# PROTOCOL for *"Development and characterization of a non-human primate model of disseminated synucleinopathy"*

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## PROTOCOL

### *Study design*

This study was aimed to develop and characterize a NHP model of disseminated synucleinopathy mimicking the known neuropathological signatures of PDD and DLB to the best possible extent. Accordingly, we sought to determine whether the intraputaminal delivery of a retrogradely spreading AAV9 coding for mutated alpha-synuclein (AAV9-SynA53T) is able to induce a widespread synucleinopathy throughout cortical and subcortical brain areas innervating the putamen. Two adult juvenile NHPs

(*Macaca fascicularis*) were injected with AAV9-SynA53T into the left putamen and sacrificed four weeks post-AAV deliveries. Upon animal sacrifices, brain tissue samples were processed for histological analysis and up to three different readouts were considered, comprising number of transduced neurons, rostrocaudal distribution and location.

***Experimental animals***

A total of two adult juvenile naïve *Macaca fascicularis* NHPs (34 months-old; both females; body weight between 2.35 and 2.49 Kg) were used in this study. Animal handling was conducted in accordance to the European Council Directive 2010/63/UE as well as in full keeping with the Spanish legislation (RD53/2013). The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra (ref: CEEA095/21) as well as by the Department of Animal Welfare of the Government of Navarra (ref: 222E/2021).

***Viral vector production (FIGURE 1)***

A recombinant AAV vector serotype 2/9 expressing the SNCA gene with A53T mutation driven by the human synapsin promoter was produced by Vector Builder (www.vectorbuilder.com; catalog No. P211024-1010fpa; lot No. 211221AAVJ03). The virus was formulated in PBS buffer (pH 7.4) supplemented with 200 nM NaCl and 0.001% pluronic F-68. Obtained vector concentration was $9.62 \times 10^{13}$ GC/ml. Virus purity was determined by SDS-PAGE followed by silver staining, resulting in >80% pure. Plasmid map for pAAV-synapsin-SynA53T and sequence are provided in Supplementary Figure 1.

***Stereotaxic surgery for AAV deliveries (FIGURE 2)***

Surgical anesthesia was induced by intramuscular injection of ketamine (5 mg/Kg) and midazolam (0.5 mg/Kg). Local anesthesia was implemented just before the surgery with a 10% solution of lidocaine. Analgesia was achieved with a single intramuscular injection of flunixin meglumine (Finadyne®, 5 mg/Kg) delivered at the end of the surgical procedure and repeated 24 and 48 h post-surgery. A similar schedule was conducted for antibiotic coverage (ampicillin, 0.5 ml/day). After surgery, animals were kept under constant monitoring in individual cages with ad libitum access to food and water. Once animals showed a complete post-surgical recovery (24 h), they were returned to the animal vivarium and housed in groups.

Stereotaxic coordinates for AAV deliveries into the left putamen were calculated from the atlas of Lanciego and Vázquez (2012). During surgery, target selection was assisted by ventriculography. Pressure deliveries of AAVs were made through a Hamilton® syringe in pulses of 5 μl/min for a total volume of 25 μl each into three sites in the left putamen, each deposit spaced 1 mm in the rostrocaudal direction to obtain the highest possible transduction extent of the putamen. Once injections were completed, the needle was left in place for an additional time of 10 min before withdrawal to minimize AAV reflux through the injection tract. Coordinates for the more rostral deposits in the left putamen of AAV9-SynA53T were 1 mm rostral to the anterior commissure (ac), 1 mm dorsal to the bi-commissural plane (ac-pc plane) and 11 mm lateral to the midline. Second deposits were performed at the ac level (ac = 0 mm), 1 mm dorsal to the ac-pc plane and 11.5 mm lateral to the midline, whereas the more caudal injections were conducted 1 mm caudal to ac, 1.5 mm dorsal to the ac-pc plane and 12.5 mm lateral to the midline.

***Necropsy, tissue processing and data analysis***

Anesthesia was firstly induced with an intramuscular injection of ketamine (10 mg/Kg), followed by a terminal overdose of sodium pentobarbital (200 mg/Kg) and perfused transcardially with an infusion pump. Both animals (MF04 and MF05) were sacrificed four weeks post-AAV deliveries. The perfusates consisted of a saline Ringer solution followed by 3,000 ml of a fixative solution made of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.125 M phosphate buffer (PB) pH 7.4. Perfusion was continued with 1,000 ml of a cryoprotectant solution containing 10% glycerine and 1% dimethylsulphoxide (DMSO) in 0.125 M PB pH 7.4. Once perfusion was completed, the skull was opened and the brain removed and stored for 48 h in a cryoprotectant solution containing 20% glycerin and 2% DMSO in 0.125 M PB pH 7.4. Next, frozen coronal sections (40 μm-thick) were obtained on a sliding microtome and collected in 0.125 M PB pH 7.4 as 10 series of adjacent sections. These series were used for (1) immunoperoxidase detection of α-Syn, (2) immunoperoxidase detection of α-Syn counterstained with toluidine blue (Nissl stain), (3) immunoperoxidase detection of tyrosine hydroxylase (TH), and (4) immunoperoxidase detection of TH counterstained with toluidine blue. The remaining 6 series of sections were stored at -80 $^{o}$C cryopreserved in 20% glycerin and 2% DMSO until further use, if needed. A completed list of primary and bridge antisera (secondary biotinylated antisera), together with incubation concentrations, incubation times and commercial sources is provided below in Table 1.

**Table 1: List of reagents**

| ITEM | CONCENTRATION | INCUBATION TIME | SOURCE | IDENTIFIER | RRID |
|---|---|---|---|---|---|
| **Viral vectors** | | | | | |
| (synapsin)AAV9-SynA53T | 9.62 x $10^{13}$ GC/ml | | Vector Builder | P211024-1010fpa | |
| **Antibodies** | | | | | |
| Goat anti-Tyrosine Hydrox. (polyclonal) | 1:500 | Overnight | My Biosource | Cat# MBS421729 | N.A. |
| Mouse anti-α-synuclein (monoclonal) | 1:40 | Overnight | Leica | Cat# NCL-L-ASYN | AB_442103 |
| Donkey anti-Goat (Biotin-SP) | 1:600 | 120 min | Jackson | Cat# 705-065-147 | AB_2340397 |
| Donkey anti-Mouse (Biotin-SP) | 1:600 | 120 min | Jackson | Cat# 715-066-150 | AB_2340787 |
| **Commercial assays** | | | | | |
| ABC kit standard | | 60 min | Vector Labs | Cat# PK4000 | |
| Toluidine blue (Nissl stain) | 0.25% | 2 min | Sigma | Cat# 88930 | |

### *Quantification of neurons expressing a-Syn (FIGURE 3)*

Every tenth section was processed for the immunoperoxidase detection of $\alpha$-Syn and used for estimating the number of transduced neurons with AAV9-SynA53T. For this purpose, a deep-learning dedicated algorithm was prepared with Aiforia® (Penttinen et al., 2018), validated and released (resulting in an error of 1.67% for quantifying $\alpha$-Syn+ neurons). Sixty-nine equally spaced coronal sections covering the range of ac +10 mm to ac -14 mm were sampled per animal, including all ROIs containing $\alpha$-Syn+ neurons at the cortical (left and right cerebral cortices) and subcortical (left hemisphere) levels. Sections were scanned at 20x in an Aperio CS2 slide scanner (Leica, Wetzlar, Germany). Selected cortical ROIs comprised the anterior cingulate gyrus (ACgG), superior frontal gyrus (SFG), middle frontal gyrus (MFG), inferior frontal gyrus (IFG), precentral gyrus

(PrG), fronto-orbital gyrus (FoG), medial orbital gyrus (MOrG), lateral orbital gyrus (LOrG) posterior cingulate gyrus (PCgG), postcentral gyrus (PoG), insular gyrus (Ins), superior parietal lobule (SPL), supramarginal gyrus (SMG), precuneus (PCu), superior temporal gyrus (STG) and middle temporal gyrus (MTG), whereas subcortical ROIs included the amygdaloid complex (AMG), ventral anterior thalamic nucleus (VAL), ventral lateral thalamic nucleus (VL), ventral posterior thalamic nucleus (VPO), centromedian-parafascicular thalamic complex (CM-Pf), substantia nigra pars compacta (SNpc) and dorsal raphe nucleus (DRN). Segmentation of cortical areas was conducted in keeping with the stereotaxic atlas of Martin and Bowden (1996). In brief, scanned sections were uploaded to the Aiforia® cloud. Next, the boundaries of each selected ROI were outlined at low magnification and the dedicated algorithm was then used as a template quantifying the desired neuronal population (e.g. $\alpha$-Syn+ neurons). Representative images illustrating the accuracy of the conducted procedure are provided in Figure 1.

***Quantification of the nigrostriatal system***

At the level of the substantia nigra pars compacta (SNpc), the number of TH+ neurons were quantified with a different Aiforia® algorithm designed to disclose TH+ cells (resulting in an error of 4.82%). In both animals, the analysis was conducted in thirteen TH-stained, equally-spaced coronal sections covering the whole rostrocaudal extent of the SNpc. Sections were scanned at 20x in a slide scanner (Aperio CS2; Leica), and uploaded to the Aiforia® cloud where TH+ neurons were quantified according to a similar procedure described above for estimating the number of $\alpha$-Syn+ neurons. Regarding the density of TH+ terminals at the level of the putamen, up to 26 equally-spaced coronal sections stained for TH and covering the whole rostrocaudal extent of the left and right putamen (pre- and post-commissural) in each animal were scanned at 20x and used for measuring TH optical densities with Fiji Image J (NIH, USA) and coverted to a logarithmic scale according to available protocol (Ruifrok and Johnston, 2011).

**LITERATURE REFERENCES**

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**FIGURE LEGENDS**

**Figure 1. Viral vector (synapsin)AAV9-Syna53T.** Plasmid map and sequence for the adeno-associated viral vector used here (produced by Vector Builder).

**Figure 2. Ventriculography-assisted stereotaxic surgery:** X-ray images illustrating the conduced procedure for the delivery of (synapsin)AAV9-SynA53T into the putamen (3 sites; 25 μl each) in both animals (panels A-D referencing injections performed in animal MF04 and panels E-H for animal MF05). Stereotaxic surgery starts with an adequate identification of the anterior (ac) and posterior (pc) commisures, that both together define the ac-pc plane of reference. This procedure is illustrated in panels A & E. For this purpose, an intrathecal needle is located in the left frontal projection of the lateral ventricle. Once in place, 0.3 ml of an x-ray contrast solution (Omnipaque®, GE Healthcare) is infused and the x-ray obtained. The ac-pc length is measured and used to calibrate the stereotaxic atlases used for the stereotaxic deliveries of AAVs (Martin and Bowden, 1996; Lanciego and Vázquez, 2012). Next, by taking advantage of a Hamilton® syringe, a viral suspension of (synapsin)AAV-SynA53T is delivered into the left putamen at an ac distance of 0, +1 and -1 mm (panels B, C and D for animal MF04; panels F, G and H for animal MF05). Each injection is made of 25 μl, delivered in pulses of 5 μl/min.

**Figure 3. Quantification of α-Syn+ neurons.** A dedicated deep-learning algorithm identifying α-Syn transduced neurons was prepared with Aiforia® (www.aiforia.com). Scanned sections were uploaded to Aiforia® cloud and several different ROIs were

outlined throughout cortical and subcortical territories. Panel A illustrates the delineation of the left supramarginal gyrus at low magnification (0.169x). Labeling intensity, reflecting the number of transduced neurons is represented color-coded in panel A'. Panel B shows the same area at a magnification of 2x, together with the accompanying result, color-coded as illustrated in panel B'. A higher-magnification view (5x) of the obtained labeling is shown in panel C, together with the accurate identification of every single $\alpha$-Syn+ neuron, as observed in panel C'. All transduced neurons were quantified within the selected ROI. Illustrated images are taken from animal MF04 and correspond to coronal section #61. In summary, all $\alpha$-Syn+ neurons were counted, and indeed the algorithm was fully accurate and specific (a total of 414 neurons were identified in this ROI). Scale bars are 10 mm for panels A & A'; 1 mm in panels B & B' and 250 $\mu$m in panels C & C'.