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Research Paper

Depressive-like phenotype induced by AAV-mediated overexpression of human α-synuclein in midbrain dopaminergic neurons

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a progressive loss of nigral dopaminergic neurons and by the presence of aggregates containing α-synuclein called Lewy bodies. Viral vector-induced overexpression of α-synuclein in dopaminergic neurons represents a model of PD which recapitulates disease progression better than commonly used neurotoxin models. Previous studies using this model have reported motor and cognitive impairments, whereas depression, mood and anxiety phenotypes are less described. To investigate these psychiatric phenotypes, Sprague–Dawley rats received bilateral injections of a recombinant adeno-associated virus (AAV) vector expressing human α-synuclein or GFP into the substantia nigra pars compacta. Behavior was assessed at two timepoints: 3 and 8 weeks post-injection. We report that nigral α-synuclein overexpression led to a pronounced nigral dopaminergic cell loss accompanied by a smaller cell loss in the ventral tegmental area, and to a decreased striatal density of dopaminergic fibers. The AAV-α-synuclein group exhibited modest, but significant motor impairments 8 weeks after vector administration. The AAV-α-synuclein group displayed depressive-like behavior in the forced swim test after 3 weeks, and reduced sucrose preference at week 8. At both timepoints, overexpression of α-synuclein was linked to a hyperactive hypothalamic–pituitary–adrenal (HPA) axis regulation of corticosterone. The depressive-like phenotype was also correlated with decreased nigral brain-derived neurotrophic factor and spinophilin levels, and with decreased striatal levels of the activity-regulated cytoskeleton-associated protein. This study demonstrates that AAV-mediated α-synuclein overexpression in dopamine neurons is not only useful to model motor impairments of PD, but also depression. This study also provides evidence that depression in experimental Parkinsonism is correlated to dysregulation of the HPA axis and to alterations in proteins involved in synaptic plasticity.

1. Introduction

Parkinson's disease (PD) is clinically diagnosed based on the presence of hypokinesia, rigidity, resting tremor and impaired postural control (Jankovic, 2008, Lima et al., 2012). The neuropathological substrate of these motor symptoms is the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta which projects to the striatum (Ehringer and Hornykiewicz, 1960, Lang and Lozano, 1998). However, these motor symptoms are often accompanied by non-motor symptoms (Langston, 2006, Chaudhuri and Schapira, 2009), including autonomic, mood, cognitive and sensory dysfunctions, as well as sleep disturbances. These non-motor symptoms significantly contribute to the reduced quality of life in PD patients, but are frequently undiagnosed and left untreated (Den Oudsten et al., 2007, Soh et al., 2011, Hemmerle et al., 2012). Depression is the most frequent psychiatric complication in PD and affects around 35% of the patients (Aarsland et al., 2011). Although depression often precedes motor symptoms in PD (Aarsland et al., 2011) it may still reflect impairments of the nigrostriatal dopaminergic circuit (Frisina et al., 2009). Anxiety is also comorbid with PD and can affect around 40% of patients, sometimes together with depression (Martinez-Martin and Damian, 2010). Despite the high incidence of depression and anxiety in PD, there is yet no established animal model to study them.

PD is known to be strongly associated with an α-synuclein related pathology. Indeed, the discoveries that point mutations, duplications or triplications in the α-synuclein gene cause PD, and that α-synuclein is the main component of protein aggregates (i.e. Lewy bodies) in both familial and idiopathic forms of PD, suggest that α-synuclein plays a central role in the disease process (Feany and Bender, 2000; Martin et al., 2006; Dauer and Przedborski, 2003). Under normal conditions, α-synuclein has been proposed to play a role in synaptic plasticity

Abbreviations: ACTH, adrenocorticotropic hormone; AAV, adeno-associated virus; Arc, activity-regulated cytoskeleton-associated protein; BDNF, brain-derived neurotrophic factor; GFP, green fluorescent protein; HPA, hypothalamic-pituitary-adrenal; KPBS, potassium-phosphate buffer; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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and to regulate dopamine (DA) release and uptake (Clayton and George, 1998, Abeliovich et al., 2000). Accumulating evidence suggest a role of α-synuclein in the presynaptic SNARE complex (Burré et al., 2014). However, in pathological conditions, there is an accumulation of α-synuclein inclusions in Lewy bodies that interferes with several cellular functions at the nerve terminal and in the cell nucleus (Decressac et al., 2012a).

Several groups have used viral vectors, lentiviruses or adeno-associated viruses (AAV), to overexpress α-synuclein in animals as a way to induce a PD-like progressive synucleinopathy (Kirik et al., 2002, Klein et al., 2002, Lo Bianco et al., 2002, Lauwers et al., 2003, Mochizuki et al., 2006). Viral overexpression of α-synuclein, specifically in the nigrostriatal system, induces a progressive dopaminergic neuronal death, reminiscent of what occurs in some transgenic α-synuclein models (Fernagut and Chesselet, 2004) and in human PD (Kirik et al., 2002, Klein et al., 2002, Yamada et al., 2004, Lee and Trojanowski, 2006, Gorbatyuk et al., 2008, Azeredo da Silveira et al., 2009, Ulusoy et al., 2010, Decressac et al., 2011). In this study, we overexpressed wild-type human α-synuclein with AAV vectors driven by the neuron-specific synapsin-1 promoter in the SN pars compacta. This model of α-synuclein overexpression is known to induce degeneration of dopaminergic neurons in the nigrostriatal pathway and to cause motor impairments (Kirik et al., 2002, Decressac et al., 2012b, 2012c). However, changes in mood and anxiety, have, so far, not been thoroughly investigated in this model.

In the present study we therefore addressed whether α-synuclein overexpression in nigral DA neurons is sufficient to induce depressive- or anxiety-like symptoms in rats. The behavior was assessed at two timepoints post-injection: at week 3, corresponding to the pre-motor phase of PD, and at week 8, corresponding to an early motor phase of PD (Decressac et al., 2012c). We report that α-synuclein overexpression in dopaminergic neurons of the SN pars compacta is accompanied by a depressive-like phenotype. This behavioral phenotype is paralleled by hyperactivity of the hypothalamic–pituitary–adrenal (HPA) axis reflected by higher peripheral ACTH and corticosterone concentrations, as well as by decreased striatal activity-regulated cytoskeleton-associated protein (Arc) levels and decreased nigral brain-derived neurotrophic factor (BDNF) and spinophilin levels.

2. Methods

2.1. Production of recombinant AAV viral vectors

We used an AAV6-α-synuclein vector construct to overexpress α-synuclein, in which the expression of the transgene is driven by the human synapsin-1 promoter and enhanced using a woodchuck hepatitis virus posttranscriptional regulatory element, as described previously (Decressac et al., 2011). Genome copy titers were determined using real time quantitative PCR. The genome copy titers for AAV6-GFP and AAV6-α-synuclein were 7.7×10^{14} genome copies/mL, and an equivalent number of genome copies (2.3×10^{11} genome copies in 3 μL) were then injected in both groups.

2.2. Animals

Adult female Sprague–Dawley rats were used in this longitudinal study since they gain weight and body size much more slowly than male rats, thus making them more suitable for long-term studies of fine behavioral analyses. Rats, weighing 200–250 g at the time of surgery, were housed 4 per cage with ad libitum access to food and water during a 12 h light/dark cycle. The experiments were performed in agreement with the European Council Directive (86/609/EEC) and approved by the local Animal Ethics Committee (Stockholms Norra Djurförsöksetiska Nämnd, ethical permits N524/11 and N62/13). All efforts were made to minimize suffering and the number of animals used.

2.3. Surgical procedures

Surgical procedures were adapted from a previous protocol developed by the Björklund group (Decressac et al., 2011) and were performed under general anesthesia using a mix of 90 mg/kg ketamine and 10 mg/kg xylazine (Apoteket, Solna, Sweden), diluted in 0.9% saline and injected i.p. Rats were placed in a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA) and vector solutions were injected using a 10 μL Hamilton syringe fitted with a glass capillary with an outer diameter of 250 μm. 3 μL of viral solution (AAV6-GFP or AAV6-α-syn) were slowly infused manually at a rate of 0.2 μL/min. The coordinates (flat skull position) for bilateral injections in the SN were −5.3 mm (antero-posterior), ±1.7 mm (medio-lateral) and −7.2 mm (dorso-ventral), as calculated relative to bregma and according to the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 2007). The capillary was left in place for 1 min before it was slowly moved 1 mm upwards and left in place for one additional minute. The capillary was cleaned between each injection with 30% H_2O_2 diluted in dH_2O, 70% EtOH and dH_2O. The anesthesia was reversed by a combination of atipamezole (0.264 mg/kg) and buprenorphine (0.036 mg/kg) (Antisedan® and Temgesic®, respectively, Apoteket).

2.4. Tissue processing and immunohistochemistry

Eight weeks after vector injection, rats were deeply anesthetized with a mix of 90 mg/kg ketamine and 10 mg/kg xylazine i.p., perfused through the ascending aorta with 40 mL 0.1 M phosphate buffer (PB) and then by 80 mL ice-cold paraformaldehyde (4% w/v in 0.1 M PB). Brains were removed, post-fixed for 24 h in 4% paraformaldehyde and cryoprotected in sucrose (30% w/v in 0.1 M PB) for 72 h. They were then sectioned into 35 μm-thick coronal sections which were collected in 6 different series using a freezing microtome (Leica, Wetzlar, Germany). Immunohistochemical stainings were performed on free-floating sections using antibodies raised against tyrosine hydroxylase (TH) (rabbit 1:2000; Chemicon AB 152), GFP (chicken 1:1000; AbCam ab13970) and α-synuclein (mouse 1:1000; Santa Cruz sc12707). Sections were rinsed three times in phosphate buffered saline with potassium (KPBS) after each incubation. All incubation solutions contained 0.25% Triton X-100 in KPBS. The sections were quenched for 10 min in 3% H_2O_2 and 10% methanol. One hour of pre-incubation with 5% normal goat serum, normal horse serum and normal rabbit serum was followed by incubation overnight with the primary antibody in 2% serum at room temperature and incubation with 1:200 dilutions of biotinylated goat anti-rabbit (BA 1000 for TH), goat anti-chicken (BA9010, for GFP) or horse anti-mouse (BA 2001 for α-synuclein) antibodies (Vector Laboratories, Burlingame, CA, USA), followed by avidin–biotin–peroxidase complex (ABC Elite; Vector Laboratories), and visualized using 3,3-diaminobenzidine as a chromogen and H_2O_2 as a catalyst, mounted and cover-slipped with DPX mounting medium.

2.5. Cell counting and optical densitometry analysis

Assessment of the total number of TH + neurons in the SN was made manually from pictures obtained with a microscope (Axioskop 2, Zeiss, 10× magnification). Every six sections covering the SN were included in the counting procedure. We excluded three brains that were not correctly injected in one hemisphere (two AAV-GFP brains and one AAV-α-synuclein brain). Striatal TH + fiber density was measured by densitometry at four different levels relative to bregma (1.4, 1.0, 0.4 and 0.0 mm) using ImageJ software (Version 1.43u, NIH, USA), after converting each image to 8-bit and transforming it into a binary picture. Data was expressed as a percentage of the corresponding AAV-GFP control animals.

2.6. Behavioral evaluation

Assessment of behavior was performed 3 and 8 weeks after AAV-α-synuclein or AAV-GFP vector injection in the SN.

2.7. Ledged beam-walking test

Animals were trained on a tapered/ledged beam-walking test, adapted from the procedure previously described (Schallert and Woodlee, 2005, Zhao et al., 2005). This test is sensitive to dopaminergic function (Feeney et al., 1982, Walsh and Wagner, 1992). Rats walked along a 165 cm-long, progressively narrowing (6.5 cm wide at the wide end, 1.5 cm at the narrow end) Plexiglas beam, elevated above the floor with an incline of 15°, to reach their home cage. The main surface of the beam was covered in rubber matting to provide traction. Two centimeters below the beam was a 2.5 cm wide Plexiglas ledge that provided a platform to step on when there was a motor deficit. This ledge allowed rats to express their motor deficit, and removed the need for postural compensation to prevent falling off the beam. Taking a step with only one or two toes on the main surface of the beam (and the other four or three toes overhanging the ledge) was scored as a half foot-fault, whereas stepping with the entire foot on the ledge rather than on the main surface of the beam was scored as a full foot-fault. Before testing, each animal was allowed one refresher trial, which was not videotaped. One test consisted of 3 consecutive trials videotaped from the rear to allow a clear observation of the hind limbs.

2.8. Forced swim test

Rats were gently placed in a vertical glass cylinder (45 cm-high and 20 cm-diameter) filled to 35 cm with 25 ± 1 °C water during 15 min for the habituation phase. A 5 minute test session took place the next day and was video-recorded. The water was changed after each animal to avoid the influence of decreased water temperature and olfactory traces made by the previous animal. Immobility and climbing behaviors were scored manually from video files by a trained observer blind to the groups. Animals were considered immobile when they were motionless or only made brief and non-rhythmical movement of the paws or tail in order to keep their nose above the water level, and were considered to be climbing when they were making active movements with their forepaws in and out of the water, directed against the walls (Porsolt et al., 1977, Lucki, 1997). Swimming (i.e. active swimming motions, more than necessary to maintain their head above water) and activity behavior (all other behaviors different from immobility, climbing and swimming) were also recorded. During analysis, the test session was divided into time segments.

2.9. Sucrose preference

Sucrose preference is a locomotor-independent test in which the relative preference for a sucrose-sweetened solution (vs. water) gives a measure related to the anhedonia observed in depressive patients (Willner et al., 1987). Rats were given a choice between two bottles containing either tap water or a 2% sucrose solution in their home cages during 48 h. The position of the two bottles was switched after 24 h to avoid a side preference. Water and sucrose consumption were measured each day at the end of the afternoon by weighing the bottles. Sucrose preference (sucrose solution consumption (g) / water consumption (g) + sucrose solution consumption (g)) was calculated over the 48 h-period and compared between both groups.

2.10. Elevated plus-maze

The elevated plus-maze was used to measure the level of anxiety-like behavior. The maze was made of gray plastic and consisted of 4 arms, two with high walls and two without walls. The maze was elevated 1 m above the floor. At the start of the test, each rat was placed in the center of the maze and was allowed to explore it for 10 min. Each trial was videotaped, and the number of arm entries and the time spent in the opened and the closed arms were measured using Noldus EthoVision XT 9 (Noldus Information Technology, The Netherlands). The maze was cleaned with 70% ethanol between trials.

2.11. Stress-induced hyperthermia

Stress-induced hyperthermia (SIH) is a locomotor-independent, physiological anxiety-related measure (Van der Heyden et al., 1997). This test addresses the activation of the autonomic nervous system by measuring the increase in core body temperature in response to isolation and mild physical stress (Van der Heyden et al., 1997). The test was conducted as previously described (Fitzgerald et al., 2010), by measuring the core body temperature with a lubricated thermometer probe (Physitem RET-2) inserted 2 cm into the rectum for about 5 s. Rats were then isolated in a smaller cage for 10 min before a second measurement. The measure for anxiety is defined as the increase in rectal temperature over the 10 minute time window in response to the mild stress of measuring rectal temperature and isolation.

2.12. Hormonal assays for corticosterone and ACTH

We collected feces in the morning, and plasma samples at the time of euthanasia which occurred between 9 AM and 3 PM. Corticosterone (feces and plasma) and ACTH (plasma) levels were measured by ELISA (Enzo Life Sciences, Inc., Farmingdale, NY) and RIA (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), respectively. Briefly, for the ELISA assay, blood was centrifuged at 4 °C for 15 min at 1500 rpm to isolate the plasmatic fraction, and 500 mg of feces of each sample were homogenized in 80% ethanol, incubated overnight on a rotator, and centrifuged for 15 min at 1500 rpm. Supernatants were processed accordingly to the manufacturer's recommendations, after adding 2.5% of steroid displacement reagent to displace the steroid binding proteins from corticosterone. RIA assay was used for measuring ACTH in plasma samples and was based upon the competition of 125I-ACTH and ACTH binding to a limited quantity of ACTH-specific antibodies. The sensitivities for the corticosterone and ACTH assays were 1.6 ng/mL and 64.7 pg/mL, respectively.

2.13. Sample processing and analysis of protein expression by Western blot

Animals were sacrificed after the last behavioral test, 8 weeks after the AAV administration. Brains were rapidly removed after decapitation and stored at −80 °C until processed. Using hole punchers of either 0.25 mm or 1 mm diameter (Harris Uni-core™), samples from different brain regions were extracted from 100 μm thick brain sections in a cryostat (HM-500 M, Microm) at −20 °C. The striatum was sampled from bregma 1.70 mm to −0.40 mm, and the SN from bregma −4.80 mm to −6.04 mm. Samples were immediately sonicated in 1% sodium dodecyl sulfate (SDS), 10 mM NaF and 1 mM Na_3VO_4, centrifuged at 1000 g for 10 min at 4 °C to remove any cellular debris, and boiled for 10 min. Protein concentrations were determined with a BCA kit in 96 well plates (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each sample was resuspended in Laemmli homogenisation buffer (Tris-base 250 mM, glycerol 40%, SDS 8%, β-mercaptoethanol 20%, bromophenol blue 0.1%).

Fifteen μg of each sample was separated using 9% or 12% SDS-PAGE gels and transferred to a 0.2 μm PVDF membrane (Merck Millipore, Darmstadt, Germany). Membranes were then incubated for 30 min at room temperature in blocking buffer (0.1% TBS-Tween 20, 5% milk). Immunoblotting was carried out overnight at 4 °C with specific antibodies against BDNF (rabbit, 1/500, Alomone Labs, Jerusalem, Israel), pro-BDNF (rabbit, 1/500, Alomone Labs), Arc (mouse, 1/500, BD Transduction Laboratories, New Jersey, USA), spinophilin (mouse, 1/200, Santa Cruz Biotechnology inc., Dallas, Texas, USA) and Actin (rabbit, 1/250,

Sigma-Aldrich, St. Louis, MO, USA). Membranes were washed three times with 0.1% TBS-Tween 20 and incubated with secondary HRP anti-rabbit antibody or HRP anti-mouse antibody (dilutions 1/1000, Dako Sweden AB, Stockholm, Sweden) for 1 h at room temperature. At the end of the incubation, membranes were washed three times with TBS-Tween 20 and the immunoreactive bands were detected by chemiluminescence (Clarity Western ECL kit, Bio-Rad Laboratories, Inc., Berkeley, CA, USA). A series of primary, secondary antibody dilutions and exposure times were used to optimize the experimental conditions for the linear sensitivity range of the autoradiography films (Kodak Biomax MR). Films were scanned and the density of each band was quantified using the NIH ImageJ 1.29 software.

2.14. Statistical analysis

Statistical analyses were performed in Statistica (StatSoft Inc., Tulsa, OK, USA). All data were initially analyzed for normal distribution by the Shapiro–Wilk test. Normally distributed data was analyzed using a repeated measures ANOVA. When data was not normally distributed comparisons across time were analyzed using the non-parametric repeated Friedman ANOVA. Upon detection of significant effects, pairwise analyses were made by Newman Keuls post hoc test. Corticosterone and ACTH levels were analyzed using Mann–Whitney tests since data were not normally distributed. Histological and immunoblotting data from both AAV-α-synuclein and AAV-GFP groups were analyzed with a two tailed Student's *t*-test after a comparison of variances with an F-test (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA, USA). $p < 0.05$ was used as the threshold for statistical significance.

3. Results

3.1. Loss of dopaminergic neurons and striatal fiber degeneration following α-synuclein overexpression in the SN

Animals used for behavioral studies were sacrificed 8 weeks after AAV-α-synuclein injection in the SN. Forebrain and midbrain sections were stained for TH in order to assess striatal TH + fiber density and TH + neuronal cell body counting, respectively. Histological analysis revealed a clear loss of nigral dopaminergic neurons and their striatal terminals in the animals injected with AAV-α-synuclein in the SN, compared with the AAV-GFP animals (Fig. 1A and B). Quantifications revealed that α-synuclein overexpression in the SN induced a 43% loss of TH + neurons in the SN (unpaired *t*-test: $t = 4.62$; $p < 0.01$), a 30% loss in the VTA ($t = 2.97$; $p < 0.05$). Although expecting the VTA being affected, it was somewhat surprising that the difference in TH + loss between SNc and VTA was rather small. The reductions in TH + neurons were paralleled by a 30% decrease of striatal TH + fiber density ($t = 2.86$; $p < 0.05$) (Fig. 1B). The reduction of TH + fiber density was more prominent in the dorsolateral striatum, primarily innervated by SN, when compared to the ventromedial striatum, mainly innervated by VTA.

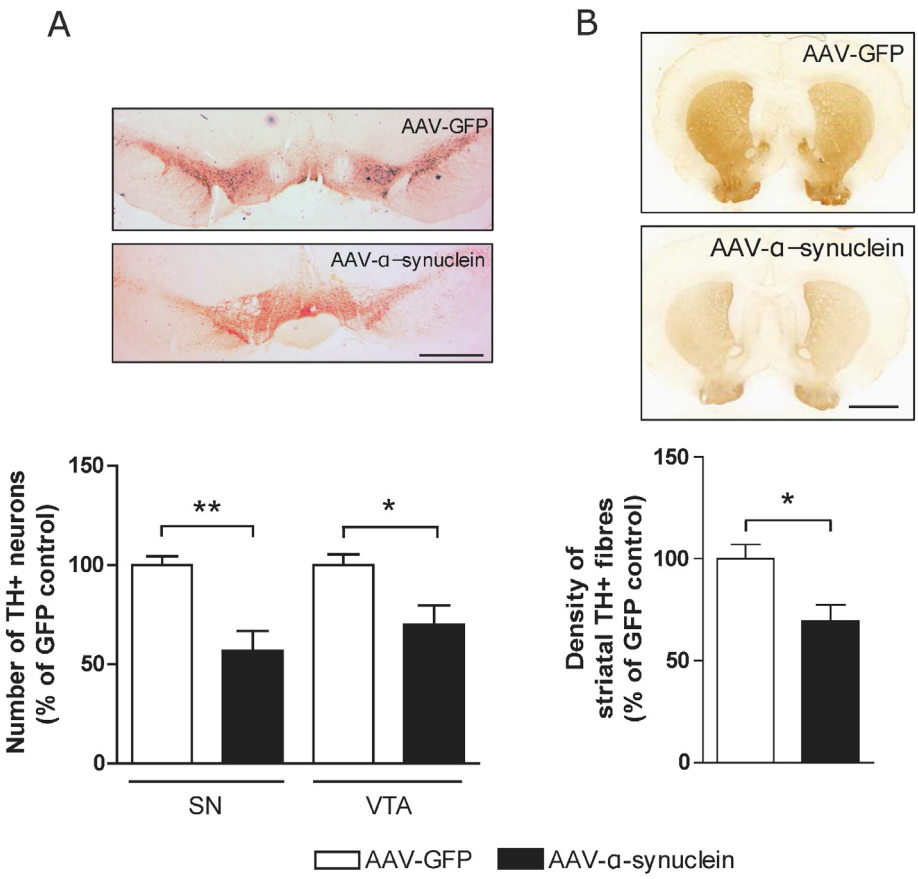

Fig. 1. Stereological estimates of dopaminergic neurons quantity in the SN and optical densitometry of striatal dopaminergic terminals in response to nigral AAV-GFP or AAV-α-synuclein. (A) Immunostaining of nigral TH + neurons in animals injected with AAV-GFP or AAV-α-synuclein into the SN, 8 weeks after injection. In AAV-GFP injected rats, the SN and the VTA contained densely packed TH + neurons, but in AAV-α-synuclein animals a reduction in the number of dopaminergic neurons was observed. (B) Striatal TH immunostaining in animals injected with AAV-GFP or AAV-α-synuclein in the SN, 8 weeks after injection. A reduction in striatal TH + fiber density was observed after 8 weeks in animals injected with the AAV-α-synuclein. Data are presented as a percentage of TH + neurons number and as a percentage of TH striatal immunoreactivity, both compared to the AAV-GFP group. Data are expressed as mean ± SEM. Scale bar: 1 mm (A), 2 mm (B). $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ (Student's *t*-test).

3.2. Motor impairment in α-synuclein overexpressing animals

To assess the functional consequences of human α-synuclein overexpression in midbrain dopaminergic neurons we examined the animals at two timepoints, 3 and 8 weeks after bilateral vector injections, corresponding to the pre-motor and motor stages, respectively (Decressac et al., 2012c). The ledged beam-walking test revealed a weak progressive motor impairment in the α-synuclein overexpressing animals, expressed as an increased number of errors per step at week 8 compared with week 3 (Fig. 2A). The Shapiro–Wilk test revealed a significant effect at both time points ($p < 0.001$ at week 3; $p < 0.001$ at week 8), demonstrating that the data were not normally distributed. Therefore, a Friedman ANOVA was performed and revealed a significant effect in the AAV-α-synuclein group at week 3 when compared with week 8 ($p < 0.05$), but not in the AAV-GFP group ($p = 0.206$). Post hoc analysis revealed a significant difference between week 3 and week 8 in the AAV-α-synuclein group ($p < 0.05$). However, the time spent to cross the beam was not significantly affected either in the AAV-α-synuclein or in the AAV-GFP groups. For these data, the Shapiro–Wilk test was significant at week 3, revealing that the data were not normally distributed ($p < 0.05$ at week 3; $p = 0.140$ at week 8). A Friedman ANOVA was therefore used but showed no significance ($p = 0.317$ for the AAV-GFP group; $p = 0.763$ for the AAV-α-synuclein group) (Fig. 2B).

3.3. Depressive-like phenotype occurring 3 and 8 weeks after nigral injection of AAV-α-synuclein

In parallel with the motor evaluation, the animals were tested for a potential depressive-like phenotype in the locomotor-dependent forced swim test and the locomotor-independent sucrose preference test. The forced swim test revealed that AAV-α-synuclein injected animals displayed decreased climbing behavior at 3 weeks after vector administration when compared to the AAV-GFP controls, indicating a depressive-like phenotype (unpaired *t*-test: $p < 0.05$) (Fig. 3A). Importantly, locomotor activity evaluated in an open field arena was not affected at this time-point (AAV-GFP: 4464 ± 352.4 vs. AAV-α-synuclein: 4363 ± 412.2; Student's unpaired *t*-test: $p = 0.856$). It is therefore reasonable to assume that the reduced climbing in the forced swim test was not a reflection of hypolocomotion.

We also assessed the preference for sucrose, reflecting the hedonic drive of animals in a locomotor-independent manner (Fig. 3B). The Shapiro–Wilk test revealed a significant effect at both time points ($p < 0.05$ at week 3; $p < 0.001$ at week 8), thus indicating that data were not normally distributed. Therefore, a Friedman ANOVA was performed and revealed a significant effect of time in both groups ($p < 0.01$ for the AAV-GFP group and $p < 0.05$ for the AAV-α-synuclein group). Post hoc analyses demonstrated a significant effect of AAV-α-synuclein between week 3 and week 8 ($p < 0.001$). Moreover, at week 8 there was a significant difference ($p < 0.001$) between the AAV-α-synuclein and AAV-GFP groups.

To examine whether this effect could be due to drinking and licking impairments, we also measured total fluid (water and sucrose) consumption during the 48 h duration of the test. At week 3, the AAV-GFP group consumed similar amounts of fluid as the AAV-α-synuclein group (719.4 ± 31.8 g vs. 616.7 ± 61.6 g; Student's unpaired *t*-test: $p = 0.276$). At week 8, the AAV-GFP group tended to consume more fluid than the AAV-α-synuclein group (821.2 ± 71.9 g vs. 552.1 ± 97.6 g; Student's unpaired *t*-test: $p = 0.156$).

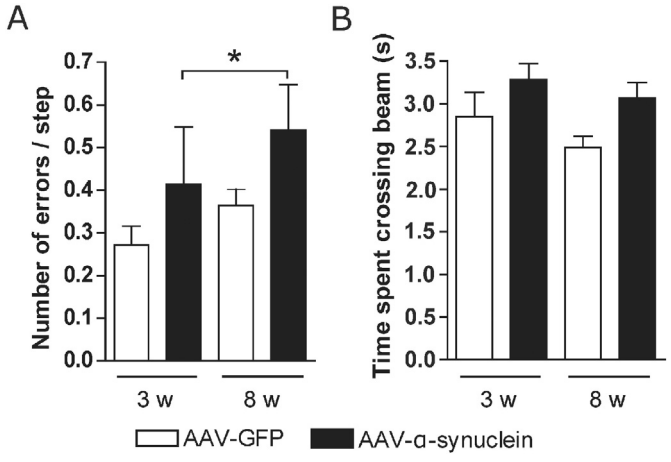

Fig. 2. Motor functions in the ledged-beam walking test after nigral AAV-GFP or AAV-α-synuclein. (A) Number of errors per step and (B) time spent to cross the beam in animals 3 weeks and 8 weeks after injection with AAV-GFP or AAV-α-synuclein. AAV-α-synuclein administration induced a higher number of errors per step at week 8 when compared with week 3, but had no significant effect on crossing time. Data are expressed as mean ± SEM. *$p < 0.05$ (Friedman ANOVA followed by Newman Keuls post hoc test).

3.4. Anxiety assessment after nigral injections of AAV-α-synuclein and AAV-GFP

To investigate whether the AAV-α-synuclein animals displayed a potential anxiety-like phenotype, the locomotor-dependent elevated plus-maze and the locomotor-independent SIH test were performed. AAV-α-synuclein injected animals and AAV-GFP control animals spent a similar percentage of time in the open arms at 3 and 8 weeks following AAV injection (Fig. 3C). The Shapiro–Wilk test showed that our data was not normally distributed ($p < 0.05$ at 3 weeks and $p < 0.001$ at 8 weeks). A Friedman ANOVA revealed a significant effect in the α-synuclein group over time ($p < 0.05$), but not in the control group ($p = 0.366$). However, the Newman–Keuls post hoc test did not reveal any differences.

Moreover, AAV-α-synuclein injected animals and AAV-GFP control animals performed a similar percentage of open arms entries (i.e. the number of open arm entries divided by the total number of arm entries). Shapiro–Wilks test was significant in both groups at week 8 (AAV-GFP 3W: $p = 0.400$; AAV-α-synuclein 3W: $p = 0.220$; AAV-GFP 8W: $p < 0.001$; AAV-α-synuclein 8W: $p < 0.001$), so a non-parametric was chosen for further analysis. A Friedman ANOVA revealed significant effects over time in both groups (AAV-GFP 3W: 29.650 ± 4.954 vs AAV-GFP 8W: 7.069 ± 3.310 and AAV-α- synuclein 3W: 31.33 ± 2.812 vs. AAV-α-synuclein 8W: 6.475 ± 2.940; AAV-GFP: $p < 0.05$; AAV-α-synuclein: $p < 0.05$), and post hoc analysis by Newman–Keuls test revealed a significant difference ($p < 0.001$) in both groups between week 3 and week 8. These data demonstrate that animals from both groups explored the open arms less at week 8, likely due to habituation.

The stress-induced hyperthermia (SIH) test was used to assess anxiety independently of motor functions, and revealed no significant differences between AAV-α-synuclein and AAV-GFP control animals at 3 or 8 weeks after AAV injection. The Shapiro–Wilk test showed that this data was normally distributed ($p = 0.179$ at week 3 and $p = 0.303$ at week 8), so a parametric test was chosen for further analysis. A repeated two-way ANOVA did not detect any significant effects (effect of AAV-α-synuclein: $F_{(1,20)} = 0.643$, $p = 0.432$; effect of time: $F_{(1,20)} = 0.387$, $p = 0.541$; interaction: $F_{(1,20)} = 1.600$, $p = 0.221$) (Fig. 3D).

Overall, these data indicate that no anxiety-like phenotype was induced in animals injected with AAV-α-synuclein.

3.5. Corticosterone and ACTH levels after nigral injections of AAV-α-synuclein and AAV-GFP

We measured corticosterone levels to study the response of the HPA axis following α-synuclein overexpression. To avoid invasive blood sampling at the 3 week timepoint, we measured the concentration of corticosterone extracted from feces samples, revealing elevated levels in feces in the AAV-α-synuclein group compared with AAV-GFP controls (unpaired *t*-test: $t = 2.13$; $p < 0.05$) (Fig. 4A). At week 8, plasma

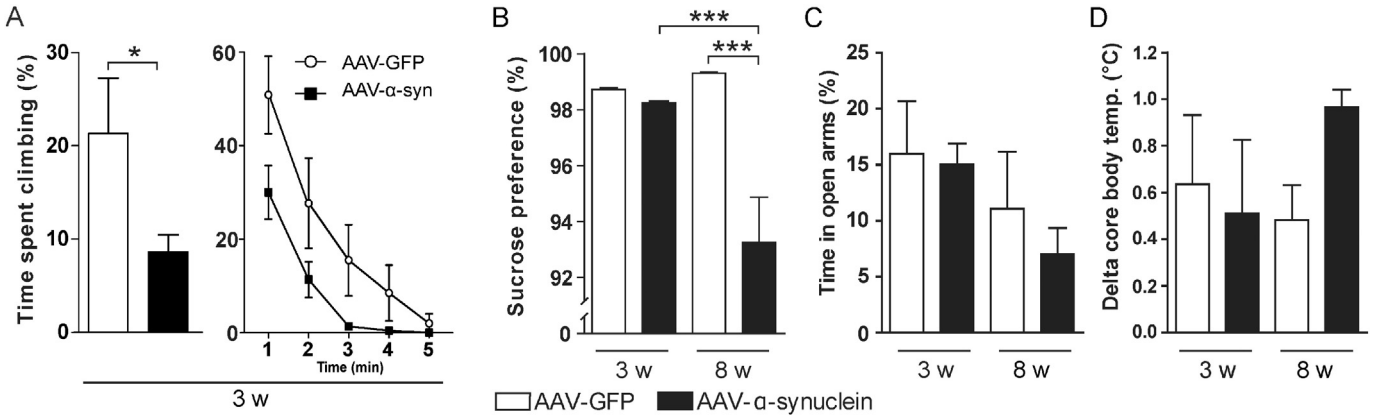

Fig. 3. Depressive-like and anxiety-like behaviors in response to nigral AAV-GFP or AAV-α-synuclein. (A) Time spent climbing in the forced swim test in animals 3 weeks after injection with AAV-GFP or AAV-α-synuclein. AAV-α-synuclein administration induced a decrease in climbing time compared with the AAV-GFP group. (B) Sucrose preference was decreased in α-synuclein overexpressing animals at week 8 when compared with week 3. (C) Time spent in the open arm of the elevated plus maze was not affected by AAV-GFP or AAV-α-synuclein administration. (D) Stress-induced hyperthermia was not affected by AAV-GFP or AAV-α-synuclein administration. Data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (Friedman ANOVA or repeated measures ANOVA followed by Newman Keuls post hoc test).

ACTH and corticosterone levels were significantly increased in the AAV-α-synuclein group compared to AAV-GFP group ($t = 2.19$; $p < 0.05$ and $t = 2.13$; $p < 0.05$, respectively) (Fig. 4B and C).

3.6. Arc, BDNF, pro-BDNF and spinophilin levels in the striatum and SN induced by α-synuclein overexpression

The levels of candidate proteins for depressive-like states; Arc, BDNF, pro-BDNF and spinophilin, were studied in the striatum and SN of AAV-GFP and AAV-α-synuclein injected animals. At the 8 week timepoint α-synuclein overexpression caused a decrease of Arc levels in the striatum (unpaired *t*-test: $t = 2.33$; $p < 0.05$), but not in the SN ($t = 1.09$; $p = 0.33$) (Fig. 5A). Moreover, reduced BDNF and spinophilin levels were found in the SN of AAV-α-synuclein animals compared to AAV-GFP controls ($t = 6.887$; $p < 0.001$ and $t = 2.34$; $p < 0.05$), whereas no significant changes were observed in the striatum for these markers ($t = 0.53$; $p = 0.61$ and $t = 0.41$; $p = 0.69$) (Fig. 5B and C). In contrast, no significant changes in pro-BDNF were detected in the striatum or in the SN ($t = 0.17$; $p = 0.87$ and $t = 0.35$; $p = 0.73$) (Fig. 5D).

4. Discussion

In this study we investigated the effects of bilateral AAV-mediated overexpression of human α-synuclein in dopaminergic neurons from the SN. In the bilaterally injected rats we report a 43% loss of TH in SN, and a 30% loss of TH in VTA dopaminergic neurons, along with a 31% reduction of striatal TH+ innervation 8 weeks after the vector injection, accompanied by a mild motor deficit measured by the ledged beam-walking test. Indeed, the nigrostriatal dopaminergic pathway is known to function in the dexterity of movement (Takakusaki, 2013; Takakusaki et al., 2008), which can be correlated with the number of errors performed upon crossing the beam in the present α-synuclein overexpression model. This motor impairment was significantly more severe at 8 weeks compared with at 3 weeks post-injection. This progressive loss of motor functions is in accordance with previous results using a unilateral α-synuclein overexpressing model, revealing a loss of motor functions in the cylinder test emerging at 5–8 weeks after AAV-α-synuclein injection (Decressac et al., 2012c). Moreover, the motor impairment revealed in our experiments was mild, and thus less likely to bias the assessment of emotional behaviors, which in many tests is dependent on mobility.

About 35% of PD patients exhibit clinically significant depressive symptoms contributing to severe disability, impaired quality of life, and shortened life expectancy (Reijnders et al., 2008, Aarsland et al., 2011). Relatively few studies have used animal models to examine depressive-like behavior and their associated neurochemical mechanisms in experimental Parkinsonism (Schintu et al., 2012). The forced swim test is a well-established paradigm for rodent studies on depression-like states. The decreased duration of climbing activity and increased duration of immobility in this test are considered as

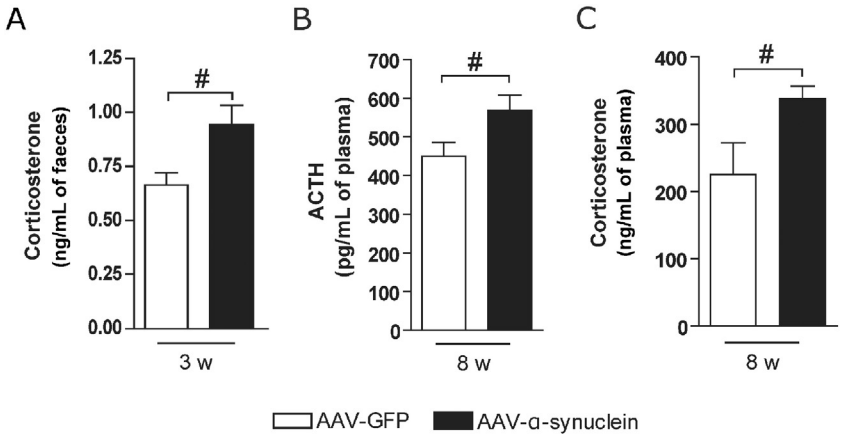

Fig. 4. Hormones levels of corticosterone and ACTH after nigral AAV-GFP or AAV-α-synuclein. (A) Fecal corticosterone concentrations were elevated in animals injected with AAV-α-synuclein compared with the AAV-GFP control group 3 weeks following AAV administration. (B, C) ACTH (B) and corticosterone (C) concentrations were elevated in plasma in animals injected with AAV-α-synuclein 8 weeks after the AAV administration compared with the AAV-GFP control group. Data are expressed as mean ± SEM. #$p < 0.05$ (Student's *t*-test).

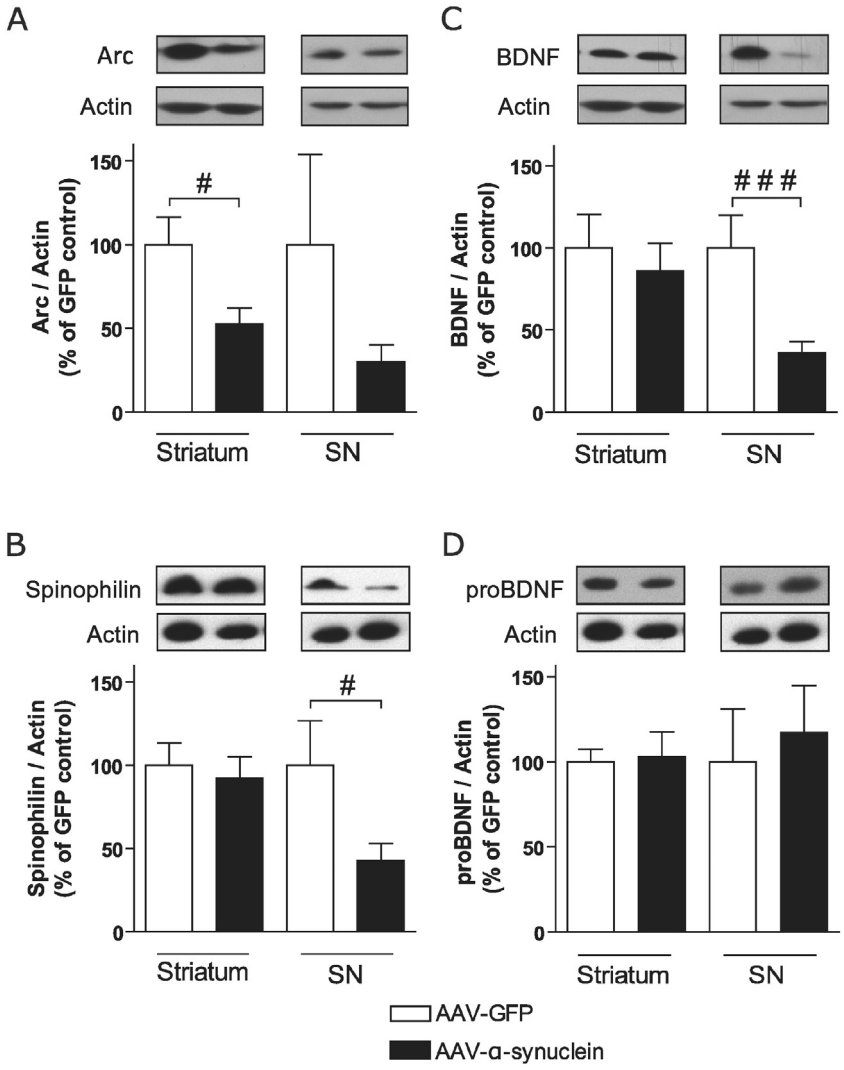

Fig. 5. Effects of nigral AAV-GFP and AAV-α-synuclein administration on striatal or nigral Arc, spinophilin, BDNF and pronBDNF levels. (A–D) Immunoblots and histograms of the ratio between the amount of Arc (A), spinophilin (B), BDNF (C) and pro-BDNF (D) and of actin band intensities, in the striatum and SN of rats 8 weeks after injection with AAV-GFP or AAV-α-synuclein. Upper immunoblots illustrate Arc (50 kDa), spinophilin (130 kDa), BDNF (14 kDa) and pro-BDNF (28 kDa) levels of the proteins (left: AAV-GFP group, right: AAV-α-synuclein group). AAV-α-synuclein induced a decrease of striatal Arc levels (A), of spinophilin (B) and BDNF (C) nigral levels compared to the AAV-GFP control group. Data are normalized to the AAV-GFP group and expressed as mean ± SEM. $^{\#}p < 0.05$ (Student's *t*-test).

"behavioral despair". Many rodent models of depression exhibits behavioral despair in the forced swim test, a behavior that is reversed by clinically used antidepressants. However, results from this acute test need to be interpreted cautiously when extrapolated to human depression, a complex multifactorial chronic disorder. Nonetheless, here we demonstrate that the α-synuclein group displayed a reduced climbing behavior already 3 weeks after the viral vector administration. This is the first time a depressive-like behavior is observed at a premotor stage in the AAV-α-synuclein model, recapitulating human PD. It has previously been shown that climbing behavior in the forced swim test can be increased by norepinephrine reuptake blockers (Detke et al., 1995, Lucki, 1997, Cryan and Lucki, 2002). Noradrenergic cell loss and combined depletion of noradrenaline and dopamine levels have been associated with depression in PD (Delaville et al., 2011). Our present data indicates a role for the dopaminergic system in the depressive-like phenotype, perhaps via direct interactions between α-synuclein and TH (Perez et al., 2002) or with the dopamine transporter (Lee et al., 2001), but we cannot exclude an indirect effect also on the noradrenergic system. The depression-like behavior in the forced swim test in response to nigral α-synuclein overexpression could also indicate an altered stress reactivity. It would be interesting in future studies to explore whether animals with nigral α-synuclein overexpression are more susceptible to other stressors, such as social defeat stress.

To measure anhedonia in α-synuclein overexpressing animals, we used the sucrose preference test, reflecting the hedonic drive in a locomotor-independent manner (Cryan and Holmes, 2005). Anhedonia, defined as a "markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day", is considered as one of the core symptoms of depression in PD (Chaudhuri et al., 2006, Aarsland et al., 2011). The preference for the sucrose solution was found to be significantly reduced in animals overexpressing α-synuclein at 8 weeks post-injection, further supporting that this model manifests a depressive-like phenotype which is maintained upon aging. Our data agree with a recent study showing that sucrose preference was reduced 22 days after nigral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) (Santiago et al., 2015). Moreover, this toxin model has also been shown to induce a depressive-like behavior in the forced swim test along with neurotransmitter alterations that are similar to those observed in PD (Santiago et al., 2014). Another study also demonstrated motivational deficits and affective impairments after 6-OHDA lesioning (Drui et al., 2014). Specifically, 3 weeks after the toxin infusion, there was an increased immobility time in the forced swim test and a decreased percentage

of time spent in the open arms of the elevated plus-maze. No parallel alterations were found on sucrose preference or on motor functions. In another PD toxin model, using 1-methyl-4-phenyl-1,2,3,6-tetrahyropyridine (MPTP) intranigral infusions, it was shown that animals displayed an increased immobility time in the forced swim test 14 days after the surgery (Castro et al., 2013). This was later confirmed in another study, in which animals demonstrated an increased immobility time and decreased swimming time 22 days after the MPTP administration (Barbiero et al., 2014).

It is important to note that a recent study using nigral human α-synuclein overexpression did not find behavioral deficits in the depressive-like tests (Campos et al., 2013). However, compared to our study, Campos et al. (2013) used different coordinates for α-synuclein injection (−5.3 mm; ±1.8 mm; −7.4 mm instead of −5.3 mm; ±1.7 mm; −7.2 mm), a different serotype for the vector (AAV2 instead of AAV6) and a different volume of injection (2 μL instead of 3 μL). Moreover, using their protocol, Campos et al. (2013) did not find any cognitive deficits, which is in contrast to several reports using α-synuclein models (Freichel et al., 2007, Masliah et al., 2011, Magen et al., 2012).

In addition to depression, many studies suggest comorbidity between anxiety and PD (Menza et al., 1993, Shiba et al., 2000, Marinus et al., 2002) and a role for dopamine in these anxiety-like behaviors (Pogorelov et al., 2005). From a clinical point of view, PD-associated depression can be distinguished from major depression by a higher rate of anxiety symptoms (Cummings, 1992). In order to assess the potential anxiety-like phenotype of animals overexpressing α-synuclein, we used the elevated plus-maze and SIH test. However, no significant effects were found on anxiety-like behaviors using these paradigms.

Hyperactivation of the HPA axis is commonly found in depression and anxiety (McEwen, 2005). It is therefore interesting that the depressive-like phenotype observed in α-synuclein overexpressing animals is associated with increased corticosterone and ACTH levels, pointing to a hyperactivation of the HPA axis. In accordance with an early depression-like phenotype, corticosterone levels were higher in feces already 3 weeks after vector injection. Normal functioning of the limbic system and hypothalamus relies on dopamine supplied by projections from the SN and VTA, which is in line with the maladaptive response to stress and the abnormal HPA axis activation observed here. Clinical studies have demonstrated that acute treatment with levodopa can reduce plasma cortisol levels in PD patients (Müller et al., 2007), thus supporting a link between dopaminergic dysfunction and increased activity of the HPA axis.

The AAV-α-synuclein model used here causes less severe dopamine degeneration than the classic 6-OHDA toxin model. Indeed, α-synuclein overexpressing animals exhibit less cell and terminal loss of TH. The progressive nature of the AAV-α-synuclein model replicates the human pathology more closely than the 6-OHDA lesion model. The neuropathological and temporal differences between the models may explain the discrepancies observed here with other studies using classic toxin models, and also why the motor deficits we observed are weaker than in the toxin models (Barbiero et al., 2014; Castro et al., 2013; Drui et al., 2014; Santiago et al., 2014; Santiago et al., 2015). It may be interesting in future studies to explore the possibility of using higher titers of AAV-α-synuclein as well as targeting other monoaminergic nuclei.

Previous work using this AAV-α-synuclein model has described an early premotor phase in which dopaminergic neurons show synaptic dysfunction, with impairments of dopamine release and reuptake, preceding overt axonal damage and neuronal degeneration in the SN (Lundblad et al., 2012, Decressac et al., 2012a, 2012b, 2012c). The depressive-like phenotype demonstrated in the forced swim test at the 3 week timepoint and in the sucrose preference test at the 8 week timepoint may therefore partly rely on a synaptic dysfunction of the surviving neurons. In this context, it is highly relevant that the current understanding of the neurobiology of depression emphasizes dysregulation of synaptic plasticity (Duman and Aghajanian, 2012). To understand the molecular mechanisms underlying this behavioral deficit we performed studies of candidate proteins involved in synaptic plasticity. We found a downregulation of Arc levels in the striatum of animals with nigral α-synuclein overexpression. The functions of Arc are multifaceted and are involved in cytoskeletal rearrangements during synaptic plasticity mechanisms in the striatum, hippocampus, amygdala, hypothalamus and cortex (Steward and Worley, 2002). Arc is enriched in neuronal dendrites, where it localizes in a distribution resembling that of F-actin (Lyford et al., 1995), interacts with cytoskeletal proteins (Fujimoto et al., 2004), AMPA receptors (Chowdhury et al., 2006) and promotes CaM kinase II-dependent neurite extension (Donai et al., 2003). It was previously demonstrated that a 6-OHDA lesion could inhibit acute cocaine-induced Arc mRNA expression in the striatum (Fosnaugh et al., 1995, Tan et al., 2000). Therefore, the reduced striatal Arc expression observed here may be involved in the inhibitory effects on motor functions and depressive-like behavior induced by α-synuclein overexpression.

We also report a reduction of mature BDNF levels in the SN α-synuclein overexpressing animals, but no significant modifications of proBDNF levels. BDNF, which is one of the most abundant neurotrophins expressed in the brain (Conner et al., 1997), promotes the survival, proliferation, and differentiation of neurons (Zhou et al., 2005), and modulates synaptic plasticity (Huang and Reichardt, 2001). The decreased BDNF levels observed here may contribute to the phenotype induced by α-synuclein overexpression. For instance, infusing antisense BDNF oligonucleotides into the rat SN pars compacta lead to animals exhibiting classic PD features (Porritt et al., 2005). Moreover, mice expressing half the normal levels of BDNF display a compromised striatal dopaminergic output and are behaviorally impaired (Fumagalli et al., 2003). It has been demonstrated that decreased TH and dopamine levels were correlated with motor dysfunctions in mutant mice lacking BDNF (Dluzen et al., 1999). This suggests a role of reduced BDNF in the motor impairments we reported in the ledged-beam transversal test. Vice versa, in some animal models of PD, BDNF delivery can rescue degenerating dopaminergic neurons in the SN pars compacta (Murer et al., 2001). For instance, in the MPTP model, cell-mediated delivery of BDNF can increase dopamine levels (Isacson et al., 1995). Moreover, post mortem clinical studies have demonstrated a reduction of BDNF levels in the SN pars compacta of Parkinsonian patients, both at the transcriptional (Howells et al., 2000) and the translational (Mogi et al., 1999, Parain et al., 1999) levels. Moreover, pathogenic mutations of α-synuclein associated with early-onset familiar PD are linked to a loss of BDNF production (Kohno et al., 2004). Interestingly, previous work from many labs indicate that BDNF underlies the antidepressant response in rats, suggesting that the decreased BDNF levels found here in the SN of AAV-α-synuclein injected animals may also be linked to the depressive-like behavior. For instance, chronic infusion of BDNF into posterior midbrain nuclei has the same effects as antidepressants in the forced swim test and in the learned helplessness paradigm (Siuciak et al., 1997), whereas hippocampal BDNF administration has antidepressant properties (Shirayama et al., 2002).

In addition to BDNF, we also found decreased spinophilin levels in the SN from rats overexpressing α-synuclein. Spinophilin is a cytoskeletal protein associated with the postsynaptic density, thus supporting the morphology of dendritic spines (Allen et al., 1997, Satoh et al., 1998, Muly et al., 2004). This protein is required for normal dopamine signaling. It interacts with the dopamine D_2 receptor (Smith et al., 1999), modulates D_2 receptor signaling efficacy, and might also serve to stabilize D_2 receptors at the cell surface (Allen et al., 2006).

In conclusion, the present study provides evidence that α-synuclein overexpression in the SN induces depressive-like behaviors which are subsequently followed by motor deficits. These behavioral dysfunctions are associated with HPA axis hyperactivation and with changes in BDNF, Arc and spinophilin levels in the nigrostriatal circuitry. Thus, our results offer a novel framework for explaining effects of α-synuclein

overexpression in emotional disturbances in experimental Parkinsonism, recapitulating important aspects of human PD.

Acknowledgments

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