Replication of Alpha-Synuclein Propagation Mouse Model

Sylvie Ramboz[1], *Andrea Kudwa*[1], *Mike Olsen*[1], *Jan Kehr*[3], *Afshin Ghavami*[1], *Kelvin C. Luk*[2], *Virginia M. Y. Lee*[2], *Robert Switzer*[4], *Syed Ahmad*[4], *Terina Martinez*[5], *Mark Frasier*[5] & *Kuldip D. Dave*[5]

[1]PsychoGenics, Tarrytown, NY; [2]University of Pennsylvania, Philadelphia, PA; [3] Pronexus Analytical AB, Stockholm, Sweden; [4]NeuroScience Associates, Knoxville, TN; [5]MJFF, New York, NY

Introduction

Alpha-synuclein (aSyn) is a 140 amino acid protein implicated both genetically and neuropathologically in Parkinson's disease (PD). Increased levels of aSyn lead to neurodegeneration both *in vitro* and *in vivo* and aggregated aSyn is the primary component of Lewy bodies, the histopathological hallmark of PD. aSyn pathology has been reported in discrete areas of the brain at early stages of disease, but exhibits an ascending pattern of progression as the disease develops, suggesting propagation or transfer of aSyn (Braak and Del Tredici, *Neurology*, 2008). Findings in PD patient brains of host-to-graft transfer in fetal transplants (Kordower et al, *Nature Medicine*, 2008) and transfer in cellular and animal models (for review see Olanow and Brundin, *Movement Disorders*, 2013) have further supported this hypothesis. Even a single intracerebral inoculation of misfolded aSyn has been shown to induce Lewy-like pathology in cells that can spread from affected to unaffected regions and can induce neurodegeneration with motor dysfunction in normal wildtype mice (Luk et al., *Science*, 2012). The Michael J. Fox Foundation for Parkinson's research (MJFF) partnered with Psychogenics, in collaboration with Dr. Luk and Dr. Lee's labs, to independently replicate these seminal findings.

Methods

Animals:
C57BL6/C3H F1, alpha-synuclein heterozygote mice (C57BL/6N-*Snca*^{tm1Mjff}/J; Stock number #016123) were purchased from Jackson laboratories (Bar Harbor, ME). Alpha-synuclein heterozygote mice were bred to generate WT, Heterozygote and Homozygote mice to support study described below. Mice were housed at PsychoGenics. Animals received an unique identification numbers and were housed in polycarbonate Optimouse cages of 4-5 animals prior to infusion. All animals were examined, manipulated and weighed prior to initiation of the study to assure adequate health and suitability and to minimize non-specific stress associated with manipulation. After surgery animals were single housed unless group housing is requested. During the course of the study, 12/12 light/dark cycles was maintained. The room temperature was maintained between 20 and 23°C with a relative humidity maintained around 50%. Chow and water were provided *ad libitum* for the duration of the study. Wet chow was placed on the cage floor and was changed daily.

Alpha-synuclein monomers and preformed fibrils were generously prepared and provided by Dr. Kelvin Luk from Dr. Virginia Lee's lab.

Surgical Methods: Mice were anesthetized with ketamine hydrochloride (100mg/kg ip) and xylazine (10mg/kg ip) and stereotaxically injected in one hemisphere with recombinant alpha-synuclein fibrils (5ug). Control animals received sterile PBS. A single needle insertion (coordinates: +0.2mm relative to Bregma, +2.0mm from midline) into the right forebrain was used to target the inoculum to the dorsal neostriatum (+2.6mm beneath the dura). Injections were performed using a 10ul syringe (Hamilton, NV) at a rate of 0.1ul per min (2.5ul total per site) with the needle in place for ≥ 5 min at each target. Animals were monitored regularly following recovery from surgery, and sacrificed at various determined time points (30, 90 or 180dpi) by overdose of ketamine/xylazine.

Behavioral tests: Group size of 14 animals per treatment group and genotype

- **Wire Hang (WH):** The wire hang test of motor function was conducted by following a modified protocol described by Santa-Maria et al. Neurobiol. Aging, 2012. Mice were placed on the top of a standard wire cage lid. The lid was lightly shaken to cause the animals to grip the wires and then turned upside down. The latency of mice to fall off the wire grid was measured and average values were computed from two trials (15min apart). Trials were stopped if the mouse remained on the lid after 15min.

- **Rotorod (RR):** Mice were tested with four trials per day. Animals were loaded on the continuous rotating rod (Rotamex, Columbus, OH) 12 animals at a time. They were given a 5-min training period at a slow speed of 4 rpm. If an animal fell off the rod, it was placed back on the rod for the duration of the 5-min training period. Animals were then placed back into the home or test cage for at least one hour prior to actual testing. In the actual trial, mice were placed on the rotorod and the speed was gradually and uniformly increased to a speed of 40 rpm by 300 s. The time that each mouse remained on the rotating rod before falling 20 cm was recorded. After rotorod testing animals were placed back into the home cage.

Biochemistry Assessments: Group size of 8 animals per treatment group and genotype

- **Western blot assay:** Striatum of one side of the brain was weighed and harvested in RIPA buffer containing protease and phosphatase inhibitors (Roche). Five ml of buffer were used per gram of tissue. Samples were sonicated and cleared at 100,000xg for 30min. Protein concentrations were determined using the BCA assay (Pierce) and samples (20ug total protein) were separated on SDS-polyacrylamide gels (4-20% gradient) and transferred onto nitrocellulose membranes. Blots were blocked in 5% non-fat milk in TBS and probed using various primary antibodies as per Luk et al; Science –Supplemental Material 2013. Target antigens were detected using Luminex analyzer following incubation with the appropriate infrared secondary antibodies.

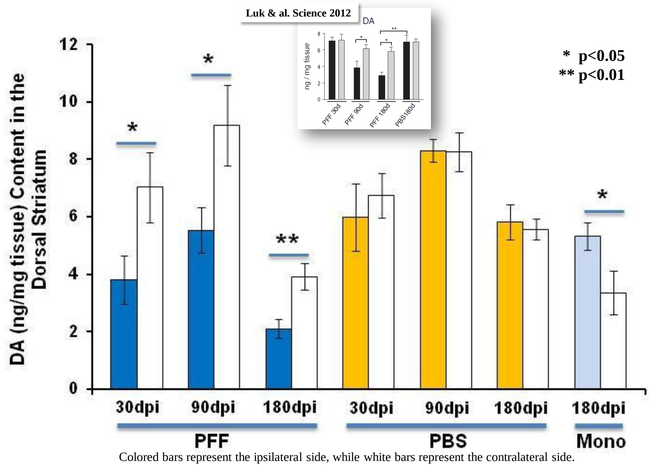
- **HPLC analysis:** Dorsal striata from the other side of the brain was dissected and homogenized in 0.2M perchloric acid including 100uM EDTA-2Na (1:10, w/v) at 0ºC. After 10 minutes on ice, homogenates were centrifuged for 15 minutes at 3,000g at 4ºC. The supernatant was mixed with 0.4M sodium acetate buffer (pH 3.0; 1:2, v/v) and filtered through a 0.22um centrifugal filter (4 minutes, 14,000g at 4ºC). Filtrates were stored at -80ºC prior to HPLC analysis. Monamines DA, NA, 5-HT and their acidic metabolites DOPAC, HVA and 5-HIAA were determined by HPLC with electrochemical detection.

Immunohistochemistry and pathology Assessments: Group size of 6 animals per treatment group and genotype

- **Immunohistochemistry:** Mice were perfused with 4% paraformaldehyde, and fixed brains were multiple embedded in gelatin matrix using MultiBrain Technology by NeuroScience Associates. Blocks of brains were sections at 35 micron thickness and stored in antigen preserve solution. Sections were stained free floating with pS129 alpha-synuclein antibody (81A, provided by Dr. Kelvin Luk) at 1:150K dilution and DAB was used to visualize the reaction product. Immunohistochemistry analyses with antibodies directed against total alpha-synuclein, GFAP, IBA1, and ubiquitin as well as stereological analyses with tyrosine hydroxylase are ongoing.

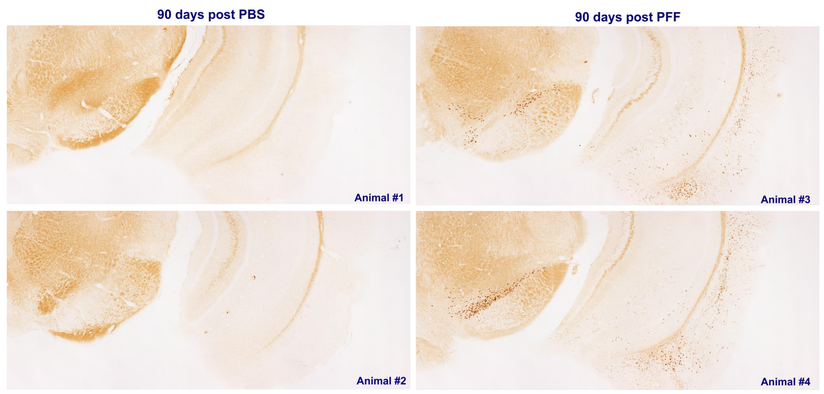
Statistics Methods: Behavioral data was evaluated using repeated measures analysis of variance (age as dependent factor) carried out with SAS (SAS Institute Inc.) using Mixed Effect Models; Genotype and Gender were the independent factors This approach is based on likelihood estimation which is more robust to missing values than moment estimation. The models were fitted using the procedure PROC MIXED (Singer, 1998). In vitro data were analyzed using two way ANOVA with Bonferroni multiple comparaisons test. Within each behavioral test, statistical significance was evaluated for each line separately using the appropriate age matched control. For in vitro studies, statistical significance was evaluated for ipsilateral side versus contralateral side.

Synthetic pff inoculation results in significant reduction of striatal dopamine concentration

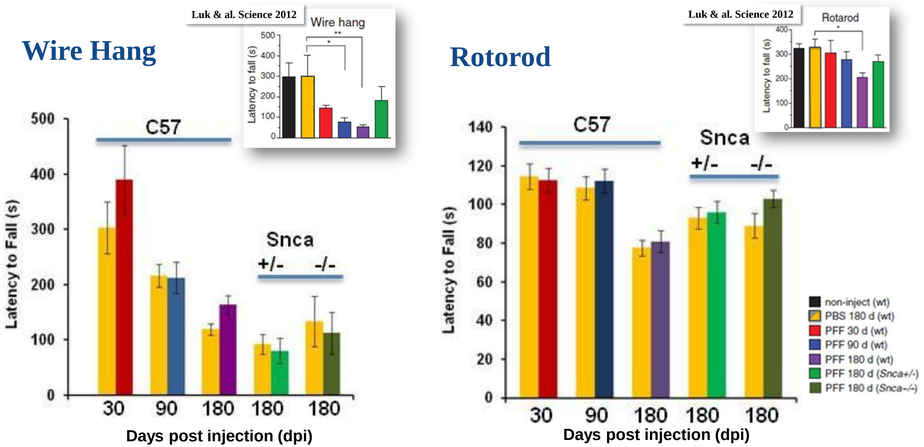


Colored bars represent the ipsilateral side, while white bars represent the contralateral side.

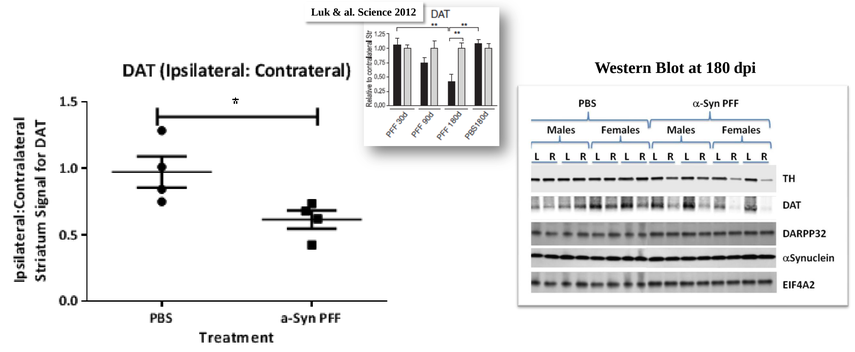
Striatal pff inoculation leads to pS129 aSyn pathology in substantia nigra and cortex



No behavioral dysfunction observed in mice up to 180 days post-inoculation with aSyn pff's



Reduction of striatal dopamine transporter post aSyn pff inoculation suggests loss of presynaptic terminals



No change in Tyrosine hydroxylase, DARPP32 nor in total alpha-synuclein.

Summary - Discussion

The presented study was meant to replicate the recent findings from Luk et al Science paper showing pathological transmission of alpha-synuclein (α-Syn) preformed fibrils single injection in striatum provoking Parkinson-like neurodegeneration and motor dysfunctions.

Our current data confirmed Luk's findings with a detection of approximately 40% reduction in dopamine and DAT concentration in the striatum. No changes in TH, DARPP32 or total α-Syn levels in the striatum were observed. Most important aspects of the replication include the immunohistological, pathological and stereological readouts which are still underway at NeuroScience Associates. Initial pilot data with pS129 α-Syn shows pathology spread to nigra and cortical areas, indicating that there is replication of the spread/transfer of α-Syn pathology.

Regarding motor dysfunction, we were not able to confirm Luk et al.'s finding who had reported significant deficits in the wire-hang and rotarod tests. No significant behavioral abnormalities were observed in any of the behavioral measures tested (Rotorod, Wire Hang, Open field, Ymaze).

Taken together, these data can help inform the PD community of the utility and reproducibility of this critical model of α-Syn transfer and may be of value to researchers seeking appropriate models in which to test potential therapeutics targeting alpha-synuclein.

Reference:

Kelvin C. Luk, Victoria Kehm, Jenna Carroll, Bin Zhang, Patrick O'Brien, John Q. Trojanowski, Virginia M.-Y. Lee. **Pathological a-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice.** ***Science***: 338, pg 949 Nov. **2012**

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