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Gene therapy for neurodegenerative disease on rodent and primate models by single to triple transduction of rAAV vectors

Shin-ichi Muramatsu[1,*], Madoka Yoshimura[1], Lijun Wang[1,2], Kunihiko Ikeguchi[1], Ken-ichi Fujimoto[1], Takashi Okada[2], Hiroaki Mizukami[2], Imaharu Nakano[1] and Keiya Ozawa[2,*]

[1] *Division of Neurology, Department of Medicine*
[2] *Division of Genetic Therapeutics, Center for Molecular Medicine*
Jichi Medical School, 3311-1 Yakushiji, Minami-kawachi, Tochigi 329-0498, Japan

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* Corresponding authors. E-mails: muramats@ms.jichi.ac.jp and kozawa@ms.jichi.ac.jp

1. INTRODUCTION: RECOMBINANT ADENO-ASSOCIATED VIRAL VECTORS

Wild type adeno-associated viruses (AAVs) are small, non-enveloped, single-stranded DNA viruses of the *Parvoviridae* family assigned to the genus *Dependovirus*, as productive infection requires co-infection with a helper virus such as adenovirus or herpesvirus. AAVs have been isolated from a variety of different species, including primates, dogs, cows, horses, sheep, and chicken, and appear to be non-pathogenic. To date, eight primate AAVs (AAV-1 to 8) have been cloned and sequenced (Srivastava *et al.*, 1983; Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997; Rutledge *et al.*, 1998; Bantel-Schaal *et al.*, 1999; Chiorini *et al.*, 1999; Xiao *et al.*, 1999; Gao *et al.*, 2002). Unlike other viral vector systems, recombinant adeno-associated viral (rAAV) vector is based on a non-pathogenic replication-defective virus. Efficient and long-term *in vivo* gene expression has been achieved without causing a substantial immune response or toxicity (Monahan and Samulski, 2000).

We used the helper virus-free triple plasmid transfection method (Matsushita *et al.*, 1998) to produce high titer rAAV vectors devoid of undesirable contamination with wild type adenovirus. The method requires a vector plasmid containing an expression cassette of a therapeutic gene flanked by AAV-2 inverted terminal repeats, a packaging plasmid directing expression of viral proteins in *trans* to supply required AAV helper functions, and a helper plasmid containing the minimum adenovirus genomic sequences necessary for helper functions. Vectors are purified by two sequential ultracentrifugations (CsCl continuous gradient or iodixanol discontinuous gradient); recently affinity or ion-exchange chromatography methods have been developed for viral particle purification (*e.g.* Zolotukhin *et al.*, 2002 and Kaludov *et al.*, 2002), although separation of empty capsids from genome-containing particles is difficult in these methods. Vector titer is determined by quantitative DNA dot-blot hybridization of DNase I-treated vector stocks, and is routinely 10^{12} to 10^{13} genome copies per milliliter.

2. GENE THERAPY FOR PARKINSON'S DISEASE

a) Parkinson's disease

Idiopathic Parkinson's disease (PD) is the second most common neurodegenerative disorder among the elderly, with an estimated 1% of the population over 60 years old suffering from PD (Olanow *et al.*, 2001). The pathological hallmarks of PD are the presence of Lewy bodies (cytoplasmic proteinaceous inclusions that contain α-synuclein and ubiquitin-proteasomal proteins) in nigrostriatal dopaminergic neurons and the loss of these neurons. Progressive reduction in the dopamine (DA) content of the striatum is closely related to the manifestation of motor problems. Cardinal symptoms including resting tremor, muscular rigidity, and bradykinesia become apparent after 40-50% of the neurons in the substantia nigra pars compacta (SNc) are lost and striatal DA is reduced to about 20% of normal levels (Kish *et al.*, 1988).

The causes of PD remain largely unknown, although genetic causes have been elucidated in some familial cases (Gwinn-Hardy, 2002) including mutations in the gene encoding α-synuclein (Polymeropoulos *et al.*, 1997) or ubiquitin carboxy-terminal hydrolase-L1 (UCH-L1) (Leroy *et al.*, 1998) in families with an autosomal dominant pattern of inheritance, and mutations in the genes *parkin* (Kitada *et al.*, 1998) or *DJ-1* (Bonifati *et al.*, 2003) in families with an autosomal recessive pattern of inheritance. Purely genetic causes probably account for only a small number of PD patients; multiple factors including environmental factors may contribute to the development of sporadic PD. Oxidative stress and dysfunction of the proteasome system are each hypothesized to be involved in the development of Parkinsonism, and they are not mutually exclusive (Cookson, 2003). They may, however, be shared by late events in the neurodegenerative pathway of many different neurodegenerative disorders. The mechanism of selective susceptibility of nigral dopaminergic neurons and the temporal sequence of events leading to cell loss in PD remain to be elucidated.

Current therapy of PD is aimed at replacement of DA in the striatum to alleviate motor dysfunction. Unlike DA, which does not cross the blood-brain barrier, the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) penetrates the brain and is the most effective drug in pharmacotherapy for PD. Virtually all patients experience clinically meaningful benefit of L-DOPA treatment. However, as the disease progresses, L-DOPA is less efficiently converted to DA in the striatum, and it loses efficacy. Frequent systemic administration of high doses of L-DOPA causes oscillations in motor performance (Jenner, 2000; Langston *et al.*, 2000) and some deleterious complications such as hallucinations due to dopaminergic stimulation of the mesolimbic system (Carey *et al.*, 1995). Patients eventually become disabled. Novel therapeutic interventions substituting for oral L-DOPA administration are therefore required.

b) Biosynthesis of DA in the striatum

In normal striatum, DA is synthesized almost exclusively in the terminals of nigrostriatal DA neurons. Three enzymes are necessary for efficient DA synthesis: tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), and guanosine triphosphate cyclohydrolase I (GCH) (Fig. 1). In the rate-limiting step of DA synthesis, L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by TH. AADC then converts L-DOPA to DA. GCH is the rate-limiting enzyme for synthesis of the essential TH co-factor tetrahydrobiopterine (BH4). Since low levels of endogenous BH4 do not yield sufficient TH activity, GCH is considered to regulate TH activity via regulation of BH4 biosynthesis, thus indirectly controlling DA production in TH-containing DA neurons (Nagatsu *et al.*, 1997; Nagatsu and Ichinose, 1999). These enzymes are transported in an anterograde manner from the substantia nigra to the striatum.

A severe loss of dopaminergic nerve terminals in advanced PD is associated with an 80-95% depletion of striatal TH and AADC activity (Zhong *et al.*, 1995; Kaddis *et al.*, 1997) leading to a profound decrease of

DA. Loss of AADC activity and decreased capacity for DA storage in synaptic vesicles leads to failure of L-DOPA therapy. AADC is present in DA-denervated striatum within non-dopaminergic neurons and glial cells, but endogenous AADC activity in the striatum is considered insufficient, at least in primates (Nakamura *et al.*, 2000). Along with decreases in the levels of BH4, TH, and DA, the activity of GCH in the striatum is also decreased in PD (Nagatsu *et al.*, 1987 and 1997; Nagatsu and Ichinose, 1999). Although BH4 can cross the blood brain barrier, uptake of exogenous BH4 from the blood is low (Hoshiga *et al.*, 1993). The primary source of BH4 in the brain is intracellular biosynthesis. GCH gene transfer into striatal cells may thus offer a more efficient method of supplying BH4 than administration of exogenous BH4.

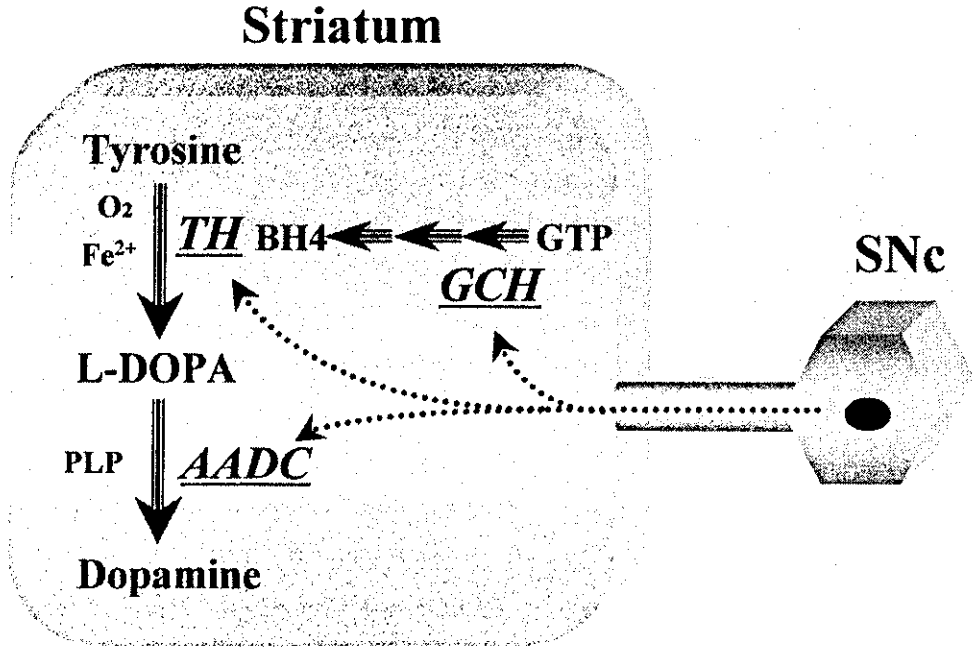

Figure 1. Dopamine biosynthetic pathway. For efficient dopamine production, guanosine triphosphate cyclohydrolase I (GCH) is necessary in addition to tyrosine hydroxylase (TH) and aromatic L-amino-acid decarboxylase (AADC). GCH is a rate-limiting enzyme for biosynthesis of tetrahydrobiopterin (BH4), an essential TH cofactor. These enzymes are transported from the substantia nigra to the striatum in an anterograde manner, and thus are reduced in the striatum in Parkinson's disease. GTP, guanosine triphosphate; SNc, substantia nigra pars compacta; PLP, pyridoxal 5'-phosphate.

c) Neurotoxin-induced animal models

One of the reasons that PD is regarded as a suitable candidate for gene therapy is the availability of well-characterized rodent and primate PD models. These models use neurotoxins that selectively elicit DA neuronal death in the SNc and deplete nigrostrital DA.

The 6-OHDA rat model. The most popular animal model to date is intraparenchymal lesioning of the striatum or nigrostriatal pathway using 6-hydroxydopamine (6-OHDA) in rats (Deumens *et al.*, 2002). 6-OHDA is transported into the cell bodies and fibers of catecholaminergic neurons and exerts its toxic effects by inhibiting mitochondrial respiratory enzymes, complexes I and IV (Blum *et al.*, 2001). Pretreatment with a noradrenalin transporter blocker and direct injection of 6-OHDA into discrete brain regions allows reasonable selectivity for destruction of nigral dopaminergic neurons. Injection of 6-OHDA into the medial forebrain bundle results in a broad loss of neurons, because the medial forebrain bundle contains axons from dopaminergic neurons in the ventral tegmental area as well as the SNc. In contrast, injection of 6-OHDA into the striatum results in more chronic and selective loss of neurons in the nigrostriatal pathway. Behavioral deficits in response to 6-OHDA lesions can give an indication of the extent of the lesions. In the unilateral lesioned model, administration of a low dose of the postsynaptic dopaminergic agonist apomorphine induces rotation contralateral to the lesioned side when the striatum dopaminergic fiber density has been decreased by 80-90% and the concomitant depletion of dopaminergic neurons in the SN has reached 50% (Barneoud *et al.*, 1995; Lee *et al.*, 1996). Denervation-induced up-regulation of D2 receptors in the lesioned striatum causes supersensitivity in the response to apomorphine only on the lesioned side and leads to this rotational behavior. Other behavioral changes have been observed when lesioning is less complete. For example, when striatal DA levels were reduced by 80%, a deficit in step adjustment was evident (Chang *et al.*, 1999) and there was asymmetry in the

use of forelimbs in a cylinder (Tillerson *et al.*, 2001). The 6-OHDA rat model is valuable for testing new therapeutic strategies including gene therapy, although it should be noted that compensatory responses (*e.g.*, sprouting from remaining intact dopaminergic fibers and elevated DA biosynthesis, metabolism, and release by the remaining dopaminergic neurons) could contribute to recovery of function noted after therapy, and any damage to the striatum or motor cortex that is caused by the therapeutic intervention might also decrease apomorphine-induced rotation.

The MPTP primate model. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is another toxin that selectively targets dopaminergic neurons. First identified as a causative agent for Parkinsonism in drug addicts (Speciale, 2002), MPTP is a by-product of chemical synthesis of a meperidine analog that has potent heroin-like effects. It is highly lipophilic and readily cross the blood-brain barrier. MPTP is converted to the active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), in non-dopaminergic cells by the enzyme monoamine oxidase B (MAO-B). MPP^+ is released into the extracellular space, binds to the DA transporter, and is taken up into dopaminergic neurons. There it is actively concentrated within the mitochondria, where it inhibits complex I, leading to oxidative stress including formation of peroxynitrite and activation of poly-ADP-ribose polymerase, and eventually cell death (Beal, 2001; Blum *et al.*, 2001). MPTP treatment reproduces many features of PD in a variety of animal species including monkeys, rodents, cats, and pigs. The MPTP primate model of PD is particularly useful for evaluating therapeutic effects on motor functions because it replicates all the cardinal signs of PD including tremor, rigidity, bradykinesia, and postural instability. Systemic repeated administration of MPTP in smaller doses over a prolonged period provides progressive depletion of nigrostriatal DA. The loss of dopaminergic terminals is greatest in the putamen, which is similar to the pattern of dopaminergic terminal loss in human PD. Neuronal inclusions resembling Lewy bodies have also been

reported in aged treated primates (Forno *et al.*, 1986; Kowall *et al.*, 2000). At present, MPTP-treated lesioned monkeys are the best available animal models for testing new therapeutic strategies in PD.

d) Behavioral recovery in a primate model by triple transduction of rAAV vectors encoding DA-synthesizing enzymes

We have attempted to restore local production of DA and produce behavioral recovery in PD model animals by rAAV vector-mediated gene transfer of DA-synthesizing enzymes. Previously we have shown that long-term (>18 months) behavioral recovery was achieved in a rat 6-OHDA model by cotransduction with rAAV-TH, rAAV-AADC, and rAAV-GCH (Shen *et al.*, 2000). Triple transduction resulted in more effective DA production and markedly increased behavioral recovery compared with transduction with rAAV-TH vector alone or a combination of rAAV-TH and rAAV-AADC vectors. This encouraging result in parkinsonian rats prompted us to extend preclinical explorations to a primate model of PD (Muramatsu *et al.*, 2002).

Bilateral striatal lesions were made in cynomolgus monkeys (*Macaca fascicularis*) by intravenous injection of MPTP once a week until a stable parkinsonian syndrome was achieved. Mixtures of three rAAV vectors (rAAV-TH/-AADC/-GCH) were then stereotaxically injected unilaterally into the putamen. Each monkey received nine unilateral injections of rAAV vectors in three tracks in the unilateral putamen. Each injection comprised 5 μl with a 1:1:1 mixture of rAAV-TH/-AADC/-GCH (1×10^{13} copies of each vector genome per ml). Thus, in total, 1.5×10^{11} genome copies of each vector were injected. Coexpression of the enzymes in the unilateral putamen resulted in marked improvement in manual dexterity on the side contralateral to rAAV-TH/-AADC/-GCH vector injections. Tremor disappeared in the contralateral limb, and the ability of the monkeys to pick up raisins improved (Fig. 2). The ipsilateral limb remained disabled, suggesting that behavioral recovery on the contralateral side cannot be explained by spontaneous recovery that might be expected to occur over time.

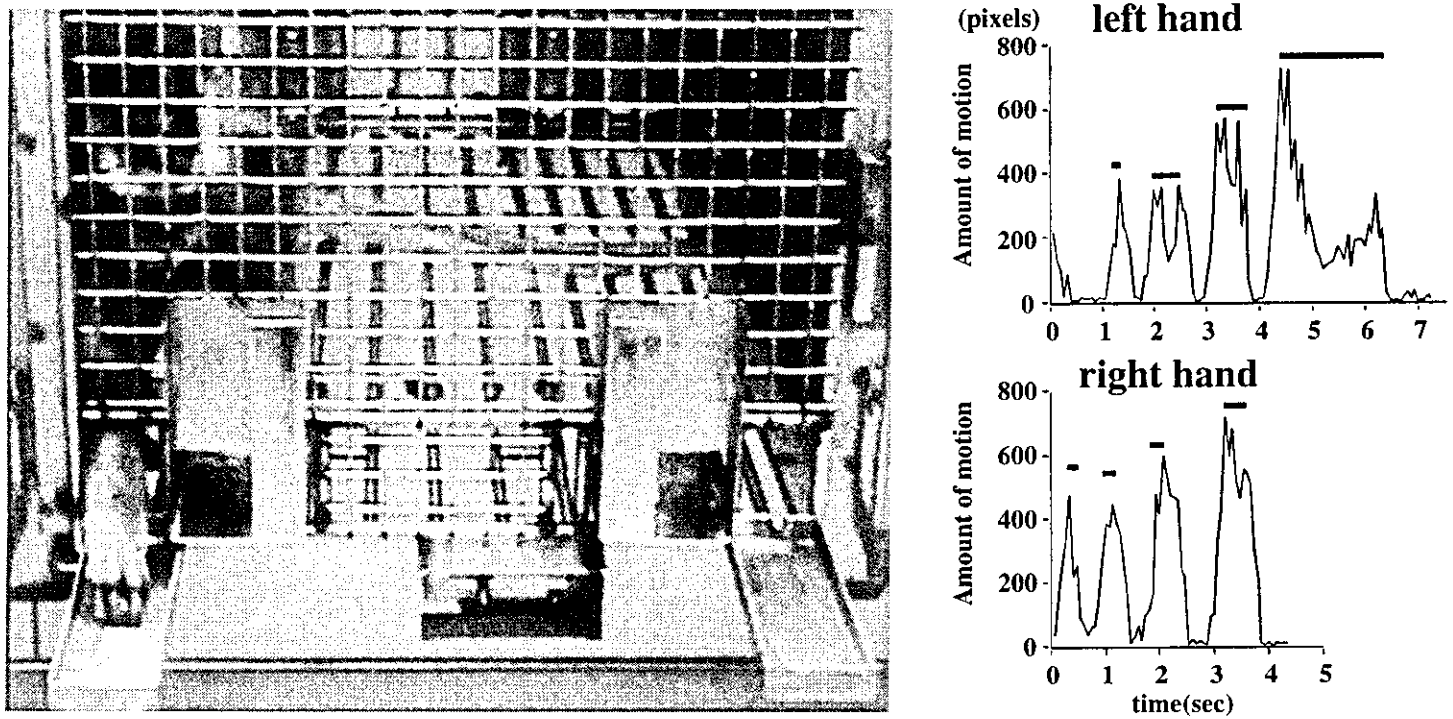

Figure 2. Improvement of performance on a fine motor task in a monkey treated with rAAV vectors expressing dopamine-synthesizing enzymes. a). The monkey is picking up four raisins sequentially with each of the two hands 1 month after rAAV-TH/-AADC/-GCH injection into the left putamen. **b).** Examples of a video-analyzed pattern representing hand movement. The horizontal black bar above each major peak represents hand movement to pick up each raisin. With the left (ipsilateral to the vector injection) hand, the parkinsonian monkey took longer to pick up raisins, and this action was often disturbed by tremor expressed as multiple small peaks on major peaks. In contrast, the time spent and pattern of grabbing the four raisins improved with the right (contralateral side) hand after vector injection. (Reprinted from Muramatsu *et al.*, *Human Gene Therapy*, **13**: 345-354, 2002 with permission from Mary Ann Liebert, Inc. Publishers).

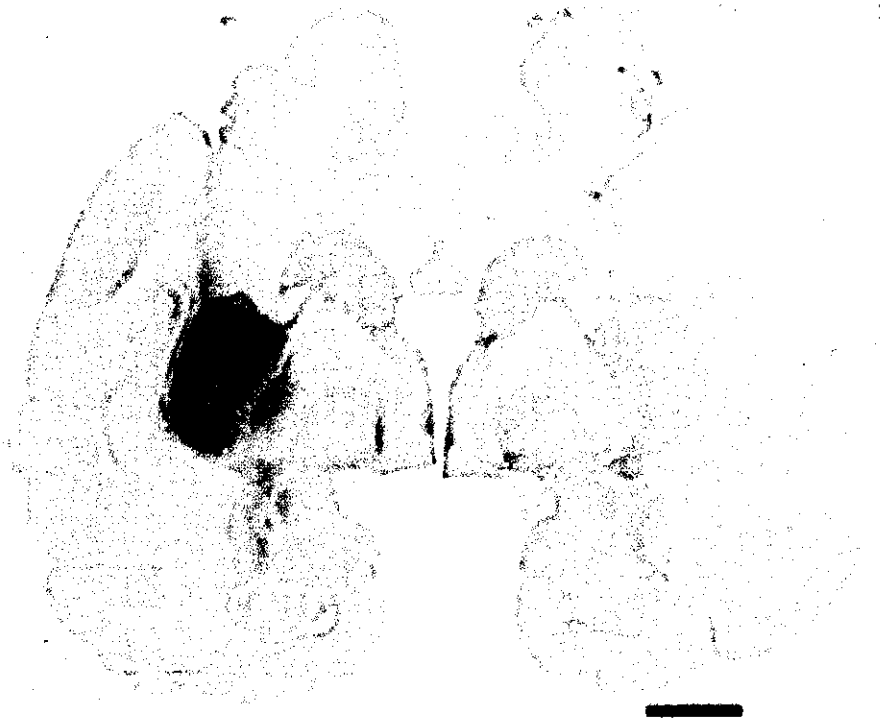

Figure 3. TH-immunostaining in the brain of MPTP-treated monkey 65 days after stereotaxic injection of rAAV-TH/-AADC/-GCH. Dense TH-immunoreactivity in the putamen was observed on the rAAV-TH/-AADC/-GCH-treated side. Immunoreactivity in striatum on the control side was nearly completely lost. Scale bar = 0.5cm. (Reprinted from Muramatsu *et al.*, *Human Gene Therapy*, **13**: 345-354, 2002 with permission from Mary Ann Liebert, Inc. Publishers).

Behavioral recovery persisted throughout the observation period (three monkeys were sacrificed for histological evaluation at 48 days, 65 days and 50 days, respectively, and one monkey was kept alive more than 30 months after injection of the rAAV vectors). TH-immunoreactive (IR), AADC-IR, and GCH-IR cells were present in a large region of the putamen (>90% of the putamen; Fig. 3). Microdialysis demonstrated that concentrations of DA in the rAAV-TH/-AADC/-GCH vector-injected putamen were increased compared with the control side. Monkeys did not show any complications such as dyskinesia after injection of the rAAV

vectors. Except for a slight infiltration of mononuclear cells and residual hemosiderin around the needle tract, hematoxylin and eosin staining revealed no signs of cytotoxicity in the rAAV vector-injected putamen.

e) Neuroprotective GDNF gene therapy in a rat model

An alternative approach to the treatment of PD is the protection of nigrostriatal pathways from progressive degeneration (Dunnett and Bjorklund, 1999) by providing genes for growth factors, antioxidant molecules, or antiapoptotic substances. GDNF (glial cell line-derived neurotrophic factor) is a small glycoprotein that provides strong trophic support for dopaminergic neurons and is a candidate for this neuroprotective strategy (Bjorklund *et al.*, 2000; Bohn, 2000). However, GDNF protein has limited usefulness as a therapeutic agent because of its short duration of activity and poor ability to cross the blood-brain barrier. Repeated injections of GDNF protein into the human brain are not practical, and implanting infusion devices is likely to increase patient morbidity and risk of infection over the long term. Recombinant viral vectors are powerful tools for providing sustained GDNF production in the brain. Previous studies have demonstrated that GDNF gene delivered via viral vectors protects nigral dopaminergic neurons in rodent and primate models of PD, when the GDNF gene was administrated prior to or shortly after injections of neurotoxin (Bilang-Bleuel *et al.*, 1997; Choi-Lundberg *et al.*, 1997; Mandel *et al.*, 1997; Connor *et al.*, 1999; Mandel *et al.*, 1999; Kirik *et al.*, 2000; Kordower *al.*, 2000; Kozlowski *et al.*, 2000; Connor *et al.*, 2001; Natsume *et al.*, 2001).

Because substantial numbers of DA neurons are already lost before characteristic symptoms appear in PD, we studied the effects of GDNF gene delivered in a delayed manner in a rat model of PD (Wang *et al.*, 2002a). Four weeks after creation of a unilateral striatal lesion using 6-OHDA, animals received injections of rAAV vectors expressing GDNF tagged with FLAG peptide (rAAV-GDNF) or β-galactosidase (rAAV-LacZ) in the lesioned striatum (Fig. 4a). At that time point, the number of TH-IR neurons on the

lesioned side had been reduced to 35% of the number on the intact side. At 20 weeks after rAAV vector injection, extensive loss of TH-IR cells in the SN (to 20% of the number on intact side) and TH-IR fibers in the striatum (to 14% of the number on the intact side) was observed in rats injected with rAAV-LacZ. In contrast, injection of rAAV-GDNF significantly increased the number of TH-IR neurons in the SN and the density of TH-IR fibers in the striatum, reaching nearly 57% and 49% of control values for the contralateral side, respectively (Fig. 4b-d). Levels of DA and its metabolites in the striatum were markedly higher in the rAAV-GDNF group compared with the control rAAV-LacZ group. Significant behavioral recovery was observed 4-20 weeks following rAAV-GDNF injection. Dual-immunostaining for FLAG and TH demonstrated double positive cells in the SN.

In conclusion, our results indicate that delayed delivery of the GDNF gene using rAAV vectors halts the ongoing degeneration of the nigrostriatal pathway, producing functional recovery even after substantial numbers of DA cells have been depleted.

3. GENE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS

a) Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis is a chronic neurodegenerative disease characterized by the progressive loss of motor neurons, leading to profound weakness and eventual death of affected individuals, on average, four years following onset. This is usually a disease of middle and later life, with an incidence of two to three per 100,000 of the population, and ALS is responsible for the death of approximately 1 in 1,000 adults. The disease is mainly sporadic, but ten percent of cases are familial, and one-fifth of these cases are caused by dominantly inherited mutations in the gene on chromosome 21q encoding the cytosolic Cu/Zn form of superoxide dismutase (SOD1) (Rosen *et al.*, 1993). A second causative gene was identified in a rare recessive inherited form of juvenile ALS (Hadano *et al.*, 2001; Yang *et*

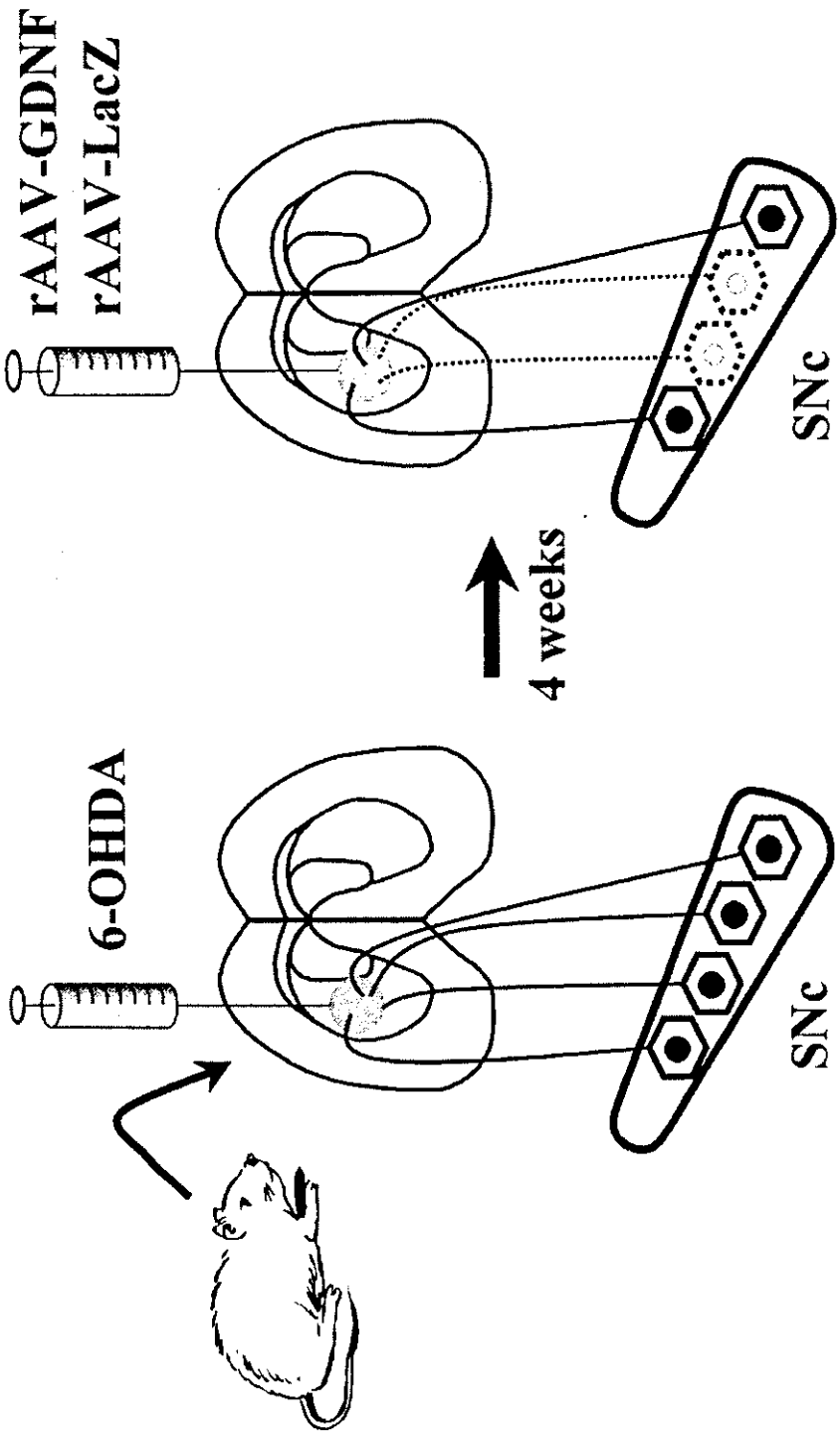

Figure 4. Neuroprotection of nigral neurons with rAAV-GDNF vector in a rat PD model. a). Schematic drawing of the neuroprotective strategy for the nigrostriatal pathway. Four weeks after injection of 6-OHDA, rAAV-GDNF or rAAV-LacZ was injected into the unilateral striatum.

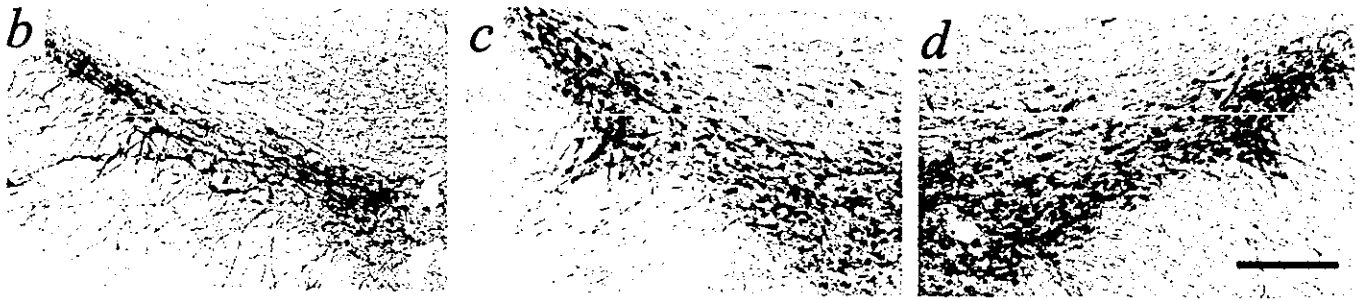

Figure 4. Neuroprotection of nigral neurons with rAAV-GDNF vector in a rat PD model. b-d). Coronal sections through the midbrain showing TH-immunoreactive (IR) nigral neurons 20 weeks after rAAV vector administration. The number of TH-IR neurons was remarkably reduced on the lesioned side of rAAV-LacZ-injected animals (b). In contrast, rAAV-GDNF-injected animals showed increased numbers of TH-IR fibers in the treated striatum (c). SN from an intact hemisphere is shown as control (d). Scale bar = 200μm. SNc, substantia nigra pars compacta. (Reprinted from Wang *et al.*, *Gene Ther*, **9**: 381-389, 2002 with permission from Nature)

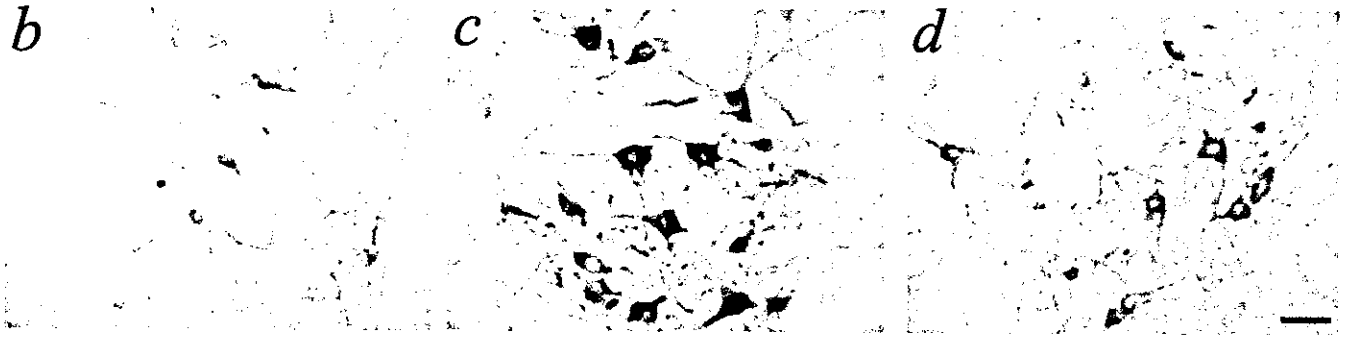

Figure 5. Neuroprotection of spinal motoneurons with rAAV-mediated gene delivery of GDNF into skeletal muscles of ALS mice. b-d). To assess surviving motoneurons that retained functioning neuromuscular projections to the injected muscles, a neural tracer cholera toxin B (CTB) was injected bilaterally into the gastrocnemius muscles of mice 1 week before sacrifice. At 110 days of age, there were markedly fewer CTB-labeled motoneurons in control ALS mice (b) than in wild-type mice (c). However, with rAAV-GDNF vector treatment, more CTB-labeled motoneurons were maintained (d) than in the control ALS group (b). Scale bar = 50μm. (Copyright 2002 by the Society for Neuroscience, reprinted with permission from Wang *et al.* 2002b).

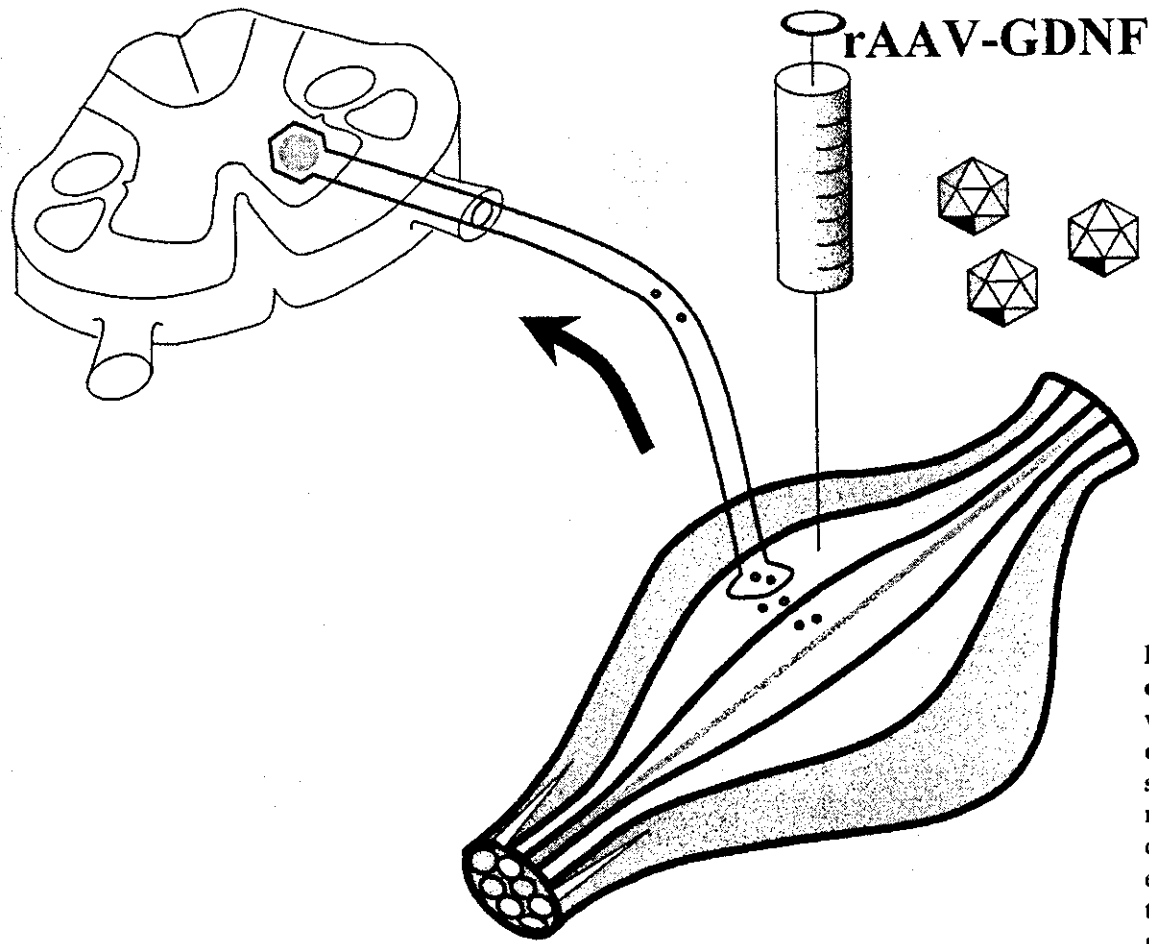

Figure 5. Neuroprotection of spinal motoneurons with rAAV-mediated gene delivery of GDNF into skeletal muscles of ALS mice. a). Schematic drawing of the strategy. GDNF is expressed in the muscle and transported to spinal motoneurons in a retrograde manner.

al., 2001). This gene, *ALS2*, encodes a protein with domains homologous to those in GTPase regulatory proteins.

Transgenic mice overexpressing mutant SOD1 exhibit spontaneous motor neuron degeneration with progressive clinical weakness, providing an excellent *in vivo* animal model of human familial ALS (Gurney *et al.*, 1994; Elliott, 1999). Since knockout mice with targeted deletions of both SOD1 alleles do not develop spontaneous motor neuron loss, toxic gain of function rather than diminished function of the mutant SOD1 protein is believed to cause motor neuron degeneration. To date, the molecular mechanisms leading to the selective motoneuron degeneration in ALS remain poorly understood, although activation of a specific death pathway involving Fas and NO was demonstrated (Raoul *et al.*, 2002). The only proven therapy for ALS in humans, a glutamate inhibitor Riluzole, extends survival by approximately three months. It is therefore critical to identify new therapeutic strategies for ALS.

b) Neuroprotective GDNF gene therapy in ALS mice

One potential strategy of gene therapy for ALS aims at providing neuroprotection to block or slow ongoing degenerative processes through supplying genes for growth factors, antioxidant molecules, or antiapoptotic substances. GDNF has been demonstrated to be a potent neurotrophic factor for the survival of spinal motoneurons in cell culture experiments, and it holds great potential for treating the disease. However, intra-ventricular delivery of GDNF is associated with weight loss, nausea, hallucinations, tingling, depression and abnormal sexual behavior (Kordower *et al.*, 1999), and as in therapy for PD, systemic administration of GDNF as a recombinant protein is not beneficial. Fortunately, skeletal muscle is a good target for gene delivery of neurotrophic factors. Proteins secreted by transduced muscle cells can be captured by motoneuron terminals at neuromuscular junctions and retrogradely transported to the motoneuron cell bodies in the ventral horn of the spinal cord (Lu *et al.*, 2003; Fig. 5a). Intra-

muscular injections are much safer and easier than intra-thecal or intra-spinal injections.

We explored the therapeutic efficacy of intramuscular delivery of the GDNF gene mediated by a rAAV vector in a mouse model of ALS (Wang *et al.*, 2002b). We used a rAAV-GDNF vector directing expression of an epitope-tagged GDNF-FLAG fusion protein that can be readily distinguished from endogenous GDNF. At 9 weeks of age, male transgenic mice overexpressing the G93A human SOD1 mutation (SOD1G93A) were injected with rAAV-GDNF vector, rAAV-LacZ, or vehicle in four limbs (gastrocnemius and triceps brachii muscles). All treatment groups of ALS mice showed similar motor performance, as quantified with a Rotarod, until 12 weeks of age. Thereafter, it deteriorated quickly in control ALS mice (average age of motor deficit onset: 101.3 ± 5.4 days, n = 11), while the performance deterioration was significantly delayed in rAAV-GDNF vector-treated mice (onset: 114.0 ± 4.0 days, n = 12). rAAV-GDNF vector treatment prolonged the mean survival by 16.6 ± 4.1 days (mean survival times were 138.9 ± 9.2 days in rAAV-GDNF vector-treated mice versus 122.3 ± 5.7 days in control ALS mice, n = 8, $P < 0.01$). However, once motor symptoms appeared, weakness and atrophy of the skeletal muscles, especially in the hindlimbs, ultimately developed in all mice in each group. The duration of the disease, evaluated as the number of days that elapsed from the onset to the end stage, did not differ between the rAAV-GDNF vector-treated and control ALS mice (24.0 ± 3.5 days versus 21.0 ± 3.5 days, $P > 0.05$). rAAV-GDNF vector treatment led to high levels of GDNF accumulation at neuromuscular junctions and substantial and long-lasting expression of transgenic GDNF in a large number of myofibers. GDNF-FLAG, but not β-galactosidase expressed as a control, was detected in the anterior horn neurons, indicating that most of the transgenic GDNF observed there is retrogradely transported GDNF protein from the transduced muscles. This transgenic GDNF protects motoneurons from

degeneration, preserves their axons innervating the muscle, and inhibits atrophy of the treated muscle (Fig. 5b-d).

These findings indicate that rAAV-mediated GDNF delivery to muscle is a promising approach to gene therapy for ALS, although further improvements are necessary to extend the duration of the protective therapeutic effect.

4. CONCLUSIONS

Advances in gene transfer methods, and particularly in the development of improved viral vectors, have expanded the potential of gene therapy to tackle neurodegenerative diseases. At present, the rAAV vector represents one of the most powerful vehicles for delivering therapeutic genes into mammalian brain and muscle. Using rAAV vectors, we have demonstrated 1) that significant functional recovery was achieved by direct gene delivery of DA-synthesizing enzymes into the striatum in a primate model of PD and 2) that delayed delivery of a GDNF gene prevents nigral neurodegeneration and promotes functional recovery in a rat model of PD. We have also shown that the intramuscular administration of a rAAV vector harboring the GDNF gene to a mouse model of ALS can significantly delay the onset of disease, lengthen the life-span, abate the behavioral impairment, and promote motoneuron survival via retrograde transportation of the transgene product to motoneurons in the spinal cord. Although it will probably be necessary to develop vector constructs that allow some regulation of gene expression to avoid excess production of DA or GDNF before going into clinical arena, gene therapy using rAAV vectors will offer a novel and feasible approach to the treatment of neurodegenerative disorders in the near future. Strategies aimed at correcting the gene defect will be developed especially for gene deletion-based genetic disorders, and the elucidation of mechanisms whereby genetic mutations lead to the loss of neurons in familial PD or ALS will provide new targets for gene therapy. A

combination of gene therapy and cell transplantation appears to be the next therapeutic strategy in some diseases.

Acknowledgments

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