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Michael J. Fox Foundation

Expression Analysis of Rats Genetically Modified to Express the Human Alpha-Synuclein Gene

Final Report

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Goal: The goal of this project was to perform the following analyses on the samples of cortex, hippocampus and kidney tissues from three (3) different rat lines genetically modified to express the human alpha-Synuclein gene (four rats per line):

1. SNCA copy number
2. SNCA mRNA expression (by qRT-PCR)
3. SNCA protein expression by Western Blot.

I. Materials and Methods.

Animals

Three genetically modified rat lines (four rats per line) have been received from the Taconic Farms, Inc.: NTac:SD-Tg(SNCA)446Cjli (line 10680, wild type human SNCA), NTac:SD-Tg(SNCA*A53T)268Cjli (line 10678), and NTac:SD-Tg(SNCA*E46K)70Cjli (line 10679) – Table 1. Tissues from cortex, hippocampus and kidney were excised and stabilized in RNAlater solution (a portion of each tissue was snap-frozen and stored at -80°C for Western Blot analysis). Total RNA was isolated from the tissues (~100 mg) using Invitrogen's Trizol reagent (Cat# 15596-018).

Table 1. MJFF0011: Rats Genetically Modified to Express the Human Alpha-Synuclein Gene .

Animal ID	Line Name	Line #	DOB	Sex
GT1-M000267	NTac:SD-Tg(SNCA*A53T)268Cjli	10678	07/30/2012	M
GT1-M000268	NTac:SD-Tg(SNCA*A53T)268Cjli	10678	07/30/2012	M
GT1-M000269	NTac:SD-Tg(SNCA*A53T)268Cjli	10678	07/30/2012	M
GT1-M000271	NTac:SD-Tg(SNCA*A53T)268Cjli	10678	07/30/2012	M
GT1-M000312	NTac:SD-Tg(SNCA*E46K)70Cjli	10679	08/06/2012	M
GT1-M000313	NTac:SD-Tg(SNCA*E46K)70Cjli	10679	08/06/2012	M
GT1-M000316	NTac:SD-Tg(SNCA*E46K)70Cjli	10679	08/06/2012	M
GT1-M000317	NTac:SD-Tg(SNCA*E46K)70Cjli	10679	08/06/2012	M
GT1-M000263	NTac:SD-Tg(SNCA)446Cjli	10680	07/30/2012	M
GT1-M000265	NTac:SD-Tg(SNCA)446Cjli	10680	07/30/2012	M
GT1-M000266	NTac:SD-Tg(SNCA)446Cjli	10680	07/30/2012	M
GT1-M000268	NTac:SD-Tg(SNCA)446Cjli	10680	07/30/2012	M

qRT-PCR Expression Analysis

The expression analysis of the SNCA transgenic lines was performed using a Quantitative Reverse Transcriptase PCR (qRT-PCR) technique, with a SNCA-specific TaqMan® Gene Expression Assay purchased from Applied Biosystems (assay# HS00240906_m1).

Total RNA was isolated from the tissues (~100 mg) using Invitrogen's Trizol reagent (Cat# 15596-018). The DNA-free RNA was purified using the Ambion's Turbo DNase (Cat #AM 1907) according to the manufacturer's protocol. The purified RNA was then quantified by measuring the absorbance at 260 nm and the quality was assessed by the A260/280 ratio.

All the qRT-PCR expression analysis data were analyzed by a "ΔCT - ΔΔCT" method (ABI).

$\Delta Ct = Ct\ (FAM\text{-}SNCA) - Ct\ (VIC\text{-}GAPDH)$
$\Delta\Delta Ct = \Delta Ct_{cal} - \Delta Ct_{sample}$,

ΔCT_{cal} represents a value calculated for a calibrator, i.e. a sample with the lowest expression. The amount of target, normalized to an endogenous reference (GAPDH, a "housekeeping" gene) and relative to a calibrator, is calculated by:

$2^{-(\Delta\Delta Ct)}$

Copy Number Analysis.

A TaqMan copy number assay for this project was developed earlier and described in detail in Interim Report#1 for MJFF-9. Briefly: the DNA copy number were determined by a standard curve method where for the standard curve the calculated number of copies of the wild type SNCA BAC DNA (designed and built for MJFF-9 project) were mixed with 5 ng of DNA prepared from a wild type Sprague-Dawley rat. A TaqMan® Copy Number Assay (Hs02236645_cn, Chr.4:90758137, located within exon 2 of SNCA gene) was purchased from Invitrogen. The data were analyzed by plotting ΔCt values against the known copy number.

Western Blot Expression Analysis.

Western Blot analysis was performed on the protein lysates from the cortex, hippocampus and kidneys of the mice described in the previous sections. The tissue samples were homogenized in Tissue Extraction Reagent I (Invitrogen, FNN0071) with 1X Complete Protease Inhibitor Cocktail (Roche, 04 693 124 001). The homogenates were held on ice, and then centrifuged twice at 15000g, for 10 min at 4°C. The clear supernatant was collected as the protein sample. Total protein concentration was determined by the Micro BCA Protein Assay Kit (Pierce, 23235). Vertical gel electrophoresis was performed using XCell4 SureLock™ Midi-Cell Runner (Invitrogen, Cat.# WR0100), and Western Blot was performed using The iBlot® Gel Transfer Device (Invitrogen, Cat.# IB1001). The following antibodies and positive control protein were used:

Primary Antibody: α-Synuclein (Syn204) Mouse mAb (#2647) from Cell Signaling Technology
Secondary Antibody: Peroxidase labeled anti-mouse antibody (Part#NIF825, GE Healthcare/Amersham ECL Western Blotting Analysis System RPN2108).
Positive Control Protein: Recombinant full length human α-Synuclein E46K from Abcam (#ab51188).

II. Results

IIIa. qRT-PCR Expression Analysis.

The results summary of the qRT-PCR expression analysis is presented in Table 2 and Fig. 1 (individual data – in Table 3). The expression of the transgenes was relatively high in the cortex and hippocampus of all Tg rats in all three lines, very low in the kidneys and not detectable in the wild type control. The highest expression level was detected in 10679-SNCA*E46K rats, the lowest – in 10678- SNCA*A53T line. Rat 10679-312 was originally genotyped as Tg but after the expression and copy number data were recorded showing no expression and zero copies of the transgene, it was re-typed and identified as WT.

Table 2. qRT-PCR Expression (Summary).

	10678-SNCA*A53T		10679-SNCA*E46K		10680-SNCA	
	Mean	STDEV	Mean	STDEV	Mean	STDEV
Cortex	105.37	21.12	578.63	129.00	346.46	35.19
Hippocampus	68.01	12.45	253.12	58.33	332.22	49.90
Kidney	1.00	0.37	1.65	0.13	4.43	1.57

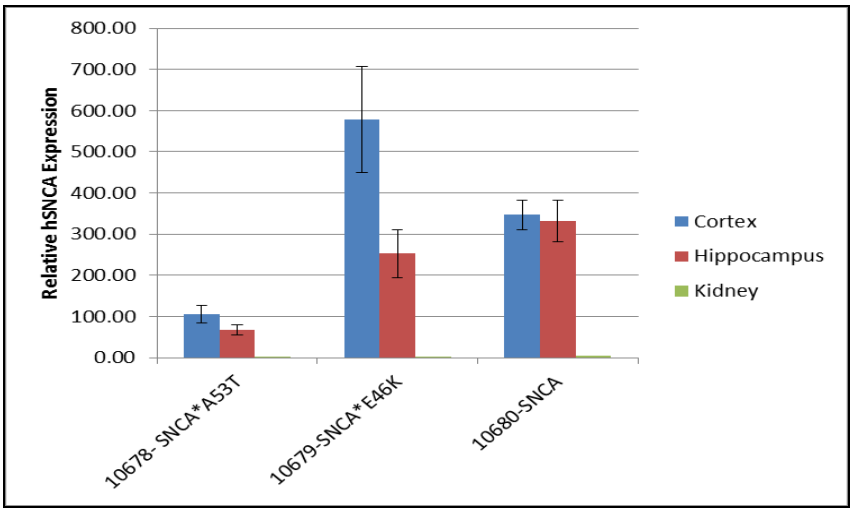

Fig. 1 qRT-PCR Expression Analysis

IIIb. Copy Number Analysis.

The copy number values were not detectable in the wild type control; in the lines NTac:SD-Tg(SNCA)446Cjli (line 10680), NTac:SD-Tg(SNCA*A53T)268Cjli (line 10678), and NTac:SD-Tg(SNCA*E46K)70Cjli (line 10679) average copy numbers were respectively 3.65 ± 1.8, 42.08 ± 4.8, and 25.62 ± 3.1 (Table 3).

Table 3. qRT-PCR Expression and Copy Number (Individual Data).

Line	Animal ID	Genotype	Sex	DOB	Tissue	Relative hSNCA Expression		Copy number	
						Mean	STDEV	Mean	STDEV
10678	267	Tg(SNCA*A53T)/-	M	7/30/2012	Cortex	114.31	21.30	40.28	3.08
					Hippocampus	64.69	11.52		
					Kidney	1.01	0.08		
	268**	Tg(SNCA*A53T)/-	M	7/30/2012	Cortex	74.92	11.38	89.45	19.16
					Hippocampus	83.43	13.04		
					Kidney	1.46	0.06		
	269	Tg(SNCA*A53T)/-	M	7/30/2012	Cortex	109.10	26.07	47.55	3.53
					Hippocampus	53.51	10.84		
					Kidney	0.95	0.17		
	271	Tg(SNCA*A53T)/-	M	7/30/2012	Cortex	123.16	5.76	38.41	4.91
					Hippocampus	70.41	19.68		
					Kidney	0.57	0.15		
		WT	M		Cortex	0.01	0.00	0.00	0.00
					Hippocampus	0.01	0.00		
					Kidney	0.00	0.00		
10679	312	Tg(SNCA*E46K)/-	M	8/6/2012	Cortex	499.13	57.07	22.66	1.72
					Hippocampus	298.87	3.17		
					Kidney	1.63	0.52		
	313	Tg(SNCA*E46K)/-	M	8/6/2012	Cortex	727.47	269.21	25.26	6.29
					Hippocampus	187.43	35.20		
					Kidney	1.78	0.11		
	316**	Tg(SNCA*E46K)/-	M	8/6/2012	Cortex	0.16	0.02	0.02	0.01
		WT			Hippocampus	0.41	0.06		
					Kidney	0.01	0.00		
	317	Tg(SNCA*E46K)/-	M	8/6/2012	Cortex	509.30	35.45	28.95	3.71
					Hippocampus	273.06	21.92		
					Kidney	1.54	0.12		
		WT	M		Cortex	0.12	0.02	0.00	0.00
					Hippocampus	0.12	0.06		
					Kidney	0.00	0.00		
10680	263	Tg(SNCA)/-	M	7/30/2012	Cortex	389.51	26.51	2.44	0.52
					Hippocampus	296.99	13.08		
					Kidney	2.73	0.34		
	265	Tg(SNCA)/-	M	7/30/2012	Cortex	360.38	41.57	3.21	0.80
					Hippocampus	307.19	51.88		
					Kidney	6.03	0.94		
	266	Tg(SNCA)/-	M	7/30/2012	Cortex	322.72	39.99	6.41	1.80
					Hippocampus	405.86	22.55		
					Kidney	5.48	0.93		
	268	Tg(SNCA)/-	M	7/30/2012	Cortex	313.24	38.83	0	0.00
					Hippocampus	318.84	1.27		
					Kidney	3.50	0.51		
		WT	M		Cortex	0.01	0.00	0	0.00
					Hippocampus	0.01	0.00		
					Kidney	0.00	0.00		

**Notes: (1) WT – wild-type Sprague-Dawley control; (2) rat 10678-0268, for which the copy number was twice as large as for the rest three animals of the group, was excluded from the calculations of the average and standard deviation values; (3) rat 10679-316 was originally shipped as a Tg(SNCA*E46K)/- transgenic but later identified as a wild-type.

IIIb. Western Blot Expression Analysis.

The results of the Western Blot expression analysis are presented in Fig. 2. hSNCA-specific immunoreactivity as revealed in all three lines with the highest expression level in line 10678 - NTac:SD-Tg(SNCA*A53T)268Cjli. The Positive Control is recombinant full length human α-Synuclein E46K from Abcam (#ab51188).

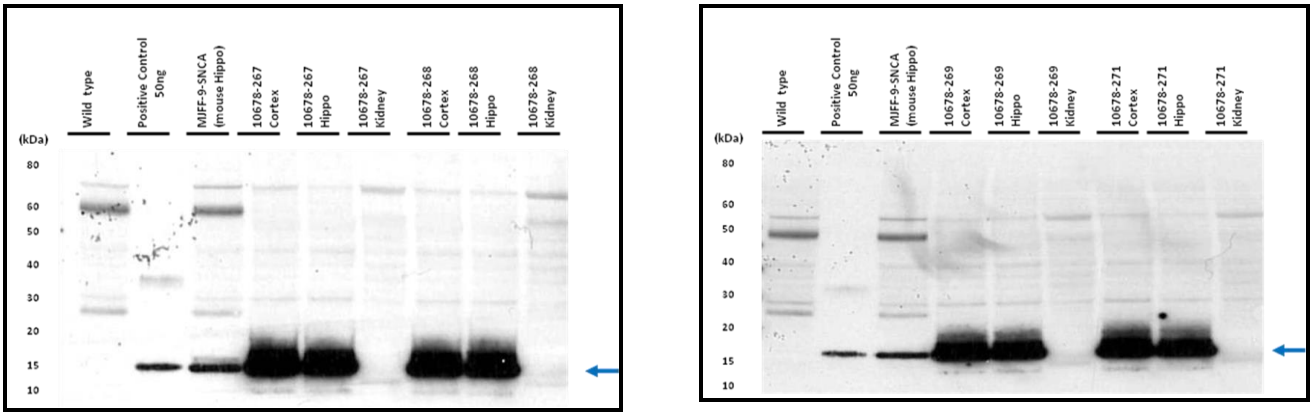

Fig. 2 Western Blot Expression Analysis.
Line 10678 - NTac:SD-Tg(SNCA*A53T)268Cjli
Each lane contained approximately 75 µg of the protein.
The position of hSCNCA-specific band is marked with blue arrow.
(Hippo=Hippocampus)

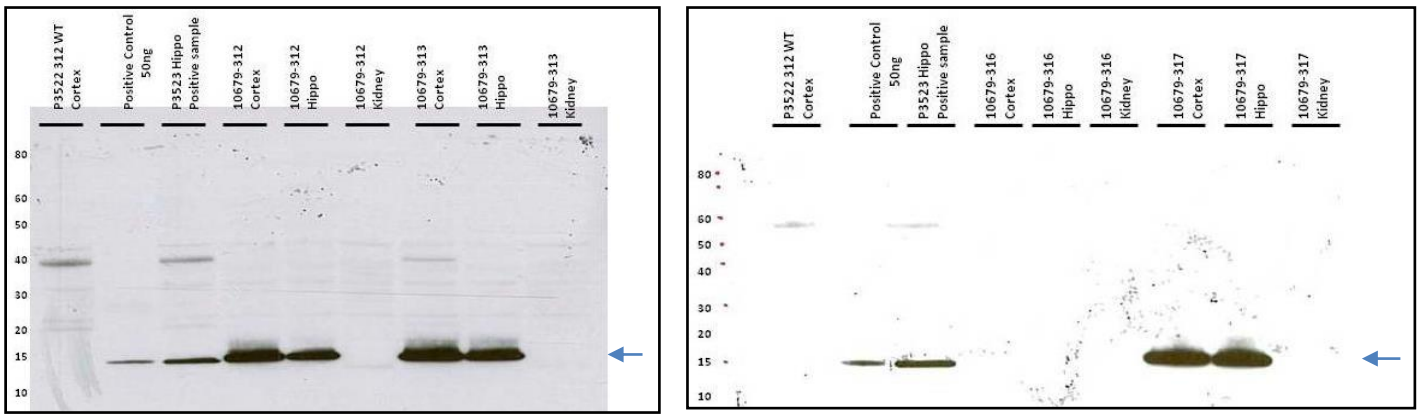

Fig. 3 Western Blot Expression Analysis.
Line 10679 - NTac:SD-Tg(SNCA*E46K)70Cjli
Each lane contained approximately 75 µg of the protein.
The position of hSCNCA-specific band is marked with blue arrow.
(Hippo=Hippocampus)

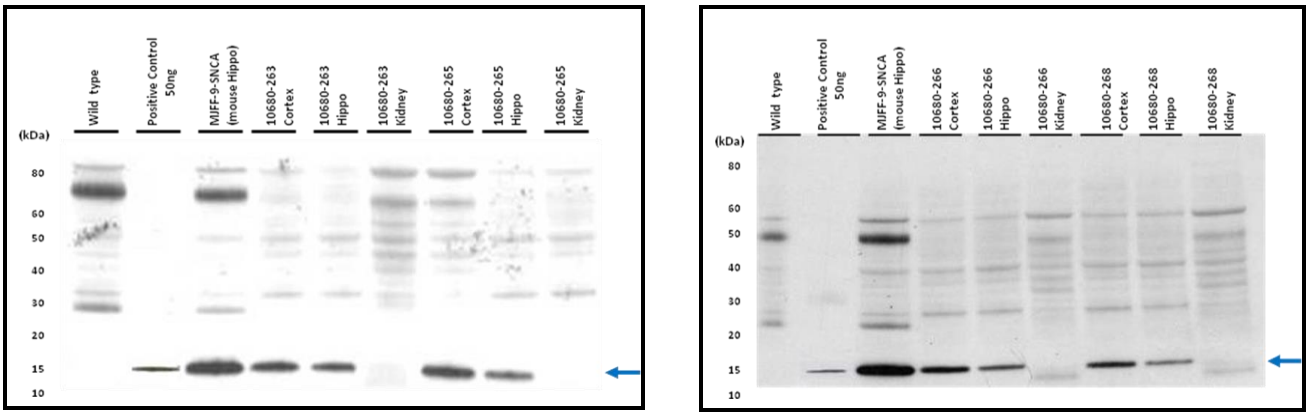

Fig. 4 Western Blot Expression Analysis.
Line 10680 - NTac:SD-Tg(SNCA)446Cjli
Each lane contained approximately 75 µg of the protein.
The position of hSCNCA-specific band is marked with blue arrow.
(Hippo=Hippocampus)

IV. Conclusions:

- The expression of the hSCNA transgenes measured by qRT-PCR was relatively high in the cortex and hippocampus of all Tg rats, very low in the kidneys and not detectable in the wild type control.
- Western Blot expression analysis revealed an hSCNA specific band in all three examined lines with the highest expression level in line 10678 - NTac:SD-Tg(SNCA*A53T)268Cjli.
- The average copy number values in the lines NTac:SD-Tg(SNCA)446Cjli (line 10680), NTac:SD-Tg(SNCA*A53T)268Cjli (line 10678), and NTac:SD-Tg(SNCA*E46K)70Cjli (line 10679) were respectively 3.65 ± 1.8, 42.08 ± 4.8, and 25.62 ± 3.1.