Molecular Therapy
Letter to the Editor

Alpha-Synuclein mRNA Is Not Increased in Sporadic PD and Alpha-Synuclein Accumulation Does Not Block GDNF Signaling in Parkinson's Disease and Disease Models

Glial cell line-derived neurotrophic factor (GDNF) protein and gene therapy are currently under clinical investigation in Parkinson's disease (PD) patients. GDNF has profound protective effects on dopamine neurons in numerous neurotoxicant PD models in rats and non-human primates.[1,2] However, in another PD model generated by recombinant adeno-associated virus (rAAV) vector transduction of human alpha-synuclein (α-syn) into the midbrain of the rat GDNF reportedly failed to exert robust neuroprotection.[3] The marked discrepancy in preclinical therapeutic efficacy between these models of PD may have relevance to the ongoing clinical studies in sporadic PD.

In a recent rat study, Decressac et al.[4] tested whether nigrostriatal overexpression of α-syn impairs GDNF signaling, rendering nigral dopamine (DA) neurons insensitive to its trophic effect. The authors used a rAAV rat model overexpressing human wild-type α-syn to levels at least 4-fold higher than endogenous rat α-syn levels.[4,5] The authors reported that the intracellular signaling response to GDNF was blocked in nigral DA neurons. The transcription factor nuclear receptor related 1 (Nurr1) and downstream target GDNF receptor tyrosine kinase (RET) were downregulated at both the transcriptional and translational levels in the rat midbrain. Overall, these data suggest that markedly increased α-syn in the rat is toxic and may result in the disruption of GDNF signaling.

Caution must be used in translating these results to clinical PD studies. The predictive value of the rAAV α-syn overexpression rat model to sporadic PD is unclear. For example, the marked overexpression of α-syn in the rAAV model fails to reproduce the pathological state of sporadic PD. Indeed, two studies of human tissue reported that α-syn mRNA expression was *decreased* in sporadic PD patients compared to age-matched controls.[6,7] The decrease of α-syn mRNA expression was due to lower mRNA expression in individual nigral DA neurons rather than to reduction in nigral cell numbers. Kinsbury et al.[6] further showed that α-syn expression decreased gradually as the disease progressed. Further, preclinical models in which synucleinopathy is induced by intracerebral injection of pre-formed α-syn fibrils similarly report a decrease in soluble α-syn in neurons possessing Lewy body (LB)-like α-syn inclusions.[8] There is also precedence for discordant observations to be made when modeling PD in rats versus primates. Viral delivery of GDNF to the nigrostriatal pathway *decreases* tyrosine hydroxylase (TH) gene expression in rats[9] but *increases* TH in primates.[1]

Herein we evaluate the association of α-syn gene SNCA with GDNF signaling molecules in PD patient brain samples, α-syn transgenic mice, and AAV-mediated α-syn transgenic rats. We demonstrate that α-syn mRNA is not increased in sporadic PD and α-syn accumulation does not block expression of GDNF signaling molecules in PD and disease models.

We observe no increase in α-syn gene (*SNCA*) expression in DA neurons laser-captured from the substantia nigra (SN) of 10 PD subjects and 8 age-matched control subjects (Gene Expression Omnibus [GEO]: GSE20141) (Figure 1A, left panel). Laser-captured samples eliminate the differences in DA neuron numbers between patients and controls. In this microarray dataset, α-syn gene (*SNCA*) expression (column ID: 236081; Figure 1B, left table) was significantly downregulated in PD subjects compared to controls ($p = 0.035$, false discovery rate [FDR] = 0.369, fold change = −4.4).

To assess whether SN neurons with high α-syn expression might selectively degenerate in the early stages of the disease compared to neurons with reduced expression represent, we analyzed another set of microarray data with samples from early preclinical PD subjects (GEO: GSE20159). These SN samples consisted of 16 cases with subclinical, PD-related, α-syn-positive, incidental LB disease and 17 age-, sex-, and postmortem interval-matched controls. In this dataset, we again failed to detect an increase in α-syn gene expression in the subclinical PD group compared to control (Figures 1A, right panel, and 1B, right table). The subclinical PD microarray analysis indicates that SN neurons with high α-syn gene expression are not present in the early stages of the disease.

Decressac et al.[4] showed a downregulation of Nurr1 and RET at the transcriptional and translational level in the rAAV-α-syn-transduced rats, which contributed to the disruption of GDNF signaling. However, by analyzing the microarray data (GEO: GSE20159 and GSE20141), which covered subclinical and clinical stages of diseases; separately, we failed to detect any significant decrease in gene expression levels for Nurr1, RET, and other associated genes (PARK7, SLC18A2, BDNF, DDC, TH, MEF2D, and PITX3) in the sporadic PD patients (Figures 1A and 1B). Furthermore, we analyzed the association between NR4A2 (Nurr1) and SNCA gene expression in individual PD patients and did not find any significant correlation between the two genes (Figure 1C), suggesting that SNCA may not affect NR4A2 expression at the transcriptional level.

To extend our analysis, we examined transgenic α-syn mice. We previously published microarray data from synuclein transgenic mice wherein a TH promoter drove overexpression of either wild-type α-syn (THsynWT) or doubly mutated (A30P and A53T) synuclein (THsynDM).[10] We observed no significant downregulation in gene expression for Nurr1 and downstream target genes, including RET, PARK7, SLC18A2, BDNF, DDC, MEF2D, and PITX3, in the transgenic mice. We further validated the gene expression for Nurr1 and RET by RT-PCR and protein expression for

A

B

GSE20141				
Column ID	Gene Symbol	p-value (PD vs. CTRL)	FDR (PD vs. CTRL)	Fold-Change (PD vs. CTRL)
236081_at	SNCA	0.0352458	0.369667	-4.4
205311_at	DDC	0.261134	0.600741	-2.0
200006_at	PARK7	0.509899	0.780874	-1.4
208291_at	TH	0.606374	0.83407	-1.3
205857_at	SLC18A2	0.750823	0.904471	-1.2
239367_at	BDNF	0.530233	0.792913	-1.2
211421_at	RET	0.77536	0.914404	-1.2
204621_at	NR4A2	0.811232	0.930226	-1.1
225641_at	MEF2D	0.787338	0.919389	1.1
214347_at	DDC	0.495237	0.771312	1.2
203003_at	MEF2D	0.258564	0.598677	1.4
208277_at	PITX3	0.124131	0.473179	1.5

GSE20159				
Column ID	Gene Symbol	p-value (PD vs. CTRL)	FDR (PD vs CTRL)	Fold-Change (PD vs. CTRL)
ILMN_1793988	BDNF	0.18673	0.999907	-1.0
ILMN_2228463	DDC	0.774231	0.999907	-1.1
ILMN_1763228	MEF2D	0.294463	0.999907	-1.1
ILMN_2339955	NR4A2	0.327751	0.999907	-1.1
ILMN_1744713	PARK7	0.798972	0.999907	-1.0
ILMN_1666526	PITX3	0.551272	0.999907	-1.0
ILMN_1666314	RET	0.293748	0.999907	-1.1
ILMN_2070003	SLC18A2	0.260118	0.999907	-1.2
ILMN_1701933	SNCA	0.0938772	0.999907	-1.3
ILMN_1677273	TH	0.784114	0.999907	1.1

C

D

E

Figure 1. Nurr1 and Associated Genes Expression in Sporadic PD Patients and α-syn Transgenic Mice

(A) Hierarchical clustering heatmaps for microarray datasets GEO: GSE20141 (left panel) and GSE20159 (right panel). (B) Expression of genes of interest in the microarray datasets (GEO: GSE20141, left table; GEO: GSE20159, right table). The p values as well as Benjamini-Hochberg false discovery rates (FDRs) and fold change were reported for every single gene. (C) The scatterplot was drawn from the robust multi-array average (RMA)-processed and logarithm 2-transformed microarray intensity data of 18 samples. The x axis represents SNCA as an independent variable, and the y axis represents NR4A2 as a dependent variable. The straight blue line is the linear regression result between the two genes SNCA and NR4A2, and the red line is gained by the local weighted scatterplot smoothing (LOWESS) regression analysis. The correlation coefficient is 0.4084 with p = 0.1442. (D) α-syn and Nurr1 protein expression in the striatum and nigra of C57, THsynWT, and THsynDM mice. Striatum (STR) and nigra (SN) tissues were microdissected from 6-month-old transgenic mice with expression of wild-type synuclein (THsynWT) or doubly mutated α-syn (A30P & A53T; THsynDM) or age-controlled C57. The expression of α-syn and Nurr1 was determined by western blot analysis. The signal density ratio of α-syn over action was quantified. (E) α-syn, Nurr1, and RET gene expression in the SN were determined by qRT-PCR.

Nurr1 by western blotting and again failed to detect significant decreases for Nurr1 and RET at the transcript level and Nurr1 at the protein level in the α-syn transgenic mice compared to wild-type controls (Figures 1D and 1E).

Finally, we also used rAAV to overexpress α-syn in a manner very similar to Decressac et al.[4] rAAV expressing human wild-type

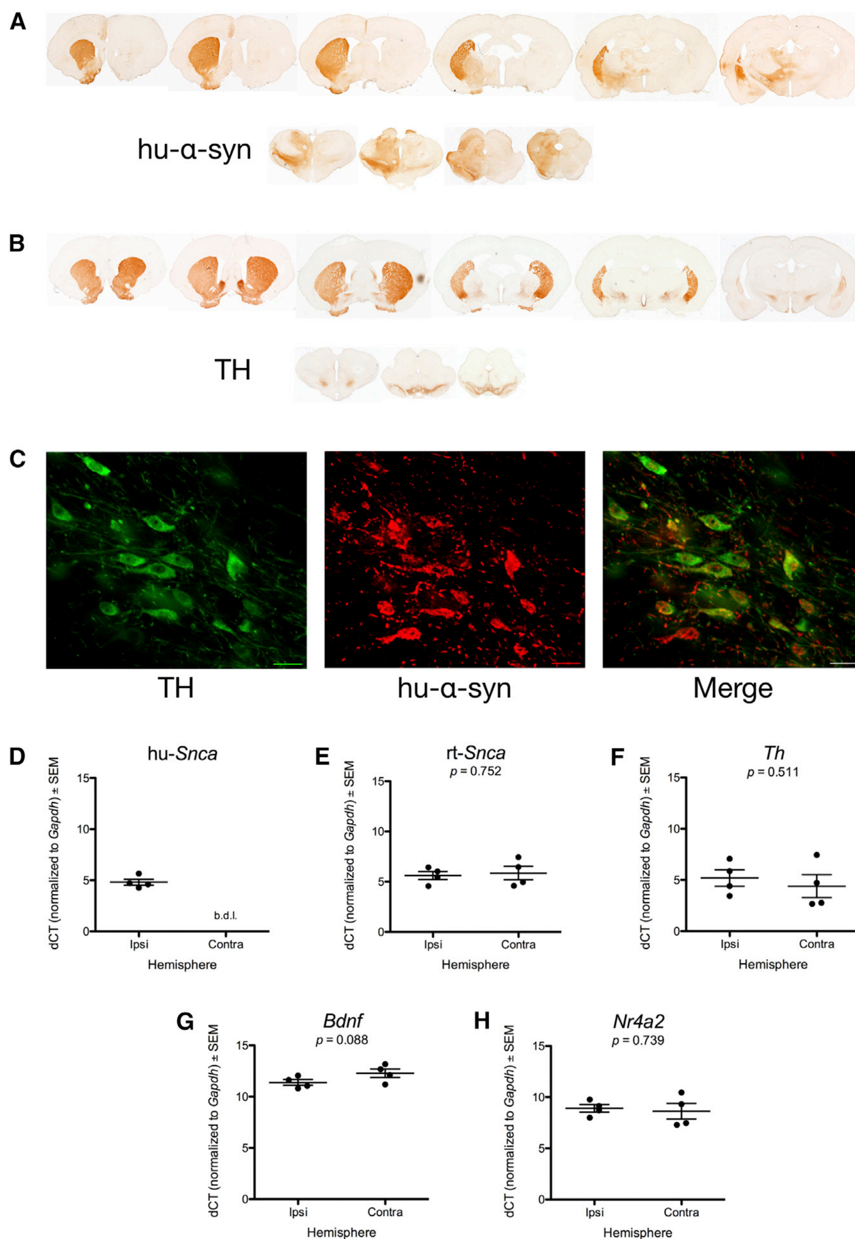

Figure 2. Moderate Viral Vector-Mediated α-syn Overexpression in the Rat Nigrostriatal System Does Not Decrease rat α-syn, BDNF, TH, or Nurr1 in the SN

Unilateral intranigral injections of recombinant rAAV2/5-α-syn results in human wild-type α-syn immunoreactivity in the nigrostriatal system. (A) Representative coronal sections demonstrating immmunoreactivity to human wild-type α-syn in the nigrostriatal hemisphere ipsilateral to injection. (B) Both hemispheres were labeled with antisera to tyrosine hydroxylase (TH). (C) Dual-label immunofluorescence identifies THir neurons of the SNpc that co-express human α-syn. A non-significant ~10% loss of SNpc neurons was observed at 8 weeks following injection. (D) SN tissue punches analyzed using qPCR demonstrate human α-syn transgene (hu-*Scna*) in the ipsilateral, but not the contralateral, SN. (E–H) No differences between the ipsilateral and contralateral SN were detected for rat α-syn (rt-*Snca*) (E), BDNF (*Bdnf*) (F), TH (*Th*) (G), or Nurr1 (*Nr4a2*) (H). Scale bars, 25 μm.

α-syn (rAAV2/5-α-syn, 2.2×10^{12} genome copies per ml) was injected unilaterally into the SN as described previously.[11] Eight weeks after nigral injection, brains were processed for immunohistochemistry for human wild-type α-syn (Figures 2A and 2C) or TH (Figures 2B and 2C). Stereological analysis of TH immunoreactive (THir) neurons in the SN pars compacta (SNpc) revealed a non-significant ~10% loss of SNpc neurons at 8 weeks following injection. The ipsilateral SNpc possessed an estimated 12,626 ± 411 THir neurons compared to 11,401 ± 561 remaining THir neurons in the contralateral SNpc ($t_{(8)} = 1.764$, $p > 0.05$). This modest level of degeneration is associated with an ~50% increase in human α-syn protein in the striatum.[11,12] In contrast, Decressac et al.[5] report an ~80% SNpc loss with transduction parameters, which resulted in an ~8-fold increase in α-syn protein in the striatum.

In a separate cohort of identical rAAV2/5-α-syn-injected rats, SN tissue ipsilateral and contralateral to rAAV2/5-α-syn were examined for human wild-type α-syn transcript levels (hu-*Snca*). Expression of human wild-type α-syn transcript was only evident in the ipsilateral SN, with none detected in the contralateral SN (Figure 2D). The SN was also examined for levels of endogenous rat wild-type α-syn (rt-*Snca*), brain-derived neurotrophic factor (*Bdnf*), TH (*Th*) and Nurr1 (NR4A2). No statistically significant differences were observed due to α-syn transduction and overexpression compared to the contralateral, non-transduced, control hemisphere.

The analysis of PD brain tissue, transgenic mouse, and rAAV-transduced rat data indicate that expression of the α-syn gene (*SNCA*) is not increased in sporadic PD and α-syn accumulation does not block GDNF signaling in PD and disease models.

The analysis of public human datasets indicates that expression of the α-syn gene (*SNCA*) is not increased in the nigral dopaminergic neurons in patients with sporadic PD, which aligns with the previous studies showing no increase of *SNCA* gene expression in sporadic PD enteric neurons,[13] CSF,[14] and blood.[15] Thus, accumulation of aggregated α-syn protein in sporadic PD is unlikely due to the enhanced *SNCA* gene expression, but, rather, is mediated by downstream failure to clear the protein, owing to either a breakdown in normal protein degradation processes and/or aberrant protein

misfolding/post-translational processes that render these conformers resistant to normal degradation processes. The analysis of public human datasets also shows no change in the transcription level of Nurr1, RET, and other associated genes (PARK7, SLC18A2, BDNF, DDC, TH, MEF2Ds and PITX3) in the sporadic PD patients as well as no positive correlation of expression of SNCA and the Nurr1 gene, NR4A2. These findings at least suggest that, at the transcriptional level, GDNF signaling molecules Nurr1, RET, and other associated genes are not affected in sporadic PD.

It is also important to assess the GDNF signaling molecule Nurr1 expression at the translational level in PD patients. Chu et al.[16] reported that SN neurons lacking α-syn inclusions from sporadic PD subjects displayed Nurr1 immunofluorescence optical density (OD) measurements that were similar to age-matched controls, whereas nigral neurons with α-syn LBs exhibited significantly decreased Nurr1 measurements. However those LB-bearing neurons were only, on average, representing 3.5%–15% of total SN neurons in sporadic PD patients, as shown by recent studies.[17] It therefore appears that the majority (>85%) of SN neurons from sporadic PD contain normal protein levels of Nurr1.

The variability of rAAV α-syn gene transfer to the rat nigrostriatal pathway to elicit changes in SN dopamine neuron numbers, alterations in signaling molecules and neurobehavioral changes suggests that vector type, particle number, packaging methods, and purifications are potential contributors. Systematic evaluation of each is required to delineate which of these may be the most important determinant of the observed variability. Because these studies all require forced expression of a gene product from a virus vector administrated intracerebrally, the clinical relevance of any such models to sporadic PD is limited, if at all relevant.

Based on our PD human data, the gene expression of GDNF signaling molecules, including RET and NUR1, are not downregulated disregarding α-syn accumulation. It should be noted that Hadaczek et al.[18] reported attenuated GNDF signaling as demonstrated by decreased phosphorylated RET (pRET) in neuronal cell lines and animals deficient in ganglio-series gangliosides. Whether pRET was decreased in PD brain remains to be investigated. Interestingly, AAV2-GDNF treatment was able to restore nigral TH-positive neurons and improve behavioral dysfunction in animals with ganglio-series gangliosides deficiency, suggesting that excess GDNF may suffice to maintain effective neuroprotective signaling, regardless of decreased pRET.

In summary, there are several important conclusions from these human transgenic mice and rAAV-transduced rat data. First, α-syn gene expression levels are not increased in the early stage of PD or in association with disease progression. Second, the majority of SN neurons in PD contain normal levels of Nurr1. Third, transgenic overexpression of human α-syn in mice did not result in downregulation of Nurr1 or RET. Fourth, rAAV transduction of rat SN producing a moderate increase in human α-syn did not result in downregulation of Nurr1, TH, or BDNF. We conclude that forced and marked overexpression of α-syn, as described in the rat rAAV model by Decressac et al.,[4] is not a relevant model for human sporadic PD. Given there is no evidence to indicate that patients with sporadic PD will be refractory to GDNF therapy, clinical equipoise is warranted for ongoing GDNF therapeutic trials.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.04.018.

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Unfounded Claims of Improved Functional Outcomes Attributed to Follistatin Gene Therapy in Inclusion Body Myositis

A recent study by Mendell et al.[1] in *Molecular Therapy* claims to have demonstrated clinical and biomarker efficacy for inclusion body myositis (IBM) from follistatin gene therapy. Although the authors are to be congratulated for performing a long and difficult study, its design could not possibly support this claim. Additionally, the publication reports a different primary outcome measure than the ClinicalTrials.gov registered primary outcome measure, uses post hoc analyses that bias efficacy evidence, presents safety data in a confusing manner, and misrepresents published IBM literature.

The study is an analysis of selected data obtained in one clinical trial[2] combined with data obtained from a neuromuscular clinical practice and analyzed using a post hoc-defined primary outcome measure (Figure 1A). The clinical trial is a phase 1A, open-label, single group assignment (there was no comparator or "control" group) study of 15 patients, 9 with IBM and 6 with Becker muscular dystrophy (BMD). Three IBM patients received unilateral quadriceps dosing and are not discussed. Analysis of 6 patients with BMD participating in the trial was previously published in *Molecular Therapy*.[3]

This new study reports on the remaining 6 IBM patients.[1] They received at least 4 potentially therapeutic interventions: follistatin gene therapy (AAV-FS344) into bilateral quadriceps muscles, high-dose prednisone for approximately 60 days, a prescribed and monitored exercise program, and the well-known placebo effects that come from both participation in a clinical study alone and the receipt of open-label candidate therapies with intended clinical efficacy. In addition, the authors use aggregate 6 min walk test data from 8 IBM patients drawn from a neuromuscular clinic as a comparator to make the claim that follistatin gene therapy has clinical efficacy.

However, it is impossible for the authors to make that conclusion. Because the "treated" group received 4 possibly therapeutic interventions and the "control" patients were not matched for any of these interventions, it is impossible to attribute any outcome differences between the two groups to any specific intervention. A hypothetical design that might have allowed such a conclusion is outlined in Figure 1B; such a design is typically reserved for phase 2 studies. The authors present circumstantial arguments as to why they attribute the apparent clinical efficacy to follistatin gene therapy rather than prednisone or exercise therapy: "A question could be raised regarding efficacy entirely related to exercise, but we believe this to be highly unlikely given the failure of exercise alone (including 10-m and 30-m walk, timed-up-and-go, stair climbing) to improve function in the absence of follistatin therapy." However, they neglected to cite a study[4] that did show statistically significant benefits from exercise for patients with IBM using 30-min walk time and stair climbing outcome measures.

Furthermore, the study did not control for placebo and related effects. Participants (patients and investigators) in this clinical trial were aware of the use of an intended therapeutic candidate based on cutting-edge science in an otherwise relentless progressive disease. Patient performance and its measurement, theoretically enhanced by placebo effects, was compared with patients from a neuromuscular clinic who had expectations of continued decline and whose performance and its measurement were theoretically reduced by nocebo responses. Empirically, placebo responses in IBM are readily apparent in several published IBM double-blind randomized clinical trials, and their magnitiude may exceed that seen in the current study (A.A. Amato et al., 2016, American College of Rheumatology Annual Meeting, abstract).[5,6]

The publication states that "The primary outcome for this trial was distance traveled for the 6-min walk test", yet the trial registration indicates its primary outcome measure is "Safety trial based on development of unacceptable toxicity defined as the occurrence of any Grade III or higher treatment-related toxicities Time Frame: 2 years." Another

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Supplemental Information

Alpha-Synuclein mRNA Is Not Increased in Sporadic PD and Alpha-Synuclein Accumulation Does Not Block GDNF Signaling in Parkinson's Disease and Disease Models

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Materials and Methods

Microarray gene expression analysis

Two microarray datasets of human brain samples were used for differential gene expression analysis in this study. They are publicly available in the Gene Expression Omnibus (GEO) under GEO accession numbers GSE20141 and GSE20159. GSE20141 used Affymetrix GeneChip Affymetrix Human Genome U133 Plus 2.0 Array, and GSE20159 used Illumina HumanHT-12 V3.0 expression beadchip. Raw data were obtained from Gene Expression Omnibus (GEO), and thereafter Pre-processing and differential expression analysis were performed. R/Bioconductor was used for data processing and analysis. For pre-processing, Robust Multi-array Average (RMA) was applied on the Affymetrix dataset (GSE 20141); Logarithm to base 2 transformation and quantile normalization were performed on the Illumina dataset (GSE20159) sequentially. Thereafter, unequal-variance, two-sample t-test was used to detect significantly expressed genes between PD subjects and controls. Hierarchical clustering was performed on datasets GSE20141 and GSE20159. Sample clustering was performed separately in both the PD subject group and Control group. An Euclidian method was used for distance metric, and average linkage was applied for linkage. Broad Institute Gene-E version 3.0.111 was used to generate heatmaps.

Human α-syn overexrepssion transgenic mice

The transgenic mouse models overexpressing human WT or DM SYN under the control of the 9-kb rat tyrosine hydroxylase promoter were previously developed in our laboratory. We have since induced homozygosity at the α-synuclein locus for these models (THsynWT and THsynDM). These transgenic mice are in C57BL6 background, male, at the age of 6 months old. The median weight was 30g (28-32 g). The animals were generated and bred in house.

Total RNA, genomic DNA and protein extraction

Total RNA, genomic DNA and protein were extracted using All-in-One kit (Norgen Biotek, Thorold, Canada). Cell media were aspirated then the cells were washed in 2mL PBS. Cell lysates were prepared by adding 350 uL of lysis solution followed by 200 uL of ethanol. Up to 600 uL of the lysate was applied to the All-in-One spin column and spun at 14,000 g for 2 min. Flow through were retained and store at -20 °C for protein purification. 400 uL of the RNA wash solution were added to the column and spin for 2 min. Total RNA were eluted with 50 uL of the RNA elution solution. Then 500 uL of the genomic DNA were added to the column and the columns were spun for 2 min. Genomic DNA were eluted with 100 uL of genomic DNA elution buffer adding to the column.

Western blot analysis

Tissue lysates were homogenized in buffer containing 50mM HEPES (pH 7.6), 150 mM NaCl, 1% Tritox X-100, 1 mM Na3VO4, 10 mM NaF, 30 mM Na4P2O7, 10% (v/v) glycerol, 1 mM microcystin. Protein was determined by the BCA (bicinchoninc acid) protein assay kit from

Piece (Thermo Fisher Scientific; Waltham, MA). Samples were resuspended in Laemmli buffer, and proteins were separated by 12% SDS-PAGE. Proteins were transferred to polyvinylidenedifluoride membranes (Millipore; Billerica, MA) and suggest to western blot analysis. After incubation with primary antibody, membranes were washed and incubated with secondary antibody linked to horseradish peroxidase (Bio-Rad; Hercules, CA). Results were quantified by densitometry using ImageJ, 1.48V (NIH).

Rats

Nine, male, Sprague-Dawley rats (Harlan, ≈250 g) were used. Animals were allowed food and water *ad libitum* and were housed in reverse dark-light cycle conditions in an AAALAC approved facility. The Michigan State University Institutional Animal Care and Use Committee specifically approved this study.

Production of Recombinant Adeno-Associated Viral Vectors

The production of the α-syn-expressing, recombinant adeno-associated viral vector pseudotype 2/5 (rAAV2/5-α-syn) was conducted as described previously [1], however unlike previous reports production was conducted in-house at Michigan State University. Briefly, human cDNA was used to produce a clone of the wildtype SNCA gene that was then inserted into the AAV plasmid backbone. The chicken beta actin/cytomegalovirus enhancer-promoter hybrid was used to drive the expression of the SNCA gene. The vectors contained AAV2 inverted terminal repeats and co-transfection was accomplished through rAAV rep and cap genes and adenovirus helper functions, thereby packaging the vector into AAV5 capsids. Iodixanol gradients and q-sepharose chromatography were used to purify vector particles and dot blot to measure the vector titer [2]. The viral vectors were stored at 4°C and were never frozen. Surfaces in contact with virus were coated beforehand with Sigmacote (Sigma-Aldrich, St. Louis, MO). The rAAV2/5-α-syn titer used in this study was estimated in-house to be 2.2 x 10^{12} genome copies per ml.

Intranigral Vector Injections

Intranigral vector injections were conducted as described previously [1]. Prior to surgery, anesthesia was induced with 5% isoflurane in O_2, and rats were maintained under anesthesia with 2% isoflurane in O_2. Rats received two unilateral, intranigral injections (AP -5.3 mm, ML +2.0 mm, DV −7.2 mm and AP -6.0 mm, ML +2.0 mm, DV −7.2 mm relative to dura mater) of rAAV2/5-α-syn (injection rate 0.5 µl/minute, 2.0 µl per site).

Euthanasia and Tissue Processing

At eight weeks post vector surgery, rats were deeply anesthetized (60 mg/kg, pentobarbital, i.p.) and perfused intracardially with heparinized normal saline at 37°C followed by ice-cold saline (rats for PCR analysis) or ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (rats for IHC analysis). PFA-perfused and postfixed brains were frozen on dry ice and sectioned at 40 µm thickness using a sliding microtome in six series.

α-Synuclein Immunohistochemistry for Transduction Verification

One series (i.e., every sixth section) was stained with antisera for α-synuclein (α-syn) using the free-floating method, as described previously[1]. Tissue was blocked in normal goat serum and incubated overnight in primary antisera directed against wild-type human α-syn (mouse monoclonal anti-human α-syn, Invitrogen AHB0261, 1:2000 dilution) in 1.0% normal goat serum (Gibco, Catalog #16210-072). Cell membranes were permeabilized with the addition of Triton-X (0.5%, Sigma X-100) to the 0.1 M Tris buffer during incubations. Sections were then incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400 dilution) and followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). α-Syn immunoreactive (α-syn-ir) neurons were visualized upon exposure to 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H_2O_2 in tris-buffered saline (TBS). Sections were mounted on subbed slides, dried flat overnight under standard temperature and pressure conditions, dehydrated with ethanol and then xylenes and finally coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Tyrosine Hydroxylase Immunohistochemistry

One series (i.e., every sixth section) was stained with antisera for tyrosine hydroxylase (TH) using the free-floating method. Tissue was blocked in serum and incubated overnight in primary antisera directed against TH (Chemicon MAB318, mouse anti-TH, 1:4000). Cell membranes were permeabilized with the addition of Triton-X (0.3%) to the 0.1 M Tris buffer during incubations. Sections were then incubated in biotinylated secondary antisera against mouse IgG (Chemicon AP124B, 1:400) and followed by the Vector ABC detection kit employing horseradish peroxidase (Vector Laboratories, Burlingame, CA). TH immunoreactive (THir) neurons were visualized upon exposure to 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H2O2 in Tris buffer. Sections were mounted on subbed slides, dried flat overnight under standard temperature and pressure conditions, dehydrated with ethanol and then xylenes and finally coverslipped with Cytoseal (Richard-Allan Scientific, Waltham, MA).

Unbiased Stereology of THir Neurons in the SNpc

The number of THir neurons in the SNpc ipsilateral and contralateral to vector injections was quantified using unbiased stereology with the optical fractionator principle. Using a Nikon Eclipse 80i microscope, Retiga 4000R (QImaging, Surrey, BC, Canada) and Microbrightfield StereoInvestigator software (Microbrightfield Bioscience, Burlingame, VT), THir neuron quantification was completed by drawing a contour around the SNpc borders at 4X, and THir neurons were counted according to stereological principles at 60X (NA 1.4); estimates of total counts per structure were extrapolated by the software. The Schmitz-Hof Coefficients of Error were less than or equal to 0.10 for all analyses.

RNA isolation, conversion to cDNA and qPCR

RNA extraction was performed using the RNA Clean and Concentrator kit (Zymo Research, Irvine, CA) and eluted into 15 µl H_2O. RNA from tissue was then converted into cDNA using SuperScript VILO Master Mix (Life Technologies, Grand Island, NY). The RNA was assumed to be converted 100% to cDNA. PCR reactions were run in 30 µl using target specific, Taqman hydrolysis probes for the gene of interest and were normalized to *Gapdh* (Ref 4351317, Applied Biosystems/Life Technologies, Carlsbad, CA). Normalized gene expression was determined by differences in the cycle thresholds (Ct) between genes of interest and *Gapdh* (ΔCt) on a ABI 7500 qPCR System (Applied Biosystems). The viral vector-injected SN ("ipsilateral") was examined for transcript expression of the transgene human, wildtype *Snca* (Applied Biosystems assay ID Hs01103386_m1). Robust expression was required on the ipsilateral side for inclusion in this experiment; no transgene expression was detected on the contralateral side. The SN was also examined for the following transcripts (Applied Biosystems Assay ID# following): rat, wildtype *Snca* (Rn00569821_m1), *Bdnf* (Rn02531967_s1), *Th* (Rn00562500_m1) and *Nurr1* (NR4a2; Rn00570936_m1). Data collected by qPCR were compared between hemispheres using the Relative Expression Software Tool 384 (REST-384 version 2) calculation software for the relative expression in real-time PCR using Pair-wise fixed reallocation randomization test [3].

Statistics

Quantitative data are presented as the mean ± SEM. Statistical significance was either assessed via an unpaired Student's t test or an ANOVA test with Student-Newman-Keuls post hoc analysis. Assessments were considered significant with a $p < 0.05$.

References

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