 7. Number of bcl-2-positive cells (including motoneurons) in the spinal cord of rAAV–bcl-2-injected mice over a 5 mm distance. G1H mice received intraspinal injection at four sites (two sites separated by 2 mm on each side of the spinal cord). Arrows refer to the injection sites. Each animal received 0.5 µl of the virus per site. Counts were made at the end-stage of disease (15 weeks following the injections). Twenty sections per point (0, 0.5, 1, 1.5 and 2 mm) and per animal were analyzed (n = 5 animals).

ALS patients (23). Moreover, caspase-1, one of several proteases functioning during apoptosis, is activated in mouse neuroblastoma cells expressing mutated, but not wild-type, *SOD1* as well as in two transgenic mouse lines (G37R and G85R) expressing mutated human *SOD1* (11). These findings are consistent with the observation that inhibition of caspase-1 slows progression of motoneuron disease and delays mortality in *SOD1*^{G93A} transgenic mice (13). Finally, transgenic overexpression of bcl-2 delayed disease onset and prolonged survival in FALS mice (12).

The present work investigates the effects of local viral-mediated expression of human bcl-2 in the spinal cord of *SOD1*^{G93A} mice on motoneuron survival, neuromuscular function and development of disease. Direct intraspinal injection of the rAAV–bcl-2 vector results in efficient and stable expression of bcl-2 in spinal motoneurons for at least 15 weeks (duration of the experiment). Moreover, rAAV–bcl-2-injected *SOD1*^{G93A} mice have significantly increased numbers of surviving motoneurons and show delayed decline of the CMAP amplitude in comparison with rAAV–EGFP-injected and other control *SOD1*^{G93A} animals. Quantification of motoneuron survival in the various experimental groups relative to normal non-transgenic littermates revealed that rAAV-mediated expression of bcl-2 rescued ~50% of the motoneurons that normally degenerate in *SOD1*^{G93A} mice (Table 1). We conclude that local bcl-2 expression in the spinal cord is able to inhibit motoneuron degeneration and improve neuromuscular function in FALS transgenic mice since the CMAP amplitude is an indicator of neuromuscular unit function (19,20). At 150 days of age, proximal muscle atrophy and paralysis become more pronounced in all groups and CMAP amplitudes are not detectable any more. Importantly, bcl-2 expression also delays the onset of motor deficiency as evaluated by the first appearance of SFPs in the gastrocnemius muscle (Fig. 3A) and improved performance in the rotarod test (data not shown). However, in contrast to the slight prolongation of the life expectancy of FALS mice carrying a neuronally expressed bcl-2 transgene (12), we did not observe an increase in the survival of rAAV–bcl-2-treated mice. This difference may be explained by several factors. Whereas SOD1/bcl-2 double-transgenic mice overexpress bcl-2 in all their neurons, including cervical motoneurons (12), we have locally targeted the motoneurons in the lumbar spinal cord. Thus, it may not be unexpected that the rescue of motoneurons was restricted to the lumbar spinal cord, whereas it is more widespread in the double-transgenic mice, possibly resulting in prolonged survival. Consistent with this, no delay in the occurrence of SFPs was observed in the diaphragm. In human ALS, respiratory failure is the major cause of death. In the G1H mice used in this study, the reduction of myelinated axons in the phrenic nerve innervating the diaphragm parallels the time course of motor neuron loss in cervical and lumbar spinal cord [60% loss at the end-stage of disease (24)], indicating that compromised respiratory function may also be involved in the death of FALS transgenic mice.

A recent study suggested that apoptosis may not be responsible for the neuronal death observed in *SOD1*^{G93A} mice (25). This conclusion was based on the failure to detect histological and biochemical markers of apoptosis in spinal cord tissue. Therefore, evidence that rAAV–bcl-2 treatment prevents cell death induced by apoptosis will be difficult to obtain. However, by regulating homeostasis across mitochondrial and other intracellular membranes and promoting mitochondrial adaptation to perturbations in cellular metabolism, bcl-2 and related proteins may have the ability to promote cell survival by means that are different from inhibition of the known apoptotic pathways (26). For example, bcl-2 has been shown to inhibit the disruption of mitochondrial membrane potential and the increase in cytosolic Ca^{2+} concentrations observed in *SOD*^{G93A} transfected neuroblastoma cells (27). Another possibility is that bcl-2 prevents motoneuron loss through its function in cellular antioxidant pathways (28–31). Increased oxygen radical production and protein oxidative damage have been detected in the spinal cord of *SOD1*^{G93A} mice (32,33). Oxidative stress can damage membranes and compromise mitochondrial function (34,35), and bcl-2 inhibits these adverse effects (36,37). Interestingly, pronounced mitochondrial degeneration has been shown to precede the functional decline of motoneurons and symptoms of ALS in *SOD1*^{G93A} mice (38,39). Taken together, these observations suggest that a mitochondrial defect triggered by chronic exposure to increased levels of oxygen radicals could underlie the pathology of ALS in *SOD1*^{G93A} mice, and that bcl-2 may inhibit this process by its antioxidant properties.

Recently, mutant *SOD1*-dependent inactivation of the glial glutamate transporter GLT-1 has been shown in a *Xenopus* oocyte expression system. This suggests calcium-mediated excitotoxicity as another mechanism by which mutant *SOD1* may contribute to neuronal death (8). Interestingly, a Ca^{2+}-dependent apoptotic pathway involving calcineurin has been described in neurons (40). Bcl-2 has been shown to increase the

calcium uptake and buffering capacity of mitochondria (41–43). Since calcium is implicated in excitotoxic neuronal death, the ability of bcl-2 to allow cells to adapt to higher concentrations of calcium may protect neurons against glutamate toxicity (44).

In summary, we believe that the pleiotropic activities of bcl-2, in particular its ability to safeguard neurons against oxidative stress and glutamate toxicity, may be important in mediating motoneuron protection in *SOD*^{G93A} mice. This interpretation is in line with results showing that enzymatic or dietary-based antioxidant therapy, as well as anti-glutamatergic drugs can delay onset of disease, progression of symptoms and/or mortality in *SOD1*^{G93A} transgenic mice (45–47). We propose that bcl-2 prevents or slows the development of mitochondrial abnormalities, although proof of this would require ultrastructural analysis of the mitochondria of rAAV–bcl-2-infected motoneurons *in vivo*. Defective oxidative phosphorylation in degenerating mitochondria could finally lead to a deficiency of ATP synthesis and contribute to neuronal death. This hypothesis is supported by the recent demonstration that oral administration of creatine prevents the loss of motoneurons and results in a dose-dependent improvement in motor performance and extended survival in *SOD1*^{G93A} mice (48).

To our knowledge, this study describes the first successful transduction and protection of spinal motoneurons by direct gene transfer in a model of progressive motoneuron disease. Although the current approach may not be directly transferable to humans, it represents a new way to evaluate the effects of candidate therapeutic proteins on motoneuron function and survival in animal models of FALS. Moreover, by expressing proteins expected to interfere with specific postulated disease mechanisms in motoneurons, it may be possible to delineate more exactly the pathological pathways and their relative contribution to disease development. In addition, in the future rAAV vectors may be used for regulated secretion of neurotrophic factors after intramuscular delivery (49,50). These combined studies may ultimately lead to improved treatment strategies for human ALS.

MATERIALS AND METHODS

Construction of the rAAV plasmids

The *Eco*RI–*Hin*dIII CMV-lacZ-polyA expression cassette from plasmid pCMV-b (Clontech, Palo Alto, CA) was filled in with Klenow polymerase and inserted between the Klenow-filled *Xba*I sites of the vector psub201 (51) to generate the plasmid psubCMV-β. Subsequently, the *Not*I–*Not*I β-galactosidase fragment of psubCMV-β was replaced by a multiple cloning site (*Not*I, *Ace*II, *Nhe*I, *Pml*I, *Mlu*I, *Acc*65I, *Kpn*I) generated by hybridization of the oligodeoxynucleotides GGCCGCAGCCATGGGCTAGCACGT-GACGCGTGGTACC and GGCCGGTACCACGCGTCACGT-GCTAGCCCATGGCTGC to produce plasmid psubCMV. Finally, the human bcl-2 coding region was amplified by PCR from plasmid pblue-bcl-2 (a kind gift of M. Cleary, Stanford University) using the primers CCGGTTCTAGAGCCACCATGGCG-CACGCTGGGAGAAC and ACTAGTGGTACCTTATCACTT-GTGGCCCAGATAG. The PCR product was digested with *Xba*I and *Kpn*I and subcloned between the *Nhe*I and *Kpn*I sites of psubCMV. The resulting rAAV vector, psubCMV-bcl-2/oK, expresses bcl-2 under the control of the CMV promoter/enhancer from an mRNA with optimized Kozak sequence (underlined). The control rAAV plasmid psubCMV–EGFP was constructed by inserting the *Sma*I–*Hpa*I EGFP fragment isolated from the vector pEGFP-N1 (Clontech) into the *Pml*I site of psubCMV.

Production and purification of rAAV particles

Thirty 175 cm^2 tissue culture plates with 80–90% confluent 293 cells in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) were co-transfected by the calcium phosphate method with rAAV plasmid (psubCMV–bcl-2/oK or psubCMV–EGFP) and AAV packaging plasmid pAAV/Ad (48). Eight hours later, the transfection medium was replaced by fresh DMEM–10% FCS containing an E1/E3-deleted adenovirus at a multiplicity of infection of 2. Virus production was allowed to proceed until the cells showed full cytopathic effect (48–60 h), at which time the cells were collected in their medium and pelleted by centrifugation at 500 *g* for 10 min at 4°C. rAAVs encoding human bcl-2 or EGFP were released from the cells and purified by two rounds of CsCl gradient centrifugation as described (52). rAAV genomes in CsCl gradient fractions were detected by slot blot hybridization and adenovirus by infection of 293 cells (52). rAAV-containing fractions with little or no adenovirus contamination were pooled, dialyzed against PBS and incubated at 56°C for 30 min to inactivate residual adenovirus. The rAAV titers were 3.9×10^{11} particles/ml for rAAV–bcl-2 and 1.9×10^{11} particles/ml for rAAV–EGFP. Contamination by adenovirus was ~1 p.f.u./10^6 rAAV particles.

Animals

Transgenic mice with the G93A human *SOD1* mutation (G1H line) were used in this study (3). This line was maintained as a hemizygote by breeding G93A males with female littermates (B6SJL/F1 females; Iffa Credo, L'Arbresle, France). The offspring were genotyped by PCR amplification of DNA extracted from the tail tissue. The primer sequences selected have previously been described (2). Mice were housed in microisolated cages at room temperature in a 12–12 h light–dark cycle. They had free access to food and water. Transgenic mice were killed when they were unable to right themselves within 30 s when placed on their sides (end-stage of disease). The experiments were carried out in accordance with the European Community Council Directive (86/609/EEC) for care and use of laboratory animals.

Intraspinal injection of rAAV vectors

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (62.5 mg/kg body wt) and received an intraperitoneal injection of the anti-inflammatory agent, Solu-Medrol (62.5 mg/kg body wt; Pharmacia and Upjohn, Kalamazoo, MI). Animals were placed in a stereotax and their spinal cords were immobilized using a spinal adaptor (Stoelting, Wood Dale, IL). rAAV was injected into the lumbar spinal cord following laminectomy. To assess transduction efficiency, 8-week-old C57BL/6 mice (n = 5) were injected with 0.5 µl of rAAV–EGFP (1.9×10^{11} particles/ml) at one site. Three weeks following rAAV–EGFP injection, wild-type C57BL6 mice were anesthetized by an intraperitoneal injection of sodium pentobarbital. The sciatic nerve was exposed at mid-thigh level and cut 5 mm proximal to the nerve trifurcation. A small cup

containing a 2% FG solution in saline was placed on the proximal segment of the transected nerve. One week after application of FG the animals were perfused transcardially with 4% paraformaldehyde. The lumbar spinal cord was dissected out and histological analysis was performed as described below. The number of FG and EGFP double-labeled motoneurons was counted 4 weeks after injection of the viral vector.

To determine the effect of bcl-2 on motoneuron survival, 5-week-old G1H transgenic mice were injected bilaterally at two sites separated by 2 mm with rAAV–EGFP ($n = 8$), rAAV–bcl-2 ($n = 8$) (for both viruses, 0.5 µl/site, 1.9×10^{11} particles/ml), PBS/glycerol ($n = 7$) or remained untreated ($n = 7$). Injections, controlled by an infusion pump (Stoelting), were at 0.1 µl/min through a 10 µl Hamilton syringe fitted with a 33 gauge needle. Following injection, the needle was left in place for 5 min before being retrieved. Electrophysiological and histological measurements were used to evaluate effects of rAAV–bcl-2 treatment on G1H mice.

Histological evaluation

To determine transduction rates, wild-type C57BL/6 mice were killed 4 weeks after injection with rAAV–EGFP. G1H transgenic mice were killed at the end-stage of disease (15 weeks following injection). Mice were perfused transcardially with 4% paraformaldehyde. The spinal cords were excised and cryoprotected in 30% sucrose for 2 days. Twenty micrometer thick cryosections were stained for Nissl and analyzed by immunohistochemistry. For immunohistochemistry, non-specific binding was blocked by a 2 h incubation with 10% goat serum and 0.1% Triton X-100 in PBS at room temperature prior to incubation with specific antibodies: (i) polyclonal rabbit anti-EGFP diluted 1:200 (Clontech, P.H. Steheling and CIE AG, Basel, Switzerland); (ii) monoclonal anti-human bcl-2 diluted 1:200 (Dako, Glostrup, Denmark); (iii) monoclonal anti-NeuN antibody diluted 1:50 (Chemicon, Lucerne, Switzerland); (iv) monoclonal anti-ChAT antibody at 2 mg/ml [ChAT-17 (53)]; and (v) monoclonal J1-31 antibody diluted 1:250 (54). Secondary antibodies were added for 2 h after washing the sections [EGFP staining: goat anti-rabbit FITC diluted 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA); bcl-2 labeling: Cy3 goat anti-mouse at a 1:400 dilution (Jackson ImmunoResearch Laboratories); ChAT staining: biotinylated goat anti-mouse at a 1:200 dilution, followed by the avidin–biotin procedure (ABC, Vector kit; Vector Laboratories, Burlingame, CA)]. Bcl-2-positive neurons and large motoneurons with distinct nuclei and nucleoli were counted in a blind manner section stained with cresyl violet and bcl-2 antibody. Twenty sections per point (0, 0.5, 1, 1.5 and 2 mm) and per mouse were analyzed ($n = 5$ animals).

Electrophysiological recordings

Evoked CMAP amplitudes were evaluated as previously described (19). Briefly, electrophysiological recordings across the sciatic nerve segment were made using a Keypoint (Dantec, Skovlunde, Denmark) electromyogram apparatus. Measurements were made at 60, 70, 80, 90, 100, 120, 130 and 140 days of age. Mice were deeply anesthetized and normal body temperature was maintained with a heating lamp. The sciatic nerve was stimulated at a paraspinal site. Stimulation consisted of single 0.2 ms, 1 Hz supra maximal pulses through a unipolar needle electrode (13R81; Dantec) The evoked CMAPs were recorded from the medial part of the gastrocnemius muscle with the same electrode. The CMAP amplitude was measured from peak to peak. SFPs were recorded (at the same days as the CMAP amplitudes) with a concentric needle electrode (13R05; Dantec) inserted through the skin into several sites of the gastrocnemius muscle and the diaphragm. Only SFPs with an amplitude ranging between 20 and 300 µV were taken into account. Traces showing voluntary contractile activity were discarded.

Statistical studies

Statistical analysis was performed using analysis of variance (ANOVA). In addition, Scheffé's test was used to check for differences between individual groups.

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