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NEURONAL SPECIFICITY OF α-SYNUCLEIN TOXICITY AND EFFECT OF PARKIN CO-EXPRESSION IN PRIMATES

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Abstract—Recombinant adeno-associated viral (rAAV) vector-mediated overexpression of α-synuclein (αSyn) protein has been shown to cause neurodegeneration of the nigrostriatal dopaminergic pathway in rodents and primates. Using serotype-2 rAAV vectors, we recently reported the protective effect of Parkin on αSyn-induced nigral dopaminergic neurodegeneration in a rat model. Here we investigated the neuronal specificity of αSyn toxicity and the effect of Parkin co-expression in a primate model. We used another serotype (type-1) of AAV vector that was confirmed to deliver genes of interest anterogradely and retrogradely to neurons in rats. The serotype-1 rAAV (rAAV1) carrying *αSyn* cDNA (rAAV1-αSyn), and a cocktail of rAAV1-αSyn and rAAV1 carrying *parkin* cDNA (rAAV1-parkin) were unilaterally injected into the striatum of macaque monkeys, resulting in protein expression in striatonigral GABAergic and nigrostriatal dopaminergic neurons. Injection of rAAV1-αSyn alone decreased tyrosine hydroxylase immunoreactivity in the striatum compared with the contralateral side injected with a cocktail of rAAV1-αSyn and rAAV1-parkin. Immunostaining of striatonigral GABAergic neurons was similar on both sides. Overexpression of Parkin in GABAergic neurons was associated with less accumulation of αSyn protein and/or phosphorylation at Ser129 residue. Our results suggest that the toxicity of accumulated αSyn is not induced in non-dopaminergic neurons and that the αSyn-ablating effect of Parkin is exerted in virtually all neurons in primates. © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; DAT, dopamine transporter; EGFP, enhanced green fluorescent protein; PB, phosphate buffer; PBS, phosphate-buffered saline; PD, Parkinson's disease; rAAV, recombinant adeno-associated virus; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; αSyn, α-synuclein; TH, tyrosine hydroxylase; VMAT2, vesicular monoamine transporter 2.

Key words: Parkinson's disease, adeno-associated virus, dopaminergic neurons, GABAergic neurons, monkey.

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting approximately 1% of people over the age of 65 (Goedert, 2001). The disease is clinically characterized by akinesia, rigidity, resting tremor, and postural instability. The pathological hallmarks of PD are the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), and the presence of eosinophilic protein inclusions termed Lewy bodies in the surviving SNpc dopaminergic neurons. Based on the finding that a point mutation in the gene encoding α-synuclein (αSyn) protein causes a rare familial form of PD (PARK1) (Polymeropoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004), αSyn has been confirmed to be a major component of Lewy bodies in patients with sporadic PD (Spillantini et al., 1997). Abnormal accumulation of αSyn protein has widely been hypothesized as a neurotoxic event in PD development (Goedert, 2001). Recent studies indicated that another dominantly inherited form of familial PD, PARK4, is caused by triplication of the *αSyn* gene locus (Singleton et al., 2003). This genetic mutation results in production of higher levels of αSyn protein, supporting the αSyn-induced neurodegeneration hypothesis. However, in cell types other than the dopaminergic one, the overexpressed αSyn protein sometimes functions as a neuroprotective molecule (da Costa et al., 2000; Xu et al., 2002). Thus, oxidative modification of αSyn by dopamine metabolites is considered responsible for the selective vulnerability to dopaminergic neurons (Conway et al., 2001; Xu et al., 2002). Based on the above studies, clearance of abnormal αSyn protein from SNpc dopaminergic neurons can be potentially used as a strategy in the treatment of PD.

The viral vector-mediated αSyn delivery causes accumulation of αSyn and subsequent degeneration of SNpc dopaminergic neurons in adult rodents and primates (Kirik et al., 2002, 2003; Klein et al., 2002; Lo Bianco et al., 2002; Lauwers et al., 2003; Yamada et al., 2004). Using a recombinant adeno-associated viral (rAAV) vector, we previously showed that αSyn protein that accumulated in rat SNpc dopaminergic neurons is phosphorylated at serine residue of 129th position (Ser129) (Yamada et al., 2004). In fact, such post-translational modification is frequently observed in human brains affected with so-called α-synucleinopathy, including PD and dementia with Lewy bodies (Fujiwara et al., 2002). Recent studies also showed that Ser129-phosphorylation of αSyn is essential for neurodegeneration in a *Drosophila* model of PD (Chen and Feany, 2005).

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The major cause of autosomal recessive juvenile Parkinsonism (ARJP) is a mutation in the gene encoding Parkin protein (PARK2) (Kitada et al., 1998). Parkin is an E3 ubiquitin ligase that catalyzes polyubiquitination of unfolded or short-lived proteins, directing the substrates to proteasomal degradation (Shimura et al., 2000). The PARK2 mutation results in loss-of-function of Parkin protein. It was reported recently that oxidative post-translational modifications of Parkin, e.g. *S*-nitrosylation (Chung et al., 2004; Yao et al., 2004) or covalent binding of dopamine (LaVoie et al., 2005), may be responsible for impairment of Parkin activity in sporadic cases of PD. Such processes could lead to accumulation of substrate proteins with subsequent dopaminergic neurodegeneration as proposed in PARK2 patients. We reported previously that rAAV-mediated overexpression of Parkin ameliorates the αSyn-induced SNpc dopaminergic neurodegeneration in a rat model (Yamada et al., 2005). Another group has shown similar results using a lentiviral vector (Lo Bianco et al., 2004). These data suggest that Parkin delivery may be therapeutically beneficial not only in PARK2 patients but also in patients with sporadic PD.

The aim of the present study was to assess the neuronal specificity of αSyn toxicity and the effect of Parkin delivery in a primate model with αSyn overexpression. While a serotype-2 rAAV vector was used in our previous studies (Yamada et al., 2004, 2005), here we applied another serotype (type-1) of rAAV (rAAV1) vector that could deliver genes of interest anterogradely and retrogradely to neurons. Injections of rAAV1 vectors producing enhanced green fluorescent protein (EGFP) (rAAV1-EGFP), αSyn (rAAV1-αSyn) and/or Parkin protein (rAAV1-parkin) into the rat and monkey striatum resulted in protein expression in striatonigral GABAergic and nigrostriatal dopaminergic neurons. Moreover, the overexpression of αSyn protein certainly decreased the density of dopaminergic axon terminals in the striatum, which was ameliorated by co-expression of Parkin protein.

EXPERIMENTAL PROCEDURES

Animals

Five adult male Sprague–Dawley rats (10-week-old) (Nihon SLC, Hamamatsu, Japan) were used for this study. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Juntendo University School of Medicine.

Two male crab-eating monkeys (*Macaca mullata*; 3.5–4.5 kg body weight) were used for this study. Intrastriatal injections of AAV vectors were carried out in a biosafety level-2 laboratory at the Tsukuba Primate Research Center which was designated for *in vivo* infectious experiments. The experimental protocol was approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience and by the Animal Welfare and Animal Care Committee of the Tsukuba Primate Research Center. All experiments were conducted according to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute for Neuroscience 2000) and the Rules for Animal Care and Management of the Tsukuba Primate Research Center. Throughout the experiments, the best efforts were made to minimize the number of animals used and their suffering.

Generation of rAAV vectors

The plasmid DNA *pAAV-MCS* (CMV promoter; Stratagene, La Jolla, CA, USA) carrying human *αSyn* cDNA (pAAV-MCS-αSyn) was constructed as reported previously (Yamada et al., 2004). Human *parkin* or *EGFP* cDNA was cloned into plasmid pAAV-MCS (pAAV-MCS-parkin or pAAV-MCS-EGFP, respectively). The plasmid DNA pAAV-MCS-αSyn, pAAV-MCS-parkin, or pAAV-MCS-EGFP was co-transfected with plasmids pHelper and Pack2/1 to HEK293 cells using a standard calcium phosphate method (Sambrook and Russell, 2001). After 48 h, the cells were harvested and crude rAAV vector solutions were obtained by repeated freeze/thaw cycles. After ammonium sulfate precipitation, virus particles were dissolved in phosphate-buffered saline (PBS) and applied to an OptiSeal centrifugation tube (Beckman Coulter, Inc., Fullerton, CA, USA). After overlaying an OptiPrep solution (Axis-Shield PoC AS, Oslo, Norway), the tube was processed by GradientMaster (BioComp Instruments, Inc., Fredericton, NB, Canada) to prepare a gradient layer of OptiPrep. The tube was then ultracentrifuged at 13,000 r.p.m. for 18.5 h. The fractions containing high-titer rAAV vectors were collected and used for injection into animals. The number of rAAV genome copies was semi-quantified by PCR within the CMV promoter region using primers 5′-GACGTCAATAATGACGTATG-3′ and 5′-GGTAATAGCGATGACTAATACG-3′. The final titers were 6.4×10^{11} genomes/mL (rAAV1-EGFP), 5.5×10^{11} genomes/mL (rAAV1-αSyn), and 7.0×10^{12} genomes/mL (rAAV1-parkin).

Stereotaxic injection of rAAV vectors

The rats were anesthetized with sodium pentobarbital (40 mg/kg body weight, i.p.). They were positioned in a stereotaxic frame. The skull was exposed, and a small portion of the skull over the striatum was removed unilaterally with a dental drill. Subsequently, rAAV1-EGFP was injected into the striatum (3 μL; 1.92×10^9 particles, 0.5 mm anterior and 2.9 mm lateral from bregma, 6.5 mm below the dural surface, tooth bar=4.0 mm) through a 5-μL Hamilton microsyringe.

After a survival period of 3 weeks, the rats were anesthetized deeply with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with PBS, followed by 4% paraformaldehyde in PBS. The brains were removed from the skull, postfixed in the same fresh fixative overnight, and immersed in PBS containing 30% sucrose until they sank. Coronal sections were cut serially at 20 μm thickness by a freezing microtome.

The monkeys were first sedated with ketamine hydrochloride (5 mg/kg, i.m.) and xylazine hydrochloride (0.5 mg/kg, i.m.), and then anesthetized with sodium pentobarbital (20 mg/kg, i.v.). After bilateral removal of skull portions over the striatum, stereotaxic injections of rAAV1-αSyn were made unilaterally into the putamen, and those of a cocktail of rAAV1-αSyn and rAAV1-parkin were made contralaterally. A total of 50 μL of each vector preparation was injected at five rostrocaudally different levels (10 μL each) through a 50-μL Hamilton microsyringe (50 μL on the side injected with rAAV1-αSyn alone; 2.75×10^{10} particles, and 100 μL on the side co-injected with rAAV1-αSyn; 2.75×10^{10} particles, and rAAV1-parkin; 3.5×10^{11} particles).

After survival periods of 4 and 7.5 months, the monkeys were anesthetized deeply with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with PBS, followed by a mixture of 8% formalin and 15% saturated picric acid in 0.1 M phosphate buffer (PB; pH 7.4). The brains were removed from the skull, postfixed in the same fresh fixative overnight, and immersed in PB containing 30% sucrose until they sank. Coronal sections were cut serially at 60 μm thickness by a freezing microtome.

Antibodies and immunohistochemistry

The primary antibodies used in this study were as follows; rabbit anti-GFP (diluted at 1:200; Chemicon International Inc., Te-