Brain-derived neurotrophic factor-mediated protection of striatal neurons in an excitotoxic rat model of Huntington's disease, as demonstrated by adenoviral gene transfer

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Running title: BDNF gene transfer in the rodent CNS

ABSTRACT

Huntington's disease (HD) is a genetic disorder leading to the degeneration of striatal GABAergic output neurons. No treatment is currently available for this devastating disorder, although several neurotrophic factors, including brain-derived neurotrophic factor (BDNF), have been shown to be beneficial for striatal neuron survival. We analyzed the effect of adenovirus-mediated transfer of the BDNF gene in a model of HD. Using a stereological procedure, three groups of rats were given an intrastriatal injection of adenovirus encoding BDNF, ß-galactosidase or sham surgery. Two weeks after treatment, the animals were lesioned with quinolinic acid (QUIN), a toxin which induces striatal neuron death by an excitotoxic process. One month after the lesion, histological study revealed that striatal neurons were protected only in rats treated with the BDNF adenovirus. Volume measurements showed that the QUIN-induced lesions were 55% smaller in the BDNF adenovirus-treated group than in the ß-galactosidase adenovirus-treated group ($p<0.05$), and the sham-treated group ($p<0.05$). To determine the survival of striatal GABAergic output neurons after the QUIN-induced lesion, we immunostained brain sections with DARPP-32, an antibody specific for striatal output neurons. Prior treatment with the BDNF adenovirus resulted in a cell survival of 64%, whereas that after ß-galactosidase treament was 46% ($p<0.05$), showing that the BDNF adenovirus protected the striatal neurons. These results indicate that transfer of the BDNF gene is of therapeutic value for Huntington's disease.

OVERVIEW SUMMARY

Huntington's disease results from a progressive neurodegenerative process affecting primarily the neostriatum. The GABAergic output neurons are the most sensitive cell type in this anatomical structure. Recently, brain-derived neurotrophic factor (BDNF) was shown to be a potent neurotrophic factor for striatal GABAergic neurons *in vitro* and *in vivo*. We investigated whether BDNF protected striatal neurons in a rodent model of Huntington's disease, by testing the effect of a recombinant adenovirus producing this neurotrophin in rats injected with quinolinic acid. BDNF was produced for at least six weeks after intrastriatal injection of the adenoviral vector. Most importantly, recombinant BDNF reduced the size of the quinolinic acid-induced lesion in the striatum and protected striatal GABAergic output neurons from the excitotoxic damage. Thus, *in vivo* BDNF gene transfer has potential for the neuroprotective treatment of Huntington's disease.

INTRODUCTION

Huntington's disease (HD) is an autosomal dominant genetic disorder involving the neurodegeneration of primarily the striatal GABAergic output neurons (i.e. medium-sized spiny neurons). This results in excessive involuntary movements (chorea) accompanied by cognitive deficits and personality changes. In 1993, it was shown that HD is caused by the expansion of a polymorphic CAG/polyglutamine repeat located in the IT15 gene (The Huntington's Disease Research Collaborative Group, 1993). The protein encoded by IT15, huntingtin, is present throughout the central nervous system and in peripheral tissues, but neurodegeneration is restricted to striatal GABAergic output neurons (Sharp *et al.*, 1995 ; Trottier *et al.*, 1995). It is now established that expansion of the polyglutamine tract causes the intranuclear aggregation of the mutant huntingtin, known as neuronal intranuclear inclusion (Davies *et al.*, 1997 ; Ordway *et al.*, 1997). Inclusions are found in the brains of both patients (DiFiglia *et al.*, 1997) and transgenic mice bearing the mutated HD gene (Davies *et al.*, 1997), but their

contribution to the pathogenicity of the mutant huntingtin is unclear (Saudou *et al.*, 1998 ; Sisodia, 1998). Nevertheless, in two recent studies, it has been suggested that the expanded polyglutamine repeat-induced cell death might be due to activation of caspases, a family of pro-apoptotic proteins (Ona *et al.*, 1999 ; Sanchez *et al.*, 1999). Although these recent findings constitute progress towards the elucidation of the pathological mechanism, they have not yet led to any obvious treatment, and HD remains an incurable disease.

Several strategies are currently being explored to develop treatments for HD, including both restorative and preventive approaches. The restorative strategy involves the intracerebral grafting of fetal striatal cells and is undergoing clinical trials in France and the USA (Horellou and Mallet, 1998 ; Kopyov *et al.*, 1998). The development of a preventive treatment to protect neuronal cells from the neurodegenerative process would be of great value for a clinical application by preventing the onset of symptoms. Animal models that allow to test possible treatments have been set up. These are based on excitotoxic cell death, which is thought to be involved in the pathological development of HD. Thus, several models involve the use of excitotoxins to induce neuronal cell death mimicking the pattern of neurodegeneration observed in HD (DiFiglia, 1990). Quinolinic acid (QUIN) has been widely used in models of HD. QUIN acts on the NMDA receptor and if injected intrastriatally, it reproduces the cell loss and neurochemical deficits of HD (Beal *et al.*, 1986). In this model of HD, various neurotrophic factors have been shown to be effective for the protection of striatal GABAergic output neurons. Nerve growth factor (NGF) (Martinez-Serrano and Björklund, 1996), ciliary neurotrophic factor (CNTF) (Anderson *et al.*, 1996 ; Emerich *et al.*, 1997) and glial cell line-derived neurotrophic factor (GDNF) (Araujo and Hilt, 1997) protect striatal GABAergic neurons from excitotoxic cell death *in vivo*. Several observations have suggested that brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, protects striatal GABAergic output neurons. In striatal primary culture, BDNF increases the survival, soma size and branching point number of GABA-expressing neurons (Mizuno *et al.*, 1994 ; Ventimiglia *et al.*, 1995 ; Widmer and Hefti, 1994). The neuroprotective effect of BDNF has also been demonstrated in two *in vitro* models of HD: firstly, BDNF protects striatal cells exposed to an excitotoxic stress in culture (Nakao *et al.*, 1995) ; secondly, BDNF abolishes the apoptosis observed in striatal cells transfected with the coding sequence of the mutant huntingtin (Saudou *et al.*, 1998). Thus, determining the protective potential of BDNF in *in vivo* models of HD is of major importance.

In this study, we tested the effect of BDNF using a recombinant adenovirus. This vector was injected into the striatum of rats. Lesions were then produced two weeks later in the same area with QUIN. The adenovirus-mediated transfer of the BDNF gene led to the strong expression of this neurotrophin gene in the rodent striatum, and the recombinant BDNF protected the striatal GABAergic output neurons from excitotoxic cell death induced by QUIN.

METHODS

In vitro adenoviral gene transfer.

Ad-BDNF and Ad-ßGal stocks were obtained as previously described (Gimenez y Ribotta *et al.*, 1997 ; Stratford-Perricaudet *et al.*, 1992). For *in vitro* infection, HeLa cells were plated at a density of 5.10^5 cells per well in 6-well culture plates and infected as described below. The culture medium (Dulbecco's modified eagle medium containing 10% fetal calf serum) was removed and cells were infected with either Ad-BDNF or Ad-ßGal at a multiplicity of infection (MOI) ranging from 20 to 80 plaque-forming units (pfu) per cell in 0.5 ml of serum-free medium for one hour. Afterwhich, the culture medium was added back to the cells. Three days later, cells were extensively rinsed with phosphate-buffered saline (PBS) and were then incubated for 24 hours in fresh medium. The amount of BDNF in the culture medium was determined with the BDNF Emax ImmunoAssay System (Promega) according to the manufacturer's instructions. Primary striatal cultures were obtained by dissection of E14 rat fetuses lateral ganglionic eminence and subsequent culture in serum free condition, as previously described (Barkats *et al.*, 1996). This method of culture allow to obtain purified neurons (>95%) as demonstrated by immunocytochemistry against glial fibrillary acidic protein and neurofilament (Weiss *et al.*, 1986). The primary cells were plated in 12-well culture plates at a density of 2.10^5 cells per well and incubated for three days. They were then infected at a MOI of 100 pfu per cell as described for Hela cells. Four days after infection, cells were fixed for 20 minutes with 4% paraformaldehyde (PFA) in PBS. Immunological detection of calbindin was performed as described below in "Histological procedures". The anti-calbindin monoclonal antibody (Sigma) was used at a 1:5000 dilution. For each well, calbindin-positive cells were counted on twelve randomly selected microscope fields.

In vivo adenoviral gene transfer in a rat model of HD.

Recombinant adenoviruses were diluted in PBS. Female Sprague-Dawley rats (Charles River, France) under ketamine/xylazine anesthesia, were injected in the left striatum with a total amount of $5x10^7$ pfu (0.25 $\mu l.min^{-1}$) in three equal aliquots of 1 μl each in the head of the caudate putamen, at the following coordinates: 1.2 mm anterior to bregma ; 2.5 mm lateral to bregma ; 5, 4.6 and 4.2 mm ventral to the dural surface. Sham-treated rats were injected with PBS only. Two weeks later, QUIN (120 nmoles in 2 μl PBS) was injected at a rate of 0.4 $\mu l.min^{-1}$ at the same site (1.2 mm anterior to bregma ; 2.5 mm lateral to bregma ; 4.6 mm ventral to the dural surface).

Quantification of BDNF in vivo.

BDNF levels were determined on striatal lysates from rats injected as described above. Rats were sacrificed by chloral hydrate injection, striata were dissected and homogenized at 4°C in 500 μl of a lysis buffer containing 137 mM NaCl, 20 mM Tris pH 7.5, 1% NP40, 10% Glycerol, 1mM PMSF, 10 μg.ml^{-1} aprotinin, 1 μg.ml^{-1} leupeptin, and 0.5 mM Vanadate. After 15 minutes centrifugation at 13,000 g, BDNF was measured in the supernatant using BDNF Emax Immunoassay system (Promega).

Histological procedures.

Four weeks after the QUIN lesion, the rats were killed by deep anesthesia with chloral hydrate and transcardial perfusion with 100 ml of 0.9% NaCl, followed by 250 ml of ice cold 4% PFA in PBS. Brains were postfixed in the same fixative for one hour and were cryoprotected by immersion in 15% saccharose dissolved in PBS. Brains were then cut into 16-μm thick coronal sections with a cryostat and were stored at -80°C.

For *in situ* hybridization, an oligonucleotide probe complementary to the coding sequence of the FLAG peptide (Brizzard *et al.*, 1994) was synthesized ($5'$CTT CTA GTC ATC GTC GTC CTT GTA GTC GCC$_{3'}$) and radioactively labeled using ^{35}S dATP and terminal deoxynucleotidyl transferase (Promega) according to the manufacturer's instructions. For hybridization, sections were dehydrated and incubated overnight at 42°C in 50% formamide, 1x Denhardt's solution, 4x SSC, 100mM dithiothreitol, 10% dextran sulfate, 250 μg.ml^{-1} *E coli* tRNA, 250 μg.ml^{-1} poly A$^+$ RNA, 250 μg.ml^{-1} salmon sperm DNA and 0.2 pmole of labeled oligonucleotide per section. Sections were then rinsed twice in 1x SSC for 15 minutes at 53°C, twice in 0.5x SSC for 15 minutes at 53°C, and once in 0.5x SSC for 15 minutes at room temperature. They were then dehydrated and dipped in Ilford K5 emulsion. Sections were exposed for two month, then developed in Kodak D19 developer, lightly counterstained with cresyl violet and mounted.

ß-galactosidase activity was detected enzymatically by incubating the sections with 5-bromo-4-chloro-3-indolyl ß-D-galactoside as previously described (Barkats *et al.*, 1996). After two hours of incubation, the sections were counterstained with neutral-red and mounted.

For immunohistochemical detection of DARPP-32, endogenous peroxidase activity in the sections was quenched with 0.3% H_2O_2 in methanol. Subsequently, sections were preincubated in PBS containing 10% normal horse serum. Mouse monoclonal anti-DARPP-32 antibody (generously donated by Drs Greengard and Hemmings) was diluted 1:20,000 in the preincubation solution. The sections were incubated overnight at room temperature, and the primary antibody was detected using the Vectastain *Elite* ABC kit (Vector Labs) according to the manufacturer's instructions. Peroxidase activity was detected with Vector SG substrate kit (Vector Labs).

Lesion volume measurement and cell counting.

Lesion volume and striatal volume were determined by camera lucida drawings of DARPP-32 immunostained sections taken every 400 μm from 2.2 mm anterior to bregma to 1.0 mm posterior to bregma.

The lesion and striatal areas of each section were integrated to obtain lesion and striatal volumes. Cells were counted on three sections located at the antero-posterior level where the lesion area was maximal. Using the PC-based Biocom software coupled to a Leitz microscope (Hirsch *et al.*, 1992), ipsi- and contralateral striata were encircled through a 4x objective, and, in this defined area, DARPP-32-positive cells were counted in 15 randomly selected square fields of $4x10^4$ μm^2 through a 10x objective. This enabled us to deduce the total number of cells from the measurements of area and cell density.

Statistical analysis.

All values are expressed as means ±SEM. Groups were compared with Statview software by one-way analysis of variance (ANOVA) followed by Scheffé's post-hoc test, except for the data in Figures 6*a* and 6*b* for which an unpaired Student's *t*-test was used. In all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Recombinant adenovirus harboring BDNF produces a functional protein.

The recombinant vectors used in this study, Ad-BDNF and Ad-ßGal, were derived from a human type 5 adenovirus with the E1 and E3 regions deleted. They encode rat BDNF and *E coli* ß-galactosidase, respectively. The BDNF cDNA was inserted upstream from the coding sequence of FLAG (Brizzard *et al.*, 1994). This construct yields a BDNF-FLAG fusion protein which made it possible to distinguish the recombinant BDNF from the endogenous BDNF (Figure 1).

We investigated whether Ad-BDNF directed the synthesis of the BDNF protein by infecting Hela cells with various doses of Ad-BDNF. Four days after infection, the amount of BDNF in culture media was determined by ELISA assay. The smallest multiplicity of infection (MOI) used, 20 plaque-forming units (pfu) per cell, was sufficient for BDNF to be detected. Moreover, the amount of BDNF increased linearly with the MOI (r^2=0.975 ; p=0.0001), up to 18.5 ng of BDNF/$5x10^5$ cells/day for 80 pfu per cell, the highest MOI tested (Figure 2*a*).

We next assayed the biological activity of the recombinant BDNF. Primary striatal cultures obtained by dissection of the lateral ganglionic eminence of E 14 rat

fetuses were infected with either Ad-BDNF or Ad-ßGal on day three (after plating). They were fixed on day seven and processed for calbindin immunohistochemistry. Calbindin-positive cells were counted and twice as many such cells were detected in the Ad-BDNF infected cultures as in the Ad-ßGal-infected or non-infected cultures (Figure 2*b*).

Experimental paradigm of the in vivo study.

We tested the neuroprotective effect of BDNF using an excitotoxic rodent model of HD. Female Sprague-Dawley rats were assigned to three groups: Ad-BDNF treatment (n=10), Ad-ßGal treatment (n=8) and sham treatment(n=8). As described in the methods, each rat received a stereotaxic injection in the neostriatum of Ad-BDNF, Ad-ßGal or vehicle (PBS). Two weeks after treatment, two rats from the Ad-BDNF group were killed for evaluation of recombinant BDNF expression. At the same time, the remaining animals received a QUIN injection (120 nmoles) in the neostriatum. Four weeks later, the animals were killed and the lesion was studied histologically.

In vivo expression of recombinant BDNF.

In vivo expression of the BDNF transgene was analyzed by *in situ* hybridization six weeks after Ad-BDNF injection. An antisense oligonucleotide probe directed against the FLAG peptide coding sequence detected the BDNF-FLAG transcript in a large region of the neostriatum, surrounding the injection site (Figures 3*b* and 3*d*). The BDNF-FLAG transcript was also detected in the corpus callosum, mimicking the pattern of ß-galactosidase staining in Ad-ßGal-treated rats (Figures 3*e* and 3*f*). Expression of the transgene was stable over the time period studied because the amount of BDNF-FLAG mRNA in rats killed four weeks after the lesion were similar to those in two rats killed at the time of the QUIN lesion (data not shown). The BDNF-FLAG transcript was not detected on the contralateral side or in the striatum of Ad-ßGal-treated rats.

To determine if recombinant BDNF expression, evidenced by anti-FLAG *in situ* hybridization, corresponds to an overexpression of BDNF in the striatum, the level of this protein was measured *in vivo*. Rats were injected in the striatum with either Ad-BDNF (n=5) or Ad-ßGal (n=3) or vehicle (n=2). Two weeks later, animals were sacrificed, striata were dissected, and BDNF content of striatal lysates was quantified using an ELISA assay. The level of BDNF increased from 6 ±1.3 pg of BDNF/mg of protein in vehicle- and Ad-ßGal-injected striata to 16.6 ± 2.7 pg of BDNF/mg of protein in Ad-BDNF-injected striata ($p<0.01$).

BDNF gene transfer reduces QUIN lesion size.

The lesion induced by QUIN injection was examined on DARPP-32 immunostained sections. DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein of 32 Kd) is a marker for the soma and axons of striatal GABAergic output neurons (Ouimet *et al.*, 1984). The QUIN-induced lesion was defined as the area devoid of immunoreactivity. Four weeks after QUIN injection, the rats were killed, cryostat serial sections encompassing the whole striatum were prepared, and one series of sections was processed for the immunohistochemical detection of DARPP-32. Lesions appeared smaller in the Ad-BDNF-treated group than in the Ad-ßGal- and sham-treated groups (Figure 4). The lesion area was quantified for each rat by camera lucida drawings of sections taken every 400 micrometers through the neostriatum. Areas were then integrated to obtain striatal and lesion volume. This quantification (Figure 5*a*) showed that lesion volume for the Ad-BDNF-treated group (1.69 ±0.34 mm^3) was 55% smaller than that for the Ad-ßGal-treated group (3.8 ±0.87 mm^3) and 75% smaller than that for the sham-treated group (6.24 ±1.76 mm^3). The lesions of the Ad-BDNF-treated group were significantly smaller than those of the other two groups ($p<0.05$), whereas the Ad-ßGal and sham treated groups were not significantly different ($p=0.26$).

We studied the overall effect of the lesion on the striatum by determining the volume of this structure. Absolute striatal volume was measured (Figure 5*b*) and it was found that the QUIN lesion caused a shrinkage of the neostriatum that was more pronounced, although non significant ($p=0.11$), in the Ad-ßGal-treated group (20.27 ±0.89 mm^3) than in the Ad-BDNF-treated group (22.27 ±0.75mm^3). In the sham-treated group, the shrinkage was intermediate (21.01 ±1.35 mm^3 ; $p=0.43$ vs Ad-BDNF treatment ; $p=0.66$ vs Ad-ßGal treament). Taken together, these results suggest that in the Ad-BDNF- and Ad-ßGal-treated groups, the neostriatum shrinkage was due not only to the QUIN lesion, but also partly to the adenovirus injection which is associated with a certain degree of toxicity.

Histological processing may cause differences between animals. Therefore, the relative lesion volume, defined as the percentage of the total striatum occupied by the lesion, was calculated for each rat. The relative lesion volume in the Ad-BDNF-treated group was significantly smaller than those of the Ad-ßGal- ($p<0.05$) and sham-treated groups ($p<0.05$; figure 5*c*). This confirmed the results obtained by absolute lesion volume measurement. The Ad-ßGal and sham-treated groups were again not statistically different ($p=0.21$).

Rescue of DARPP-32-positive neurons by BDNF gene transfer.

The effect of recombinant BDNF on the QUIN lesion was examined at the cellular level by quantifying the survival of DARPP-32-positive neurons. For each rat, at the rostrocaudal level where the area of the QUIN lesion was maximal, three sections were selected and DARPP-32-positive neurons were counted on these sections on the ipsi- and contralateral sides. Cell density and striatal area were calculated for each rat and expressed as a percentage of the intact side (Figure 6). As previously observed with camera lucida drawings, the shrinkage of the neostriatum was more pronounced in the

Ad-ßGal-treated group (87 ±1.5%) than in the Ad-BDNF-treated group (92.5 ±2% ; p=0.03 vs Ad-ßGal treatment) whereas shrinkage in the sham-treated group (92 ±3% ; p=0.49 vs Ad-BDNF treatment) was similar to that in the Ad-BDNF-treated group (Figure 6*b*). In contrast, cell density measurements showed that DARPP-32-positive cells were only protected in the Ad-BDNF group (69 ±4%) and not in Ad-ßGal (54 ±7% ; p=0.04 vs Ad-BDNF treatment) and sham (55 ±10% ; p=0.12 vs Ad-BDNF treatment) groups (Figure 6*a*). The total cell number was deduced from area and cell density measurement in each animal. Ad-BDNF was found to have significantly protected the medium spiny projection neurons of the neostriatum. We found that 46% (±5%) of cells survived in Ad-ßGal-treated animals, whereas 64% (±4%) survived in Ad-BDNF-treated animals (Figure 6*c*, p=0.01). In terms of total cell number, comparison between sham- and Ad-BDNF-treated animals resulted in a p value of 0.19. This lack of significance could be explained by the QUIN lesion variability which appeared much more pronounced in the sham-treated animals than in the adenoviral-treated animals.

DISCUSSION

In this article, we have described the effective *in vivo* transfer of the BDNF gene using a recombinant adenovirus vector. In a rodent excitotoxic model of HD, we demonstrated that the adenovirus-mediated transfer of BDNF protected the medium-sized spiny neurons of the neostriatum, the principal target of neurodegeneration in HD. This is the first *in vivo* evidence that BDNF is of value for HD treatment.

Effect of BDNF gene transfer in vitro.

BDNF has been reported to promote the differentiation of GABAergic neurons. In particular, it increases the number of calbindin immunoreactive cells in primary striatal cultures (Mizuno *et al.*, 1994 ; Ventimiglia *et al.*, 1995). Consistent with these observations, we found that the infection of primary striatal cultures with Ad-BDNF resulted in an increase in the number of calbindin-immunoreactive neuronal cells (Figure 2*b*). Thus, striatal cells produced the protein encoded by the transgene after adenovirus-mediated gene transfer. This result also demonstrated that the addition of the FLAG peptide to the carboxy-terminal part of the recombinant BDNF did not affect its biological activity.

Effect of BDNF gene transfer in an in vivo model of HD.

Several observations suggest that BDNF is an important factor for mature striatal neurons and that it should therefore be considered as a putative therapeutic agent for HD. Endogenous BDNF and the mRNA coding for its high-affinity receptor TrkB have been detected in the adult neostriatum (Altar *et al.*, 1994 ; Conner *et al.*, 1997). The endogenous BDNF is transported to the cortical nerve terminals by anterograde transport (Altar *et al.*, 1997), and the TrkB receptor is primarily located in the post-synaptic densities (Wu *et al.*, 1996). Moreover, BDNF has been reported to be implicated in the induction of the DARPP-32 phenotype in the developing striatum (Ivkovic *et al.*, 1997).

We tested the therapeutic value of BDNF for HD using an excitotoxic model of the disease obtain by intrastriatal injection of QUIN. This procedure reproduces the cell loss observed in HD and can be used to evaluate experimental treatments by comparing QUIN-induced lesion in treated and non-treated animals. Thus, this model of HD is highly relevant for testing experimental therapy, and we have shown here that the prior treatment by intracerebral injection of an adenovirus expressing the BDNF gene protected the striatal neurons from the QUIN-induced lesion. More recently, transgenic models of HD have been developed. In these models, despite the reduced life span and the behavioral abnormalities, there is no clear neuronal cell death (Price *et al.*, 1998) which makes them difficult to use for testing a neuroprotective approach. Nevertheless, they could be taken into account to test the ability of BDNF to prevent the neuronal dysfunction which occurs in these models.

The mechanism by which BDNF protects against excitotoxic insult is unknown. However, it has been suggested that the protection results from the action of BDNF on the transcription of calcium-binding proteins (Nakao *et al.*, 1995). Studies *in vitro* have shown that BDNF increases the expression of calbindin, calretinin and parvalbumin (Mizuno *et al.*, 1994 ; Ventimiglia *et al.*, 1995 ; Widmer and Hefti, 1994). Calbindin levels in BDNF knockout mice are also lower than normal (Jones *et al.*, 1994). As excitotoxicity challenged with an NMDA agonist, such as QUIN, are known to increase intracellular calcium concentration (reviewed in Choi, 1994), upregulation of the expression of calcium-binding protein levels may rescue striatal cells from excitotoxic cell death by increasing their capacity to buffer intracellular calcium (Mattson *et al.*, 1991).

The neuroprotective effect of Ad-BDNF treatment reported in this study could also be explained by the anti-apoptotic property of BDNF. Apoptotic cell death has been implicated in both HD and the QUIN-induced rat model of HD (Portera-Cailliau *et al.*, 1995 ; Ona *et al.*, 1999 ; Sanchez *et al.*, 1999). Although the effect of BDNF on anti-apoptotic proteins is not documented, BDNF was shown to counteract apoptosis in several models of neurodegeneration (Bhave *et al.*, 1999 ; Kaal *et al.*, 1997). In particular, in an *in vitro* model of HD, the apoptosis observed after transfection of primary striatal neurons with a polyglutamine construct was abolished when BDNF was added in the culture medium (Saudou *et al.*, 1998).

Efficiency of adenovirus-mediated transfer of the BDNF gene.

The transfer of the BDNF gene in the QUIN

model of HD has previously been reported. However, although neural rescue tended to be greater in BDNF-treated animals, Martinez-Serrano and Björklund have described that the effects were not significant for several parameters, including lesion size and DARPP-32 neuron survival (Martinez-Serrano and Björklund, 1996). In another study by Frim *et al.*, it has been reported that exogenous BDNF has no effect on the excitotoxic lesion (Frim *et al.*, 1993). The lack of agreement of these two reports with our results may be due to differences in the gene transfer procedure.

In these studies, the BDNF gene was delivered by an *ex vivo* gene transfer method involving retroviral vectors. However, different types of transplanted cells were used for the two studies. Frim *et al.* used transplants of immortalized fibroblast cells, whereas Martinez-Serrano and Björklund used transplants of conditionally immortalized neural progenitor cells. The two studies confirm the importance of the local production of BDNF in that the immortalized fibroblasts remained at the injection site (i.e. the corpus callosum) and did not protect the neostriatum (Frim *et al.*, 1993), whereas conditionally immortalized neural progenitor cells migrated throughout the neostriatum, giving a mild protection (Martinez-Serrano and Björklund, 1996).

In this report, we used a gene transfer procedure consisting of the direct intracerebral injection of a recombinant adenoviral vector. This procedure leads to strong expression of the transgene in a large area around the injection site (Le Gal La Salle *et al.*, 1993). Thus, in the paradigm of neuroprotection by BDNF in the QUIN lesion model, the *in vivo* gene transfer method used here appears to be more efficient than *ex vivo* gene transfer. This may be because more BDNF is produced *in situ* after *in vivo* gene transfer, resulting in better diffusion of the factor. We and others have also demonstrated that the neuroprotection provided by the adenovirus-mediated transfer of genes encoding neurotrophic factors is not restricted to the transfected cells, but extends to neighboring cells (Baumgartner and Shine, 1997 ; Bilang-Bleuel *et al.*, 1997 ; Castel-Barthe *et al.*, 1996).

Potential of adenoviral vectors for gene transfer.

Consistent with previous studies (Bilang-Bleuel *et al.*, 1997), we report herein that a first generation adenoviral vector drove the *in situ* production of a therapeutic protein. However, this vector appeared to have a toxic effect on the cerebral parenchyma after intracerebral injection (Byrnes *et al.*, 1995). In our study, the striatum was slightly, but not significantly, smaller in Ad-ßGal-treated animals than in sham-treated animals (Figures 5*b* and 6*b*). The striatal size measured for Ad-BDNF-treated animals suggests that the recombinant BDNF protects against both the QUIN lesion and adenoviral toxicity. The reasons for adenoviral toxicity are unclear, but several mechanisms, including the production of viral proteins and antigen-mediated cytotoxicity, are thought to be involved (Byrnes *et al.*, 1995 ; Byrnes *et al.*, 1996). Use of new-generation adenoviruses, which are less toxic and less immunogenic (Dedieu *et al.*, 1997), or of lentiviral vectors, which are efficient agents for gene transfer in the CNS (Blömer *et al.*, 1997), is likely to increase the therapeutic value of neurotrophic gene transfer. This type of vector, which should allow safe and sustained transgene expression, will be of particular value for testing transfer of the BDNF gene in preclinical animal models of HD such as 3-nitropropionic acid chronic intoxication of primates. These models will permit the evaluation of potential clinical benefits of BDNF gene transfer in restorative or neuroprotective approaches for HD over the longer term.

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FIGURES

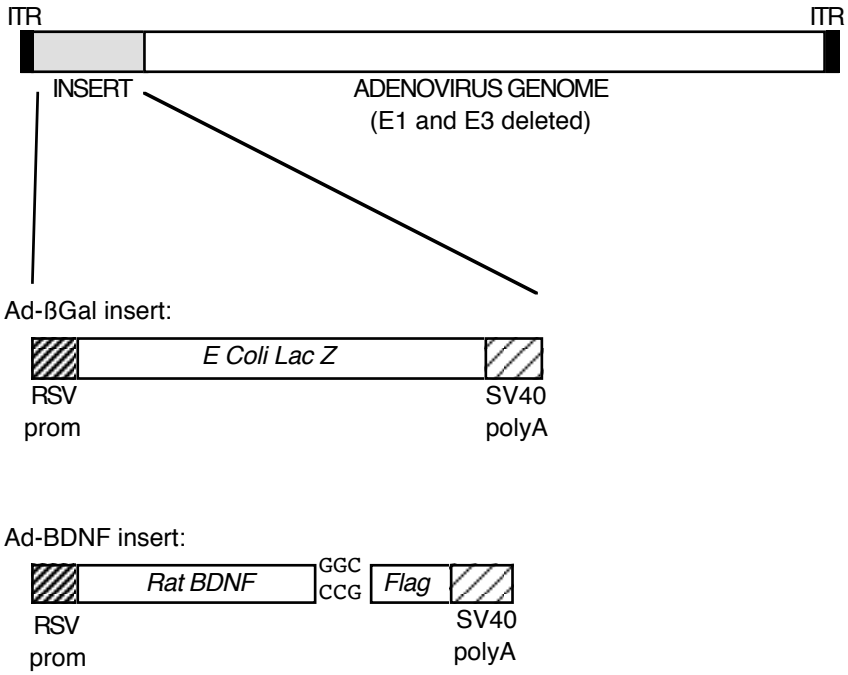

Figure 1: Map of the adenoviral constructs. *E coli* Lac Z and rat BDNF genes are under the transcriptional control of the Rous sarcoma virus long terminal repeat promoter. The rat BDNF gene is fused with the coding sequence (30 base pairs) of the FLAG peptide. BDNF and FLAG are separated by a glycine codon.

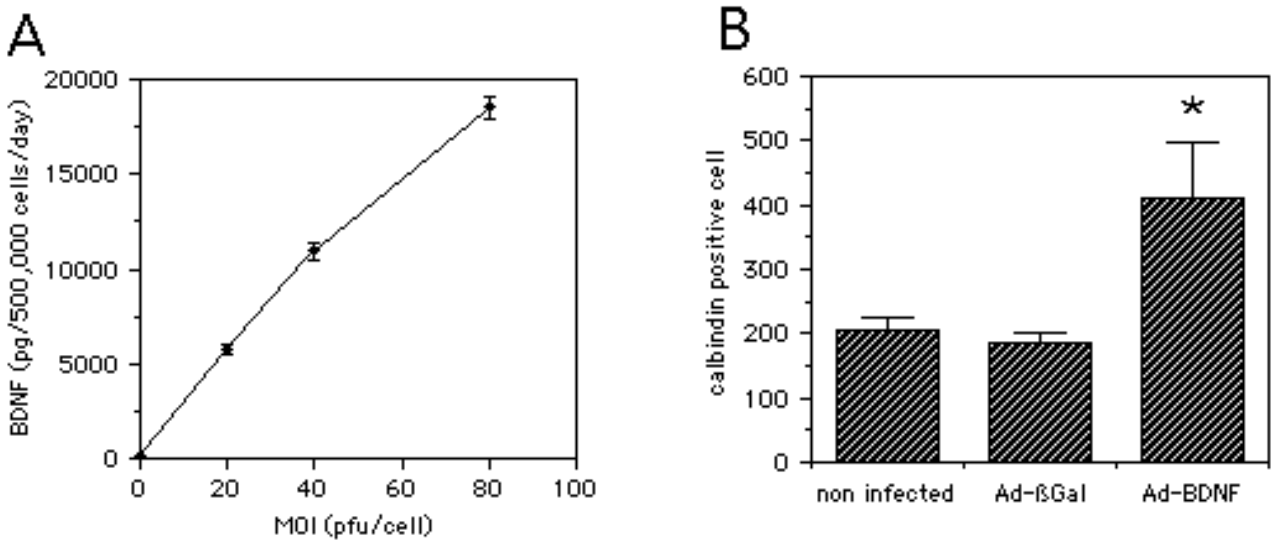

Figure 2: *In vitro* expression of recombinant BDNF. *a*, BDNF amount in the culture medium three days after infection of Hela cells with various doses of Ad-BDNF. The quantity of BDNF detected increased linearly with MOI (r^2=0.975, p=0.0001). *b*, Effect of recombinant BDNF release on calbindin-positive cells after Ad-BDNF infection of primary striatal cultures. Four days after the infection with Ad-ßGal or Ad-BDNF of primary striatal cells from sister cultures, calbindin-positive cells were detected. Values are means for total calbindin-positive cell counts determined for each well on 12 randomly selected microscope fields through a 10x objective. Similar results were obtained from three independent experiments. *, p<0.001 vs non-infected or Ad-ßGal-infected cells.

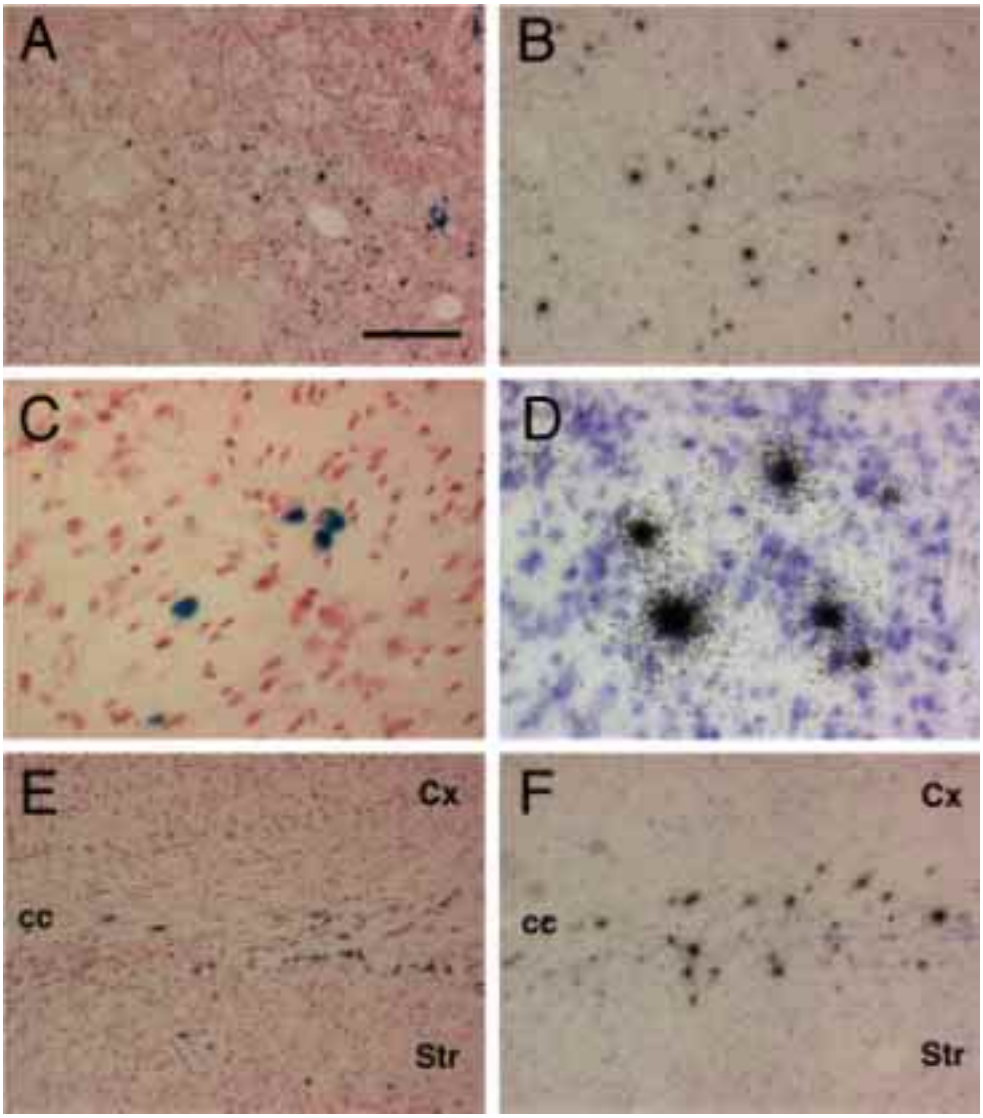

Figure 3: ß-galactosidase (*a, c, e*) and recombinant BDNF (*b, d, f*) levels six weeks after a single intrastriatal injection of Ad-ßGal or Ad-BDNF respectively. ß-galactosidase activity was detected enzymatically with 5-bromo-4-chloro-3-indolyl ß-D-galactoside as a substrate, resulting in a blue color. Recombinant BDNF mRNA was detected by anti-FLAG *in situ* hybridization (see Methods). Transgene expression was detected throughout the caudate putamen (*a, b*) and the corpus callosum (*e, f*). *c-d,* higher magnification of ß-galactosidase- and recombinant BDNF- containing cells, respectively. Due to the nuclear localizing sequence, only the nucleus of ß-galactosidase-containing cells is stained, and these cells therefore appear much smaller than the cells containing recombinant BDNF. Cx: cortex ; cc: corpus callosum ; Str: striatum. Scale Bar: 200 μm in *a, b, e* and *f* ; 50 μm in *c* and *d*.

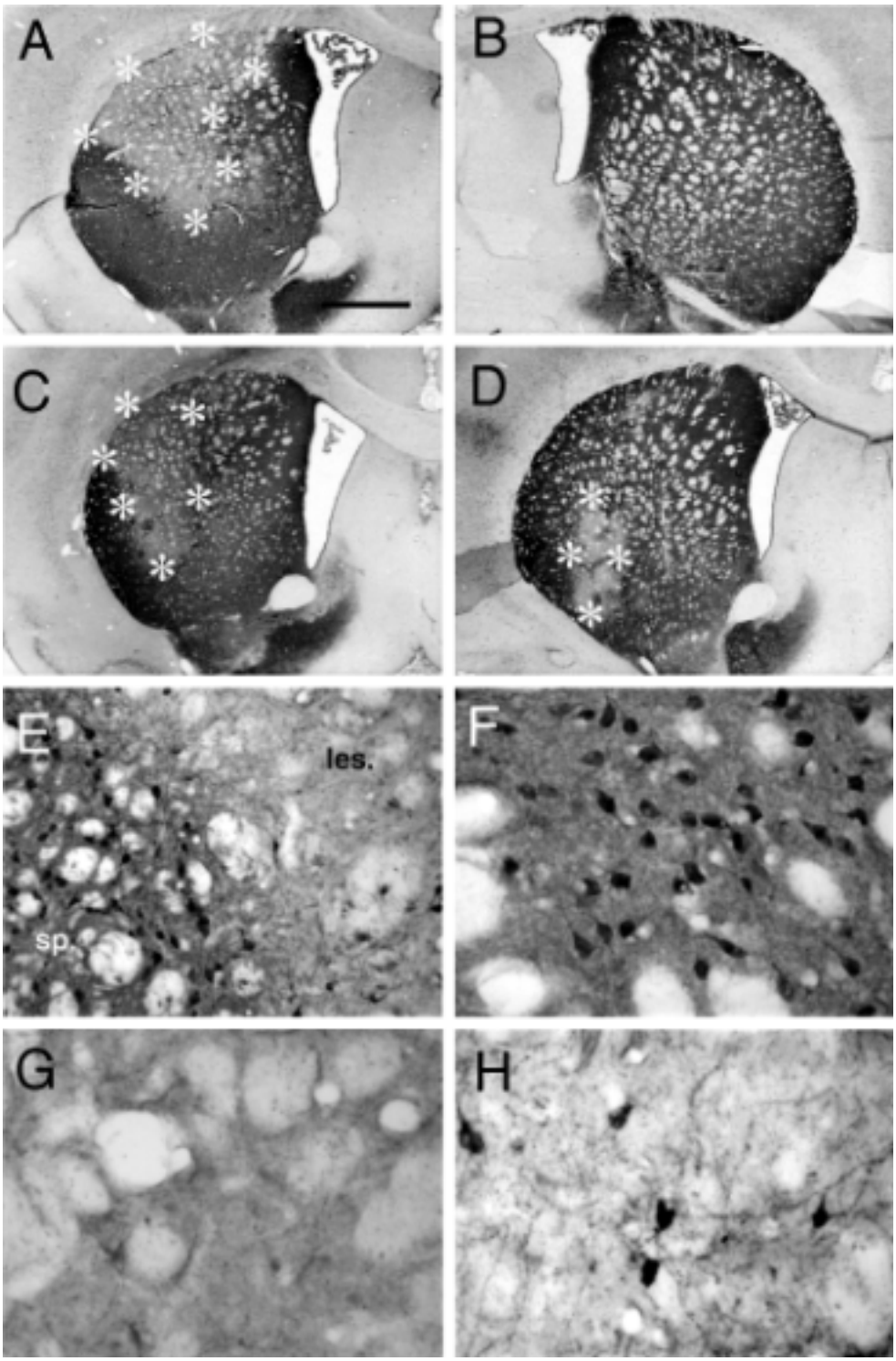

Figure 4: Representative photomicrographs showing DARPP-32-immunostained sections of sham- (*a*), Ad-ßGal- (*c, e, g*), Ad-BDNF- (*d, f, h*) injected, or intact (*b*) striatum. *a, c, d,* The QUIN-induced lesion (indicated by asterisks) was clearly identified by the absence of DARPP-32 staining. The lesion was smaller in Ad-BDNF-treated animals than in Ad-ßGal- or sham-treated animals. *e*: higher magnification of the boundary between the spared (sp.) and QUIN lesioned (les.) areas. *f*: higher magnification of the spared part of the neostriatum. *g, h*: higher magnification of the lesioned areas shown in *c* and *d* respectively. Scale bar: 1 mm in *a, b, c* and *d* ; 200 μm in *e* ; 100 μm in *f, g* and *h*.

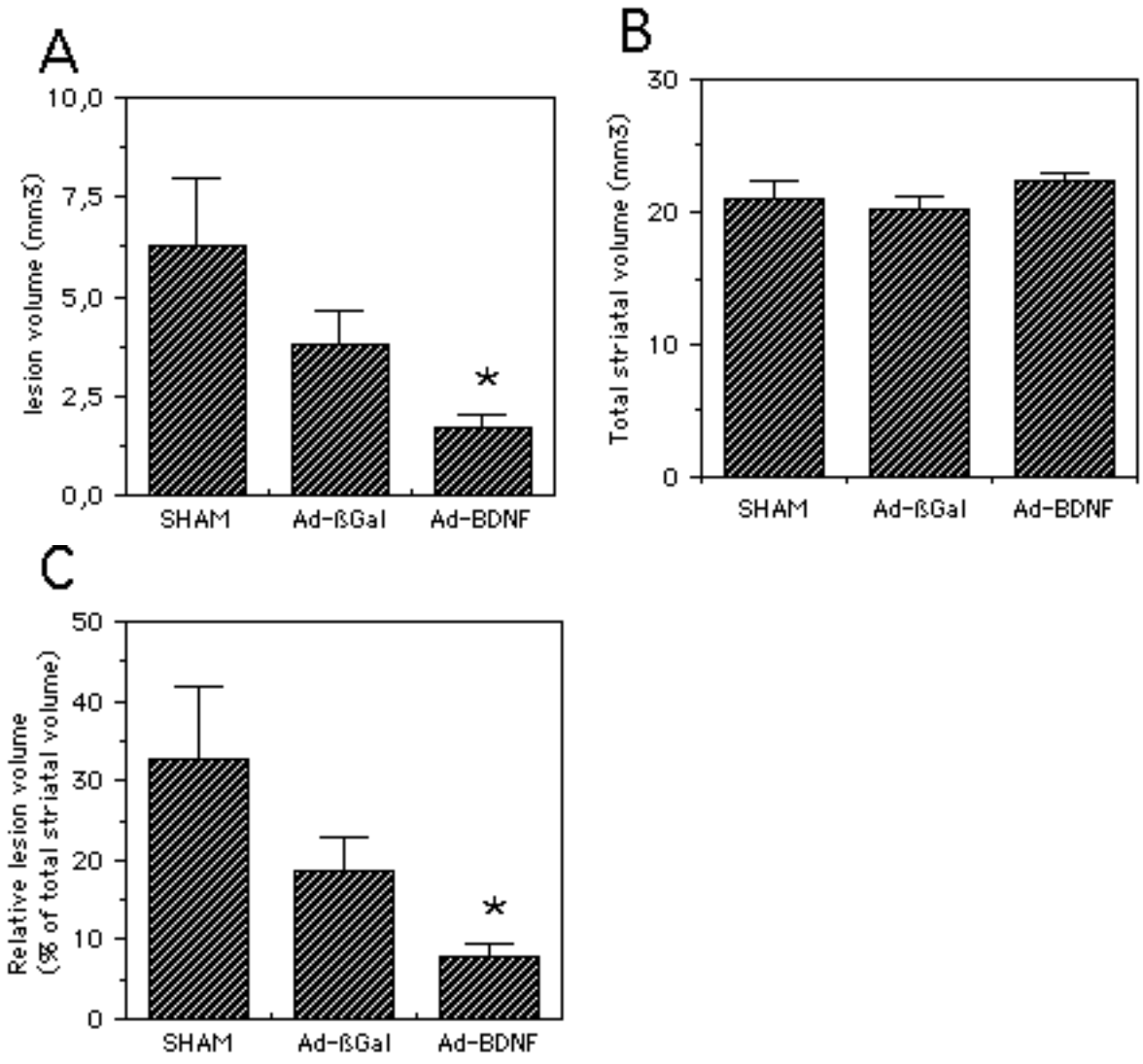

Figure 5: Quantification of lesion and striatal volume based on DARPP-32 immunostained sections. *a*, Absolute lesion volume deduced by integration of lesioned areas determined every 400 μm across the neostriatum. *b*, Total striatal volume (lesioned + spared areas) expressed in mm^3, and determined on the same sections as for *a*. *c*, Relative lesion volume, expressed as a percentage of whole neostriatum volume. *$p<0.05$ versus Ad-ßGal- and sham-treated groups.

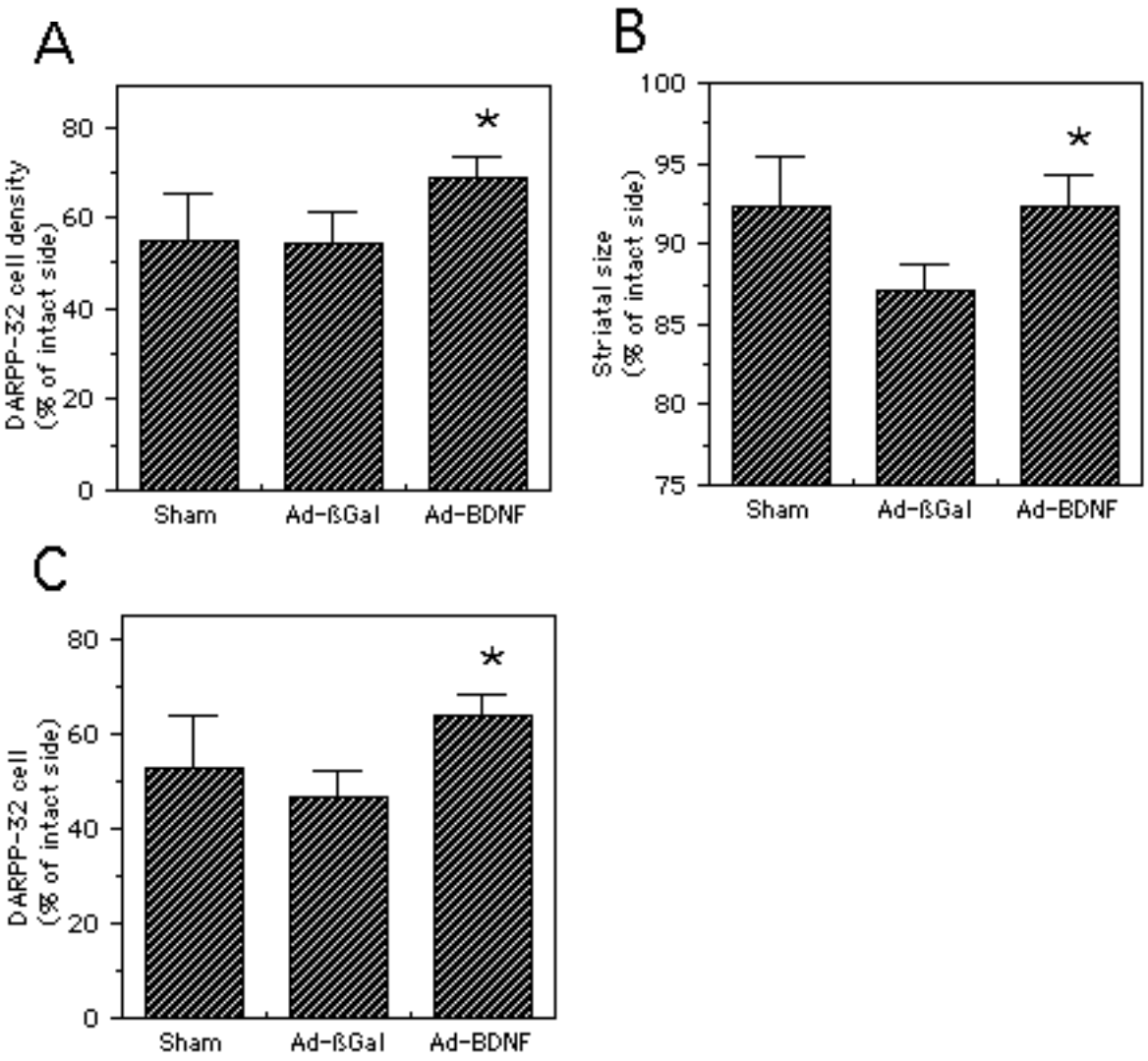

Figure 6: Rescue of DARPP-32-positive cells by adenovirus-mediated transfer of the BDNF gene. *a*, Density of the DARPP-32 cell population evaluated for each rat from three sections selected at the rostrocaudal level where lesion area was maximal. *b*, evaluation of the striatal size on the same three sections. *c*, Total number of cells in the DARPP-32-positive population, deduced from cell density and striatal area values. Data are expressed as a percentage of the intact side. **p*<0.05 versus Ad-ßGal. In *a* and *b* comparisons were made using Student's *t*-test.