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Generation of a α-synuclein-based rat model of Parkinson's disease

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Two missense mutations (A30P and A53T) in the gene for alpha-synuclein (α-syn) cause familial Parkinson's disease (PD) in a small cohort. There is increasing evidence to propose that abnormal metabolism and accumulation of α-syn in dopaminergic neurons play a role in the development of familial as well as sporadic PD. The complexity of the mechanisms underlying α-syn-induced neurotoxicity, however, has made difficult the development of animal models that faithfully reproduce human PD pathology. We now describe and characterize such a model, which is based on the stereotaxic injection into rat right substantia nigra pars compacta of the A30P mutated form of α-syn fused to a protein transduction domain (TAT). The TAT sequence allows diffusion of the fusion protein across the neuronal plasma membrane and results in a localized dopaminergic loss. Dopaminergic cell loss was evaluated both by tyrosine hydroxylase immunohistochemistry and by HPLC analysis of dopamine and its catabolite 3,4 dihydroxyphenylacetic acid. Infusion of TAT-α-synA30P induced a significant 26% loss in dopaminergic neurons. This dopaminergic loss was accompanied by a time-dependent impairment in motor function, evaluated utilizing the rotarod and footprint tests. In comparison to chemical neurotoxin-based (e.g. 6-hyroxydopamine, MPTP) animal models of PD, the α-syn-based PD animal model offers the advantage of mimicking the early stages and slow development of the human disease and should prove valuable in assessing specific aspects of PD pathogenesis in vivo and in developing new therapeutic strategies.

Keywords: α-Synuclein; Parkinson's disease; Neurodegeneration; Animal models; Fusion protein; Dopaminergic loss; Motor impairment; Substantia nigra pars compacta

Introduction

Parkinson's disease (PD) is a multicentric, progressive neurodegenerative disorder that strikes ~1% of people beyond 65 years of age (Tanner, 1992). PD is characterized clinically by severe motor symptoms including uncontrollable resting tremor, muscular rigidity, and bradykinesia (Lotharius and Brundin, 2002), together with secondary symptoms such as postural instability, cognitive dysfunction (dementia, psychosis), sleep abnormalities and mood disorders (depression, anxiety), as well as gastrointestinal and genitourinary disturbances (Berrios et al., 1995; Schapira et al., 2006). The pathophysiology of PD results from the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Tanner, 1992), which leads to the depletion of dopamine (DA) in its striatal projections, and other brainstem neurons, with consequent disruption of the cerebral neuronal systems responsible for motor functions (Dawson and Dawson, 2003; Lotharius and Brundin, 2002). Motor symptoms become manifest only after the loss of >70% of dopaminergic neurons in the SNpc and may be preceded by cognitive impairments, likely related to alterations in brain regions other than the SNpc (Stiasny-Kolster et al., 2005). The PD brain is characterized also by the presence of cytoplasmic (Lewy bodies) and neuritic (Lewy neurites) inclusions (Gómez-Tortosa et al., 1999) in the surviving DA neurons and other affected regions of the CNS (Forno, 1996; Shults, 2006).

PD is an essentially sporadic neurodegenerative disease whose pathogenesis remains largely unknown, although a complex interaction among multiple predisposing genes and environmental risk factors has gained more attention of late as possible causes of late-onset idiopathic PD (Siderowf and Stern, 2003; Warner and Schapira, 2003). In addition, several rare monogenic familial forms of PD (<10% of all cases), characterized by early-onset and an autosomal dominant or recessive pattern of inheritance, have been identified. Mutations in seven genes have now been linked to early-onset PD, encoding for α-synuclein (α-syn) (Polymeropoulos et al., 1997), ubiquitin carboxy-terminal hydrolase L-1 (Leroy et al., 1998), parkin (Shimura et al., 2001), DJ-1 (Bonifati et al., 2003), pink1

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(Bonifati et al., 2005), PARK8: LRRK2/dardarin (Zimprich et al., 2004) and PARK13: Omi/HtrA2 (Strauss et al., 2005). Duplication and triplication in the α-syn gene can also be a cause of PD (Singleton et al., 2004).

Interest in α-syn has intensified following the discovery that two missense mutations, A53T (Polymeropoulos et al., 1997) and A30P (Krüger et al., 1998), in the α-syn gene appear to account for rare cases of autosomal dominant early-onset PD in families of European origin. Recently, a short triplication containing the *α-syn* gene plus flanking regions on chromosome 4 and a novel E46K mutation in α-syn have been identified in separate kindreds in which individuals manifest classical PD or dementia with Lewy bodies (Singleton et al., 2003). The aggregation and accumulation of these abnormal α-syn proteins in dopaminergic neurons have been postulated to be responsible for the subsequent neurodegeneration (Krüger et al., 1998). α-Syn aggregation is present also in the classic form of PD and in other CNS disorders, called α-synucleinopathies, which include Alzheimer's disease (for a recent review, see Recchia et al., 2004). α-Syn is a major component both of Lewy body filaments and of dystrophic Lewy neurites (Conway et al., 1998; Goedert, 2001). A recent study has shown that aggregated α-syn mediates dopaminergic neurotoxicity in vivo (Periquet et al., 2007). The mechanism of α-syn-induced toxicity is unclear but may be related to the propensity of normal α-syn and its mutated forms (A53T, A30P, E46K) to self-aggregate at higher concentrations, producing fibrils (Wood et al., 1999; Greenbaum et al., 2006) with amyloid-like cross-beta conformation (Serpell et al., 2000). Monomeric α-syn aggregates in vitro to form stable fibrils via a metastable oligomeric (protofibril) state (Volles and Lansbury, 2002), and PD-associated α-syn is more fibrillogenic than β- and γ-synuclein (Biere et al., 2000). The protofibrillization rate of both α-syn mutants is higher than that of the wild-type protein (Conway et al., 2000a,b). This complex behavior of α-syn has made difficult the development of models for studying synucleinopathies in neurodegenerative diseases, particularly PD. More 'traditional' chemical toxin models of PD (MPTP, 6-hydroxydopamine) have focused on the nigrostriatal pathway and loss of dopaminergic neurons in this region. While contributing much to our understanding of PD and the development of symptomatic therapeutics, they do not reproduce the full pathology and progression seen in PD. Considering the important role that α-syn may play in the pathogenesis of sporadic as well as familial PD, there is clearly a need to develop well-characterized α-syn-based PD animal models to further clarify disease etiopathogenesis and for designing new and more efficacious therapeutics. In the present study, we describe a new α-syn-based PD animal model, which relies on the stereotaxic intranigral injection of A30P mutated form of α-syn fused to a protein transduction domain (TAT), thereby allowing passage across the neuronal plasma membrane (Albani et al., 2004). The administered fusion protein induced a localized and modest dopaminergic loss, together with long-term motor deficits.

Materials and methods

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. All other chemical were of the highest commercial grade available.

Synthesis of α-syn A30P and TAT-α-synA30P

The fusion protein TAT-α-synA30P was generated as described (Albani et al., 2004). A human brain cDNA library (CLONTECH, Heidelberg, Germany) was used to amplify the human *α-syn* gene by polymerase chain reaction (PCR), using a specific primer complementary to the double-stained cDNA and based on the published sequence of the human *α-syn* gene: primer a, 5′ATGGCTAGCATGGATGTATTCATGAAAGGAC3′; primer b, 5′CGAAGCTTAGGCTTCAGGTTCGTAGTCTGG3′. The purified PCR product was cut at the *Nhe*I and *Hin*dIII restriction sites and cloned directly into the bacterial expression vector pRSETB in the same restriction sites (plasmid pSyn). Site-directed mutagenesis of pSyn was then carried out to introduce the A30P α-syn mutation (plasmid pA30P-α-syn). A pTAT vector was constructed as reported for pTAT-HA (Becker-Hapak et al., 2001), and the sequence of α-synA30P was cloned after the TAT sequences between the *Nhe*I and *Hin*dIII restriction sites (plasmid pTAT-A30P-α-syn).

A green fluorescent protein (GFP)-α-syn fusion protein was constructed using plasmid pGFP-CNTF (Negro et al., 1997) as vector and replacing the CNTF sequence with that of α-syn. The coding sequence for GPF fused to α-syn was subcloned between the *Nhe*I and *Hin*dIII restriction sites of expression vector pTAT-HA to give pTAT-GFP-α-syn. All clonings were verified by sequence analysis at the CRIBI core sequencing facility.

Purification of TAT-α-synA30P

The fusion protein expression construct pTAT-A30P-α-syn was transformed into *Escherichia coli* strain BL21 (DE3) pLysS; 2 l was grown in LB/ampicillin broth for 12 h with shaking at 37 °C. Fusion protein production was induced by the addition of 500 μM isopropyl β-thiogalactoside. After 3 h of induction at 37 °C, the cells were harvested. Because TAT-α-synA30P was produced as inclusion bodies, pelleted cells were resuspended in inclusion body sonication buffer (500 mM NaCl, 20 mM Tris/HCl pH 8.0) and then heated for 10 min at 100 °C. The lysate was then centrifuged at 11,000×*g* for 20 min and the supernatant retained. Twenty millimolars of imidazole was then added to the lysate and loaded onto a 2.5 ml Ni^{2+}-agarose column (Qiagen). After washing with 20 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl pH 8.0, the recombinant protein was eluted with 0.25 M imidazole. The recovered protein was dialysed against PBS and aliquots flash frozen in liquid nitrogen and stored at −80 °C.

Animals

Adult male Sprague–Dawley rats (280±20 g) were obtained from Harlan Italy, (S. Pietro al Natisone, UD). Rats were housed under controlled conditions of temperature (23±2 °C) and illumination (12-h light; 12-h darkness; darkness: 7 p.m.–7 a.m.) and had free access to a standard diet and water. All experiments were carried out from 10 am to 5 pm and followed guidelines governing animal experimentation (European Union decree of 24/11/1986 (86/609/IIC)).

6-OHDA and TAT-α-synA30P lesion induction

Rats (8–10 animals per group) were anesthetized i.m. with a solution of ketamine (87 mg/kg) plus xylazine (13 mg/kg) and secured in a Kopf stereotaxic apparatus, with the tooth bar set +5 mm above

the interaural line. Lesions were made by unilateral injection of 6-OHDA (2.7 μg/μl) or TAT-α-synA30P (1 μg/μl) into the right SNpc at the following coordinates: AP: −5.7, LM: −2.0: DV: −8.7 mm from the Bregma (Paxinos and Watson, 1986). Control sham-operated animals received the same volume of vehicle (0.1% ascorbate in 0.9% saline solution) at the same coordinates. Administrations were performed using a 27-gauge Hamilton syringe connected to an infusion minipump (Harvard Compact Infusion Pump, Holliston; MT) at a rate of 0.5 μl/min. The syringe was left in place for 3 min before slowly retracting it to allow substance infusion and prevent reflux.

Immunohistochemistry

Rats were sacrificed and their brains were removed, snap-frozen in a dry ice/isopentane bath and stored at −80 °C. Serial coronal sections of 10 μm were cryostat cut from each SNpc, mounted on gelatin-coated slides and fixed/dehydrated in acetone (30 min at −20 °C). Sections were incubated with 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidase activity followed by 20 min incubation with normal goat serum to block non-specific binding sites. Alternate sections were then incubated with a mouse anti-tyrosine hydroxylase (TH) antibody (Clone TH16, Sigma, 1:4000) for 1 h at room temperature followed by a 10 min rinse with PBS and incubation with a peroxidase-conjugated anti-mouse secondary antibody (Histofine Simple stain MAX PO, Nichirei Corp., Japan). The sections were finally treated with 3,3-diaminobenzidine to visualize the reaction product. Adjacent sections were processed for Nissl stain.

Morphometric analysis

A series of regularly spaced sections across the SNpc were analyzed, starting with the first section on which the region appeared and continuing every 150 μm until the structure terminated. Images of the right and left SNpc from each section were digitized at a primary magnification of ×5 using a digital camera (Leica DC 200, Leica Microsystems GmbH, Wetzlar, Germany) mounted on a light microscope (Leica DM-R, Leica Microsystems GmbH, Wetzlar, Germany) and saved as TIFF files. The number of TH-positive profiles was then evaluated on both sides of each section using a computer-assisted image analysis software (QWin, Leica Imaging Systems Ltd., Cambridge, UK), as reported previously (Zoli et al., 1992; Guidolin et al., in press). For each animal, the sum of the cell counts over all sections analyzed was taken as an estimate of the total number of TH-positive neurons in the right and left SNpc separately.

Western blotting analysis

Rat brain tissues (pooled from 8 animals per experimental condition) were homogenized in 50 mM Tris–HCl, pH 7.0, 1 mM EDTA, 0.1 mM DTT using a Polytron and centrifuged at 11,000×*g* for 20 min. The supernatant was brought to 0.5 M NaCl and placed in a boiling water bath for 10 min and centrifuged again at 11,000×*g* for 20 min. Supernatant proteins were precipitated with 90% ethanol, separated by 12% SDS–PAGE and electroblotted onto Immobilon transfer membranes (Millipore, USA). Blots were preincubated with 3% bovine serum albumin before immunodetection. We used a monoclonal anti-α-syn antibody (#211, from Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Bioscience, USA) according to the manufacturer's instructions. Western blots shown are representative of three experiments with similar results.

HPLC analysis

The tissue content of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) was measured by high-performance liquid chromatography (HPLC) 15, 30 and 90 days after 6-OHDA or TAT-α-synA30P stereotaxic injection. Left and right striata were rapidly dissected, weighed and sonicated in 1 ml (w/v) of cold 0.4 M perchloric acid. Homogenates were centrifuged at 13,000×*g* for 15 min at 4 °C. Aliquots (450 μl) of supernatant were added to 50 μl of 1 mM 3,4-dihydroxybenzylamine as internal standard and 50 μl subjected to HPLC analysis (Waters, Milford, Mass.). The HPLC system was equipped with an electrochemical detector (BAS LC-4B Bioanalytical Systems, West Lafayette, Ind.) and a reverse-phase chromatography column (Bio-Sil C18HL 90–10, 250×4.6 mm; Bio-Rad Lab, Richmond, USA) heated at 30 °C. The mobile phase consisted of KH_2PO_4 (50 mM), sodium acetate (100 mM; pH 4.8), EDTA (0.2 mM), octylsodium sulfate (0.14 mM) and methanol (10%) filtered and degassed before use. The flow rate was 0.88 ml/min.

Apomorphine rotational behavior testing

DA agonists such as apomorphine are highly effective in measuring rotational asymmetry in animals with unilateral lesions to the DA system (Ungerstedt, 1971; Pycock, 1980; Carman et al., 1991). Sham, 6-OHDA and TAT-α-synA30P treated rats were injected subcutaneously with 1.0 mg/kg *R*-(−)-apomorphine hydrochloride dissolved in 0.1% ascorbate saline solution and placed in individual cylindrical cages (240 mm in diameter, 300 mm high). The number of rotations over a 1 h period, both ipsilateral and contralateral, was recorded using an automatic rotometer (Rota-Count 8; Columbus Instruments, Columbus, Ohio). This test was performed 1 day before sacrifice for HPLC analysis.

Rotarod motor coordination test

Motor performance was assessed as described (Hamm et al., 1994), with modification. Briefly, the rotarod device consists of a plastic frame with a motorized, rotating assembly of rods (Geramotor, Bedine Electric Co., Chicago. IL, USA) on which the animal must walk. Sham animals were first assessed at five speeds of rotation: 8, 10, 12, 14 and 16 rpm. Each rat was allowed to remain stationary at 0 rpm for 10 s, after which the speed was suddenly increased to 8 rpm for 10 s and then again in the same manner to 10, 12 and 14 rpm for 10 s each. The highest speed, 16 rpm, was then maintained for 200 s until the 4 min test period elapsed. These rotational speeds were chosen so that sham (uninjured) animals would not fall off during the test. A rat was considered failing the 4-min period test if (a) it fell completely off the device before the time period elapsed or (b) it simply gripped the rungs and spun for two consecutive revolutions rather than actively walking on the rotating rods. Rats from all groups (sham, 6-OHDA, TAT-α-synA30P) were then tested twice daily (once each morning and afternoon) over a 5-day period. The best of five performances was utilized for data analysis.

Curves generated from these experiments were fitted using the following equation:

$$Y = (\mathrm{Tmax}*\mathrm{Tpr}^{\mathrm{p}})/(\mathrm{Tpr}^{\mathrm{p}} + K^{p})$$

where Tmax is the maximal time a rat was allowed to remain on the rod for any trial (240 s), *K*/2 is the number of trials needed for a

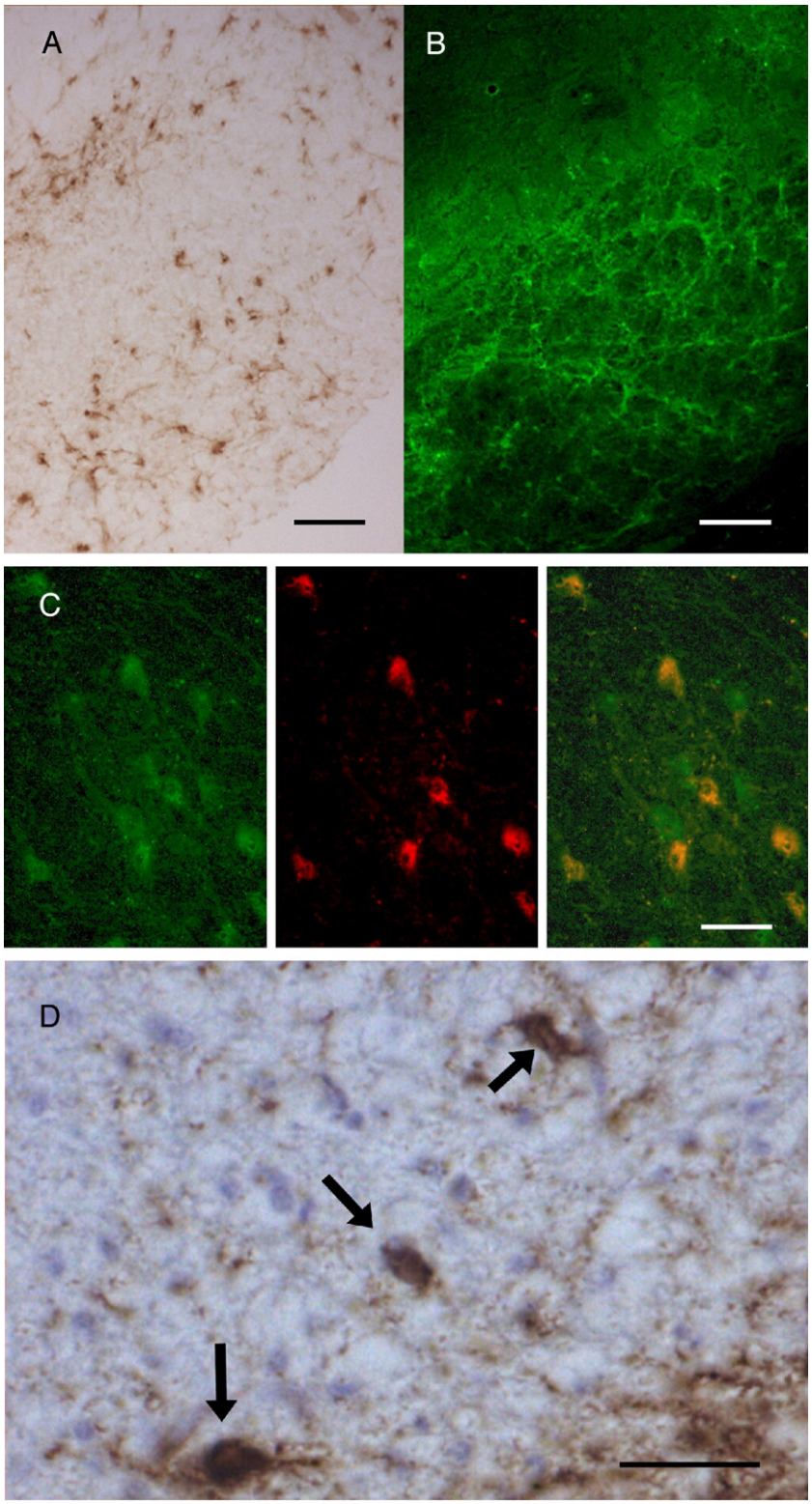

Fig. 1. Distribution of TAT-α-synA30P fusion protein and its colocalization with DA neurons. (A) TH immunostaining of DA neurons in the right SNpc. (B) GFP fluorescence in an adjacent section of the right SNpc showing distribution of the injected GFP-conjugated TAT-α-synA30P. (C) Higher magnification of the section in panel B, showing GFP fluorescence (left panel), TH^+ cells (center panel) and colocalization of GFP with DA neurons (right panel, formed by overlaying the left and center panels). (D) Internalization of TAT-α-synA30P was confirmed by α-syn immunohistochemistry. Note cytoplasmic staining of neurons (arrows). Scale bars: A and B, 100 μm; C and D, 50 μm.

rat to remain on the device for 120 s and p is a shape factor. The rotarod test was performed 15, 30 and 90 days after intranigral injection, and the time each rat remained on the rod (Tpr) was recorded for each trial.

Footprint analysis of motor function

Motor function can be assessed by analyzing footprint patterns and walking tracks (Klapdor et al., 1997). Briefly, this test was conducted in groups of rats treated with vehicle, 6-OHDA or TAT-α-synA30P after 15, 30, 90, 180 and 365 days. Each rat had its hind feet dipped in food dye and was then placed on a gangway 100 cm long, 12 cm wide, and with 40 cm high side walls. The floor of the gangway was lined with ordinary paper. Rats were allowed to walk up the gangway five times without prior conditioning. The footprints were then scanned, stored as black/white bitmaps and evaluated using the FOOTPRINT program, kindly provided by F.J. Van der Staay (Institute of CNS Research, Bayer, Wuppertal, Germany). This program permits evaluation of five different parameters: area touched, stride with, stride length, foot length and angle (defined in relation to the longitudinal axis).

Statistical analysis

Data are expressed as the mean±SD, in spite of the probable non-normality of the distribution of scores. Differences in TH-positive cell number between the right and the left SNpc were tested by Student's *t*-test for paired data. HPLC data were analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. Behavioral apomorphine and rotarod test data were subjected to Kruskall–Wallis nonparametric ANOVA followed by a two-tailed Mann–Whitney *U* test. Because the footprint test comprises five parameters of foot postural assessment during walking, it was important to describe the overall pattern of difference between *treated* and *control* populations across all variables. A multivariate approach using Canonical Discriminant Analysis (Kshirsager, 1972) was therefore applied. The latter is a data reduction technique that computes linear combinations (weighted sums) of the original parameters to yield a single new variable, called the canonical variable, having the highest possible correlation with the groups. Bartlett's χ^2-test was then applied to evaluate the statistical significance of such a correlation, i.e. to evaluate if the considered set of measured parameters allowed a significant discrimination between the two groups.

For all analyses statistical significance was taken as $p \leq 0.05$.

Results

CNS distribution of injected TAT-α-synA30P

Stereotaxic injection of α-syn A30P (which lacks the protein transduction domain) into the right SNpc failed to result in neuronal detection of the mutant protein (data not shown). In contrast, the TAT-α-synA30P conjugated to GFP was readily taken up by neurons and colocalized with TH^+ cells (Fig. 1). Approximately 80% of the TH^+ cells (DA neurons) displayed incorporation of α-syn A30P, with more of these cells being localized to the vicinity of the injection site. Not unexpectedly, there was no evidence for the formation of either Lewy bodies or Lewy neurites as a consequence of single injection of α-syn A30P because the fusion protein's presence declines with time and does not influence endogenously expressed natural synuclein (Fig. 2). The overall distribution of TAT-α-synA30P (Western blotting) is shown in Fig. 2. Within 24 h of administration the fusion protein was found in SNpc as well as in striatum and contralateral cortex (not directly connected to the right SNpc). After 15 days, TAT-α-synA30P was detected only in the right SNpc and remained so even after 30 days. By 90 days TAT-α-synA30P had disappeared from the site of injection.

TAT-α-synA30P and 6-OHDA-induced dopaminergic cell loss

Injection of the DA neurotoxin 6-OHDA into the right SNpc resulted, after 15 days, in the loss of 81.2±2.1% ($n=10$, $p<0.01$ compared to contralateral side) of the dopaminergic cell popula-

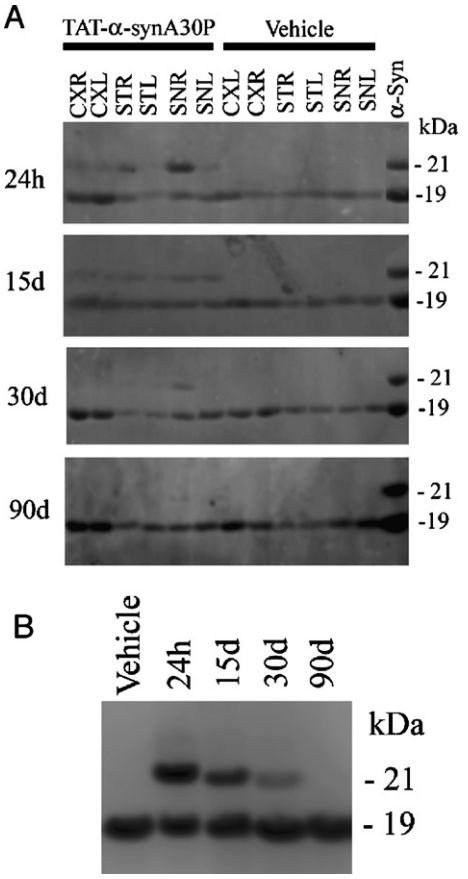

Fig. 2. Western blot analysis of TAT-α-synA30P brain distribution at different times following injection into the right SNpc. (A) Twenty-four hours, 15 days, 30 days and 90 days post-injection (5 μl/rat), the indicated brain areas were dissected, extracted and proteins separated by 12% SDS–PAGE then immunoblotted using α-syn antibodies. Endogenously expressed α-syn (19 kDa band) was present in all brain areas examined at all times. TAT-α-synA30P (21 kDa) was initially found in SNpc, as well as cortex and striatum, but at later times was only evident in SNpc until disappearing by day 90. TAT-α-synA30P and vehicle refer to rats injected with TAT-α-synA30P and vehicle, respectively; far right lane, internal standard of α-syn (19 kDa) and TAT-α-synA30P (21 kDa). CXR and CXL, right and left cortex; SNR and SRL, right and left substantia nigra; STR and STL, right and left striatum. (B) Samples from the right substantia nigra at different times were run on a separate blot for more quantitative comparison. Note comparable amounts of endogenous α-syn (19 kDa) in all samples, as an index of equal protein loading.

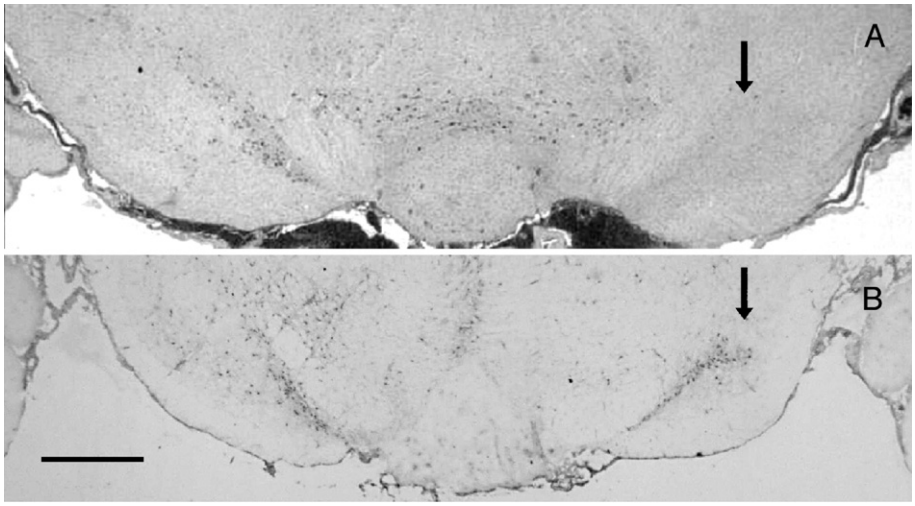

Fig. 3. TH immunostaining of rat SNpc dopaminergic neurons 15 days after intranigral administration of 6-OHDA (A) or TAT-α-synA30P (B). Note the more robust loss of TH immunoreactive neurons in 6-OHDA-treated animals. Arrow indicates site of injection. Scale bar: 400 μm.

tion, as evidenced by TH immunostaining (Fig. 3A). At 30 and 90 days post-lesion the dopaminergic loss was 68±12.3% and 74±11.2% (n=10, $p<0.01$), respectively. Neither TAT nor α-synA30P injection produced a significant decrease in TH immunoreactivity at 15, 30, 90, 180 and 365 days post-injection (data not shown). Intranigral injection of the membrane-diffusible TAT-α-synA30P fusion protein caused a 26.3±5.0% loss (n=10, $p<0.01$) of dopaminergic neurons after 3 days. The effect of TAT-α-synA30P on dopaminergic neuron loss was dose-dependent (Table 1). No further loss was observed at 15 (Fig. 3B), 30, 90, 180 and 365 days post-injection (data not shown).

Effect of TAT-α-synA30P and 6-OHDA on striatal DA and DOPAC levels

The effect of TAT-α-synA30P and 6-OHDA intranigral injection on striatal DA and DOPAC content was analyzed by HPLC 7, 15, 30, 90, 180 and 365 days post-toxin administration (Table 2). DA and DOPAC levels in right striatum of sham animals were 32.0±3.4 and 2.7±0.1 mg/mg of tissue (n=10), respectively, and did not change significantly up to 365 days (data not shown). Similar values for DA and DOPAC were obtained for the contralateral side (32.0±1.6 and 2.7±0.1 μg/mg of tissue) (n=60). In 6-OHDA-treated rats DA and DOPAC levels decreased significantly as a function of the TAT-α-synA30P dose administered (Table 1) and over time (Table 2), with a 40% decrease at 7 days. Greatest reductions (>80%) occurred between 15 and 90 days after neurotoxin administration. Interestingly, the two catecholamines had recovered significantly 180 and 365 days post-lesion (Table 2), being ~2.5-folder higher after 1 year as compared to the 90-day values. Even so, DA and DOPAC levels remained significantly below contralateral striatal values (34.8±4.5 and 35.8±3.9%, respectively). In contrast to 6-OHDA, intranigral administration of TAT-α-synA30P resulted in a more modest reduction in striatal DA and DOPAC contents after 7 days (−20.8±3.6 and −16.6±2.5 for DA and DOPAC, respectively) (Table 2). Again in contradistinction to the action of 6-OHDA, the α-synA30P fusion protein-induced decrease in DA and DOPAC remained at the 7-day

Table 1
Effect of intranigral TAT-α-synA30P administration on TH^+ cell loss in SNpc and DA and DOPAC striatal content

TAT-α-synA30P (μg)	SNpc	Striatum	
	Cell loss (%)	DA (%)	DOPAC (%)
1.25	11.6±3.6^b	89.5±3.5^b	92.5±1.7^b
2.5	13.5±4.6^b	90.2±1.8^b	91.3±2.8^b
5	26.3±5.0^a	79.2±2.8^a	78.6±3.9^a
10	28.4±3.8^a	78.3±2.6^a	79.5±2.8^a

Rats received 5 μl of TAT-α-synA30P (amount as indicated) or vehicle to the right SNpc and were sacrificed after 15 days. Data are expressed as percent TH^+ cell loss, and DA and DOPAC content relative to the left striatum, in which DA and DOPAC values were 32.0±1.6 (=100±5.0%) and 2.7±0.1 (=100±3.7%) μg/mg of tissue, respectively. Data are means±SD. Different superscript letters indicate statistically significant differences ($p<0.01$, one-way ANOVA followed by Bonferroni's Multiple Comparison Test).

Table 2
Effect of 6-OHDA and TAT-α-synA30P administration on striatal content of DA and DOPAC

Days	6-OHDA		TAT-α-synA30P	
	DA (%)	DOPAC (%)	DA (%)	DOPAC (%)
Left striata	100±5.0^a	100±3.7^a	100±5.0^a	100±3.7^a
7	39.4±3.6^b	40.3±4.5^b	79.2±3.6^b	83.4±2.5^b
15	14.6±5.2^c	20.1±5.7^c	79.4±3.8^b	78.3±4.6^b
30	13.8±4.9^c	22.5±4.3^c	77.3±4.1^b	68.6±8.9^b
90	14.4±3.9^c	16.4±7.1^c	76.5±4.6^b	75.6.±7.9^b
180	28.6±5.1^b	29.6±2.7^b	77.5±3.9^b	79.3±2.9^b
365	34.8±4.5^b	35.8±3.9^b	78.8±4.5^b	81.5±4.6^b

Rats received 6-OHDA, TAT-α-synA30P or vehicle (5 μl each) to the right SNpc and were sacrificed after 7, 15, 30, 90, 180 or 365 days. DA and DOPAC levels in the left striata remained constant over the time course of the experiment and all samples (60 rats) and were thus pooled. Data are expressed as percent of DA and DOPAC content relative to the left striatum, in which DA and DOPAC values were 32.0±1.6 (=100±5.0%) and 2.7±0.1 (=100±3.7%) μg/mg of tissue, respectively. Data are means±SD. Different superscript letters indicate statistically significant differences ($p<0.01$, one-way ANOVA followed by Bonferroni's Multiple Comparison Test).

Table 3
Apomorphine induces circling behavior in rats with unilateral 6-OHDA intranigral lesion

Days after intranigral injection of 6-OHDA	Rotations/h	
	Contralateral	Ipsilateral
15	726±20[b]	2.3±4.9[b]
30	753±99[b]	2.1±0.4[b]
90	730±40[b]	4.2±6.9[b]
180	635±56[c]	8.8±9.8[b,c]
365	605±44[c]	12.3±6.5[b,c]
90+L-DOPA	2.2±1.3[a]	21±4.3[a]
Sham	2.4±1.0[a]	23±1.1[a]

Rats (n=10 per group) received a stereotaxic injection (5 μl) of 6-OHDA (lesion) or vehicle (sham) into the right SNpc. The number of rotations during a 1 h period immediately following administration of apomorphine (1 mg/kg s.c.) or vehicle was recorded on the days indicated. Row '90+L-DOPA' is rats that received, 90 days after 6-OHDA, L-DOPA (4 mg/kg, s.c.) 1 h before apomorphine injection. Data are means±SD. Different superscript letters indicate statistically significant differences (p<0.01, one-way ANOVA followed by Bonferroni's Multiple Comparison Test).

level for the duration of the experiment (30 days: reductions of −22.7±4.1 and −31.4±8.9, respectively, for DA and DOPAC; 365 days: −21.4±4.5 and −18.5±4.6 for DA and DOPAC, respectively) (Table 2).

Apomorphine behavioral test

Administration of apomorphine to rats with 6-OHDA lesions of the nigro-striatal dopamine system results in a stereotypical contralateral rotational behavior (Ungerstedt, 1971). The apomorphine effect was already evident 15 days after 6-OHDA administration (Table 3) and continued up to 1 year; this was accompanied by a corresponding decrease in ipsilateral rotations for 365 days. Apomorphine administration to TAT-α-synA30P-injected rats resulted in a slight, albeit significant, increase in the number of contralateral rotations (Table 4). As with 6-OHDA-lesioned rats, the apomorphine effect in TAT-α-synA30P-treated animals was observed at 15 days and remained 1 year later. No changes in apomorphine-induced ipsilateral rotations were noted in the TAT-α-synA30P-treated group, illustrating the difficulty in using this test to assess motor dysfunction when DA loss is of a small magnitude (the ipsilateral response to apomorphine is less sensitive than the contralateral one). Interestingly, L-DOPA (4 mg/kg, s.c.), given 1 h before apomorphine completely prevented the turning behavior in both 6-OHDA- and TAT-α-synA30P-treated rats (Tables 3 and 4).

Table 4
Effect of apomorphine on circling behavior in rats with unilateral TAT-α-synA30P stereotaxic injections

Days after intranigral injection of TAT-α-synA30P	Rotations/h	
	Contralateral	Ipsilateral
15	14±2.3[b]	14±7.1[b]
30	16±1.2[b]	12±5.9[b]
90	20±4.8[b]	14±5[b]
180	18±4.8[b]	13±6.2[b]
365	19±3.9[b]	15±2.3[b]
90+L-DOPA	2.6±1.1[a]	19±3.3[a]
Sham	2.4±1.4[a]	22±1.6[a]

Rats (n=10 per group) received a stereotaxic injection (5 μl) of TAT-α-synA30P (lesion) or vehicle (sham) (5 μl each) into the right SNpc. The number of rotations during a 1 h period immediately following administration of apomorphine (1 mg/kg s.c.) or vehicle was recorded on the days indicated. Row '90+l-DOPA' is rats that received, 90 days after TAT-α-synA30P, L-DOPA (4 mg/kg, s.c.) 1 h before apomorphine injection. Data are means±SD. Different superscript letters indicate statistically significant differences (p<0.01, one-way ANOVA followed by Bonferroni's Multiple Comparison Test).

Motor performance: rotarod test

The rotarod test, in which animals walk on a rotating drum, is widely used to assess motor status in laboratory rodents. Sham and 6-OHDA-treated rats exhibited the same behavioral patterns when tested at 15, 30 and 90 days after intranigral injection; therefore only one curve per group is shown in Fig. 4A. Sham rats reached Tpr (equal to Tmax, or 240 s) at trial 3 or 4, whereas the 6-OHDA-lesioned rats achieved their best Tpr (195±32 s) only after 8 trials (Fig. 4A). In the TAT-α-synA30P-treated group, rats were able to remain on the rod for 240 s after 9 trials, but their performance diminished when tested at 30 and 90 days (best Tpr of 190±12 and 192±17 s, respectively; Fig. 4B).

The $K/2$ values (number of trials needed to remain 120 s on device) were derived from the data in Fig. 4. Both the TAT-α-synA30P- and 6-OHDA-treated rats displayed significantly higher

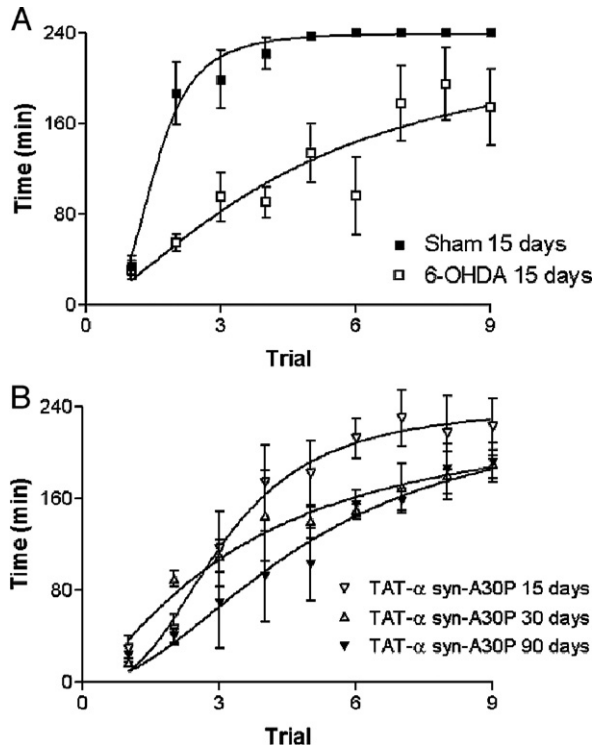

Fig. 4. Rotarod test in rats receiving intranigral injection (5 μl per rat) of vehicle (sham), 6-OHDA (A) and TAT-α-synA30P (B) at different times. For further details, see Materials and methods.

Table 5
K/2 values of groups of rats subjected to the rotarod test

Treatment	*K*/2 values (number of trials)	
	–	L-DOPA
Sham	1.5±0.2[a]	1.4±0.2[a]
6-OHDA (15 days)	4.7±1.5[b]	1.7±0.2[a]
TAT-α-synA30P (15 days)	3.0±0.4[c]	1.6±0.3[a]
TAT-α-synA30P (30 days)	3.5±0.7[c]	1.5±0.2[a]
TAT-α-synA30P (90 days)	4.9±0.6[b]	1.6±0.2[a]

The rotarod test was performed as described in Materials and methods. *K*/2 is the trial number in which permanence on the rod reached 120 s (for further details see, Materials and methods). The L-DOPA column refers to rats that received 4 mg/kg s.c. of the dopamine precursor 1 h before the morning rotarod test, for each of the 5 days. Data are means±SD. Different superscript letters indicate statistically significant differences ($p<0.01$, one-way ANOVA followed by Bonferroni's Multiple Comparison Test).

values compared to sham. Moreover, in the TAT-α-synA30P group, *K*/2 increased with time: the 90-day value was significantly greater than either the 15- or 30-day value, and *K*/2 after 90 days was not different from 6-OHDA at day 15 (Table 5). Interestingly, L-DOPA (4 mg/kg, s.c.) given 1 h prior to the morning trial for each of the 5 days significantly improved motor performance in both 6-OHDA- and TAT-α-synA30P-treated rats, confirming that motor dysfunction was due to dopaminergic loss (Table 5). The 6-OHDA and TAT-α-synA30P groups reached their best Tpr (240 s) after 3–4 trials (data not shown), with *K*/2 values similar to sham animals (Table 5). Increasing animal weight rendered the rotarod test impractical at 180 and 365 days.

Motor performance: footprint analysis

The FOOTPRINT program provides for single evaluation of five different parameters: area touched, stride with, stride length, foot length and angle. While some of these parameters were significantly affected in both 6-OHDA- and TAT-α-synA30P-treated rats (data not shown), a more accurate assessment was obtained with Canonical Discriminant Analysis, which allowed the simultaneous scoring of all five parameters (see Materials and methods), thereby giving a better idea of walking performance. As Fig. 5 shows, the 6-OHDA and TAT-α-synA30P treatment groups each demonstrated a significant impairment in walking behavior, starting 30 days after intranigral injection. In TAT-α-synA30P-treated rats, walking performance continued to worsen between 30 and 90 days. Administration of L-DOPA (4 mg/kg, s.c.), 1 h before testing significantly improved motor performance in both 6-OHDA and TAT-α-synA30P-treated rats, being very similar to the sham groups (data not shown).

Discussion

The pathogenetic mechanism(s) underlying the nigro-striatal neurodegeneration of idiopathic and familial PD is likely to be complex and may encompass interactions among several predisposing genes and environmental factors. Mutations in the gene coding for α-syn have received a great deal of attention with the discovery that fibrillar α-syn aggregates are the major components of both Lewy bodies and Lewy neurites (Conway et al., 1998; Goedert, 2001) and characterize most familial and sporadic PD brains. Although α-syn mutations are infrequent in PD, other cellular processes leading to abnormal metabolism and accumulation of this protein may play an important role in disease pathogenesis (for a review, see von Bohlen und Halbach, 2005). Indeed, aggregated α-syn is capable of mediating dopaminergic neurotoxicity in vivo (Periquet et al., 2007). We describe here a novel, α-syn-based rat model of PD which reproduces, in particular, the early stages of the disease. Fusion of the A30P mutated form of α-syn to a protein transduction domain derived from the human immunodeficiency TAT protein allowed diffusion through neuronal membranes following stereotaxic injection into right SNpc, which resulted in a localized dopaminergic loss and long-term motor deficits. The A30P mutated form α-syn was used because in vitro it is more toxic than A53T or wild-type forms (Feany and Bender, 2000).

The dopaminergic toxin 6-OHDA was used in comparison to TAT-α-synA30P for purposes of model validation. Immunohistochemical analysis revealed a clear dopaminergic neuronal loss in the right SNpc, being more modest in TAT-α-synA30P (−26% already at 3 days post-lesion) compared to 6-OHDA (−81% at 15 days post-lesion) treated rats. No further loss was observed in the TAT-α-synA30P group after 30, 90, 180 and 365 days. These findings were confirmed by HPLC analysis of the striatal contents of DA and DOPAC, which showed a 40% decrease at 7 days, and >80% reduction between 15 and 90 days after 6-OHDA administration. Striatal DA and DOPAC had recovered significantly 180 and 365 post-lesion, being □2.5-fold higher after 1 year as compared to the 90-day values (although remaining below contralateral values). In contrast to 6-OHDA, intranigral administration of TAT-α-synA30P resulted in a more modest reduction in striatal DA and DOPAC contents after 7 days (−20.8% and −16.6% for DA and DOPAC, respectively). Again in contradistinction to 6-OHDA, the α-synA30P fusion protein-induced decrease in DA and DOPAC remained at the 7-day level for up to 1 year.

The overall distribution of TAT-α-synA30P, analyzed by Western blotting, showed the fusion protein within 24 h of administration to be in SNpc, as well as in striatum and contralateral

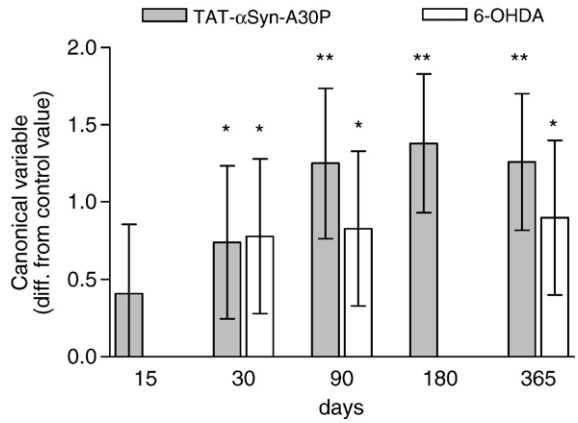

Fig. 5. Walking performance in rats receiving intranigral injection of 6-OHDA or TAT-α-synA30P: Canonical Discriminant Analysis of footprint test data. The plot shows the mean values (±SD) of the differences in the Canonical Variable between the test and control groups (10 rats per treatment group, 5 μl injected per rat). The canonical variable is the linear combination (weighted sum) of the five parameters (area touched, stride width, stride length, foot length, and angle) used to characterize walking performance. Significant differences between treatment and control groups were observed starting at 30 days. *=$p<0.01$, **=$p<0.001$ (Bartlett's χ^2-test).

cortex (not directly connected to the right SNpc). In principle, the TAT-α-synuclein fusion protein should be able to translocate to any cellular compartment. For example, Schwarze et al. (2000) showed that TAT-β-galactosidase injected via the tail vein in rats becomes detectable throughout the brain within a few hours. It is not necessarily surprising therefore that TAT-α-synuclein injected in one brain area would be detected in other areas or the opposite hemisphere. The study of Schwarze et al. (2000) suggests that this can occur independent of axonal transport (retrograde and/or anterograde), although we cannot distinguish between the possibility and that resulting from diffusion on the injected fusion protein.

A series of behavioral tests were utilized to demonstrate that the dopaminergic loss observed in rats receiving 6-OHDA or TAT-α-synA30P was accompanied by an impaired motor performance. In rats with 6-OHDA lesions of the nigro-striatal dopamine system, the resulting severe imbalance in dopaminergic innervation causes a postural asymmetry with rotation away from the non-lesioned side following apomorphine administration (Ungerstedt, 1971). 6-OHDA-induced ipsiversive rotational behavior was not observed in TAT-α-synA30P-lesioned animals after apomorphine, perhaps due to the fact that the present model makes use of a unique injection of α-synA30P, while lentivirus delivery (Lauwers et al., 2007) is characterized by a continuous synthesis of the protein which can accumulate over time. It was thus necessary to employ other behavioral tests that would permit assessment of any underlying motor dysfunction(s).

The rotarod test is frequently used to evaluate motor deficits, e.g. following traumatic brain injury (Hamm et al., 1994). The rotarod test can provide also a robust tool for the qualitative analysis of movement in rodents with nigro-striatal lesions (Whishaw et al., 2003). Unlike 6-OHDA-lesioned rats, the rotarod test did not show any alterations in TAT-α-synA30P-treated rats. The test was thus modified to provide for a gradual increase in the demand for muscular coordination. Under these conditions, the rotarod test showed that stereotaxic injection of TAT-α-synA30P induced a motor impairment which became progressively more evident with time, while in 6-OHDA-lesioned rats motor impairment was maximal at 15 days (Fig. 4). Extrapolated values of trial numbers at test half time (120 s) confirmed this conclusion (Table 5). Moreover, L-DOPA administration restored normal motor performance in both the TAT-α-synA30P and 6-OHDA treatment groups, indicating that the observed motor impairment was indeed a consequence of the toxin-induced destruction of dopaminergic neurons.

Motor function was also evaluated using the footprint test (Klapdor et al., 1997). The analysis of footprint patterns and walking tracks permits assessment of motor function in a manner distinct from that of the rotarod test, in that the former does not impose on the animal a requirement for either motor ability or coordination. Canonical Discriminant Analysis of the footprint test data demonstrated a significant deterioration in the walking pattern of rats > 30 days after administration of 6-OHDA or TAT-α-synA30P, in comparison to controls. Footprint test scores in the TAT-α-synA30P group appeared to worsen at time points beyond 30 days (Fig. 5).

While this work was ongoing, Lauwers et al. (2007) reported on lentivirus-mediated overexpression of A30P α-synuclein in a rat model using non-invasive imaging of neuropathology. Other groups have reported as well on viral vector-mediated overexpression of α-synuclein in the substantia of the rat (Lo Bianco et al., 2002; Kirik et al., 2002; Klein et al., 2002) or the monkey (Kirik et al., 2003). Klein et al. (2002) monitored rats over a time course of 1 year after adeno-associated virus vector-mediated expression of A30P α-synuclein. Apart from Lewy-like inclusions of α-synuclein and dystrophic neurites, no abnormal behavior or motor dysfunction was found in either rotational or rotating rod testing despite a 53% loss of DA neurons. In contrast, after AAV-mediated overexpression of wild-type and A53T mutant α-synuclein in the substantia nigra of the rat, significant motor impairment was observed in a subgroup of animals in which DA neuron cell loss exceeded 50–60% (Kirik et al., 2002). The rats were monitored for up to 6 months, but a maximal effect was seen at 8 weeks. At 8 months, there was a recovery of the total number of nigral TH^+ cells and striatal TH^+ fibers. However, striatal dopamine levels were still reduced by 50% (Kirik et al., 2002). In line with this observation, the rats showed impaired skilled paw use at 4 and 5 months after vector injection. DA cell loss was also observed after lentivirus-mediated overexpression of different forms of human but not rat α-synuclein in the substantia nigra of the rat (Lo Bianco et al., 2002). This study reported no behavioral changes. In Lauwers et al. (2007), there was no aberrant behavior at any time point in the stepping test, while the number of amphetamine-induced rotations varied considerably between rats. These findings are in agreement with the results of Kirik et al. (2002) where about 25% of the animals were impaired. Interestingly, in Lauwers et al. (2007), in the group expressing modest levels of α-synuclein, fewer rats were positive in the amphetamine-induced rotation test, and the impairment started at a later time point. In the high expression group, there was an apparent reversal of behavioral impairment at the latest time point (46 weeks).

An important distinction between the present model and viral transduction (e.g. lentivirus) is that the former allows one to administer a precise quantity of TAT-α-synuclein. We know from previous studies that the effect of TAT-α-synuclein on cell survival in vitro can vary as a function of the amount of TAT fusion protein administered. This is an advantage over viral transduction, where protein expression depends on factors such as the promoter (relative strength) and mRNA stability, thus making it almost impossible to synthesize a predetermined amount of protein. In principle tight control of expression might be achievable, but one would need to confirm the amount of protein synthesized (requiring much additional effort), and this in turn may depend upon the context in which the protein is expressed (e.g. cortex may differ from substantia nigra).

Neurotoxins such as 6-OHDA, MPTP, rotenone and paraquat have traditionally been utilized to induce PD-like conditions in animals (Dawson et al., 2002). Such neurotoxin-based models are limited by the fact that they produce an acute and precipitous dopaminergic loss without LBs (except for rotenone) and thus mimic the terminal disease condition with its characteristic drastic (> 80%) loss of DA neurons. The α-syn-based model described here, while expressing only a modest dopaminergic loss, is accompanied by a clear motor dysfunction. Because this PD animal model more closely approximates the early stages and slow development of the human disease, it may be of utility in identifying therapeutic agents which halt or retard dopaminergic neurodegeneration rather than those providing symptomatic relief. Such molecules could, for example, be designed to promote the maintenance and/or recovery of the native and therefore soluble conformation of α-syn, preventing both its precipitation and accumulation into LBs, thereby impeding aggregation-induced neurotoxicity.

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