**PROTOCOL for “Neuromelanin accumulation drives endogenous synucleinopathy in non-human primates”**

**STUDY DESIGN**

This study was aimed to develop and characterize a non-human primate (NHP) model of Parkinson’s disease mimicking the known neuropathological hallmarks of Parkinson’s disease to the best possible extent. Accordingly, we sought to determine whether AAV-mediated enhanced expression of human tyrosinase (hTyr) in the substantia nigra (SNpc) of non-human primates (NHPs) is able to induce a time-dependent accumulation of neuromelanin (NMel) in dopaminergic neurons, further triggering and endogenous synucleinopathy, progressive cell death and a pro-inflammatory scenario, in keeping with what was formerly reported in rats by taking advantage of a similar strategy (Carballo-Carbajal et al., 2019). Furthermore, the potential prionoid spread of endogenous alpha-synuclein (-Syn) species towards the prefrontal cortex was analyzed, in an attempt to evaluate to what extent there is a propagation of endogenous -Syn by permissive trans-synaptic templating (e.g. the so-called Braak hypothesis). Adult juvenile NHPs (*Macaca fascicularis*) were injected with adeno-associated viral vectors (AAVs) encoding either the hTyr gene (AAV-hTyr; delivered into the left SNpc) or a null construct for control purposes (AAV-null; injected into the right SNpc). In order to delineate a timeline for the underlying processes, one group of NHPs was sacrificed four months post-AAV deliveries (animals M308F4 and M310M4), whereby the follow-up timing for second experimental group was settled at eight months post-AAVs surgeries (animals M307F8 and M309M8). Neuroimage studies (MRI and MicroPET) were conducted *in vivo* at different time points. Upon animal sacrifices, brain tissue samples were processed for histological analysis comprising intracellular NMel levels, intracellular aggregates, nigrostriatal degeneration and neuroinflammation.

**EXPERIMENTAL ANIMALS**

A total of four adult juvenile naïve *Macaca fascicularis* non-human primates (36-40 months-old; two males and two females; body weight 2.3-4.5 Kg) were used in this study. Animal handling was conducted in accordance to the European Council Directive 2010/63/UE as well as in keeping with 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). Animals numbered as M307F8 and M308F4 are females, whereas animals M309M8 and M310M4 are both males. Animal records are provided below.

**Animal records**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Monkey | Gender | Weight & date of arrival | date of birth – date of sacrifice | Follow-up | Supplier |
| M307F8 | Female | 2.373 Kg (16/06/2020) | 20/11/2017 – 02/03/2021 | 8 months | BioPrim (France) |
| M308F4 | Female | 2.430 Kg (16/06/2020) | 21/11/2017 – 15/12/2020 | 4 months | BioPrim (France) |
| M309M8 | Male | 4.508 Kg (11/06/2020) | 02/07/2017 – 02/03/2021 | 8 months | BioPrim (France) |
| M310M4 | Male | 3.783 Kg (11/06/2020) | 07/07/2017 – 15/12/2020 | 4 months | BioPrim (France) |

**PROTOCOL #1: VIRAL VECTOR PRODUCTION**

Recombinant AAV vector serotype 2/1 expressing the human tyrosinase cDNA driven by the CMV promoter (AAV-hTyr) and the corresponding control empty vector (AAV-null) were produced at the Viral Vector Core Production Unit of the Autonomous University of Barcelona (UPV-UAB). In brief, AAVs were produced by triple transfection of 2 x 108 HEK293 cells with 250 g of pAAV, 250 g of pRepCap, and 500 g of pXX6 plasmid mixed with polyethylenimine (Sigma-Aldrich). The UPV-UAB generated a pAAV plasmid containing the ITRs of the AAV2 genome, a multi-cloning site to facilitate cloning of expression cassettes, and ampicillin resistance gene for selection. Two days after transfection, cells were harvested by centrifugation, resuspended in 30 ml of 20 mM NaCl, 2 mM MgCl2, and 50 mM Tris-HCl (pH 8.5) and lysed by three freeze-thawing cycles. Cell lysate was clarified by centrifugation and the AAV particles were purified from the supernatant by iodixanol gradient as previously described (Zolotukhin et al., 1999). Next, the clarified lysate was treated with 50U/ml of benzonase (Novagen; 1 h at 37 ºC) and centrifuged. The vector-containing supernatant was collected and adjusted to 200 mM NaCl using a 5-M stock solution. To precipitate the virus from the clarified cell lysate, polyethylene glycol (Sigma-Aldrich) was added to a final concentration of 8% and the mixture was incubated (3 h, 4 ºC) and centrifuged. AAV containing pellets were resuspended in 20 mM NaCl, 2 mM MgCl2 and 50 mM Tris-HCl (pH 8.5) and incubated for 48 h at 4 ºC. The AAV titration method used was based on the quantitation of encapsulated DNA with the fluorescent dye PicoGreen®. Obtained vector concentrations were 1.7 x 1013 gc/ml for AAV-hTyr and 2.48 x 1013 for AAV-null. Plasmid map for pAAV-CMV-hTyr and sequence are provided below.

**Plasmid map for pAAV-hTyr:**



**Sequence for pAAV-hTyr:**

CAGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTC

AGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTTGTAGTTAATGATTAACCCGCCAT

GCTACTTATCTACGTAGCCATGCTCTAGACATGGCTCGACAGATCTCAATATTGGCCATTAGCCATATTATTCATTGGTT

ATATAGCATAAATCAATATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCA

TGTCCAATATGACCGCCATGTTGGCATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAG

CCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGA

CGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAA

ACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTCCGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGC

CTGGCATTATGCCCAGTACATGACCTTACGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCA

TGGTGATGCGGTTTTGGCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATT

GACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTGCGATCGCCCGCCCCG

TTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCACTAG

AAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGTGCTTCTGACACAACAGTCTCGAACTTAAGCT

GCAGTGACTCTCTTAAGGTAGCCTTGCAGAAGTTGGTCGTGAGGCACTGGGCAGGTAAGTATCAAGGTTACAAGACAGGT

TTAAGGAGACCAATAGAAACTGGGCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTG

ACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGTTCAATTACAGCTCTTAAGGCTAGAGTACTTAATACGAC

TCACTATAGGCTAGCctcgacctcgagacgcgtgataaacttaagcttggtaccgagctcggatccactagtccagtgtg

gtggaattctgcagatatccagcacagtggcggccgctcgaccGACCTTGTGAGGACTAGAGGAAGAATGCTCCTGGCTG

TTTTGTACTGCCTGCTGTGGAGTTTCCAGACCTCCGCTGGCCATTTCCCTAGAGCCTGTGTCTCCTCTAAGAACCTGATG

GAGAAGGAATGCTGTCCACCGTGGAGCGGGGACAGGAGTCCCTGTGGCCAGCTTTCAGGCAGAGGTTCCTGTCAGAATAT

CCTTCTGTCCAATGCACCACTTGGGCCTCAATTTCCCTTCACAGGGGTGGATGACCGGGAGTCGTGGCCTTCCGTCTTTT

ATAATAGGACCTGCCAGTGCTCTGGCAACTTCATGGGATTCAACTGTGGAAACTGCAAGTTTGGCTTTTGGGGACCAAAC

TGCACAGAGAGACGACTCTTGGTGAGAAGAAACATCTTCGATTTGAGTGCCCCAGAGAAGGACAAATTTTTTGCCTACCT

CACTTTAGCAAAGCATACCATCAGCTCAGACTATGTCATCCCCATAGGGACCTATGGCCAAATGAAAAATGGATCAACAC

**PROTOCOL #2: STEREOTAXIC SURGERY FOR AAV DELIVERIES**

Surgical anesthesia was induced by intramuscular injection of ketamine (5 mg/Kg) and midazolam (5 mg/Kg). Local anesthesia was implemented just before 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 SNpc 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 1 L/min for a total volume of 10 L each into two sites in the SNpc, each deposit spaced 1 mm in the rostrocaudal direction to obtain the highest possible transduction extent of the SNpc. 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 SNpc of AAV-hTyr (left SNpc) and AAV-null (right SNpc) were 7.5 mm caudal to the anterior commissure (ac), 5 mm ventral to the bicommissural plane (ac-pc plane) and 4 mm lateral to the midline, whereby the more caudal deposits were placed 8.5 mm caudal to ac, 5.5 mm ventral to the ac-pc plane and 4 mm lateral to the midline.

**PROTOCOL #3: NEUROIMAGE STUDIES (MICROPET SCANS)**

MicroPET scans with (+)--[11C]Dihydrotetrabenazine (11C-DTBZ; a selective VMAT2 ligand) were performed on each animal at baseline and 1, 2, 4, 6 and 8 months post-AAV deliveries (6 and 8 time points only applying to animals with 8 months of follow-up). (+)--[11C]Dihydrotetrabenazine was synthesized by [11C]CH3 methylation of the corresponding (+)-9-O-desmehtyl-a-dihydrotetrabenazine precursor. [11C]methane was obtained by the wet chemistry method starting from [11C]CO2 porduced in the Cyclone 18/18 cyclotron at the Department of Nuclear Medicine, Clínica Universidad de Navarra, with a radiochemical purity of >95%. Images were acquired on a dedicated small animal Philips mosaic tomograph (Cleveland, OH, USA). The standard adquisition and quantification of the radiotracer binding potential was conducted as previously described (Blesa et al., 2010). In brief, a dynamic study of 40 min was acquired after the intravenous injection of the radiotracer. Obtained scans were analyzed by a radiotracer kinetic model using PMOD v3.2 software (PMOD Technologies, Ltd., Adliswil, Switzerland) to obtain parametric images containing the information of the binding potential of VMAT2. Parametric images were spatially normalized into standard stereotaxic space using a specific template (Collantes et al., 2009). The binding potential was measured using a predefined map of regions of interest (ROIs) defined over MRI images comprising the putamen nucleus. Changes in radiotracer binding potential were calculated for each animal at each time point.

**PROTOCOL #4: NEUROIMAGE STUDIES (MRI SCANS)**

Studies were conducted at the Department of Radiology, Clínica Universidad de Navarra, in keeping with available protocols (Ariz et al., 2019; Castellanos et al., 2015). In brief, animals were scanned on a 3T MRI scanner (Siemens, Erlangen, Germany), using a 12-channel head array and consisted of the acquisition of an anatomical dataset and the NMel-sensitive sequence dataset with a total duration of 30 minutes. The anatomical T1-weighted image was acquired with MPRAGE sequence of 5 min duration. The following parameters were employed: 1 mm-isotropic resolution, FOV = 256 x 192 mm2, matrix = 256 x 192 voxels, 160 axial slices, repetition time/echo time = 1620/3.09 ms, inversion time = 659 ms, flip angle = 15º. Images of the SNpc were obtained with an NMel-sensitive T1-weighted fast spin-echo sequence with the following parameters: repetition time/echo time, 600/15 ms, two-echo train length, 11 slices, 2.0 mm slice thickness, 0.2 mm gap, 512 x 408 acquisition matrix, 220 x 175 field of view (pixel size 0.43 mm2, interpolated to 0.21 mm2), bandwidth 110 Hz/pixel, four averages, and a total scan time of 12 min. MRI studies were conducted four months post-AAV deliveries in all animals and eight months post-injection of viral vectors in animals M30F87 and M309M8.

**PROTOCOL #5: 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. Animals M308 and M310 were sacrificed four months post-AAV deliveries, whereas animals M307 and M309 were euthanized eight months post-injection of AAVs. 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% dimethylsulphoxyde (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 m-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) direct NMel visualization, (2) immunoperoxidase detection of TH, (3) dual immunofluorescent detection of tyrosine hydroxylase (TH) and P62 combined with brightfield visualization of NMel, (4) triple immunofluorescent detection of -Syn, TH and P62 combined with brightfield visualization of NMel, (5) dual immunofluorescent detection of ubiquitin and P62 combined with brightfield visualization of NMel; (6) triple immunofluorescent detection of P62, Iba-1 and CD68 combined with brightfield visualization of NMel, (7) triple immunofluorescent detection of -Syn (pre-digested with proteinase K), TH and P62 combined with brightfield visualization of NMel, (8) triple immunofluorescent detection of NeuN, TH and P62 and (9) multiple immunofluorescent detection of DAPI, -Syn, P62 and TH.

A complete list of the used primary and bridge antisera (secondary antisera; either biotinylated or Alexa®-conjugated), together with incubation concentrations, incubation times and commercial sources is provided below.

**List of reagents**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **ITEM** | **DILUTION** | **INCUBATION TIME** | **SOURCE** | **IDENTIFIER** |
| **Antibodies** |  |  |  |  |
| Goat anti-Tyrosine Hydrox. (polyclonal) | 1:500 | Overnight | My Biosource | Cat# MBS421729 |
| Mouse anti--synuclein (monoclonal) | 1:40 | Overnight | Leica | Cat# NCL-L-ASYN |
| Mouse anti-P--syn (monoclonal) | 1:1000 | Overnight | Fujifilm Wako | Cat# 015-25191 |
| Guinea Pig anti-P62 (polyclonal) | 1:1000 | Overnight | PROGEN | Cat# GP62-C |
| Mouse anti-Ubiquitin (monoclonal) | 1:500 | Overnight | Abcam | Cat# ab7254 |
| Rabbit anti-NeuN (monoclonal) | 1:1000 | Overnight | Abcam | Cat# ab177487 |
| Rabbit anti-Iba 1 (polyclonal) | 1:500 | Overnight | Fujifilm Wako | Cat# 019-19741 |
| Mouse anti-CD68 (monoclonal) | 1:300 | Overnight | Dako | Cat# M0814 |
| Donkey anti-Goat (Biotin-SP) | 1:600 | 120 min | Jackson | Cat# 705-065-147 |
| Donkey anti-Goat (Alexa488) | 1:200 | 120 min | Invitrogen | Cat# A-11055 |
| Donkey anti-Goat (Alexa350) | 1:200 | 120 min | Invitrogen | Cat# A-21081 |
| Donkey anti-Goat (Alexa633) | 1:200 | 120 min | Invitrogen | Cat# A-21082 |
| Donkey anti-Guinea Pig (Alexa594) | 1:200 | 120 min | Jackson | Cat# 706-585-148 |
| Donkey anti-Mouse (Alexa488) | 1:200 | 120 min | Invitrogen | Cat# A-21202 |
| Donkey anti-Mouse (Alexa546) | 1:200 | 120 min | Invitrogen | Cat# A-10036 |
| Donkey anti-Rabbit (Alexa488) | 1:200 | 120 min | Invitrogen | Cat# A-21206 |
| **Commercial assays** |  |  |  |  |
| ABC kit standard |  | 60 min | Vector Labs | Cat# PK4000 |
| Neutral Red | 0.40% | 1 min | Sigma | Cat# 72210 |
| V-VIP |  | 1 min | Vector Labs | Cat# SK-4600 |
| Proteinase K | 1 µg/ml | 10 min | Invitrogen | Cat# 25530049 |
| DAPI | 1:50000 | 5 min | Invitrogen | Cat# D1306 |

**PROTOCOL #7: QUANTIFICATION OF PIGMENTED SNpc NEURONS**

Every tenth section was counterstained with neutral red (NR) and used for estimating the number of transduced neurons with AAV-hTyr in the left SNpc. For this purpose, a deep-learning dedicated bi-layer algorithm was prepared with Aiforia® ([www.aiforia.com](http://www.aiforia.com)), validated and further released (resulting in an error of 1.65% for quantifying either NMel+/ NR+ or NMel-/ NR+ neurons). Ten equally-spaced coronal sections covering the whole rostrocaudal extent of the SNpc were sampled per animal. Sections were counterstained with NR and scanned at 20x in an Aperio CS2 scanner (Leica, Wetzlar, Germany) and uploaded to Aiforia cloud. The boundaries of SNpc were outlined at low magnification (excluding neighboring areas such as the ventral tegmental area and the retrorubral field). The algorithm was then used as a template quantifying the desired neuronal populations. Representative images illustrating the accuracy of the conducted procedure are provided in Figure 3C1.

**PROTOCOL #8: QUANTIFICATION OF NMel INTRACELLULAR LEVELS**

Quantification of the intracellular density of NMel was achieved by measuring optical densitometry at the single-cell level with Fiji ImageJ software (NIH, USA) and converted to a logarithmic scale according to available protocol (Ruifrok et al., 2001). Scanned images comprising the entire rostrocaudal extent of the SNpc were inspected at a magnification of 80x. Analyses were conducted in 2,202 neurons for animal M307F8, 2,498 neurons in animal M308F4, 1,967 neurons in animal M309M8 and in 2,537 neurons for animal M310M4.

**PROTOCOL #9: ASSESSMENT OF NIGROSTRIATAL DEGENERATION**

The degree of nigrostriatal lesion resulting from NMel accumulation was measured both at origin and destination (e.g. at the level of the SNpc and striatum, respectively). At the level of the SNpc, every tenth section was stained for the immunoperoxidase detection of TH and used for estimating the number of number of TH+ neurons in the left and right SNpc. For this purpose, a deep-learning dedicated algorithm was prepared with Aiforia® ([www.aiforia.com](http://www.aiforia.com)), validated and further released (resulting in an error of 4.82% for quantifying TH+ neurons). Ten equally-spaced coronal sections covering the whole rostrocaudal extent of the left and right SNpc were sampled per animal. Sections were scanned at 20x in a slide scanner (Aperio CS2; Leica), uploaded to Aiforia cloud and TH+ neurons were quantified according to a similar procedure described above for estimating the number of NMel+ neurons. Regarding nigrostriatal degeneration at destination, up to 25 equally-spaced coronal sections stained for TH covering the whole extent of the left and right caudate and putamen nuclei (pre- and post-commissural putamen and caudate) in each animal were scanned at 20x and used for measuring TH optical densities with Fiji Image J and converted to a logarithmic scale.

**PROTOCOL #10: ANALYSIS OF NMel INTRACELLULAR DENSITY AND NEURONAL INCLUSIONS**

In order to evaluate to what extent intracellular NMel levels correlate to the presence of intracellular inclusions, sections comprising all SNpc levels and stained for TH and P62 were used. The immunofluorescent detection of TH and P62 was combined with brightfield visualization of neuromelanized neurons under the confocal microscope at a magnification of 63x. Intracellular NMel levels were measured as described above by taking advantage of confocal Z stacks of similar thickness. For every single animal, a minimum of 25 pigmented dopaminergic neurons showing P62 aggregates were randomly selected from each section (comprising 12 equally-spaced sections per animal covering the whole rostrocaudal extent of the SNpc) and compared with a similar number of NMel+ / TH+ neurons without P62 intracellular inclusions.

**PROTOCOL #11: QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was done in GraphPad Prism version 9.0.2. for Windows and Stata 15 (Stata Corp. 2017. Stata Statistical Software Release 15, College Station, TX; StataCorp LLC). Relevant tests are listed in figure legends. Species with *p* < 0.05 were considered statistically significant.

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