TIMECOURSE ASSESSMENT OF PATHOLOGY FOLLOWING BILATERAL INOCULATION OF ALPHA-SYNUCLEIN PREFORMED FIBRILS IN C57BL/6 MICE

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INTRODUCTION

Alpha-synuclein (αSyn) is a 140 amino acid protein implicated both genetically and neuropathologically in Parkinson's disease (PD). Increased levels of αSyn lead to neurodegeneration and aggregated αSyn is the primary component of Lewy bodies, the histopathological hallmark of PD. Synthetic αSyn (murine and human) are capable of 'seeding' and propagating αSyn pathology in both αSyn transgenic and non-transgenic (WT) neuronal cultures and mice (Luk, et al., 2012a; Luk, et al., 2012b; Volpicelli-Daley, et al., 2014). Unilateral striatal administration of synthetic murine alpha-synuclein pre-formed fibrils [PFFs] in WT mice has been shown to induce reliable pS129 αSyn-positive pathology at 30, 90, and 180 days post inoculation [DPI]. However, motor disruption at any timepoint hasn't been a reliable outcome measure.

We sought to improve on previously published Parkinson's disease models based on αSyn fibril inoculation by studying the behavior and pathology that develops over time post bilateral inoculation (30, 60, 90 and 180DPI) of *in vitro* generated mouse alpha-synuclein preformed fibrils into wild type mice. We used unilaterally injected mice as in previous studies, and PBS injected mice, at 90DPI as reference. From the bilaterally injected animal, one hemisphere was subjected to histology and the second one was used to produce tissue homogenates of affected brains. The latter were used to show seeding capacity and propagation of pathological features in rat primary neuronal cultures.

METHODS

Animals: A total of 24 (n=4 per treatment group and dpi), six-week-old gender mixed C57Bl6/J mice were purchased from The Jackson Laboratories (Bar Harbor, ME). All animals were examined, manipulated, and weighed prior to study initiation to ensure adequate health and suitability for the study.

Alpha-synuclein pre-formed fibrils: Mouse alpha-synuclein preformed fibrils (referred to as mPFFs) (2.5 µg/µl) were prepared as per Luk's preparation protocol (Luk et al, Science, 2012a).

Surgical method: Mice were anesthetized with isoflurane and stereotactically injected into the striatum, either the right hemisphere (for unilateral injections) or both hemispheres (for bilateral injections) - coordinates (anteroposterior: +0.2, mediolateral: ±2.0, dorsoventral: -2.6), with recombinant mPFF (5.0 µg; 2.0 µl total volume). Control animals received inoculations with PBS. Injections were performed with a 10 µl syringe (Hamilton, NV) at a rate of 0.1 µl/min with the needle left in place for 5 minutes following administration at each target. All mice received analgesia both pre- and post-operatively. Carprofen (5 mg/kg), subcutaneous (SC) was administered immediately before surgery and Buprenex (0.1 mg/kg, SC) was administered after surgery once the mouse was fully awake. In addition, 1 ml of Ringer's solution was administered (SC) at the conclusion of surgery. Wet feed was provided for post-surgical animals for a period of 3 consecutive days following surgery.

Behavior assessment: At each timepoint, all the animals except the unilateral group [n=4], were tested in wire hang and tapered balance beam tasks. The wire hang test, Santa-Maria et al. (2012)'s modified protocol in which mice were placed on top of a standard wire cage lid and the lid was then turned upside down. The latency of mice to fall off was measured, and average values were computed from three trials (30 seconds apart). Trials were stopped if the mouse remained on the lid after 5 minutes. Tapered balance beam consisted of a 100 cm in length black acrylic strip tapered from a width of 1.5 cm to 0.5 cm. The beam was angled of 17° from horizontal running from low to high [58 cm from the floor] topped with a goal box. After training, animals are tested 24 hours later. During testing, mice received 3 trials with an intertrial interval (ITI) of 30 seconds. The following measures are captured: the latency to turn [animal turn to face the goal box]; latency to traverse; total number of steps and footslips for each paw and expressed as a ratio of the total number of footslips to the total number of steps.

Brain collection and preparation for histology: Brain hemispheres were collected according to PGI' standard procedures. In short, mice were flush perfused with saline to remove blood cells before harvesting brains. Hemibrains were cryoprotected in 15% sucrose after overnight post fixation in 4% phosphate-buffered PFA. Brains were frozen within OCT in molds in dry ice cooled liquid isopentane. Tissue blocks were cut coronally in a uniform systematic random protocol on a Leica CM3050 cryotome at 10-microns section thickness.

Labeling, imaging and quantification: 8 sections per sample were immunohistochemically labeled with pSer129 αSyn [EP1536Y] and TH [1B7] primary antibodies. AlexaFluor-conjugated secondary antibodies were used to visualize primary binding, all sections were counterstained with DAPI to visualize nuclei. Whole slices images were acquired on an Axio.Scan Z1 slide scanner. Dorsal striatum (CPu) and substantia nigra (SN) were manually delineated. The saved ROIs were used to count immunoreactive objects above threshold and size restrictions fully automatedly using Image Pro Premier image analysis software (v9.1 or higher).

Brain tissue preparation and rat neuronal culture experiments: Total homogenates were prepared from fresh frozen, brain tissues of PFF or PBS inoculated mice. Hemibrains were regionally dissected to isolate the cortex and midbrain including the substantia nigra. Tissues were lysed in 9x volume of homogenization buffer complemented with cOmplete™ protease inhibitor cocktail (Sigma) and sonicated on ice prior to storage in single-use aliquots.

Rat cortical neurons at DIV5 (days *in vitro*) were exposed to brain homogenates from the cortex and midbrain regions. The formation of pS129 αSyn positive aggregates was observed upon fixation of cells on DIV22 or DIV25. An antibody for MAP2 [ab5392] was used to visualize neurons and pS129 αSyn [EP1536Y] positive-area was normalized to MAP2-positive area.

References:
Kelvin C. Luk,1 Victoria Kehm,1 Jenna Carroll,1 Bin Zhang,1 Patrick O'Brien,1 John Q. Trojanowski,1 and Virginia M.-Y. Lee1, **Pathological α-Synuclein Transmission Initiates Parkinson-like Neurodegeneration in Non-transgenic Mice;** *Science. 2012 Nov 16; 338(6109): 949–953.* PMCID: PMC3552321

BEHAVIORAL RESULTS

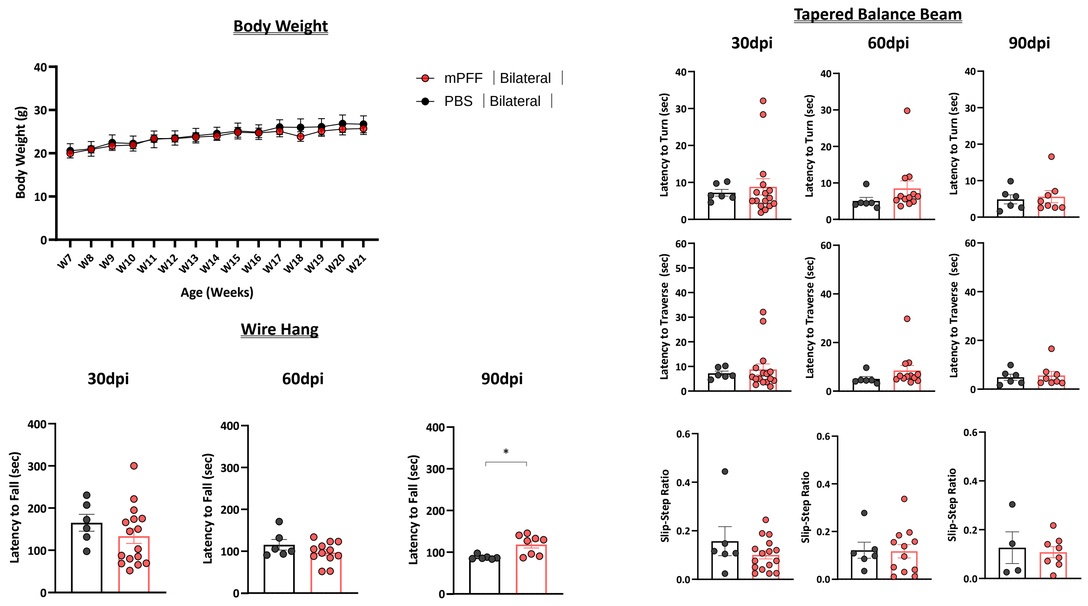

Figure 1: Body weight and motor assessments:
Body weights were recorded once per week starting from 7 weeks of age until tissue collection at either 30-, 60-, 90-, or 180-days post inoculation. All animals went under surgery at 8 weeks of age.
Wire Hang assessment was conducted at 30-, 60-, and 90-dpi with data expressed as an average of 3 trials. There were no significant differences in latency to fall between the treatment groups at 30 and 60dpi; however, there were significant differences in latency to fall between the treatment groups at 90dpi ($t(12) = 3.138$, $p = 0.0086$).
Tapered Beam task – Latency to turn with data expressed as an average of 3 trials. There were no significant differences in latency to turn between the treatment groups at any timepoint.
Latency to traverse with data expressed as an average of 3 trials. There were no significant differences in latency to traverse between the treatment groups at any dpi.
Total Footslips. There were no significant differences in footslips between the treatment groups at any dpi timepoint.
Means +/- SEM are displayed in all graphs.

HISTOPATHOLOGY

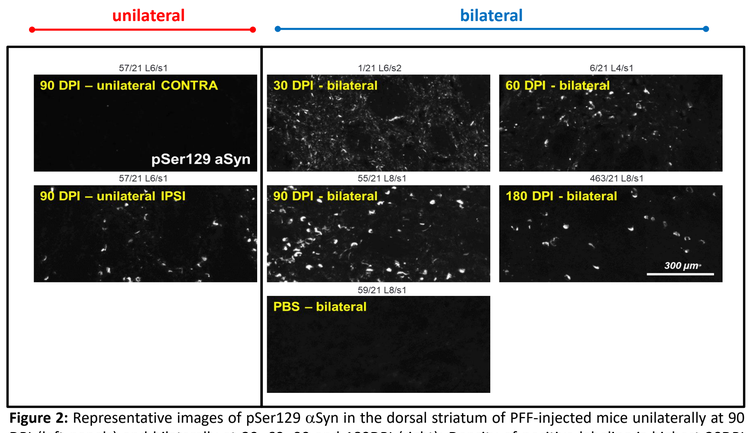

Figure 2: Representative images of pSer129 αSyn in the dorsal striatum of PFF-injected mice unilaterally at 90 DPI (left panels) and bilaterally at 30, 60, 90 and 180DPI (right): Density of positive labeling is highest 30DPI consisting of many small sized fibrils that accumulate over time to larger size Lewy body-like aggregates. Those decrease with time due to the loss of positive neurons. No positive fibrils are present in PBS injected controls and only very few in contralateral side at 90DPI in the unilateral model (top left).

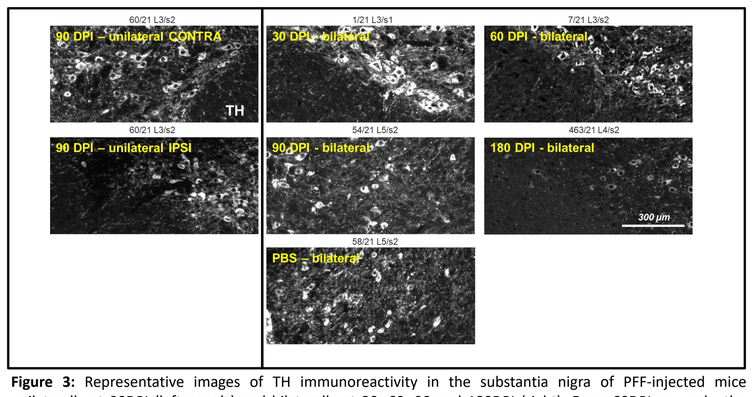

Figure 3: Representative images of TH immunoreactivity in the substantia nigra of PFF-injected mice unilaterally at 90DPI (left panels) and bilaterally at 30, 60, 90 and 180DPI (right): From 60DPI onwards, the number of the TH+ neurons starts to drop and this leads to significantly lower positive cells in both the uni- and bilateral model at 90DPI. At 180DPI, only few cells were found positive for TH in the SN.

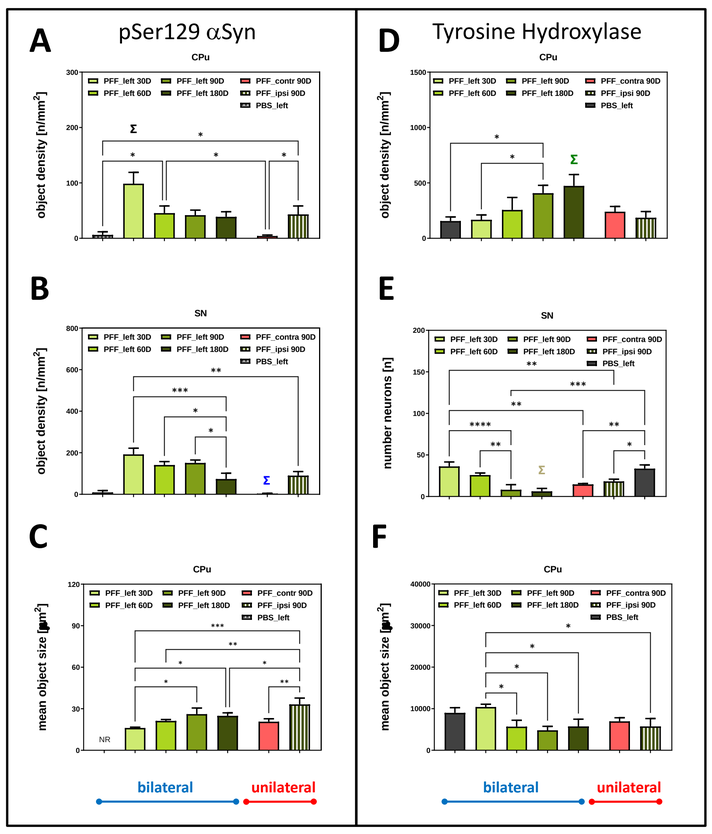

Figure 4: (A,B,C) pSer129 αSyn-positive object density in the CPu and SN and mean object size in the CPu. Highest load of pSer129 αSyn is seen 30DPI and then drops significantly. In the SN, the significant drop is seen between 90 and 180 DPI in the bilateral groups. No difference at 90DPI was found between uni-and bilateral injection. Average object size increases towards 90DPI (uni- and bilateral). **(D,E,F) TH object density in the CPu, TH cell number in the SN and mean object size in the CPu.** TH-positive area surface drops from 60DPI onwards (not shown), while measured object density increases as a sign of fragmentation of TH labeling. This is also seen as a significant drop in average object size. In addition, the TH+ cell number in the SN drops significantly from 90DPI onwards. No difference at 90 DPI was found between uni-and bilateral injection. ∑ = $p<0.05$ vs. all; ∑ = $p<0.05$ vs. all but PBS; ∑ = $p<0.05$ vs. all but 90 DPI bilateral.

RAT NEURONAL CULTURES EXPOSED TO PFF MOUSE TISSUES

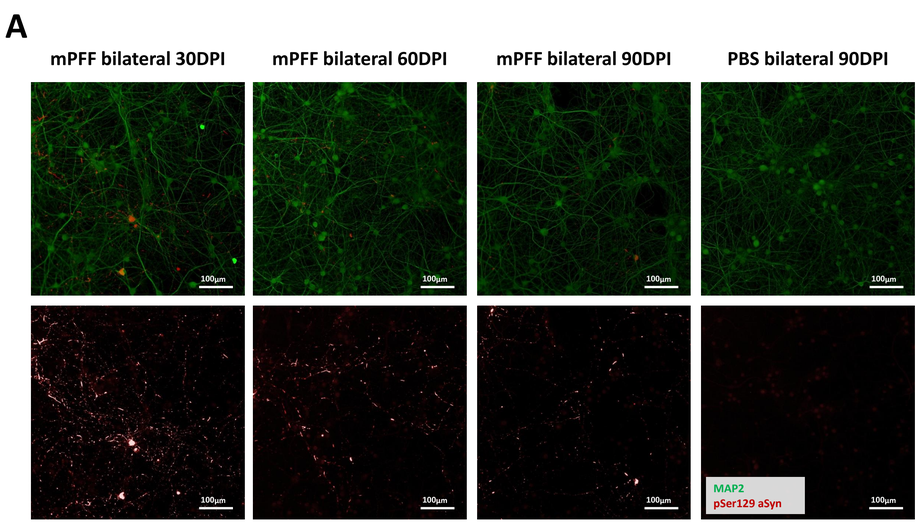

Figure 5: (A) Images of phosphorylated αSyn (red) associated with MAP2 (green) labeling in primary rat neurons fixed on DIV25 following exposure to brain tissues from PFF or PBS bilaterally-injected mice (B) When cells were exposed for longer and fixed on DIV25 vs DIV22, the pS129 αSyn signal was increased. The highest burden of pS129 αSyn positive puncta was observed in lysates from the 30DPI group. No aggregates were formed in untreated neurons or in neurons exposed to homogenates from the PBS-injected group.

CONCLUSIONS

- This new, bilateral model of α-synuclein mPFF inoculation, does not significantly alter the behavioral phenotype when examined at 30-, 60-, or 90-days post inoculation at a dose of 10 µg per mouse.
- No significant differences observed in tyrosine hydroxylase levels or pSer129 αSyn pathology between bilaterally injected animals at 90 DPI and the ipsilateral side of the unilateral model at 90 DPI.
- Highest load of pSer129 αSyn at 30DPI while size of aggregates increase with time; Reduction in pSer129 αSyn over time likely linked to cell loss as it's accompanied by drop of TH-positive cells after 90DPI.
- Brain homogenates from animals inoculated with α-synuclein PFF can induce the accumulation of pSer129 αSyn-positive inclusions in a neuronal culture system of rat cortical neurons demonstrating the seeding capacity of the *in vivo* formed aggregates.
- Importantly, the *in vitro* pathology load correlated with *in vivo* data, as the largest load of pSer129 αSyn aggregates was observed in neurons exposed to brain homogenates collected at 30 DPI.
- Newly developed model of PFF bilateral inoculations results in overall higher load of αSyn aggregates (bilaterally) and allows parallel biochemical and histological investigations in drug efficacy studies.