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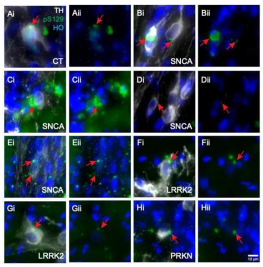
Quantification of pathological protein accumulation (aSyn and tau) in transplanted human iPSC-derived dopamine neurons



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We use this protocol and it's working

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Abstract

This protocol details the immunohistochemistry protocol and subsequent imaging analysis protocols for quantifying pathological protein accumulation in human derived iPSC dopaminergic neurons transplanted into athymic mice. Tissue sections were stained free floating for human dopamine cells (tyrosine hydroxylase antibody) and pathological markers (Table 1) as per protocol.

Materials

Materials

- 0.1M PBS
- 10mM sodium citrate (pH 6)
- Triton X
- normal donkey serum
- bovine serum albumin
- gelatin
- Tween 20
- Hoechst (# B2261; Sigma-Aldrich)
- Anti-fade fluorescence mounting medium (#S3023, DAKO)
- CoverGrip coverslip sealant (Cat.# 23005, Biotium

Antibody table

	A	B	C	D
	Primary antibodies (type, Cat.#)	**Epitope (immunogen)**	**Source**	**Fluorophore**
	Tyrosine hydroxylase (TH) (mouse IgG2b mAb, Cat.# TA506549)	Full length human recombinant protein of human TH	ThermoFisher Scientific	647
	a-Syn ((Syn204) mouse IgG2a mAb, Cat.# 838201)	Syn 204 raised using recombinant a-syn and recognises aa.1-130 of a-syn	Biolegend	568
	Phospho-a-syn at S129 (rabbit mAb, Cat.# ab51253)	Synthetic peptide aa.100-140 of human a-Syn	Abcam	488
	Tau ((SP70) rabbit mAb, Cat.# SAB5500182)	Synthetic peptide N-terminus of human tau	Sigma Aldrich	568
	Phospho-tau (Ser202, Thr205) (AT8) (mouse IgG1 mAb, Cat.# MN1020)*	Partially purified PHF-tau aa. 1-758	ThermoFisher Scientific	488
	Secondary antibodies	**Epitope (immunogen)**	**Source**	

	A	B	C	D
	Goat anti-mouse IgG2b-Alexa Fluor 647, A-21242	Mouse IgG 2b	ThermoFisher Scientific	
	Goat anti-mouse IgG2a-Alexa Fluor 568, A-21134	Mouse IgG 2a	ThermoFisher Scientific	
	Donkey anti-rabbit- Alexa Fluor 488, A-21206	Rabbit IgG	ThermoFisher Scientific	
	Donkey anti-rabbit- Alexa Fluor 568, A-21134	Rabbit IgG	ThermoFisher Scientific	
	Goat anti-mouse IgG1-Alexa Fluor 488, A21206	Mouse IgG 1	ThermoFisher Scientific	

Table 1:
List of antibodies used in this study.
aa=amino acid; mAb=monoclonal antibody; pAb=polyclonal antibody

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet) for each chemical listed from the supplier.

Before start

For qualitative and quantitative analyses of iPSC-derived dopamine neurons, images of whole brain sections and higher magnification images of dopamine cells were captured using an Olympus VS200 slide scanner and a Nikon C2 confocal microscope.

- Olympus VS200 slide scanner: For both qualitative and quantitative analyses of iPSC-derived dopamine neurons, images were captured at 20x magnification with a z-range and z-spacing of 12µm and 1.5µm, respectively, and at 60x at one z-plane on the slide scanner.

- Nikon C2 confocal microscope: For z-stack qualitative analyses of iPSC-derived dopamine neurons, images were captured at multiple magnifications on the confocal microscope. A 10x overview image of the regions of interest (ROI) was captured, followed by multiple magnifications (60x-120x) of the ROI to capture cell morphology and any potential pathologies. Z-stacks at 120x (step size= 0.3µm) were also performed followed by Huygens deconvolution using Huygens Professional version 23.04 (Scientific Volume Imaging, The Netherlands, http://svi.nl) for image presentation. Parameters including laser, gain and offset were selected based on single channel labelling for each section.

For image analysis, FIJI, v. 2.9.0/1.53t (1), and QuPath, V 0.5.0 (2), software were used.

Tissue processing and immunofluorescence staining

1 ***Day 1*** 35m

Tissue Prep

1. Pour sections into well insert in well-plate to separate the cryoprotectant storage solution from
the sections.
2. Move well-insert to well-plate containing approximately 6 mL of 1 x phosphate buffered saline (PBS)
in each well.
3. Wash sections for 00:30:00 in 1 x PBS on an orbital shaker changing the buffer every 00:05:00

2 **Antigen retrieval** 25m

1. Place sections in glass vials containing 6 mL of [M] 10 millimolar (mM) sodium citrate buffer (pH 6.0).
2. Place in the steamer at 98 °C for ~ 00:20:00 .
3. Cool sections to room temperature and transfer back to corresponding wells.
4. Wash sections in 1 x PBS for 00:05:00 and repeat.

3 **Blocking and primary antibody incubation**

1. Incubate sections in blocking buffer (containing 2% donkey serum, 1% bovine serum albumin solution, 0.2% Triton X-100, 0.1% gelatin and 0.1% Tween-20 in PBS (PBST)) for 2 hours on an orbital shaker at room temperature.
2. Incubate sections in the cocktail of primary antibodies in blocking buffer as per Table 1 for ~72 hours at 4°C on the orbital shaker.

4 3h 20m

Day 2

Secondary antibodies

1. Wash sections in 0.1% PBST for 00:30:00, changing the buffer every 10 minutes.
2. Incubate sections in corresponding secondary antibodies (Table 1) and bisbenzimide H 33342 trihydrochloride (Hoechst, Cat.# B2261; Sigma-Aldrich) in blocking buffer for 02:00:00 at Room temperature on the orbital shaker.
3. Wash sections in 0.1% PBST for 00:30:00, changing the buffer every 00:10:00.
4. Wash sections in 1 xPBS for 1 00:05:00, changing the buffer every 00:05:00.

5 ***Mounting and sealing***

1. Place sections in a petri dish containing 1 x PBS and immerse slide at an angle to gently mount sections
   onto slides using a brush.
2. Place the coverslip gently on the slide using anti-fade fluorescence mounting medium (Cat.# S3023, DAKO).
3. Once dry, seal slides with CoverGrip coverslip sealant (Cat.# 23005, Biotium) and store slides away from light at 4 °C.

Identification of protein pathologies

6 Dopamine neurons were identified in scanned images and manually counted based on TH immunoreactivity together with a large dull nucleus (Fig. 1 and 2). Aggregate-like structures were identified, counted and analysed in 3D image reconstructions if phosphorylated a-synuclein (pS129)
immunofluorescence appeared within or partially within TH+ neuronal somata.
Similarly, aggregate-like structures labelled by phosphorylated tau (AT8) immunofluorescence were identified and further analysed in 3D images if they appeared within or partially within TH+ neuronal somata (Fig. 2). PS129 and AT8 tau immunofluorescence labelled morphologically diverse structures in neurons (Fig. 1 and 2).

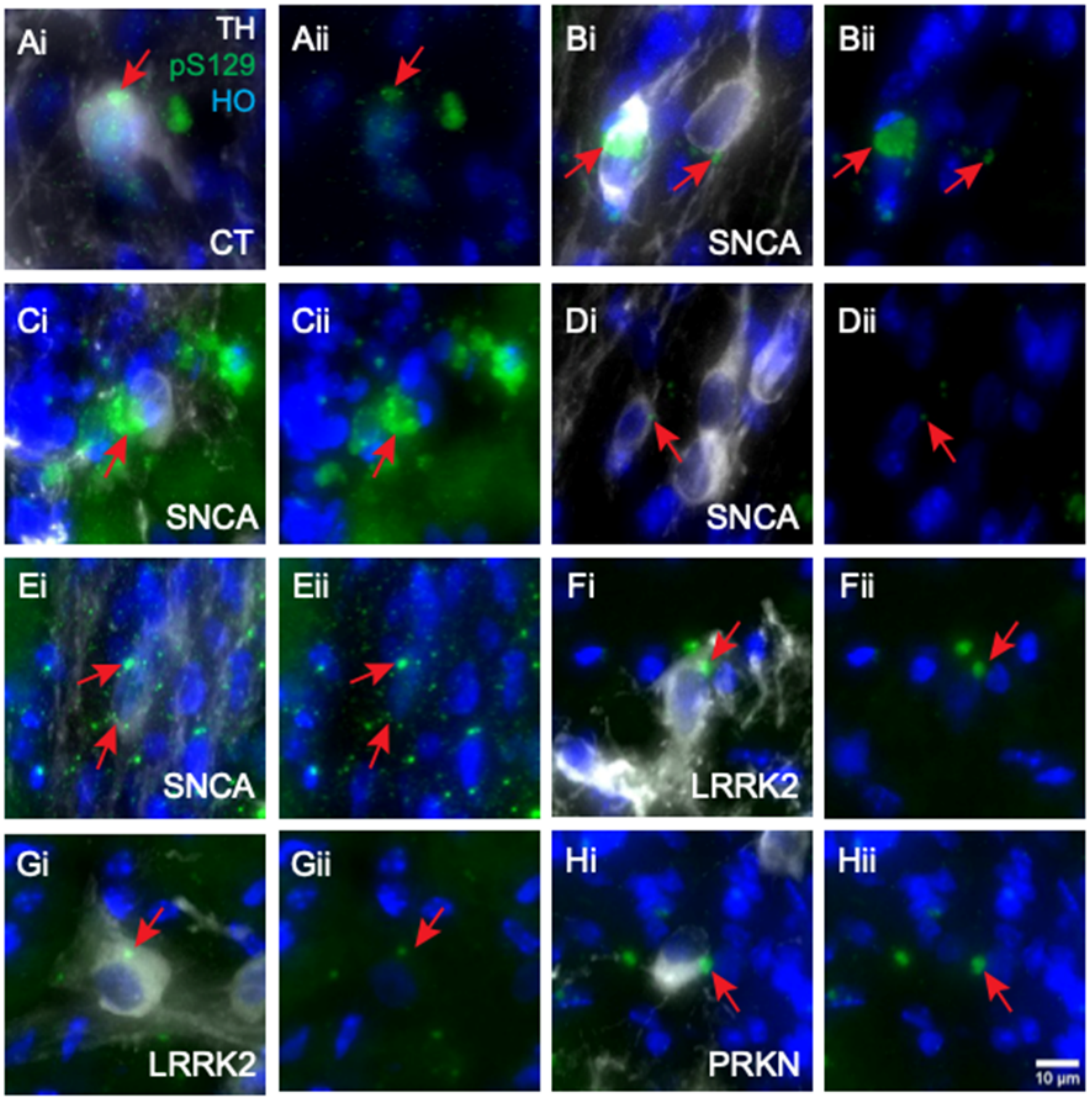

Figure 1.
Phospho-a-syn at S129 (pS129) aggregate-like structures apparent in transplanted human
iPSC-derived TH+ neurons from controls (CT) and patients carrying *SNCA, LRRK2* and *PRKN* mutations. Scanner images illustrating the various aggregate-like structures labelled by pS129
immunofluorescence (red arrows, Ai-Hii) apparent in transplanted human iPSC-derived TH+ neurons. Immunofluorescent images revealed Tyrosine Hydroxylase (TH), white; pS129, green; Hoechst (HO), blue. Images labelled with (i) show merged image, and (ii) show only pS129 and HO. Scale bar (10µm) in Hii applies to all panels.

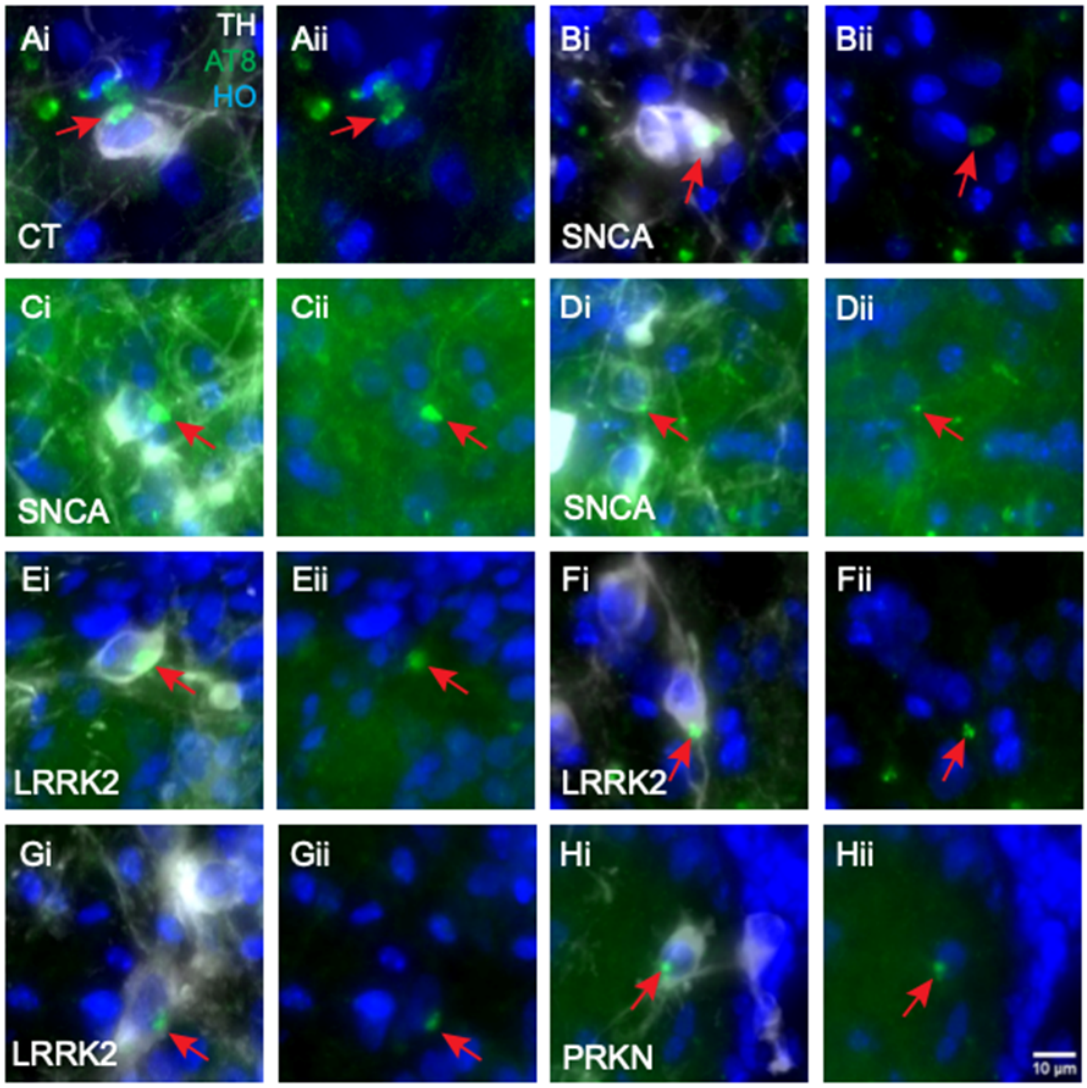

Figure 2. Phospho-tau (AT8) aggregate-like structures apparent in transplanted human iPSC-derived TH+ neurons from controls (CT) and patients carrying *SNCA, LRRK2* and *PRKN* mutations.
Scanner images illustrating the various aggregate-like structures labelled by AT8 immunofluorescence (red arrows, Ai-Hii) apparent in transplanted human iPSC-derived TH+ neurons. Immunofluorescent images revealed Tyrosine Hydroxylase (TH), white; AT8, green; Hoechst (HO), blue. Images labelled with (i) show merged image, and (ii) show only AT8 and HO. Scale bar (10µm) in Hii applies to all panels.

Imaging and analyses - QuPath analysis

7 *Contour*

1. Using the polygon annotation tool, contour the ROI based on pan a-syn and pS129 staining together with the outline observed from the camera images captured prior to staining. The

ROI contour provides the area (μm^2) of TH+ signals, which were used to detect total cell number (Hoechst), TH+ cell number, and intensity data as outlined below.

8

Cell detection and other counts

1. Annotate a small region close to the ROI contour to test the accuracy of the cell detection parameters using the Hoechst channel, in different areas to account for different nuclei intensities and morphologies. Once the parameters (nucleus parameters including sigma, minimum and maximum area, intensity, and cell expansion) are used to result in a count within 10% variance of a manual count, these parameters may then be applied to the ROI annotation for cell detection.
2. Using the acquired parameters, apply the cell detection tool to the ROI to identify the total cell number.
3. Using the point tool, manually count the TH+ neurons and create an annotation.
4. Using the point tool, manually count the TH+ neurons with pS129 immunofluorescence (see definition
above) by toggling off individual channels together with the counted “TH+ cells point annotation”.

9 ***Intensity data and masking***

1. Extract intensity data from the ROI contour using the “add intensity features” function.
2. Create thresholders using different intensity parameters guided by the detection measurements provided for the Cell: TH mean and standard deviation to generate masks within the ROI annotation.

a. Generate the first mask of the TH+ area fraction including all TH immunoreactivity within the ROI.
b. Generate the second mask utilized a much higher intensity to mask mainly the TH+ cells and major processes or projections.
c. Add intensity features to the masks created for the detection of mean intensities of other channels within the TH+ masks.

Statistical analyses

10 After identifying all TH+ neurons in the ROI, the TH+ cell proportion, density, projections and cell size were calculated. Non-parametric statistics were performed using Kruskal-Wallis tests and post-hoc Dunn’s multiple comparisons tests (Prism 10 (v.10.0.0)) to identify any differences in the above parameters between groups with $p < 0.05$ as the level of significance.

Protocol references

References

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