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Electronic Supplementary Information

Dysfunction of vesicular storage in young-onset Parkinson's patient-derived dopaminergic neurons and organoids revealed by single cell electrochemical cytometry

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1. Experimental Section

1.1 Reagents and solution

Chemicals of analytical grade were obtained from Sigma-Aldrich unless stated otherwise. The HEPES physiological saline consists of 150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, and 2 mM $CaCl_2$. All solutions were made with 18 MΩ·cm water from a Millipore purification system, and the solution pH was adjusted to 7.4 with NaOH (3.0 M).

1.2 iPSC line generation and maintenance

The iPSC line (PD52) was generated from one young-onset Parkinson's disease (YOPD) patient as reported.[1, 2] The gender of the patient is male and he was diagnosed as YOPD at age of 23 years with the *PINK1* gene mutation, and the iPSC line was generated at age of 26 years from peripheral blood mononuclear cells (PBMCs). The human sample was collected with informed consent under full ethical approval in accordance with the ethics committee at Nanjing Medical University ([2019] No. 485). Briefly, PBMCs from the YOPD patient were transfected with Sendai virus to integrate the multifunctional transcription factors SOX2, KLF4, OCT3/4, and c-Myc, which could make the peripheral blood cells transcribe into iPSCs after 2-3 weeks (Figure S1A). Then the clones were selected to domesticate and amplify early iPSCs. The obtained iPSCs could specifically express pluripotency markers NANOG, SOX2, and cell-proliferation marker KI67, indicating that iPSCs with the ability of multi differentiation and proliferation were established successfully (Figure S1B). We analyzed the targeted loci sequence in exon 8 of the *PINK1* gene of iPSC generated from the patient and PBMCs. The results indicated that there exists mutation in the *PINK1* gene and the reprogramming process did not change the targeted loci sequence (Figure S1C), which further showed that iPSC from the YOPD patient had been successfully constructed. hESC line (H9, WiCell Agreement No. 16-W0060) was used as control. For pluripotency maintenance, iPSCs/hESCs were maintained under the feeder-free condition as previously described.[1, 2] Cells were cultured on vitronectin-coated or Matrigel-coated plates in E8 medium (Essential 8, Life Technologies) and were passaged every 5-7 d with 1mL trypsin-EDTA (Stemcell Technologies). The medium was half changed every day.

1.3 Midbrain-like cultures and differentiation

hPSCs (PD52/H9) were cultured in Matrigel (Corning) with the neural induction medium including Dulbecco's modified Eagle's medium/F12, N2 supplement, nonessential amino acids (Life Technologies) with 2 μM SB431542 (Stemgent), 2 μM DMH1 (Tocris), 500 ng/mL SHH (C25II, R&D Systems) and 0.4 μM CHIR99021 (Stemgent) for 8 days. On day 9, individual epithelial cell colonies were gently blown off and expanded into floating clusters in the neural induction medium containing 2 μM SAG (Calbiochem), 100 ng/mL SHH, and 0.4 μM CHIR99021 for 4 days (D9-12). Then the progenitors in suspension were cultured in the neural induction medium containing 0.5 μM SAG and 100 ng/mL FGF8b (PeproTech) for another week (D13-19). Progenitors will be kept in 100 ng/mL FGF8b and 20 ng/mL SHH till day 34. The neurospheres were dissociated by ACCUTASE (Innovative Cell Technologies) on day 35 and plated onto glass coverslips that were coated with Matrigel in the neurobasal medium to obtain 2D midbrain DA neurons while 3D midbrain organoids were kept in the neural induction medium combined with 10 ng/mL brain-derived neurotrophic factor (PeproTech), 200 μM ascorbic acid (Sigma-Aldrich), 10 ng/mL glial-derived neurotrophic factor (PeproTech), 1 ng/mL transforming growth factor β3 (R&D Systems), 1 μM cAMP (Sigma-Aldrich) and 1 μM Compound E (Calbiochem).

1.4 Fabrication of nano-tip microelectrodes

Nano-tip carbon fiber microelectrodes were fabricated as previously described.[3, 4] Briefly, a carbon fiber (5 μm in diameter) was firstly aspirated into a borosilicate glass capillary (1.2 mm o.d., 0.69 mm i.d., Sutter Instrument Co., Novato, CA). Subsequently, the glass capillary was pulled into two separate electrodes with a commercial micropipette puller (P1000, Sutter Co., USA). Under a microscope, the fiber exposing the glass was cut into about 100 μm with a scalpel. The electrodes were held on the edge of a butane flame for about 2 s to flame etch the carbon fiber,. When the tip end turned red, the electrode was pulled out from the flame and examined under the microscope. Finally, the as-obtained electrodes with needle-sharp fiber tips were sealed with epoxy (Epoxy Technology, Billerica, MA). Scanning electron microscope images of the nano-tip microelectrodes were obtained and

recorded with S-4800 (Hitachi, Japan).

1.5 Single cell electrochemical cytometry

Electrochemical recordings from single dopaminergic neurons or organoids at 6-7 weeks were performed on an inverted microscope (BX51W1, Olympus, Japan) in a Faraday cage. The working electrode was held at +780 mV versus Ag/AgCl reference electrode by a patch-clamp amplifier (700B, Axon Instrument, CA, USA), which recorded the oxidation current and filtered the signals at 0.3 kHz with a low pass Bessel filter. The output signals were digitized at 5 kHz by a digitizer (1550A, Axon Instruments, CA, USA). The nano-tip of the microelectrode was moved to the surface of the cell membrane of the neuron (at day 45) with a micromanipulator (MP-225, Sutter Instrument Co., Novato USA), and then slowly moved forward to penetrate inside the cell body of the neurons. Three independent biological replicate experiments were conducted for each group.

1.6 Data acquisition and analysis

The amperometric data were analyzed with Igor software. The filter for the current was 1 kHz. The threshold for the peak detection was three times the standard deviation of the noise. The traces were carefully checked after peak detection and false positives were manually rejected. The parameters (I_{max} and t_{half}) could be observed from the body of the individual peak. The number of molecules from individual current transients can be quantified with Faraday's equation ($N = Q/nF$) where Q is the charge from the time integral of current transients or amperometric spikes, and n is the number of electrons exchanged in the oxidation reaction (2 for catecholamines) and F is Faraday's constant (96485 Cm^{-}). These parameters were pooled, and the mean of the average of these parameters calculated from single neurons was used. In this way, we minimized the impact of the cell-to-cell variations, as the value is less sensitive to outliers. Pairs of data sets were compared with two-tailed Mann-Whitney rank-sum test; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

1.7 Whole-cell patch-clamp

Whole-cell patch-clamp recordings were performed in dopaminergic neurons within

midbrain-like cultures at 6-8 weeks. Briefly, a series of step currents with incremental amplitude (10 pA) from -30 pA to 60 pA were injected to evoke action potential. For recording sodium or potassium current, a series of voltages with 10 mV increments from -30 mV to 80 mV were injected and the inward current was used as the voltage-dependent sodium channel current (I_{Na+}). The resistance of the electrodes was 9-15 MΩ. Voltage and current signals were recorded with an Axopatch 700B amplifier (Axon) connected to a Digidata 1550B interface (Axon). The data were digitized and stored on disks using pClamp (version 9; Axon) and analyzed by Clampfit and Origin.

1.8 Immunofluorescent staining of midbrain-like cultures

Midbrain DA neurons cultured on the Matrigel-coated cover glasses were fixed in 4% paraformaldehyde (PFA, Sigma) for 15 min, permeabilized, blocked by blocking buffer containing 0.1% donkey serum, and 0.3% Triton X-100 for 30 min, incubated with the primary antibodies overnight at 4 °C and incubated with the fluorescence conjugated secondary antibodies at room temperature for 1 h. Nuclei were counterstained with Hoechst 33342 (HO) (Sigma-Aldrich). Images were randomly taken by Nikon TS100 microscope and the data were analyzed by ImageJ and GraphPad.

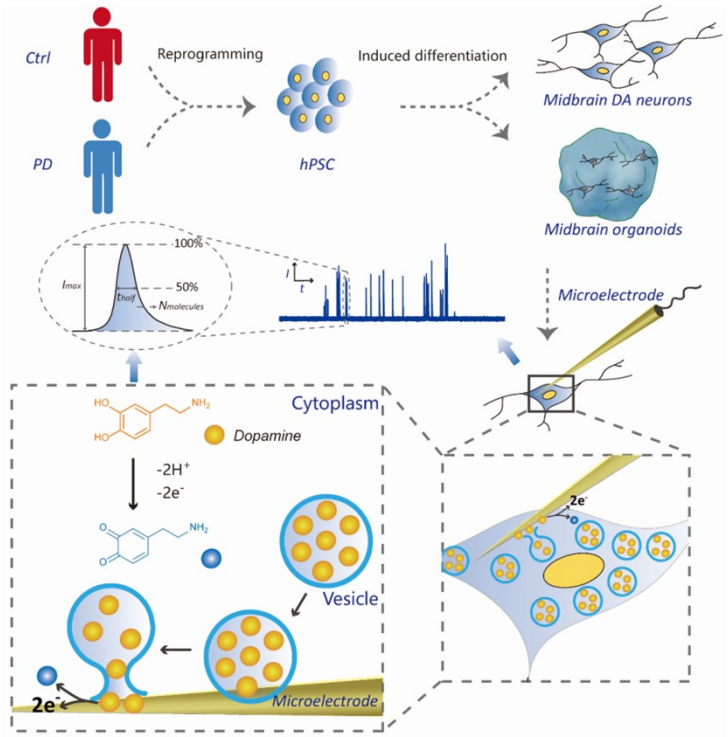
The midbrain organoids were fixed in 4% PFA for 2-4 h at 4 °C and then washed in PBS for three times. Next, the organoids were removed and immersed sequentially in 20% and 30% sucrose (Sigma) until sunk. Subsequently, the organoids were embedded in optimal cutting temperature (OCT) and cut into 10 μm sections using a cryostat microtome (CM 1950, Leica). For immunostaining, cryosections were rinsed with PBS for three times. Cryosections were treated with 1% X-100 Triton and 5% donkey serum for 1 h before incubation with primary antibodies overnight at 4 °C. Cryosections were then incubated for 1 h at room temperature with fluorescently conjugated secondary antibodies (Life Technologies). The nuclei were stained with Hoechst 33342. Images were randomly taken by Nikon TS100 microscope and the data were analyzed by ImageJ and GraphPad. All values were obtained from three independent differentiations for each group.

1.9 Western blotting

Neurons or organoids were lysed in RIPA buffer containing protease and protease inhibitor cocktail (Roche). Then lysates were sonicated by injecting syringe several times. Samples were then centrifuged for 25 min at 4 °C at 13,000 rpm. Proteins measured using Enhanced BCA Protein Assay Kit (Beyotime, P0010) beforehand were loaded into gels (SurePAGE) and separated by SDS-PAGE (GenScript, M00652) with 100 V electrophoresis. Then, proteins were transferred to polyvinylidene fluoride membranes at 300 mA for 2 hours and blocked in 5% Bovine Serum Albumin for 2 hours. Primary antibodies were incubated overnight at 4 °C. The secondary antibodies were incubated with the membranes on a shaker for 2 h at room temperature. Finally, the signals were visualized using a ChemiDoc XRS+ imaging system (BIO-RAD). Primary antibodies used were human α-synuclein (1:1,000 dilution, Abcam, ab138501), tyrosine hydroxylase (TH) (1:2,000 dilution, Pel-Freez, P40101), cleaved caspase 3 (1:1,000 dilution, CST, 9664s), GAPDH (1:4,000 dilution, Affinity, T0004). All values were then compared across at least three independent differentiations for each group.

2. Supplementary Figures

2.1 Schematic illustration



Scheme S1. Schematic illustrating the generation and analysis of midbrain-like cultures from normal control and the YOPD patient hPSC lines.

2.2 Generation of hPSCs from the YOPD patient

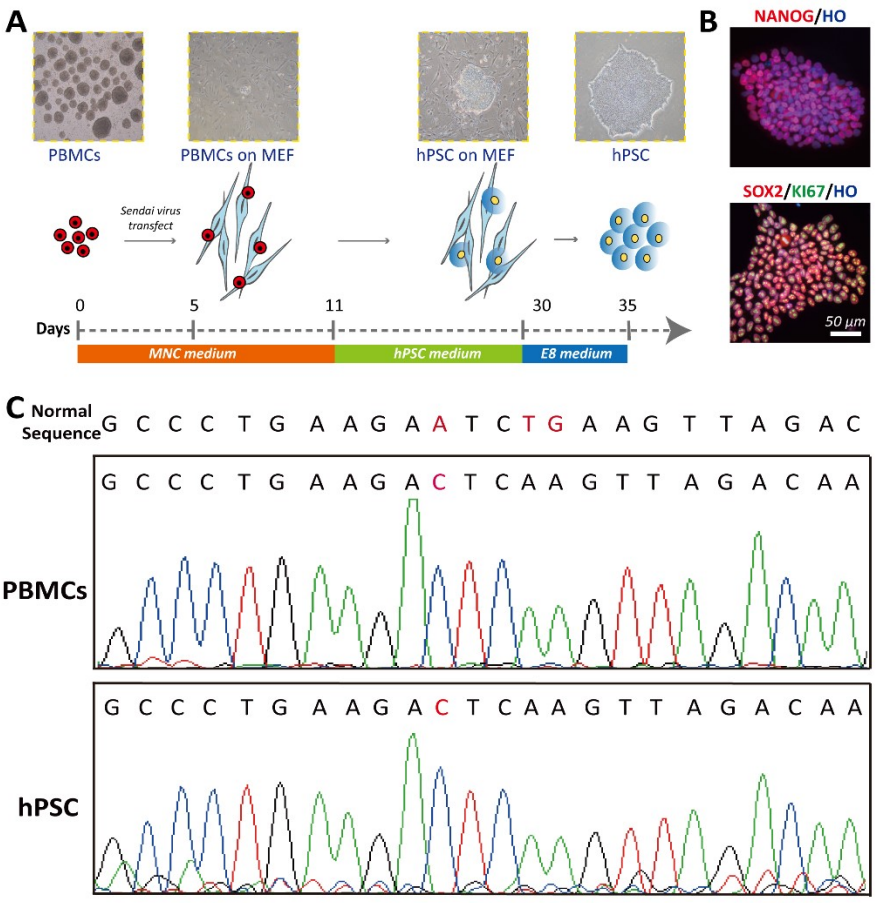


Figure S1. (A) Schematic diagram illustrating the generation of YOPD hPSCs. (B) Representative images of the stem cell pluripotency markers (NANOG and SOX2) and the cell-proliferation marker KI67 immunostaining of hPSCs. Scale bar = 50 μm. (C) Sequences of targeted loci (in exon 8 of *PINK1* gene) in hPSC clone and PBMC.

2.3 Immunostaining for the neuronal markers

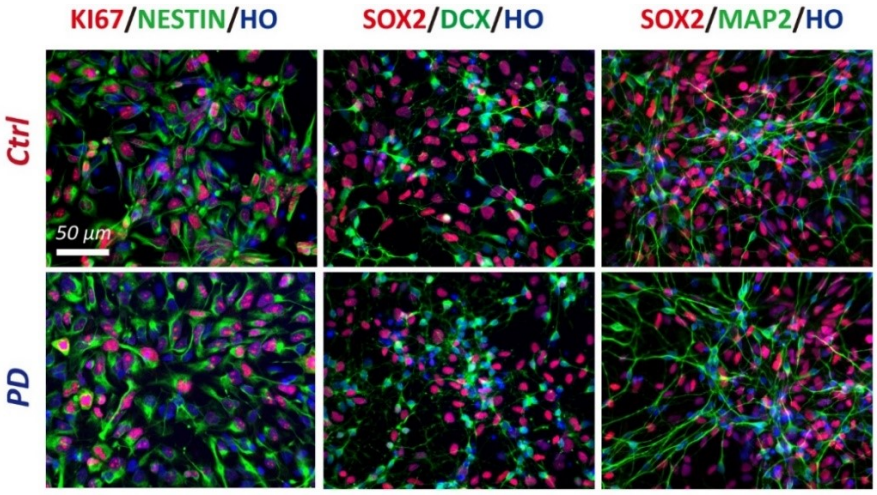


Figure S2. Immunostaining for the proliferation marker KI67, the neural precursor cell markers SOX2 and NESTIN, the immature neuronal marker DCX, mature neurons marker MAP2 at Day 18 in Ctrl or PD52 iPSCs-derived DA neurons. Scale bar = 50 μm.

2.4 Fabrication of the conical nanotip electrodes

In order to observe the micro morphology, the conical nanotip electrodes were characterized by scanning electron microscope. As shown in Figure S3A, the length of the carbon fiber exposing the glass was about 30-100 μm and the diameter of the sharp tip was about 50-100 nm, indicating that the nano tip electrode has been successfully prepared. The cyclic voltammogram of the prepared electrode in 0.1 mM dopamine solution was detected (Figure S3B). A well-defined curve with nearly sigmoidal-shaped voltammograms was obtained, demonstrating stable diffusion limiting current in the electrochemical process. The electrode with long cone, tip formation and good electrochemical performance can be used in the subsequent determination process.

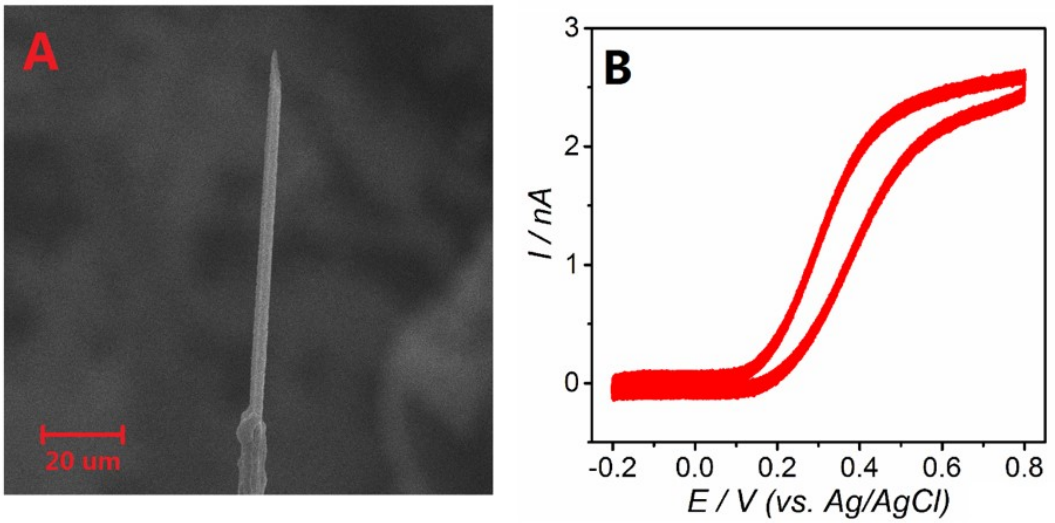


Figure S3. Characterization of nano-tip carbon fiber electrode for intracellular vesicle impact cytometry (IVIEC). (A) Scanning electron microscopy of a nano-tip electrode. Scale bar = 20 μm. (B) Representative cyclic voltammogram of nano-tip carbon fiber electrode in 0.1 mM dopamine solution.

2.5 Plasticity differs in control and DA cultures derived from PD52 iPSC

Typical peaks for vesicle rupture and amperometric detection for control cultures (curve red) and PD52-derived DA cultures (curve blue) are shown in Figure S4A. Compared to those control cultures, the single peak from PD52-derived DA cultures leads to lower amplitude and broader rupture events. Figure S4B shows the peak parameters evaluated with t_{half} and I_{max} summarized in Figure S4C and D. In our previous study, we proposed that electroporation was the main driving force for vesicle opening at a polarized electrode in IVIEC.[3,4] Herein, we suggested that t_{half} presented the formation of the pore on the vesicle membrane via the electric field. A significant increase of t_{half} was observed in DA neurons derived from PD52 iPSC (2.14 ± 0.09 ms, n = 22), as compared with that in the cells from normal controls (1.84 ± 0.05 ms, mean ± SEM, n = 24) ($p<0.05$, Figure S4C), revealing that PD52 mDA cultures virtually prolongs the duration of the pore formation on the vesicle membrane. A decrease in the value of I_{max} is observed in the DA cultures (Ctrl: 23.40 ± 2.21 pA, n = 24 vs. PD: 15.64 ± 0.81 pA, n = 22) ($p<0.01$, Figure S4D), which is in agreement with the results of depletion of single vesicle content in DA cultures derived from PD52.

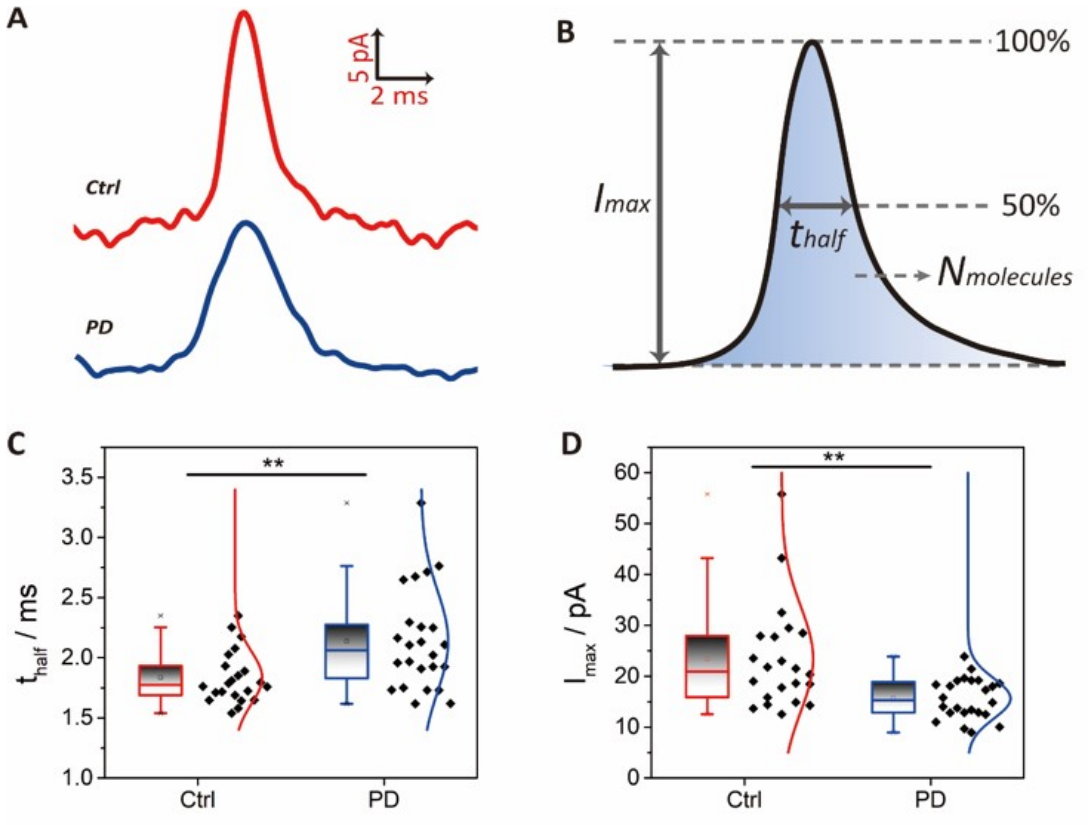


Figure S4. (A) Typical peaks obtained from IVIEC of H9 and PD52 derived DA neurons. (B) Scheme showing the different parameters used for the peak analysis. Comparisons of (C) half peak width, t_{half}; and (D) peak current, I_{max} from IVIEC with each point representing the average level of single vesicles from a separate neuron. Ctrl (24 neurons), PD (22 neurons) from three independent differentiations. **: $p<0.01$.

2.6 PD52 iPSC-derived midbrain organoids exhibited classical PD phenotype

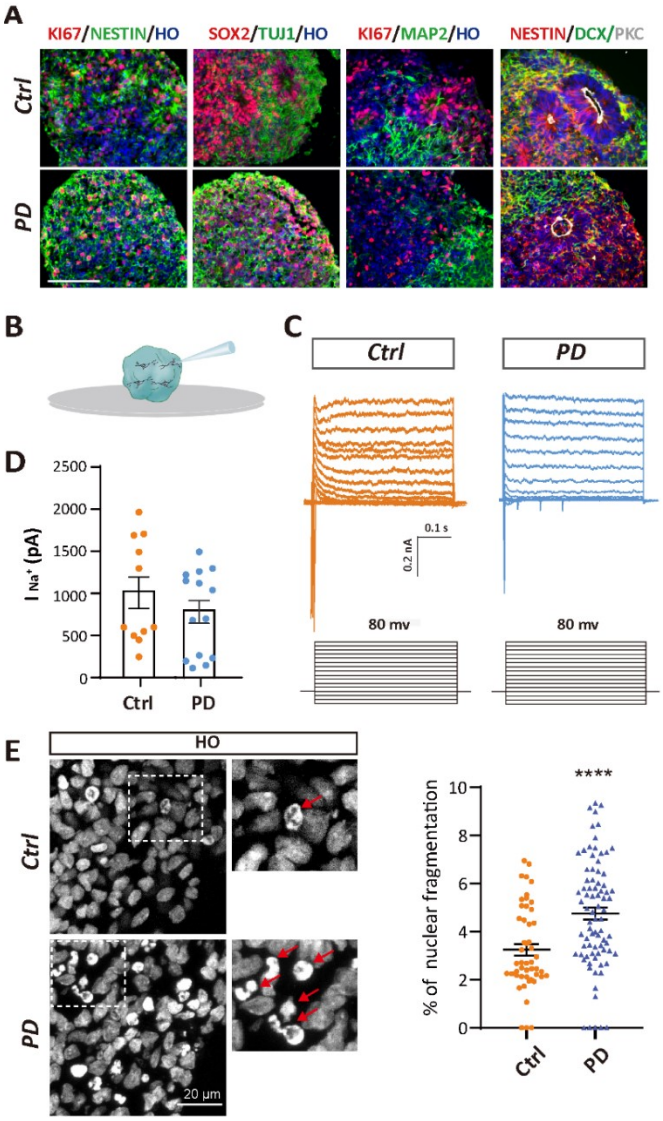


Figure S5. (A) Cryosection of midbrain organoids at days 24 and stained for KI67(proliferation marker), NESTIN (neural stem cell marker), SOX2 (neural stem cell marker), PKC-λ (adherent junction marker), and neuronal marker TUJ1, MAP2, and DCX. Scale bar = 100 µm. (B) Schematic illustration of whole-cell patch electrical experiments. (C) Representative traces showing the presence of voltage-dependent Na^+ and K^+ currents in neurons of midbrain organoids, and (D) statistical analysis of voltage-dependent Na^+. (Error bars, ± SEM; Two-tailed unpaired t-test.) (E) Representative images and quantification of nuclear fragmentation in H9 and PD52 derived midbrain organoids at day 45, respectively. The results were obtained from three independent differentiations for each group. Scale bar = 20 µm. ****: $p<0.0001$.

2.7 Pharmacological effects of AMA were evaluated in midbrain organoids

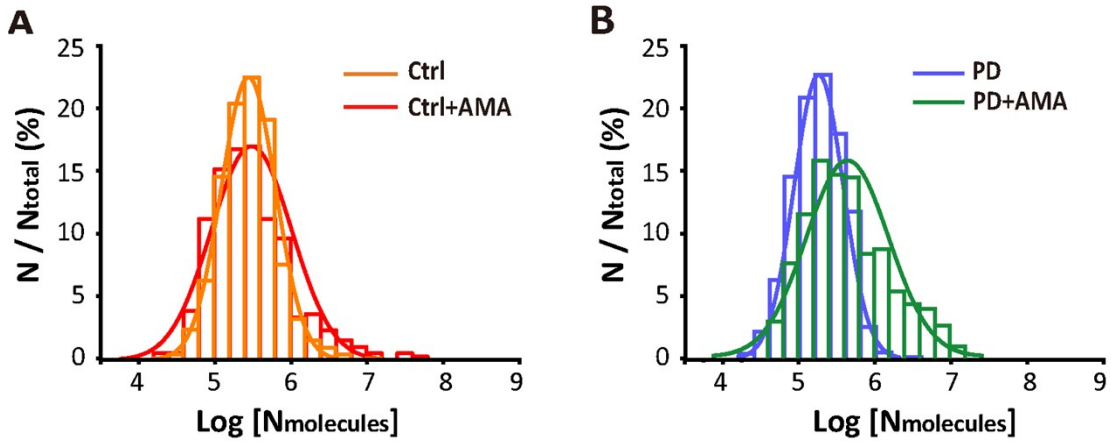


Figure S6. Normalized frequency distribution for vesicular content from (A) control (orange) and control treated with AMA (red), (B) PD52-derived organoids (blue), and PD52-derived organoids treated with AMA (green).

2.8 α-Syn accumulates specifically in DA neurons derived from PD52 iPSC

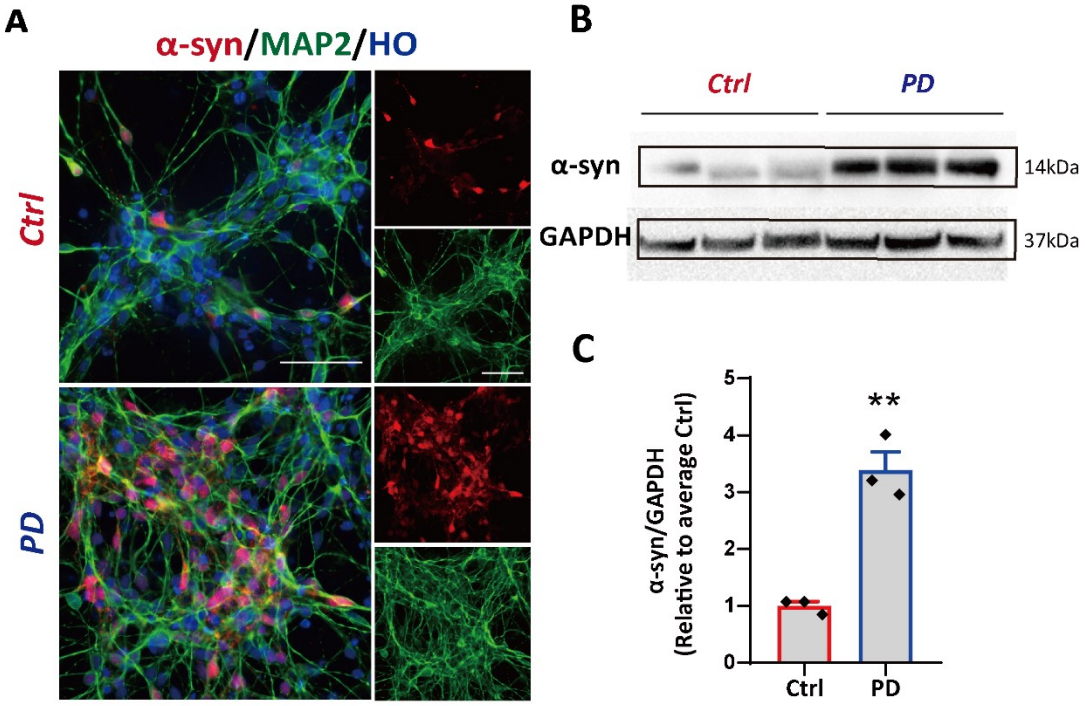


Figure S7. (A) Representative microscopy views of day 45 DA neurons derived from H9 or PD52 showing MAP2 and α-syn expression and morphology. Scale bar = 100 µm. (B) Western blots to detect the level of α-syn from three differentiations of day 45 DA cultures and GAPDH as a housekeeping control. (C) Relative intensities from

western blots from three differentiations. Intensities are given relative to the average for the control lines. **: p<0.01.

2.9 PMA were evaluated in midbrain organoids

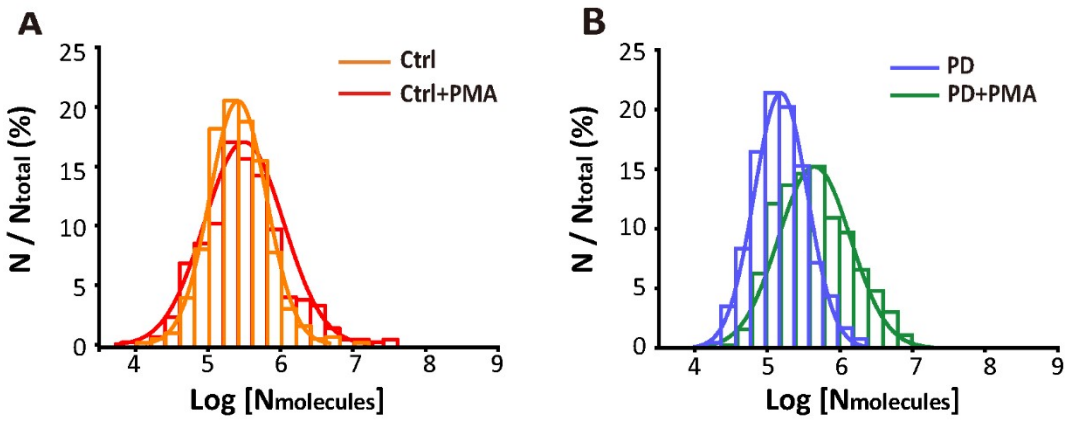


Figure S8. Normalized frequency distribution for vesicular content from (A) control (orange) and control treated with PMA (red), (B) PD52-derived organoids (blue), and PD52-derived organoids treated with PMA (green).

3. References

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