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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 2.2×10^{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

1 Gombash, S. E. *et al.* Morphological and Behavioral Impact of AAV2/5-Mediated Overexpression of Human Wildtype Alpha-Synuclein in the Rat Nigrostriatal System. *Plos One* **8**, doi:ARTN e8142610.1371/journal.pone.0081426 (2013).

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