Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of α-synuclein in midbrain dopamine neurons

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A R T I C L E  I N F O

Article history:
Received 1 August 2011
Revised 11 October 2011
Accepted 4 December 2011
Available online 11 December 2011

Keywords:
Parkinson’s disease
alpha-Synuclein
Adeno-associated viral vector
Motor deficit
Rat

A B S T R A C T

Parkinson’s disease (PD) is characterised by the progressive loss of nigral dopamine neurons and the presence of synucleinopathy. Overexpression of α-synuclein in vivo using viral vectors has opened interesting possibilities to model PD-like pathology in rodents. However, the attempts made so far have failed to show a consistent behavioural phenotype and pronounced dopamine neurodegeneration. Using a more efficient adeno-associated viral (AAV) vector construct, which includes a WPRE enhancer element and utilizes the neuron-specific synapsin-1 promoter to drive the expression of human wild-type α-synuclein, we have now been able to achieve increased levels of α-synuclein in the transduced midbrain dopamine neurons sufficient to induce profound deficits in motor function, accompanied by reduced expression of proteins involved in dopamine neurotransmission and a time-dependent loss of nigral dopamine neurons, that develop progressively over 2–4 months after vector injection. As in human PD, nigral cell loss was preceded by degenerative changes in striatal axons and terminals, and the appearance of α-synuclein positive inclusions in dystrophic axons and dendrites, supporting the idea that α-synuclein-induced pathology hits the axons and terminals first and later progresses to involve also the cell bodies. The time-course of changes seen in the AAV-α-synuclein treated animals defines distinct stages of disease progression that matches the pre-symptomatic, early symptomatic, and advanced stages seen in PD patients. This model provides new interesting possibilities for studies of stage-specific pathologic mechanisms and identification of targets for disease-modifying therapeutic interventions linked to early or late stages of the disease.

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Introduction

Parkinson’s disease (PD) is characterised by the progressive loss of specific neuronal populations and the presence of α-synuclein (α-syn)-containing inclusions, Lewy bodies and Lewy neurites, in surviving neurons (Spillantini et al., 1997). Familial forms of early-onset PD result from the overexpression or mutated forms of α-syn (Polymeropoulos et al., 1997; Singleton et al., 2003; Zarranz et al., 2004), and a recent genome-wide association study further support the notion that α-syn is critically linked to PD (Simon-Sanchez et al., 2009). In addition, observations from PD patients with duplication or triplication of the α-syn gene suggest that elevated α-syn expression is a critical factor contributing to the aberrant protein toxicity and the disease progression (Conway et al., 2001; Singleton et al., 2003; Nishioka et al., 2006).

Generation of animal models of PD that replicate the α-synucleinopathy seen in patients remains a challenge. Attempts to model the disease by overexpression of wild-type (wt) or mutated forms of human α-syn in transgenic mice have been quite instructive and greatly promoted understanding of some aspects of α-syn-induced toxicity (Magen and Chesselet, 2010). However, none of the transgenic models published so far replicate the hallmark of the disease, i.e. the progressive and severe loss of dopamine (DA) neurons in the substantia nigra (SN), and development of profound DA-dependent motor impairments.

Overexpression of α-syn using viral vectors represents an alternative approach to model PD-like pathology in rats (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002; Yamada et al., 2004). We, and others, have reported that adeno-associated viral (AAV) vector-mediated overexpression of α-syn replicates many of the neuropathological features of the human disease (Kirik et al., 2002; Yamada et al., 2004; Gorbatyuk et al., 2008; Azeredo da Silveira et al., 2009). However, these previous studies, using AAV vectors and CBA or CMV promoters to drive the expression of human α-syn, have so far resulted in moderate DA neuronal loss and highly variable behavioural deficits (Kirik et al., 2002; Yamada et al., 2004; Gorbatyuk et al., 2008). However, since the extent of neuropathology is likely to depend on the level of α-syn expression (Azeredo da Silveira et al., 2009; Ulusoy et al., 2010; Koprich et al., 2011), it is essential to boost the expression level above a critical threshold in order to induce significant pathology leading to more...
profound neuronal loss and behavioural deficits. Expression of the transgene is dependent not only on the AAV serotype (AAV2, 5 and 6 used so far), and the number of viable vector particles injected, but also on the efficiency of gene expression, which in turn is determined by the design of the vector construct, and the promoter used.

Using a more efficient AAV vector construct, which uses the neuron-specific synapsin-1 promoter to drive α-syn expression and includes a WPRE enhancer element, we have now been able to achieve sufficiently high cellular levels of α-syn to achieve profound deficits in motor function associated with down-regulation of proteins involved in DA neurotransmission, and accompanied by a time-dependent loss of nigral dopamine neurons that develops over 2–4 months after vector injection. The progressive degenerative changes seen in the transduced nigral dopamine neurons over time support the idea that the α-syn-induced pathology hits the axons and terminals first and later progresses to involve also the cell bodies, strikingly similar to the retrograde progression of neurodegeneration seen in human PD.

Materials and methods

Production of recombinant AAV6 viral vectors

Two different vector constructs were used to overexpress α-syn: (1) in the AAV6-α-syn(+WPRE) vector, the expression of the transgene is driven by the synapsin-1 promoter and enhanced using a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE); (2) in the previously used AAV6-α-syn(−WPRE) vector (Decressac et al., 2011), the expression of α-syn is under the control of a chicken beta-actin (CBA) promoter without WPRE sequence. The effect of these vectors was compared to an AAV6-GFP(+WPRE) control vector similar to that used previously (Decressac et al., 2011).

Vector production was performed as described previously (Decressac et al., 2011). Briefly, transfer plasmids carrying AAV2 ITRs coding for either a human wt α-syn or enhanced GFP, downstream to a synapsin-1 or a cytomegalovirus enhancer hybrid synthetic chicken β-actin (CBA) promoter, were generated. The transfection into HEK 293 cells was carried out using the calcium phosphate method, and included the packaging plasmids pDP6 encoding AAV6 capsid proteins (Zolotukhin et al., 1999; Grimm, 2002). The cells were treated with a lysis buffer (50 mM Tris, 150 mM NaCl, pH 8.4) and by performing freeze–thaw cycles in dry ice/ethanol bath. The crude lysates were purified first by ultracentrifugation (1.5 h at 350 000 × g at 18 °C) in a discontinuous iodixanol gradient and the virus containing fractions were purified on ion-exchange chromatography using FPLC. Genome copy titres were determined using real time quantitative PCR. The genome copy titres for AAV6-GFP, AAV6-α-syn(+WPRE) and AAV6-α-syn(−WPRE) were 2.5 × 10^{12}, 3.7 × 10^{12} and 3.0 × 10^{12} genome copies/mL, respectively. Dilution of the batches was performed so that an equivalent number of genome copies (3.1 × 10^8 gc/3 µl) was injected in the different groups.

Animals

Adult female Sprague Dawley rats, 225–250 g at the time of surgery, were housed two to three per cage with ad libitum access to food and water during a 12 h light/dark cycle. All procedures were conducted in accordance with guidelines set by the Ethical Committee for the use of laboratory animals in the Lund–Malmö region and the European Ethical Committee (86/609 EEC).

Surgical procedure

All surgical procedures were performed under general anaesthesia using a 20:1 mixture of fentanylcitrate (Fentanyl) medetomidin hypochloride (Dormitor) (Apoteksbolaget, Sweden) injected i.p. Rats were placed in a stereotactic frame (Stoelting) and vector solutions were injected using a 10 µl Hamilton syringe fitted with a glass capillary (outer diameter of 250 µm). 3 µl of AAV-GFP of AAV-α-syn vector solutions were infused at a rate of 0.2 µl/min and the needle was left in place for an additional 3 min period before it was slowly retracted. Injection was carried out unilaterally on the right side, above the SN, at the following coordinates (flat skull position): antero-posterior: −5.3 mm, medio-lateral: −1.7 mm, dorso-ventral: −7.2 mm below dural surface as calculated relative to bregma according to the stereotactic atlas of Paxinos and Watson (1986).

Behavioural testing

Assessment of behaviour was performed 1–5 days before and 10 days, 3, 5, 8, and 16 weeks after vector injection in the SN. Two different sets of experiments were carried out: in Experiment 1 the animals were injected with the AAV-α-syn or the AAV-GFP vectors (n=8 per group) and followed over time. The motor tests were repeated at the given time-points and all animals were sacrificed after the last test; in Experiment 2 the motor tests were performed on separate groups of animals at each time-point (n=8 per group) and followed by sacrifice at that time-point. Behavioural tests were performed only on animals used for histological analysis (n=10 per group). Animals used for other types of analysis (n=4–6 per analysis) were not tested for motor performance to avoid stress or drug-induced variations.

Rats were tested for forelimb use asymmetry by the cylinder test and for amphetamine-induced rotational behaviour, as described (Winkler et al., 2000). For cylinder test, rats were put in a glass cylinder, a total of 20 forepaw touches were counted and the percentage of left paw touches was determined. To study the response to dopaminergic treatment, rats were injected subcutaneously with L-DOPA (10 mg/kg i.p. DOPA methyl ester, plus 15 mg/kg benzerazide–HCl) 1 h prior to cylinder test.

Assessment of drug-induced rotational behaviour was performed in automated bowls coupled to the Rotameter software (AccuScan Instruments) as described previously (Ungerstedt and Arbuthnott, 1970). Rats received an i.p. injection of d-amphetamine sulphate (2.5 mg/kg) (Apoteksbolaget, Sweden). Right and left full body turns were recorded over a period of 90 min. Data are expressed as net full turns per minute, with turns ipsilateral to the injection side assigned a positive value.

Tissue processing and immunohistochemistry

Rats were deeply anesthetised with 1.2 ml sodium pentobarbital i.p. (Apoteksbolaget, Sweden) and then perfused through the ascending aorta with 50 ml saline (0.9% w/v) at room temperature, followed by 250 ml ice-cold paraformaldehyde (4% w/v in 0.1 M phosphate buffered saline). The brains were removed, post-fixed for 2 h in 4% paraformaldehyde and cryoprotected overnight in sucrose (25% w/v in 0.1 M phosphate buffered saline) before being sectioned on a freezing microtome (Leica). Coronal sections were collected in 8 series at a thickness of 35 µm.

Immunohistochemical stainings were performed on free-floating sections using antibodies raised against tyrosine hydroxylase (TH) (rabbit, 1:2000; Chemicon), GFP (chicken, 1:5000; R&D systems), vesicular monoamine transporter-2 (VMAT-2) (rabbit, 1:5000; Abcam, or Guinea Pig, 1:5000, Sigma), dopamine transporter (DAT) (rat, 1:500, Abcam), human phospho-Ser129-α-syn (rabbit, 1:2000, Abcam), human α-syn 211 (mouse, 1:10 000; courtesy of Dr. Virginia M. Lee, University of Pennsylvania) and Neuronal Nuclei (NeuN) (mouse, 1:500; Millipore), and Hu (mouse, 1:500; Invitrogen). Sections were rinsed three times in potassium-phosphate buffer (KPBS) between each incubation period. All incubation solutions contained 0.25% Triton X-100 in KPBS. The sections were quenched for 10 min
in 3% H2O2/10% methanol. One hour of pre-incubation with 5% normal goat serum, normal horse serum, normal rabbit serum was followed by incubation overnight with the primary antibody in 2% serum at room temperature and incubation with 1:200 dilution of biotinylated goat anti-rabbit antibody, rabbit anti-rat or horse antimouse (Vector Laboratories), followed by avidin–biotin–peroxidase complex (ABC Elite; Vector Laboratories), and visualised using 3,3-diaminobenzidine as a chromogen, mounted and coverslipped using DPX mounting medium.

Double fluorescence immunostaining was performed as described above without the quenching step. Pre-incubation was performed with 5% normal horse serum, primary antibodies were incubated overnight together at room temperature, and adequate Alexa 488 and Cy3-conjugated secondary antibodies were incubated simultaneously.

Cell counting and optical densitometry analysis

Assessment of the total number of TH+ and VMAT-2+ neurons in the SN was made according to the optical fractionator principle, using the Olympus Denmark A/S (Albertslund, Denmark) CAST-Grid system, as described (Decressac et al., 2011). Every eight sections covering the entire extent of the SN was included in the counting procedure. A coefficient of error of <0.10 was accepted.

Striatal TH+, VMAT-2+ and DAT+ fibre density was measured by densitometry at four coronal levels (+1.2, 0.8, 0.00 and −0.4 mm relative to bregma) using the ImageJ software (Version 1.32j, NIH, USA). The measured values were corrected for non-specific background staining by subtracting values obtained from the cortex. The data are expressed as a percentage of the corresponding area from the intact side.

Analysis of the α-synuclein positive axonal swellings

As previously described (Decressac et al., 2011), the number of α-syn+, TH+ or VMAT-2+ inclusions was measured by densitometry at four coronal levels (+1.2, 0.8, 0.00 and −0.4 mm relative to bregma) using the ImageJ software (Version 1.32j, NIH, USA). The measured values were corrected for non-specific background staining by subtracting values obtained from the cortex. The data are expressed as a percentage of the corresponding area from the intact side.

Determination of α-syn expression by ELISA

Animals (n = 4 per group) were sacrificed 3 weeks after vector injection, ventral midbrain and striatal tissue was quickly dissected bilaterally and frozen on dry ice. The levels of striatal α-syn (human and rat forms) were determined by ELISA (Millipore) following the supplier’s recommendations. Data are presented as fold increase compared to the level measured on the contralateral side.

Analysis of protein expression by Western blot

The animals (n = 5 per group) were sacrificed at all time-points, brains were rapidly removed, and the ipsi and contralateral striatum and ventral midbrain were dissected and snap-frozen on dry ice. Tissues were homogenised in RIPA buffer (Sigma) with phosphatase inhibitor and protease inhibitor cocktail 2 (1:100, Sigma). Homogenates were centrifuged at 1000×g for 10 min at 4 °C to remove cellular debris. Supernatants were collected and stored at −80 °C until use. Protein concentration was determined using the DC protein assay kit (Bradford method). 20 μg of protein were boiled at 90 °C for 5 min in Laemmli buffer (Biorad), separated on a 4–20% SDS-PAGE gel and then electrotransferred (100 V, 1 h) on a PVDF membrane (Bio-Rad). After blocking for 1 h in Tris-buffered saline with 0.1% Tween-20 (TBST) and 3% bovine serum albumin, membranes were incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-tyrosine hydroxylase (1:2000, Chemicon) in combination with a mouse anti-actin antibody (1:20 000, Sigma). After washing for 30 min in TBST with gentle agitation, membranes were incubated during 1 h at room temperature with a goat anti-mouse or anti-rabbit Alexa 568-conjugated secondary antibody (1:1000, Invitrogen, USA). Signal was detected using a Gel VersaDoc system (Biorad). Band intensities were quantified by densitometry using the ImageLab software (Biorad).

Study of protein expression by immunofluorescence

Brain sections from animals sacrificed 10 days after nigral injection of AAV-α-syn (+WPRE) vector were double stained for human α-syn in combination with TH or VMAT-2. Image acquisition was performed bilaterally in the VTA and SN using a confocal microscope, and similar scanning settings were use between both sides. Images were then processed using the Velocity software (Perkin Elmer). TH or VMAT-2 staining intensity was quantified only in a-syn-transduced neurons. Fluorescence intensity was determined from a minimum of 200 cells in each region (SN and VTA, ipsi and contralateral) taken from at least 6 different animals. Data are presented as percentage of intensity in the contralateral side.

Study of gene expression by RT-qPCR

Midbrain tissue was dissected and snap-frozen as described above. In an attempt to dissociate A9 and A10 neuronal populations, the lateral region comprising the nigral neurons was separated from the medial region comprising VTA neurons under the microscope. Total RNA was isolated using the RNeasy Lipid tissue Mini kit (Qiagen) according to the supplier’s recommendations. RNA concentration was determined using the NanoDrop (Thermo Scientific) and a 500 ng quantity of RNA was used for the reverse transcription performed with random primers (Invitrogen) and SuperScriptII (Invitrogen) according to the manufacturer’s recommendations. SYBR green quantitative real-time PCR was performed with LightCycler 480 SYBR Green I Master (Roche) using standard procedures. Data were quantified using the ΔΔCt-method and normalised to GAPDH and β-actin expression. GAPDH-normalised data were very similar to actin data. Primers were designed using Primer Blast (NIH, USA). Primer sequences (IDT) were as follows (5′→3′): Actin Fwd CTAACGCAACCCTGGTAAAAAGA, Actin Rev CACACCGCTGATGCTACG, Gapdh Fwd, Gapdh Rev, rat SNCA Fwd, rat SNCA Rev, human SNCA Fwd CCTACCGCTTCTCCATACGGC, human SNCA Rev TTGGTCCGAAATGGCACCCTCCA, TH Fwd, TH Rev, VMAT-2 Fwd GCCACTGGCCAAAAGATGCTCC, VMAT-2 Rev AACACGAGGTAGACAGCAGCAT, DAT Fwd CCGGGCTCTCCCGGGGAGAAAT, DAT Rev GTGAATTGCGGACCCGACCA, Gapdh Fwd ACGCCATTGGAGGAGCATGC, AADC Fwd, AADC Rev GATCAAGGCCCGAGAGTACCA, AADC Rev GATCAAGGCCCCGAGGACCA.

Determination of dopamine and its metabolites by HPLC

Rats were sacrificed at the different time-points, striatal tissues were dissected and quickly frozen on dry ice. Samples were homogenised in perchloric acid 0.1 N and after centrifugation at 14 000×g for 30 min, 200 μl of the supernatant was filtered through a glass 0.22 μm filter Avantec 13CP020AS. 20 μl of the filtered supernatant was examined for DA, DOPAC and HVA levels by reversed-phase high-performance liquid chromatography (RP-HPLC) with electrochemical detection. Briefly, the
HPLC system consisted of a HPLC pump (LC-20AD, Shimadzu, Kyoto, Japan), a degasser (LC-27A, Waters, by, Denmark), a refrigerated micro-sampler (SIL-20ACHT, Shimadzu), an amperometric detector (Antec Decade II, Antec, Leiden, The Netherlands) and a computerised data acquisition system (Empower, Waters). The electrochemical detector cell was equipped with a glassy carbon electrode operating at +0.7 V vs. Ag/AgCl reference electrode. Typically, 20 μl samples were injected onto a Prodigy C18 column (100 × 2 mm LD, 3-μm particle size, YMC Europe, Schermbek, Germany). The mobile phase consisted of 93% of 94.2 mM NaH₂PO₄, 0.98 mM octanesulfonic acid, 0.06 mM Na₂EDTA, adjusted to pH 3.7 with 1 M phosphoric acid and 7% acetonitrile (v/v). The flow-rate was 0.25 ml/min.

**In vitro TH enzyme activity assay**

The assay is based on the release of titriated H₂O following the conversion of l-[3,5-3H]tyrosine into DOPA by the TH enzyme (Reinhard et al., 1986). The striatal tissue for in vitro TH enzyme activity assay was sonicated in 10 μM of homogenisation buffer (20 mM MES, pH 6.1, 0.2% triton) and centrifuged at 20 000 × g for 10 min at 4 °C. A cocktail of l-[3,5-3H]tyrosine (Amer sham Biosciences), 500 μM l-tyrosine, 200 mM MES (pH 6.1), 84 mg catalase and dh₂O was prepared and 65 μl was added to 10 μl of brain homogenate, together with 25 μl of the cofactor biotin (0.6 μg/ml, dissolved in 3 mg/ml DTT). The samples were subsequently centrifuged at 20 000 × g for 15 min and 100 μl of the supernatant was transferred to a scintillation vial and the number of decays per min was measured in a liquid scintillation counter (Georgevsk a et al., 2004).

**Statistical analysis**

All statistics were conducted using the GraphPad Prism software (version 5.0). For the time-course study, main effects were determined using 2-way factorial analysis of variance (ANOVA) with treatment and time as between-subject factors. For all analysis, upon confirmation of significant main effects, differences among group means were analysed using the Bonferroni post-hoc test. Some experiments were analysed using a one-way ANOVA followed by a Dunnett post-hoc test (comparison with 16 weeks AAV-GFP group). Analysis of qPCR data was performed using a Student t test. All values are presented as mean ± standard error of mean. Correlation analysis was performed using linear (r coefficient of Spearman) or non-linear (one-phase decay) regressions. Statistical significance was set at P < 0.05.

**Results**

**Transgene expression following AAV-vector injection**

We first evaluated the expression of the two transgenes (α-syn and GFP) at 3 weeks after AAV vector injection, and compared the expression levels obtained with the new WPRE-containing vector construct (AAV-synapsin-α-syn-WPRE) with the one used previously (AAV-CBA-α-syn), injected at the same genome copy dose. Histological analysis showed that overexpression of wt human α-syn or GFP resulted in high immunoreactivity in the injected side of the midbrain (SN pars compacta and reticulata and VTA) (Figs. 1A, K and P), and no staining was observed on the contralateral side (data not shown). We observed that both the non-phosphorylated form and the phospho-Ser129 form of human α-syn were expressed (Figs. 1A, C and K). High levels of expression were also seen in axon terminals in the ipsilateral striatum suggesting that the proteins produced in the nigral DA neurons were actively transported anterogradely towards the synaptic terminals (Figs. 1B, J and O). Double immunofluorescent staining specific for human α-syn, the phosphorylated form, or GFP confirmed that the vast majority of the TH + or VMAT-2 + DA neurons in the SN (>90%) were transduced (Figs. 1D-F, G-I, L-N and Q-S).

Histological analysis suggested that the expression level of α-syn was higher with the WPRE-containing AAV-α-syn vector compared to the one lacking this enhancer element (Figs. 1A and K). This observation was further confirmed by quantitative analysis where α-syn mRNA and protein levels were determined by RT-qPCR and ELISA. We found that GFP overexpression did not modify the expression of the endogenous rat α-syn. The AAV-α-syn (+WPRE) vector induced a 4-fold increase in human α-syn mRNA level (relative to the expression of rat α-syn mRNA measured on the non-injected contralateral side), and a 5 and 8-fold increase in α-syn protein level in the SN and striatum, respectively (all P < 0.0001), accompanied by a 62% reduction in the rat α-syn mRNA level (P < 0.01) (Figs. 1T-U). This suggests that overexpression of human wt α-syn induces a down-regulation of the endogenous SNCA gene. The increases in α-syn mRNA and protein following injection of the WPRE-lacking AAV-α-syn vector were about 50% lower than in the animals receiving the WPRE-containing vector: a 2.1-fold increase in α-syn mRNA and 3-fold increase in α-syn protein in the SN, and a 3.8-fold increase in α-syn protein in the striatum (all P < 0.01) (Figs. 1T-U). Injection of the AAV-α-syn (−WPRE) vector also resulted in a significant decrease in the level of the endogenous rat α-syn mRNA (28 ± 6%; P < 0.05).

**α-Synuclein overexpression induces progressive behavioural impairment**

To determine the functional consequences of overexpressing human wt α-syn or GFP in nigral DA neurons, we performed a time-course study to evaluate changes in motor performance. In Experiment 1, the rats were tested for spontaneous behaviour (cylinder test) and drug-induced rotational behaviour (using amphetamine) 1–5 days before and 10 days, 3, 5, 8 and 16 weeks after vector injection. We found that delivery of the WPRE-containing AAV-α-syn vector had a significant effect on motor performance over time [F₄,₅₆ = 3.21 for cylinder test; F₄,₅₆ = 4.57 for cylinder test; F₄,₅₆ = 4.74 for amphetamine-induced rotations, P < 0.001; time × treatment interaction] (Figs. 2A–B). Post hoc analysis revealed that, compared to GFP group, α-syn overexpression induced a progressive functional deficit both in the cylinder test and the amphetamine-induced rotations test, which was significant from 5 weeks post-AAV vector injection and maintained at 16 weeks (P < 0.05) (Figs. 2A–B).

In Experiment 2, separated groups of animals were tested at the same time-points and were sacrificed afterwards for histological analysis. As in Experiment 1, we observed a progressive development of motor deficits over time [F₄,₅₆ = 3.21 for cylinder test; F₄,₅₆ = 4.61 for amphetamine-induced rotations, P < 0.001; time × treatment interaction]. In both experiments the behavioural impairments were fully developed at 8–16 weeks after vector injection. At these time-

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Fig. 1. Pattern of expression of the different transgenes in the nigro-striatal system. DAB immunostaining showing the expression of human α-syn [A and B, j and K], human phospho-Ser129-α-syn (C) and GFP (O–P) in the midbrain (A, C, K and P) and forebrain (B, j and O) 3 weeks after AAV-α-syn (+WPRE) (A–J), AAV-α-syn (−WPRE) (J–N), or AAV-GFP (O–S) vector injection in the SN. Nigral injection of vectors results in the expression of the transgenes in both the midbrain and the striatum. Double immunofluorescence staining shows that α-syn (E and M), phospho-α-syn (H) or GFP (R) is expressed in the vast majority (>90%) of the TH + (D, F, L and N) or VMAT-2 + (G, I, Q and S) nigral DA neurons. Asterisks show the site of delivery of the vector. VTA: ventral tegmental area, SNc: SN pars compacta, SNr: SN pars reticulata, T: Measurement of rat (noted R) or human (noted H) α-syn mRNA levels by RT-qPCR 10 days after AAV-α-syn (+WPRE) or AAV-α-syn (−WPRE) vector. U: α-syn protein level was assessed by ELISA in the midbrain and striatum 3 weeks after injection of AAV-GFP or AAV-α-syn vectors. Scale bar: 2 mm for DAB stained sections, 150 μm for fluorescence pictures. Data are expressed as mean ± SEM *: P < 0.05 (Student t-test).
points the animals showed a significant reduction in the percentage of contralateral (left) paw use in the cylinder test, to an average of about 30% (Figs. 2A and C) and a significant increase in the numbers of rotations following amphetamine administration reaching an average of about 5–6 turns/min at the two time-points (Figs. 2B and D). By contrast, the animals that received the WPRE-lacking AAV-α-syn vector...
did not show any impairment in the cylinder test score at 8 weeks (50.8±3.2%; P=0.51 compared to GFP group) and no significant amphetamine-induced rotational behaviour was observed in these rats (1.8±0.9 turns/min; P=0.37) (Figs. 2C–D).

In the AAV-α-syn(+WPRE) treated group subcutaneous injection of L-DOPA (10 mg/kg, plus benserazide), given 1 h before testing, abolished the deficit observed at the cylinder test (50.7±2.5%; P<0.05 compared to AAV-α-syn(+WPRE) group) indicating that the motor impairment was effectively reversed by dopaminergic treatment (Fig. 2C).

Progressive loss of dopaminergic neurons

The animals were sacrificed at 10 days, and 3, 5, 8 and 16 weeks after AAV-α-syn-WPRE or AAV-GFP vector injection, and midbrain sections were stained for TH (Fig. 3A) or VMAT-2 (Fig. 3B). Histological analysis revealed a progressive loss of nigral DA neurons after α-syn overexpression induced by injection of the WPRE-containing vector (Figs. 3A–B). Using a stereological approach, we estimated the number of TH-immunopositive nigral neurons and compared it to that recorded from the contralateral intact side. Consistent with the development of motor impairment (see above), quantifications revealed a progressive loss of TH+ and VMAT-2+ neurons in the SN [F4,50=8.26 for TH+ cells, F4,50=11.79 for VMAT-2+ cells; P<0.001; time×treatment interaction] (Figs. 3C–D). Post hoc analysis indicated a significant neuronal loss already at 3 weeks (to 58.1±6.2% of normal for TH+ cells and 63.5±5.4% of normal for VMAT-2+ cells; P<0.001 compared to GFP group), that became more pronounced at 8 weeks (19.86±1.3% for TH+ cells; 25.1±2.0% for VMAT-2+ cells; P<0.001) and maintained at 16 weeks (25.9±5.5% for TH+ cells; 21.8±3.1% for VMAT-2+ cells; P<0.001) (Figs. 3C–D).

Two separate groups of animals (n=8 per group) received an intranigral injection of the WPRE-lacking AAV-α-syn vector at the same genome copy dose, and were killed after 3 or 8 weeks. No apparent neuronal degeneration was observed in the histological analysis (Figs. 3A–B). No significant loss of nigral TH+ or VMAT-2+ dopamine neurons was observed in these animals, as compared to the AAV-GFP group at the respective time-points (at 3 weeks: 90.5±0.6% TH+ cells, P=0.17 and 89.6±2.5% VMAT-2+ cells, P=0.48; at 8 weeks: 86.9±3.0% TH+ cells, P=0.56, and 91.9±1.0% VMAT-2+ cells, P=0.45) (Figs. 3D–E).

To ascertain that the long-term loss of TH+/VMAT-2+ cells were due to degeneration of nigral DA neurons rather than down-regulation of the marker proteins, we performed double immunostaining of TH in combination with the neuronal markers NeuN or Hu. We found that 16 weeks after vector injection the loss of TH immunoreactivity was accompanied by loss of NeuN+ (Figs. 3C) and Hu+ neurons (data not shown) in the SN pars compacta, while the neurons in the VTA was relatively spared. Notably, the NeuN+ neurons located in the SN pars reticulata and dorsal to the SN remained unaffected, suggesting that the cell loss induced by the α-syn vector was quite selective, and largely confined to the DA neurons of the SN pars compacta. Stereological counting performed on the animals in the 16 week group revealed that the loss of NeuN+ neurons in
the SN pars compacta was of similar magnitude to that observed for TH+ and VMAT-2+ cells (27.0±4.3% of control, \(P<0.001\)).

Progressive degeneration of axonal terminals and dendritic arborisations

To evaluate the impact of α-syn overexpression on the survival of axonal and dendritic projections of the nigral DA neurons, forebrain and midbrain sections from the animals in Experiments 1 and 2, sacrificed at 10 days, and 3, 5, 8 and 16 weeks after vector injection, were stained for TH, VMAT-2 or DAT. Histological analysis showed a time-dependent loss of striatal TH+ fibres, as assessed by optical densitometry (Figs. 4A, C–D). α-Syn overexpression using the WPRE-containing vector had a significant effect on the density of striatal fibres over time \(F_{4,50} = 6.19\) for TH, \(F_{4,50} = 5.54\) for VMAT-2 and \(F_{4,50} = 8.51\) for DAT, \(P<0.001\), time × treatment interaction. Post hoc analysis showed that striatal fibre staining density was significantly reduced already at 3 weeks, to 69.0±7.1% for TH+ fibres, 75.6±7.4% for VMAT-2+ fibres, and 76.3±6.3% for DAT+ fibres (\(P<0.05\)), expressed as percent of the staining density recorded on the contralateral intact side, and remained stable from 8 weeks post-injection (41.8±5.9% for TH+ fibres, 47.5±6.9% for VMAT-2+ fibres, 42.1±6.7% for DAT+ fibres; \(P<0.001\)) (Fig. 4B).

In the SN, the dendrites of the DA neurons extend ventrally to form extensive terminal arborisations in the pars reticulata (Fig. 5A). In rats receiving the AAV-α-syn(+WPRE) vector this TH+ dendritic projection showed progressive changes in both morphology and density that evolved over time \(F_{1,30} = 26.6, P<0.001\). Optical densitometry showed a significant loss of TH+ dendrites at 3 weeks after vector injection (67.8±8.0% of normal, \(P<0.05\)) that was further pronounced at 8 and 16 weeks (21.9±3.8%, \(P<0.001\)) (Figs. 5A–D). Remaining dendrites, derived from the spared DA neurons, appeared truncated and had a beaded and swollen appearance (Figs. 5B and C) different from their normal smooth and slender outline (Fig. 5A).

Nigral delivery of the AAV-α-syn vector lacking WPRE led to no significant loss of striatal TH+ fibres at 3 weeks (82.3±8.2%; \(P=0.40\)) or 8 weeks (74.8±3.5%; \(P<0.05\)). Similarly, the loss of TH+ dendrites were less pronounced in the animals receiving the WPRE-lacking AAV-α-syn vector, reaching significance only at 8 weeks after vector injection (43.4±8.5%, \(P=0.001\)) (Fig. 5A).

Fig. 3. Stereological estimates of DA neuron numbers in the SN. A: Immunolabelling of midbrain TH+ neurons in AAV-GFP (16 weeks after injection), AAV-α-syn(+WPRE) (10 days, 3, 5, 8, 16 weeks after injection) or AAV-α-syn(−WPRE)-injected rats (8 weeks after injection). On the intact side (left), the SN contains numerous densely packed TH-positive neurons. On the side of injection (right), a progressive reduction in the number of DA neurons can be appreciated after injection of the AAV-α-syn(+WPRE) vector. B: Immunostaining of VMAT-2+ neurons in the SN 8 weeks after nigral delivery of AAV-GFP or the two AAV-α-syn vectors. C: Double immunofluorescence staining showing the specific loss of DA neurons (TH+/NeuN+) in the midbrain 16 weeks after AAV-α-syn(+WPRE) vector injection. D–E: Quantification of the number of TH− (C) and VMAT-2-immunoreactive neurons (D) 10 days, 3, 5, 8, 16 weeks after injection of AAV-GFP (white bars) or AAV-α-syn(+WPRE) vector (black bars), and 3 and 8 weeks after injection of the AAV-α-syn(−WPRE) vector (grey bars). Progressive and profound loss nigral DA neurons is observed after injection of AAV-α-syn(+WPRE) vector. Data are presented as percentage of neurons in the intact side and expressed as mean±SEM. Scale bar: 2 mm (A–B), 400 μm (C). *\(P<0.05\) (2-way ANOVA, Bonferroni post hoc test or Student t-test).
Fig. 4. Optical densitometry of striatal dopaminergic terminals. A: Illustration of striatal TH immunoreactivity in AAV-GFP (16 weeks after injection), AAV-α-syn(+WPRE) (10 days, 3, 5, 8, 16 weeks after injection) or AAV-α-syn(-WPRE)-injected rats (8 weeks after injection). A marked reduction in striatal TH+ fibre density is observed over time following injection of AAV-α-syn(+WPRE) vector. B: Quantification of striatal TH+ fibre immunoreactivity at all time-points for the AAV-GFP (open bars) and AAV-α-syn(+WPRE) groups (black bars), and 3 and 8 weeks after injection for the AAV-α-syn(-WPRE) group (open bars). Data are presented as percentage of TH or VMAT-2 striatal immunoreactivity compared to intact side and expressed as mean±SEM. C and D: Illustration of striatal dopaminergic fibres immunostained for VMAT-2 (C) or DAT (D) 8 weeks after nigral injection of AAV-GFP or AAV-α-syn vectors. A pronounced loss of striatal fibre density is observed in the AAV-α-syn(+WPRE) group compared to the AAV-GFP group. E and F: Quantification of VMAT-2+ (E) and DAT+ (F) fibre density at all time points for the AAV-GFP (open bars) and AAV-α-syn(+WPRE) groups (black bars), and 3 and 8 weeks after injection for the AAV-α-syn(-WPRE) group (grey bars). Data are presented as percentage of striatal fibre immunoreactivity compared to intact side and expressed as mean±SEM. Scale bar: 2 mm (A, C and D). *P<0.05 (2-way ANOVA, Bonferroni post hoc test or Student t-test).
injection (74.8 ± 3.5%, P < 0.05) (Fig. 5D). Despite the absence of overt neurodegenerative effects induced by α-syn using the WPRE lacking AAV-α-syn vector, abundant pathological signs were observed in the cell bodies, along the nigro-striatal pathway, at the level of the striatal terminals and the dendritic network (data not shown).

Correlation analysis

In Fig. 6, the performance in the AAV-α-syn(−WPRE) injected animals in the two motor tests, amphetamine-induced rotation and paw use in the cylinder test, is plotted against the percentage of TH+ cells remaining in the SN (Figs. 6A–B), and the density of remaining TH+ fibres (Figs. 6C–D) and DAT+ fibres (Figs. 6E–F) in the striatum. The animals in the 10-day, and the 3-, 5- and 8-week groups are from Experiment 1, and those at 16 weeks group are pooled from the two experiments.

We examined whether there was a correlation (linear L, or non linear N-L) between the histological parameters and the behavioural scores. We found a good correlation between the percentage of surviving of TH+ cells and amphetamine-induced rotation [r = 0.71 (L), 0.78 (N-L), P < 0.001] and somewhat less against the scores in the cylinder test [r = 0.42 (L), r = 0.48 (N-L), P < 0.01)]. Similar correlations were obtained using VMAT-2 as a marker of SN DA neurons (data not shown). The density of TH-immunoreactive fibres correlated better with the cylinder scores [r = 0.71 (L), r = 0.75 (N-L); P < 0.001] than with the rotational scores [r = 0.61 (L), P < 0.001]. Similar analysis using DAT as a marker of striatal fibre density showed good correlations with the performance in both tests (cylinder test: r = 0.56 (L), r = 0.71 (N-L), P < 0.001; rotation test: r = 0.77 (L), r = 0.79 (N-L), P < 0.001). Cell number and fibre density values obtained from the TH and VMAT-2 immunostained sections were highly correlated (r = 0.87 and r = 0.98, respectively) (data not shown).

Closer inspection of the scatter-plots showed that significant impairment in the cylinder test (≤ 35% contralateral left paw use) and significant motor asymmetry in the rotation test (≥ 3 turns/min) is seen only in animals with at least 40–50% loss of TH+ cells in the SN, and a similar loss of TH+ or DAT+ fibres in the striatum (marked by shading in Figs. 6A–F). Ten of the 14 animals in the 16-week group and 2 of the 6 animals in the 8-week group passed this criterion. By contrast, none of the animals in the 10-day, 3-week and 5-week groups showed this magnitude of impairment.

Biochemical changes following α-synuclein overexpression

To further study the impact of α-syn overexpression on the function of the nigro-striatal dopaminergic pathway, the changes in striatal TH enzymatic activity and the striatal content of DA and its metabolites, DOPAC and HVA, were assessed biochemically at the same five time points as used in the histological analysis (n = 4–6 per group). We found that α-syn overexpression lead to a progressive reduction in TH activity [F1,24 = 51.09, P < 0.001]. Enzymatic activity was significantly decreased already at 10 days after AAV-α-syn(+WPRE) vector injection (59.2 ± 3.5%), and further reduced at 3 weeks (33.4 ± 3.6%), 8 weeks (24.6 ± 3.4%) and 16 weeks (17.8 ± 2.3%) (P < 0.001 at all time points, compared to AAV-GFP group at 16 weeks) (Fig. 7A).

Assessment of striatal DA, DOPAC and HVA levels revealed that α-syn overexpression affected the level of these molecules over time [F1,34 = 13.06, P < 0.001] (Fig. 7B). Consistent with the development of motor impairment (see above), a moderate reduction in DA level was observed at 5 weeks (to 68.8 ± 6.3% of normal; P < 0.01) and a more pronounced depletion at 8 and 16 weeks (43.8 ± 4.9% and 39.3 ± 6.0% of normal, respectively; P < 0.001). The DOPAC and HVA

![Fig. 5. Optical densitometry of dopaminergic dendrites. A–C: Illustration of TH+ dendrites in the intact SN pars reticulata (A), and 3 weeks (B) and 8 weeks (C) after AAV-α-syn(+WPRE) vector injection. A marked degeneration of dendritic fibres is observed following injection of the AAV-α-syn(+WPRE) vector. D: Quantification of TH+ dendrite density in the SN pars reticulata at all time points following AAV-α-syn(+WPRE) vector injection (black bars), 3 and 8 weeks after AAV-α-syn(−WPRE) vector injection (grey bars) and 16 weeks after GFP vector injection (open bar). Scale bar: 50 μm. Data are presented as percentage of TH+ immunoreactivity compared to intact side, and expressed as mean ± SEM. *P < 0.05 (1-way ANOVA, Dunnett post hoc Student t-test).](image)
Fig. 6. Correlations between dopaminergic parameters and behavioural score. A–D: Scatter-plots showing the correlation of the percentage of TH+ nigral neurons (A and B), striatal TH+ (C–D) or DAT+ (E and F) fibre density, with the number of amphetamine induced rotations (A, C and E) or score at cylinder test (B, D and F). Shaded areas include individuals that reached the cut-off of behavioural impairment (≤35% in cylinder test and ≥3 turns/min in amphetamine-induced rotations test). Spearman coefficient ($r$) and $P$ values were calculated from the linear (L) or non-linear (N-L) regression analysis.

Fig. 7. Enzymatic activity and HPLC measurements. A: Histogram illustrating the level of TH enzymatic activity in striatum at 10 days, 3, 5, 8 and 16 weeks after AAV-$\alpha$-syn(+WPRE) vector injection (black bars), and 16 weeks after AAV-GFP injection (open bars). B: Histogram showing the striatal levels of DA (open bars), DOPAC (grey bars) and HVA (black bars) measured by HPLC at each time-point following AAV-$\alpha$-syn (+WPRE) vector injection and 16 weeks after AAV-GFP injection. Measurements revealed a significant reduction in the 3 metabolites from 8 weeks after AAV-$\alpha$-syn(+WPRE) delivery. C: Histogram representing the striatal turnover of DA, as calculated by the DOPAC/DA (open bars) and HVA/DA ratios (black bars). A significant increase in these ratios is observed from 5 weeks post AAV-$\alpha$-syn(+WPRE) delivery. Data are expressed as mean ± SEM. *$P<0.05$ (1-way ANOVA, Dunnett post hoc test or Student $t$-test).
levels were markedly reduced at 8 weeks (55.7 ± 12.5% of normal for DOPAC and 57.3 ± 5.1% of normal for HVA, P < 0.01), and at 16 weeks (53.6 ± 5.9% of normal for DOPAC and 56.1 ± 5.4% of normal for HVA, P < 0.01) (Fig. 7B). The reduction in DA synthesis and storage was accompanied by a significant, 60–80%, increase in DA turnover, as assessed by HVA/DA and DOPAC/DA ratios, from 5 weeks after AAV-α-syn(+WPRE) vector injection (Fig. 7C).

α-Synuclein overexpression inhibits the dopaminergic synthesis and release machinery

The early effect of α-syn overexpression on TH enzyme activity, seen already at 10 days after vector injection, prompted us to investigate the effect of α-syn overexpression on the expression of dopaminergic markers by qPCR analysis. As soon as 10 days after vector injection, i.e. at a time when no significant cell loss had yet occurred, we observed a significant decrease in the expression of TH mRNA levels (47.8 ± 9.8% of normal), VMAT-2 (62.6 ± 5.1% of normal), DAT (61.0 ± 8.7% of normal) and AADC (67.2 ± 6.3% of normal) (P < 0.01) in the AAV-α-syn(+WPRE) transduced animals (Fig. 8A).

To confirm the effect at the protein level, we determined the levels of striatal TH expression by western blot at the different time-points. We found that striatal TH protein levels were markedly reduced at 3 weeks after vector injection (to 46.4 ± 5.1% of normal), and more pronounced at 8 and 16 weeks (28.5 ± 4.7% and 31.2 ± 3.6%, respectively) (P < 0.001) (Fig. 8B).

The impact of α-syn overexpression on TH and VMAT-2 expression was further studied by quantification of fluorescence intensity.
in α-syn transduced DA neurons at 10 days after AAV-α-syn(+WPRE) vector injection. Measurement of fluorescence signals revealed a significant decrease of TH and VMAT-2 levels in nigral DA neurons (72.3 ± 6.1% for TH, P < 0.01, and 86.1 ± 2.0% for VMAT-2, P < 0.05). In contrast, the level of these 2 dopaminergic markers remained unaltered in VTA DA neurons (P > 0.05) (Figs. 8C–O).

Detection of the phosphorylated form of α-synuclein

In human PD, as well as in animal models of PD, α-syn toxicity and aggregation is linked to phosphorylation of serine 129. This modified version of the protein can be readily visualised with an antibody specific for human phospho-Ser129-α-syn (Abcam). Using this antibody we detected phospho-α-syn+ inclusions in the cell bodies of nigral DA neurons as soon as 10 days after AAV vector delivery (Figs. 8O–Q) and they remained prominent in the surviving nigral DA neurons at all longer time-points.

The presence of phospho-α-syn in the nucleus has been suggested to mediate the effect of α-syn on transcriptional activity (Kontopoulos et al., 2006; Azeredo da Silveira et al., 2009; Schell et al., 2009). Interestingly, we found a proportion of the transduced nigral neurons with a high level of phospho-α-syn in the nucleus. In the AAV-α-syn treated animals we observed that the pattern of α-syn and phospho-α-syn expression differs in the transduced nigral DA neurons. While α-syn expression was diffusely distributed in the cells, the phosphorylated form was either cytoplasmic, nuclear or diffusely located in both compartments (Figs. 8P–W). It seems possible that this difference in the cellular distribution of phospho-α-syn may have a direct impact on gene expression, and perhaps also the ability of the individual DA neurons to resist α-syn-induced toxicity.

Characterisation of α-synuclein-positive swellings

Toxicity of α-syn is most likely due to the formation of oligomeric forms that evolve into larger proteinaceous structures, corresponding to Lewy bodies and neurites in human PD. We examined the presence of cellular, axonal and dendritic pathology following α-syn overexpression. Pathological swellings and dystrophic axonal structures, characteristic of axonopathy, were counted in 3 parts of the striatum (Fig. 9A). Immunostaining against human α-syn showed abundant axonal pathology in the striatum (Fig. 9B). A high number of α-syn+ axonal swellings (size > 3 μm3) were detected as soon as 10 days after AAV-α-syn+ (WPRE) vector injection and their number remained stable over 16 weeks (black bars in Fig. 9C). We revealed a dynamic process of formation by different approaches. First, we calculated the number of α-syn+ axonal swellings per surviving TH+ nigral neuron (assessed in the same animal). This revealed a progressive accumulation in the terminals of the surviving DA neurons (open bars in Fig. 9C). Secondly, we observed that α-syn+ swellings become larger over time, as shown by the time-dependent increase in the number of structures larger than 20 μm3 (Fig. 9D).

We then studied the composition of these α-syn+ structures. Using double fluorescent immunostaining, we found that some of these dystrophic structures contained both TH and VMAT-2 proteins (Figs. 9E–G and G–L), but not DAT (not shown). The finding that TH and VMAT-2 are sequestered within the α-syn-positive inclusions is interestingly similar to what has been observed in Lewy bodies in the human disease (Shults, 2006; Dugger and Dickson, 2010). In contrast to what we observed with the α-syn staining, quantification of TH+ and VMAT-2+ structures showed a time-dependent increase in number (black bars in Figs. 9H and M). The dynamic process of accumulation of TH and VMAT-2 was further confirmed by a dramatic time-dependent increase in the number of TH or VMAT-2 immunoreactive axonal swellings calculated per TH+ or VMAT-2+ cell remaining in the SN (open bars in Figs. 9H and M), and in the number of large-sized structures (> 20 μm3) (Figs. 9I and N). In addition, phospho-α-syn+ punctate structures were found in the soma of nigral DA neurons (Figs. 9O–Q) and similar structures co-labelled with TH were also found in the dystrophic dendritic processes (Figs. 9R–T). Despite the absence of neurodegeneration after AAV-α-syn(−WPRE) vector injection, we observed the presence of axonal pathology at 3 and 8 weeks. However, the number of α-syn+, TH+ or VMAT-2+ axonal swellings was about 50% lower than that counted after injection of the AAV-α-syn(+WPRE) vector (data not shown).

Discussion

α-Synuclein expression

The new vector construct, containing the WPRE enhancer sequence and the synapsin promoter, resulted in a 2-fold increase in the expression of the human α-syn gene, compared to the vector with CBA promoter without WPRE. The expression level obtained with the AAV6-CBA-α-syn vector was 2–3-fold higher than the endogenous rat-α-syn level, which is similar to that reported by Azeredo da Silveira et al. (2009) using a similar vector construct (AAV6-CMV-α-syn). At this level of expression the loss of TH+ cells in the SN is small, around 10% in the present experiment and about 20% in the Azeredo da Silveira et al. study.

With the new AAV6-synapsin-α-syn-WPRE vector we obtained an expression level in the SN that was 4–5-fold higher than the endogenous rat-α-syn level (measured by qPCR and ELISA at 3 weeks post-injection). This resulted in > 70% TH+ cell loss in the SN in 16 of the 20 animals analysed at 8–16 weeks after injection, and was accompanied by marked behavioural impairments. At the relatively low dose used here, the AAV6-CBA-α-syn vector induced only marginal nigral cell loss and no significant behavioural impairments. Previous studies using higher doses of the AAV-CBA-α-syn vector (in either AAV2 or AAV5 capsid) have reported higher α-syn expression levels, i.e., a 3.6-fold increase in total α-syn protein expression (rat + human) in the SN, and a mean TH+ cell loss in the range of 40–60% (Kirik et al., 2002; Gorbatyuk et al., 2008). In the Kirik et al. study only about 25% of the injected animals showed any significant behavioural impairment. Taken together, these data suggest that the expression of human wt α-syn has to reach above a critical threshold level (around 4-fold above normal) in order to induce more profound DA neuron cell loss, and consistent motor impairments, in the rat AAV-α-syn model.

Behavioural impairments

Rats receiving the AAV6-α-syn(+ WPRE) vector as a single 3 μl injection into the SN, developed significant motor impairments that developed progressively over time. This was first seen at 5 weeks, and was fully developed at 8–16 weeks after injection. At these longer time-points 16/20 rats had developed marked motor asymmetry, ≥3 turns/min in the amphetamine-induced rotation test, and 12/20 rats showed also significant impairment in the cylinder test. Impairment in the two motor tests, i.e. more than ≥3 turns/min and ≤35% in left paw use, was associated with > 45% loss of TH+ cells in the SN, and a corresponding loss of TH+ innervation in the striatum, and high turning rates, i.e. ≥6 turns/min in the amphetamine test, was seen only in animals with > 70% TH+ cell loss. Similar to 6-hydroxydopamine-lesioned rats, however, not all animals with TH+ cell loss in the range of 50–80% were impaired. This probably reflects the ability of basal ganglia circuitry to compensate for the loss or dysfunction of part of the nigro-striatal pathway, similar to that seen in pre-symptomatic, or early-stage PD.

Effects on dopamine synthesis and release

Measurements of mRNA and protein expression levels at 10 days after vector injection revealed early signs of DA neuron dysfunction,
before any major cell loss. At this early stage, expression of VMAT-2, DAT, TH and AADC, were all reduced by 30–50%, suggesting that overexpression of human α-syn had caused a general down-regulation of DA synthesis and release in the transduced neurons. These observations are consistent with previous studies showing that α-syn plays a role in the regulation of DA biosynthesis, acting to reduce both expression and activity of TH and AADC (Perez et al., 2002; Baptista et al., 2003; Yu et al., 2004; Tehranian et al., 2006; Alerte et al., 2008), as well as in the regulation of vesicular recycling and transmitter release (Abeliovich et al., 2000; Larsen et al., 2006; Nemani et al., 2010; Anwar et al., 2011). Overexpression of α-syn in PC12 cells has been shown to inhibit catecholamine release, resulting from a reduced pool of readily releasable vesicles (Larsen et al., 2006).

The present data suggest that these cellular mechanisms, so far studied mostly in cell culture, operate also in nigral DA neurons in vivo. The early effects on DA synthesis seen within the first 3 weeks in the AAV-α-syn treated animals are thus likely to be due to inhibition of TH and AADC enzyme activity in combination with a direct effect of α-syn on TH and AADC gene expression. In line with previous in vitro studies (Larsen et al., 2006; Nemani et al., 2010) we have in a parallel series of experiments shown that striatal DA release and re-uptake, as measured by in vivo amperometry, are impaired already at 10–21 days after AAV-α-syn injection (Lundblad et al., unpublished). These early changes in DA neurotransmission may, at least in part, be mediated via α-syn-induced down-regulation of VMAT-2 and DAT, as observed in the present experiments. Interestingly, while the native form of α-syn was confined to the cytoplasm, we observed that the S129-phosphorylated form, in some of the transduced DA neurons, was preferentially located in the nucleus. This phenomenon, which has previously been reported to occur in α-syn overexpressing transgenic mice (Wakamatsu et al., 2007; Schell et al., 2009), suggests the interesting possibility that the phosphorylation of α-syn, and its translocation into the nucleus, may be directly involved in the transcriptional regulation of DA

Fig. 8. Characterisation of α-synuclein positive axonal swellings. A–D: The number of α-syn+ swellings (C and D) was quantified in 3 different regions of the striatum, as shown in A (black bars). To evaluate the progression of α-syn+ inclusions formation over time, the ratio of striatal α-syn+ swellings per TH+ surviving neuron was calculated (open bars in C) and the number of dystrophic elements larger than 20 μm³ was quantified (grey bars in D). E–N: Double immunofluorescent staining showing the colocalisation of TH (E–G) and VMAT-2 (J–L) with α-syn+ in dystrophic striatal terminals (examples indicated with white arrows). Similarly, the ratios of TH+ and VMAT-2+ swellings per surviving nigral stained for the respective marker was calculated (open bars in H, M). Quantification of TH+ (I) and VMAT-2+ (N) axonal swellings larger than 20 μm³ in the striatum. Data are expressed as mean ± SEM. O–Q: Double immunostaining showing the presence of phospho-α-syn+ punctate elements in the cytoplasm of nigral VMAT-2+ neurons. R–T: Double immunostaining showing the presence of α-syn+/TH+ inclusions in dendritic process in the SN pars reticularis. Arrows indicates double-labelled aggregates. Scale bar: 100 μm (B), 12 μm (E–G, R–T), 15 μm (J–L), 20 μm (O–Q).
neurotransmission (Kontopoulos et al., 2006). Nurr1 is an interesting candidate to mediate this effect. Nurr1 is a central player in the regulation of DA neurotransmission genes, TH, AADC, DAT and VMAT-2 (Jankovic et al., 2005). In PD, increased levels of α-syn in nigral DA neurons have been associated with reduced cellular expression of both Nurr1 and TH (Chu et al., 2006; Chu and Kordower, 2007). A similar effect on Nurr1 and TH expression has been reported in a α-syn-overexpressing neuroblastoma cell line (Baptista et al., 2003).

Progression of neurodegenerative changes over time

The structural and functional impairments seen in the AAV6-synapsin-α-syn-WPRE treated rats define three distinct stages that progress over time:

1. A pre-symptomatic stage with unaffected performance in the motor tests and no, or limited, DA neuron cell loss. With the α-syn expression level obtained here (4–5-fold above normal) this stage lasted about 3 weeks and was characterised by marked axonal and dendritic pathology, appearance of widespread α-syn + axonal swellings and dystrophic neurites, and an early loss of striatal TH + terminals. Interestingly, DA tissue levels and DA turnover rate (measured as DOPAC/DA and HVA/DA ratios) remained unaltered at these early time-points, indicating that compensatory mechanisms act to maintain striatal DA neurotransmission at this pre-symptomatic stage.

2. An early symptomatic stage, seen at 5 weeks after vector injection, when the animals start to display motor deficits on the side opposite to the vector injection. At this stage some 50–60% of the TH + and VMAT-2 + cells in the SN are lost, and axonal pathology, including α-syn + dystrophic neurites and axonal swellings, has developed further. At this stage striatal DA turnover is significantly increased as a sign of failing synaptic DA release.

3. An advanced stage, when behavioural impairments are fully developed and TH + nigral cell loss is complete (in most cases >70%). This stage was reached at 8 weeks, and remained stable over the subsequent 8 weeks. In agreement with previous studies (Kirik et al., 2002; Maingay et al., 2006) the cell loss affected mainly the A9 neurons of the SN, while the A10 neurons of the VTA were largely spared. Consequently, loss of TH +, VMAT-2 + and DAT + fibres was confined to the areas innervated by the A9 neurons, i.e., the head and tail of the caudate-putamen. Axonal pathology, measured as α-syn + swellings, >3 μm² in size, increased in number between 5 and 8 weeks and remained unchanged at 16 weeks, the longest survival time studied here. Interestingly, the spared nigral DA neurons were able to survive long-term in this pathological state, despite that the level of expression of the α-syn transgene, as judged by immunostaining, was maintained in the surviving cells. This suggests that individual midbrain DA neurons may vary in their vulnerability to α-syn overexpression, and that a sub-population of the A9 nigral neurons, as well as most A10 VTA neurons, are able to resist α-syn-mediated toxicity, perhaps by more efficient handling or degradation of toxic α-syn species.

Relevance to human PD

AAV-mediated overexpression of human wt α-syn provides an interesting model of α-syn-induced pathology that replicates many of the aspects seen in human PD. The changes that develop in the transduced nigral DA neurons over time, from the early signs of neuritic α-syn aggregation and axonal damage, and impaired DA neurotransmission, seen at 10 days and 3 weeks after vector injection, to the overt cellular pathology and degeneration that develops at 5–8 weeks, provide support for the idea that the α-syn-induced pathology hits the axons and terminals first and later progresses to involve also the cell bodies. This retrograde progression of neurodegeneration is strikingly similar to that described to occur in PD (Cheng et al., 2010), although the progression of changes seen in the AAV-α-syn model is much faster than in the human disease. The time-course of changes, however, is sufficiently spread out in time to allow us to define, within a time-span of 2–4 months after vector injection, distinct stages that matches the pre-symptomatic, early symptomatic, and advanced stages of the disease seen in PD patients, thus providing new interesting possibilities for studies of stage-specific pathologic mechanisms and identification of targets for disease-modifying therapeutic interventions.

Acknowledgments

The study was supported by grants from the Swedish Research Council (grant no. 04X-3874 and the NeuroFortis programme), the Swedish Foundation for Strategic Research, and the EU 7th Framework project REPLACES. The authors thank Björn Anzelius, Ulla Jarl, Anneli Josefsson, Elsy Ling and Michael Sparrenius for excellent technical assistance, and Dr. Agnete Kirkeby and Bogusz Ostrowski for help with molecular biology.

The authors declare no conflict of interest.

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M. Decressac et al. / Neurobiology of Disease 45 (2012) 939–953


