Methods

Generating glutamatergic neurons from human embryonic stem (hES) cells

hES cells were grown on a matrigel-coated plate in TeSR. On day 1, cells were treated with Collagenase IV to lift off colonies. Colonies were washed and resuspended in TeSR with 10 μM Y-27632 (STEMCELL Technologies) and then transferred to an ultra-low attachment plate and cultured overnight at 37ºC 5% CO_2 with regular shaking to form embryoid bodies (EBs). On day 2, the media was replaced with pan base media (DMEM F12 w/Glutamax, 1 X N2 supplement, 1 X B27 supplement; Gibco), with 0.5 μM LDN-193189, (Adooq), 10 μM SB-431542 (Adooq), and 500 ng/mL Noggin (R&D systems). Media was changed every 2-3 days. On day 17, the EBs were transferred to pan base media with 1 μg/mL laminin (Invitrogen), 0.5 μM LDN-193189, 10 μM SB-431542, and 500 ng/mL Noggin onto plates coated with 10 μg/mL ploy-L-ornithine (Sigma) and 1 μg/mL laminin for the formation of rosettes. On day 25 rosettes were picked manually and dissociated with accutase (STEMCELL Technologies) to yield a suspension of neural progenitor cells (NPC). NPCs were plated onto ploy-L-ornithine/laminin-coated plates and expanded in pan base media with 20 ng/mL FGF-2 (Joint Protein Central). For terminal differentiation into glutamatergic neurons, NPCs were plated onto ploy-L-ornithine/laminin-coated plastic coverslips and cultured in pan base medium with GDNF, BDNF (both 10 ng/mL; Peprotech), 0.2 μM ascorbic acid (STEMCELL Technologies), and dibutyryl cAMP (500 μg/mL; TOCRIS) for 3 weeks prior to iDA coculture.

Generating GABAergic neurons from hES cells

hES cells were grown on a matrigel-coated plate in TeSR. On day 1, cells were treated with Collagenase IV to lift off colonies. Colonies were washed and resuspended in TeSR with 10 μM Y-27632 (STEMCELL Technologies) and then transferred to an ultra-low attachment plate and cultured for 2 days at 37ºC 5% CO_2 with regular shaking to form embryoid bodies (EBs). On day 3, media was switched to EBM (DMEM/F-12, 1 X non-essential amino acid, 1 X GlutaMAX, 0.1 mM 2-mercaptoethanol and 20% Knock-out Serum Replacement, all from GIBCO). On day 7, media was switched to neural induction medium (DMEM/F-12, 1 X non-essential amino acid, 1 X N2 supplement, all from GIBCO, and supplemented with 2 μg/mL heparin. On day 10, EBs were transferred to plates coated with 10 μg/mL ploy-L-ornithine (Sigma) and 1 μg/mL laminin for the formation of rosettes. On day 24, rosettes were picked onto an ultra-low attachment plate in NIM media with 1 X B27 supplement, 1% Pen/Strep, 10 μM Y-27632. Cells were cultured at 37ºC 5% CO_2 with regular shaking to form neural progenitor spheres (NPS). On day 30, NPS were dissociated with accutase and replated onto ploy-L-ornithine/laminin-coated plastic coverslips in GABA neuron media (Brainphys Neuronal Medium (STEMCELL Technologies), 1 X B27 supplement, 1X Glutamax, BDNF, GDNF, IGF1 (all in 10 ng/mL), 1 μM cAMP, 200 ng/mL

Ascorbic Acid) supplemented with 200 nM compound E. Compound E was kept in the medium for 6 days. Cells were then grown in GABA neuron media for 30 days prior to iDA co-culture.

Direct conversion of adult human fibroblasts into iDAs

Primary human dermal fibroblasts (Table S1) were shared by ICM in Paris, University Hospital Erlangen and Institute of Neurology (The study approval for the cell lines from the University Hospital Erlangen was granted by the local ethics committee (No. 4485, FAU Erlangen-Nürnberg)), University College London, and obtained from Corriel Institute and a previous study (1). Fibroblasts were cultured in DMEM containing 20% tetracycline-free fetal bovine serum 1 X non-essential amino acid, 1 X GlutaMAX and 1% pen/strep. Fibroblasts were transduced with lentiviral particles for Ngn2-2A-Ascl1 (2, 3) and expanded in the presence of puromycin (1 µg/mL; GIBCO). Prior to conversion, cells were pooled into high densities and transduced with lentiviral particles for XTP-lmx1a. Two days later medium was changed to iDA conversion medium for 4 weeks. iDA conversion medium is composed of DMEM:F12/Neurobasal (1:1), 1 X N2 supplement, 1 X B27 supplement, 2 µg/mL doxycycline (STEMCELL Technologies), 1 µg/mL Laminin, 10 ng/mL BDNF, 10 ng/mL GDNF, 500 µg/mL dibutyryl cAMP, 150 ng/mL human recombinant Noggin, 0.5 µM LDN-193189, 0.5 µM A83-1 (Adooq), 3 µM CHIR99021 (Adooq), 5 µM Forskolin (Adooq), 10 µM SB-431542, 0.25 µM SAG (Adooq), 2 µM purmorphamine (Adooq) and 100ng/mL FGF-8 (Peprotech). Medium was changed every 2-3 day. At the end of week 4, iDAs were either subjected to FACSorting or fixed with 4% paraformaldehyde for immunocytochemistry.

Co-culture of iDA for electrophysiology and dopamine release recordings

Fibroblasts transduced with lentiviral particles for h-syn-RFP were converted to iDAs. iDAs were dislodged with TrypLE (GIBCO) and replated on a supporting layer of mouse astrocytes, human glutamatergic or GABAergic neurons and cultured in BrainPhys maturation media supplemented with 10 µM Y-27632. BrainPhys maturation media was based with Brainphys Neuronal Medium, supplemented with 1 X N2 supplement, 1 X B27 supplement, 10 ng/mL BDNF, 10 ng/mL GDNF, 500 µg/mL dibutyryl cAMP and 1 µg/mL laminin. For Co-culture with mouse astrocytes, 1% of Knock-out Serum Replacement was supplemented to the Brainphys Neuronal Medium. iDAs were allowed to mature for 7 weeks for electrophysiological recordings. For dopamine release recordings, iDAs were allowed to mature for 12 weeks with glutamatergic neurons. Culture media stayed with the cell for 3 days was harvested for aptamer nanopipette recording.

High-throughput whole transcriptome RNA sequencing (RNA-Seq).

RNA-Seq libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit according to the manufacturer's instructions (Illumina). Libraries were sequenced single-end 50 bp using the Illumina HiSeq2500 platform. Reads were processed with BioTuring software. Expression values, principal component analysis and corresponding plots were performed using R4.1.1 and RStudio 1.4.1717 software based on normalized counts. Differential gene expression was calculated using edgeR package (bioconductor.org). STRING 11.5 (string-db.org) was used for gene interaction analysis and gene ontology analyses with differentially expressed genes between fibroblasts and iDAs. GO term analysis for differentially expressed genes in aging and PD to healthy control

comparison was performed with DAVID v6.8.

Making of TSTD1_sh lentiviral vector and viral particle production

An open reading frame variant 1 of TSTD1 was purchased (Genescript) and cloned into the tet-inducible pLV-XTP backbone (Clontech). For TSTD1 knockdown, shRNA sequences (TSTD1_sh: CCGGGCTGCCTTCCAGGCTTTATATCTCGAGATATAAAGCCTGGAAGGCAGCTTTTTG) were cloned into the Tet-pLKO-puro vector (58, a gift from Dmitri Wiederschain, Addgene plasmid # 21915; http://n2t.net/addgene:21915; RRID:Addgene_21915). Lentiviral particles were produced in HEK-293T cells that were co-transfected with the plasmids psPAX2 and pMD2.G and the respective lentiviral vector plasmid. Viral particles were enriched by ultracentrifugation and were used fresh or stored in high concentration at -80°C.

Electrophysiology

Whole-cell patch-clamp was performed on hSyn-RFP iDA in corresponding co-culture conditions for 8-10 weeks. Coverslips with iDAs were transferred to a recording chamber in a recording medium containing (in mM): 10 HEPES, 4 KCl, 2 CaCl2,1 MgCl2, 139 NaCl, and 10 D-glucose (solution will be brought close to physiological conditions 310 mOsm, pH 7.4). Patch electrodes with a typical resistance of 8-10 Mohm were filled with internal solutions containing (in mM): 130 K-gluconate, 6 KCl, 4 NaCl, 10 Na-HEPES, 0.2 K-EGTA, 0.3 GTP, 2 Mg-ATP, 0.2 cAMP, 10 D-glucose, 0.15% biocytin and 0.06% rhodamine. Signals were digitized and recorded, and the data were analyzed using Clampfit-10 and MATLAB.

Dopamine detection with aptamer-modified nanopipette

DNA aptamers were functionalized on the inside of the quartz nanopipette using a previously reported protocol (4, 5). Thiolated dopamine aptamers were purchased from Microsynth AG (Balgach, Switzerland). The sequence is as follows: (5'/Thiol/-CGA CGC CAG TTT GAA GGT TCG TTC GCA GGT GTG GAG TGA CGT CG-3') with a molecular weight of 13,871.8 g/mol and melting point of 73.7 °C. Alternatively, scrambled sequences with the same number of nucleotides as the dopamine aptamer sequence but with pseudo-randomized order of the bases, were used as controls. The scrambled sequence was as follows: (5'/Thiol/- AGT ACG TCG ATG CTC GAT CAG TGG GCT AGG TGC GTA GCG GTC TG-3') with a molecular weight of 13,871.8 g/mol and melting point of 74.9 °C.

Dopamine sensing was conducted in the culture media of human DA neurons and the negative control from pan neuron culture medium. The baseline current was taken from blank medium that had never touched any cells with the same ionic content as the test samples. Samples were shipped from the Salk Institute for Biological Studies (San Diego, USA) to ETH Zürich (Zürich, Switzerland) overnight on dry ice and were stored immediately at -80 °C until use. For measurements, samples were thawed; freeze-thaw cycles were limited to twice per sample to

minimize dopamine degradation. To decelerate potential dopamine polymerization during the recordings, 1 wt% of ascorbic acid was added to all tested media.
Real-time recordings were conducted at a set voltage bias of +0.5 V. A baseline was first obtained by inserting the nanopipettes in the blank medium and stabilized. Then, the medium was exchanged to the negative control and, subsequently, the iDA medium. Cyclic voltammograms (N>5, averaged) were acquired after each of the exchanges by sweeping voltage from -0.3 to +0.6 V at a rate of 0.1 V s-1. To demonstrate the statistical significance of the difference between the negative control medium vs. iDA medium, the sensor response was quantified by calculating the % change in current from the blank medium at +0.5 V.

Immunocytochemistry

Cells were washed and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature and blocked with 5% normal horse serum in PBS with 0.1% TritonX-100. Primary antibodies were incubated overnight at 4°C. Cells were washed with PBS three times, blocked again with 5% normal horse serum and incubated with secondary antibodies with DAPI for 45 min at room temperature. Antibodies are listed in Supp Table 1.

Sectioning and immunohistochemistry of human substantia nigra

Formalin-fixed substantia nigra specimens were dehydrated in 30% sucrose solution in PBS for 72 hrs. Dehydrated tissues were embedded in OCT compound and sectioned into 35-µm slices using standard procedures. Before staining, sections were incubated with 0.5% sodium borohydride at room temperature, followed by sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) at 90°C for 1 hr in decloaking chamber. Decloaking Chamber NxGen (BIOCARE MEDICAL). Primary antibodies were applied for 5 days at 4°C and secondary antibodies overnight at 4°C. Background fluorescence was reduced using Autofluorescence Eliminator Reagent (Millipore) and nuclei were counterstained with DAPI. Sections were mounted onto glass specimen slides and mounted in PVA-DAPCO and imaging was performed using a Zeiss LSM880 confocal microscope. For quantification, Imaris (Oxford Instrument) was used to establish the surface object defining TH-positive cells. Volume and total TSTD1 fluorescence intensity were determined. Expression of TSTD1 was quantified in terms of fluorescence intensity per cell volume. Antibodies are listed in Supp Table 1.

Western blotting

Cells were lysed with RIPA buffer (Sigma Aldrich) containing protease inhibitors (Roche) and incubated for 30 min on ice. Lysates were clarified by centrifugation (13,000 g, 10 min, 4°C) and blotting was performed following standard procedures with pre-casted NuPage gels, nitrocellulose membranes and iBlot2 transfer system (Life Technologies). Primary antibodies were incubated overnight at 4°C and IRDye680/800 coupled secondary antibodies were incubated for 1 hr at room

temperature. Protein bands were visualized using Odyssey Imager (LI-COR Biosciences). Antibodies are listed in Supp Table 1.

Mass spectrometry analysis

Amino acids and other polar metabolites were extracted from samples according to Yuan et al., 2012 (6). Briefly, metabolites from cell pellets were extracted with 500 µL of 80 % methanol (ice cold) containing heavy internal standards (Metabolomics Amino Acids Standard, MSK-A2, Cambridge Isotope Laboratories). Samples were sonicated, vortexed and incubated for 1h at -80 °C. Samples were centrifuged (14,000 x g, 4 °C, 10 min), and supernatants were transferred to new tubes and dried in speedvac. Samples were reconstituted in 45 µL water prior to injection. Amino acids were analyzed on a Dionex Ultimate 3000 LC system (Thermo) coupled to a TSQ Quantiva mass spectrometer (Thermo) fitted with a XBridge Amide HILIC column (3.5 µm, 100 x 4.6 mm i.d., Waters). The following LC solvents were used: solution A, 95:5 water:acetonitrile, 20 mM ammonium hydroxide, 20 mM ammonium acetate, solution B, 100 % acetonitrile. The following gradient was utilized: at a flow rate of 0.4 mL/min with a gradient consisting of 85-60 % B in 2 min, 60-50 % B in 8 min, 50-2 % B in 2 min, 2 % B for 4 min, up to 85 % B in 1 min and re-equilibrate at 85 % B for 7 min, for a total run time of 24 min. The injection volume was 10 µL, the column oven temperature was set to 25 °C and the autosampler kept at 4 °C. MS analyses were performed using electrospray ionization in positive ion mode, with spay voltages of 3.5 kV, ion transfer tube temperature of 325 °C, and vaporizer temperature of 275 °C. Multiple reaction monitoring (MRM) was performed by using mass transitions between specific parent ions into corresponding fragment ions for each analyte (Supp Table 2). Skyline (7) was used to measure peak areas, absolute and relative quantitation was obtained by using heavy internal standards.

FACS analysis for cellular H_2S level and MMP

Cellular H_2S level was determined using sulfidefluor 7 AM (Tocris). iDAs or iPSC DA neurons were dissociated to single cells using Accutase, then incubated with 2.5 µM SF7-AM diluted in media for 30 min at 37 °C and 5% CO_2. iDAs were also incubated with hNCAM-APC antibody.

MMP was assayed using the JC-1 lipophilic cationic dye (Life Technologies). iDAs or iPSC DA neurons were dissociated to single cells using Accutase then incubated with 2 µM JC-1 diluted in media for 20 min at 37°C and 5% CO_2. iDAs were also incubated with hNCAM-APC antibody.

The cells in both assays were washed twice and resuspended in DPBS with 0.5% serum replacement. The green fluorescence of sulfidefluor 7 AM and the green and red fluorescence of JC-1 dye were quantitated with LSRII (Becton Dickinson), Fluorescence intensity of sulfidefluor 7 AM and red/green intensity ratio of JC-1 were determined using FlowJo 10 software (Tree Star).

Statistical analysis.

Statistical values for RNA-Seq data were corrected for adjusted p value (padj) using edgeR. Means, SD, SEM, correlation and 95%CI of all other quantitative data were calculated using GraphPad Prism 8 software. Student's t tests and one way ANOVA were performed with Prism 8 for significance evaluation.

Reference for Methods

1. Marchetto MC, Belinson H, Tian Y, Freitas BC, Fu C, Vadodaria K, Beltrao-Braga P, Trujillo CA, Mendes APD, Padmanabhan K, Nunez Y, Ou J, Ghosh H, Wright R, Brennand K, Pierce K, Eichenfield L, Pramparo T, Eyler L, Barnes CC, Courchesne E, Geschwind DH, Gage FH, Wynshaw-Boris A, Muotri AR. Altered proliferation and networks in neural cells derived from idiopathic autistic individuals. Mol Psychiatry. 2017 Jun;22(6):820-835.

2. Herdy J, Schafer S, Kim Y, Ansari Z, Zangwill D, Ku M, Paquola A, Lee H, Mertens J, Gage FH. Chemical modulation of transcriptionally enriched signaling pathways to optimize the conversion of fibroblasts into neurons. Elife. 2019 May 17;8:e41356.

3. Mertens J, Paquola ACM, Ku M, Hatch E, Böhnke L, Ladjevardi S, McGrath S, Campbell B, Lee H, Herdy JR, Gonçalves JT, Toda T, Kim Y, Winkler J, Yao J, Hetzer MW, Gage FH. Directly Reprogrammed Human Neurons Retain Aging-Associated Transcriptomic Signatures and Reveal Age-Related Nucleocytoplasmic Defects. Cell Stem Cell. 2015 Dec 3;17(6):705-718.

4. Nakatsuka N, Faillétaz A, Eggemann D, Forró C, Vörös J, Momotenko D. Aptamer Conformational Change Enables Serotonin Biosensing with Nanopipettes. Anal Chem. 2021 Mar 2;93(8):4033-4041.

5. Nakatsuka N, Heard KJ, Faillétaz A, Momotenko D, Vörös J, Gage FH, Vadodaria KC. Sensing serotonin secreted from human serotonergic neurons using aptamer-modified nanopipettes. Mol Psychiatry. 2021 Jul;26(7):2753-2763.

6. Yuan M, Breitkopf SB, Yang X, Asara JM. A positive/negative ion-switching, targeted mass spectrometry-based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. Nat Protoc. 2012 Apr 12;7(5):872-81.

7. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics. 2010 Apr 1;26(7):966-8.