Supplementary materials and methods

Generation of induced hiPSC-neurons (iNeurons) by NGN2 overexpression

Rapid induction of iPSC into human excitatory neurons was performed via a doxycycline-inducible neurogenin-2 (*NGN2*) system as previously described[1]. The lentiviral DNA pLV-TetO-hNGN2-eGFP-Puro was a gift from Kristen Brennand (Addgene plasmid # 79823) and FUdeltaGW-rtTA was from Konrad Hochedlinger (Addgene plasmid # 19780). Lentiviruses were produced in HEK293T cells (Cat# CRL-3216, ATCC) as previously described[2]. On day -1, iPSCs were dissociated into single cells with Gentle Cell Dissociation Reagent (Cat# 07174, StemCell Technologies) and plated at a density of 50,000 cells/cm^2. Lentiviruses were added in fresh mTeSR1 medium on day 0. To induce *NGN2* expression, doxycycline was added on day 1 (D1) at a final concentration of 2 μg/mL in fresh media consisting of KnockOut DMEM (Cat# 10829018), KnockOut Serum Replacement (Cat# 10828010), MEM-NEAA (Cat# 11-140-050), Glutamax (Cat# 35050061), and β-mercaptoethanol (Cat# 21985023, all from Invitrogen). On D2, puromycin was added at 5 μg/mL for selection. On D4, cells were replated on poly-ornithine (10 μg/mL; Cat# P4957, Sigma-Aldrich) and mouse laminin (5 μg/mL; Cat# 23017015, Invitrogen) co-coated T-25 flasks (2 × 10^6 cells/flask) in media consisting of Neurobasal (Cat# 21103049, Gibco), B27 (Cat# 17504044, Gibco), Glutamax, 20% dextrose (Cat# G8769, Sigma-Aldrich), MEM-NEAA supplemented with 10 ng/mL BDNF (Cat# 450-02), GDNF (Cat# 450-10) and CNTF (Cat# 450-13, all from PeproTech). Half of the culture media was replaced every three days with fresh complete media. On D14 and D21, cells were characterized using immunocytochemistry or quantitative PCR to assess the expression of specific neuronal markers.

Generation of hiPSC-microglia-like cells (iMGLs)

hiPSCs were differentiated to hematopoietic progenitor cells (HPCs) and subsequently to microglia as previously described with minor modifications[3]. Briefly, single cell hiPSCs were plated at low density and differentiated to hematopoietic progenitor cells (HPCs) using the STEMDiff Hematopoietic kit (Cat# 05310, StemCell Technologies). After 10-12 days non-adherent HPCs accumulated within the media and were gently collected and plated onto Matrigel-coated 6-well plates (200,000 cells/well) for iMGL differentiation in microglia basal medium consisting of DMEM/F12 (Cat# 11330032), 2× insulin-transferrin-selenite (Cat# 51500056), 1× Glutamax, 1× NEAA, 2% v/v B27, 0.5% v/v N2 (Cat# 17502048, all from Invitrogen), 400 µM monothioglycerol (Cat#M1753, Sigma-Aldrich), and additional 5 µg/mL insulin (Cat# I9278, Sigma-Aldrich) with fresh added 50 ng/mL recombinant human TGFβ-1 (Cat# 100-21), 25 ng/mL recombinant human M-CSF (Cat# 300-25) and 100 ng/mL recombinant human IL-34 (Cat# 200-34, all from Peprotech). After 28 days of differentiation, iMGLs were cultured in microglia basal medium supplemented with 100 ng/mL recombinant human CD200 (Cat# C311, Novaprotein) and 100 ng/mL recombinant human CX3CL1 (Cat# 300-31, Peprotech) for an additional three days before use in studies. iMGLs were characterized using immunocytochemistry or quantitative PCR to assess the expression of microglia-specific markers.

Generation of hiPSC-astrocytes (iAstrocytes)

hiPSCs were differentiated to neural progenitor cells (NPCs) and subsequently to astrocytes as previously described with some modifications[4]. Briefly, hiPSCs were plated at 10,000 cells/well in an AggreWell 800 24-well plate (Cat# 34815, StemCell Technologies) to generate the embryoid bodies (EBs) using the STEMdiff SMADi Neural Induction Kit (Cat# 08581, StemCell Technologies). After 5 days, EBs were replated onto Matrigel pre-coated 6-well plates for further

differentiation. At D12, neural rosettes were selected by Rosette Selection Reagent (Cat# 05832, StemCell Technologies) and differentiate to NPCs using neural induction medium with SMAD inhibitors. NPCs were characterized immunocytochemically for neural progenitor cell markers.

For astrocyte differentiation, single cells of passaged NPCs (15,000 cells/cm^2) were dissociated and plated on Matrigel coated culturewares in complete astrocyte medium (Cat# 1801, ScienCell). Cells were continually cultured and harvested as astrocytes after 6~8 passages. iAstrocytes were characterized using immunocytochemistry and quantitative PCR to assess the expression of astrocyte-specific markers.

Generation of hiPSC-Oligodendrocytes (iOligos) by overexpression of mouse Sox10 and Olig2

Rapid induction of NPCs into oligodendrocytes was performed via a doxycycline-inducible overexpression of oligodendroglial transcriptional factors (Sox10 and Olig2) as previously described with minor modifications[5]. The lentiviral DNA pLV-TetO-FUW-Sox10-BleoR and pLV-TetO-FUW-Olig2-BleoR were from Marius Wernig (Addgene plasmid # 45843; # 45844). Lentiviruses were produced in HEK293T cells (ATCC) as previously described[5]. On day -1, NPCs were plated with a density of 5 × 10^4 cells/cm^2 in fresh NPC medium consisting of DMEM/F12 medium supplemented with 1% v/v N2 and 20 ng/mL recombinant human bFGF (Cat# 100-18B, PeproTech). Cells were transduced with equal volumes of concentrated Lenti-rtTA, TetO-Sox10 and TetO-Olig2 virus particles for infection. At D1, doxycycline was added at a final concentration of 2 μg/mL in fresh glial induction media (GIM) consisting of DMEM-F12 with 1:200 N2 supplement, 1:100 B27 supplement without vitamin A (Cat# 12587010, Invitrogen), 1 μM SAG (Cat# 9128694, PeproTech), 10 ng/mL recombinant human PDGF-AA (Cat# 100-13A,

PeproTech), 10 ng/mL recombinant human NT3 (Cat# 450-03, PeproTech), 10 ng/mL recombinant human IGF-I (Cat# 100-11, PeproTech), 200 µM Ascorbic acid (AA, Cat# A92902, Sigma-Aldrich), 1:1,000 Trace Elements B (Cat# 25-022-CI, Corning), 10 ng/mL T3 (Triiodo-L-Thyronine; Cat# T2877, Sigma-Aldrich). On D2, Zeocin (Cat# ABT-531, Boston BioProducts) was added at 200 µg/mL for selection. GIM was replaced at day 5 with differentiation media (DM) consisting of DMEM-F12 with 1:200 N2 supplement, 1:100 B27 supplement without vitamin A, 60 ng/mL T3, 10 ng/mL NT3, 10 ng/mL IGF-1, 200 µM AA, 1:1,000 Trace Elements B, and 100 µM dbcAMP (Cat# D0627, Sigma-Aldrich). After additional 7 days of differentiation, cells were replated on poly-ornithine (10 µg/mL) and mouse laminin (5 µg/mL) co-coated T-25 flasks (2×10^6 cells/flask) to differentiate into mature oligodendrocytes in DM which was changed every other day. iOligos were characterized using immunocytochemistry and quantitative PCR to assess the expression of the specific markers.

Total RNA isolation and quantitative RT-PCR

Total RNA was extracted from cells using Qiazol (Cat# 79306, Qiagen) and miRNeasy kit (Cat# 217004, Qiagen) according to the manufacturer's protocol. The RNA was quantified using NanoDrop (Thermo Fisher Scientific), and the quality of RNA was assessed by 260/280 and 260/230 ratios. For conventional quantitative reverse transcription polymerase chain reaction (qRT-PCR), total RNA (200 ng) with specific mRNA probes were used after reverse transcription reaction according to the manufacturer (SuperScript Vilo IV Kit, Cat# 11766050, Invitrogen). All mRNA amplifications were performed with commercially available FAM-labeled Taqman probes (Applied Biosystems/Thermo Fisher Scientific). The following Taqman probes were used: Hs00258900_m1-MAP2, Hs01370653_m1 -NeuN, Hs00194572_m1-SYT1, Hs00902901_m1-

S100B, Hs00909233_m1-GFAP, Hs01003842_m1-ALDH1L1, Hs00610419_g1-IBA1, Hs00219132_m1-TREM2, Hs02786711_m1-PU.1, Hs00263981_m1-CNP, Hs00921945_m1-MBP, Hs01555268_m1-MOG, Hs99999905_m1-GAPDH. All qRT-PCRs were performed in duplicate, and the data are presented as relative expression compared to GAPDH (reference gene) as Mean ± S.E.M. Real-time PCR reaction was performed using 7900HT PCR system (Applied Bio-systems). No RT control reactions were run to confirm successful DNA contamination removal.

IL-6 ELISA (enzyme-linked immunosorbent assay) with hiPSC-derived astrocytes

hiPSC-derived astrocytes were plated at a 24-well plate in astrocyte medium at a density of 200,000 cells/well one day prior to the experiment. Cells were then treated with 50 ng/ml or 100 ng/ml of Poly(I:C) (InvivoGen, Cat# tlrl-pic), 10 µg/ml or 50 µg/ml of Lipopolysaccharide (LPS) (Sigma-Aldrich, Cat# L4391) and vehicle control (Saline) for 24 hours. The conditioned medium from astrocyte cultures (n = 3) was collected by centrifuging at 2,000 rpm for 5 min at 4˚C. Samples were analyzed with an IL-6 ELISA assay (Abcam, ab178013) according to the manufacturer's protocol.

Phagocytosis assay and mesoscale multiplex cytokine assay with iMGLs

Phagocytic activity of iMGLs was examined using a live-imaging system (Incucyte S3, Essen BioScience) as previously described with minor modifications[3]. Briefly, differentiated iMGLs (n = 4) were treated with either 1 µg/mL pHrodo tagged Zymosan A beads (Essen BioScience; Cat# 4617), or pHrodo tagged Zymosan A beads with 10 µM Cytochalasin D pre-treated for 30 min. Time-lapse images of iMGLs phagocytosis were obtained from 4 fields per culture well every hour

for total 24 hours and further processed in Incucyte software for analysis. Cellular red fluorescent signal over time in each treatment was used as an indicator of phagocytosis efficiency over time. Total integrated intensity of red fluorescence within cells per mm^2 was calculated for each time point using Incucyte software.

The production of cytokines in response to neuroinflammatory stimulation was examined in iMGLs by multiple cytokine ELISA as previously described[6]. Briefly, iMGL culture media was replaced with basal media for 2 hr prior to stimulation with LPS (100 ng/ml) for 24 hr, after which conditioned media assessed for cytokine secretion. To simultaneously assess multiple cytokine, conditioned media from iMGLs was processed and analyzed using the V-PLEX human cytokine 30-plex kit (Mesoscale) according to the manufacturer's protocol.

Processing of cell type-specific cell lysates and EV proteins and label-free quantitative LC-MS/MS proteomics

Isolated EVs were acetone-precipitated as described previously[7]. For each cell lysate or EV preparation (n = 3 per cell type), 20 μg of precipitated proteins were resuspended in 1× Laemmli sample buffer (Cat# 1610747, Bio-Rad) with 2-mercaptoethanol and heated at 95°C for 5 min. The whole lysate was loaded to 10% SDS-PAGE gel (Cat# 4561033, Bio-Rad) and run until 1 cm off the loading well. Following Coomassie staining, the entire protein region of the gel was excised and subjected to in-gel trypsin digestion after reduction with dithiothreitol and alkylation with iodoacetamide. Peptides eluted from the gel were lyophilized and reconstituted in 25-50 μL of 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid (Cat# 302031, Sigma-Aldrich) with 2 pmol ADH digest for LC-MS/MS.

Samples were analyzed on a NanoAcquity UPLC (Waters Corporation) coupled to a Q Exactive (Thermo Fisher Scientific) hybrid mass spectrometer as previously described[8]. Briefly, a 1-2 µL injection was loaded at 4 µL/min for 4 min onto a custom-packed fused silica pre-column (100 µm I.D.) with 2 cm of 5 µm (200 Å) Magic C18AQ (Bruker-Michrom, Billerica, MA). Peptides were then separated on a 75 µm I.D. fused silica analytical column packed with 25 cm Magic C18AQ (3µm, 100Å) particles to a gravity-pulled tip. Peptides were eluted at 300 nL/min using a linear gradient from 5-35% of mobile phase B (0.1% (v/v) formic acid in acetonitrile) in mobile phase A (0.1% (v/v) formic acid in water) in 120 min. Ions were introduced by positive electrospray ionization via liquid junction at 1.4-1.6 kV into a Q Exactive hybrid mass spectrometer (Thermo Scientific). Mass spectra were acquired from m/z 300-1750 at a resolution of 70,000 (m/z 200) with an AGC target of 1×10^6, and data-dependent acquisition selected the top ten most abundant precursor ions for tandem mass spectrometry by HCD fragmentation using an isolation width of an isolation of 1.6 Da, max fill time of 110 ms, and AGC target of 1×10^5. Peptides were fragmented using a collision energy of 27%, and fragment spectra acquired at a resolution of 17,500 (m/z 200).

Label-free proteomic data analysis

Raw data files were processed with Proteome Discoverer (Thermo Scientific, version 2.1) and searched with Mascot Server (Matrix Science, version 2.2) against the Human (Swissprot) FASTA file (downloaded 12/2017). Search parameters included tryptic specificity (up to two missed cleavages), a 10 ppm mass tolerance for the precursor, and a 0.05 Da mass tolerance for the fragments. A fixed modification of carbamidomethyl cysteine, and variable modifications of N-terminal acetylation, oxidized methionine, and pyroglutamic acid for Q were considered. All non-

filtered search results were processed by Scaffold (Proteome Software Inc, version 4.8.7) with threshold values set at 95% for peptides (0.2% false-discovery rate) and 99% for proteins (two peptide minimum) using the Trans-Proteomic Pipeline (Institute for Systems Biology). Protein quantitation values were exported for further analysis in Microsoft Excel.

Bioinformatics analysis of proteomes from cell lysates and cell type-specific EVs

The protein comparison across hiPSC-derived neural cell types was conducted using label-free intensity-based absolute quantification (iBAQ) method as previously described[9]. Briefly, precursor ion intensities of all peptides matching each protein were divided by the theoretical number of peptides derived from in silico tryptic digestion. Subsequently, each protein iBAQ value was further normalized by the average of ADH iBAQ across all the samples. The 0 iBAQ values were replaced with 1 for calculation purposes. Within each cell type (n = 3), fold expression of each protein was calculated from the individual median averaged protein normalized iBAQ values and the corresponding median iBAQ values of the other cell types. The 0 iBAQ values were replaced with 1. Principal component analysis (PCA) was performed on the iBAQ data using R (version 3.6.3). A volcano plot was generated using Graphpad prism 6. The unsupervised hierarchical clustering was performed after z-score normalization of the regulated data set using multiExperiment Viewer software (MEV, 4.8.1). We filtered cell type-specific EV signatures based on the hierarchical clustering of protein intensities, and input to DAVID Bioinformatics Resources (version 6.8) for GO enrichment and KEGG pathway analysis[10]. The gene symbols retrieved from UniProtKB accession numbers were mapped to cellular components (GO_CC), molecular functions (GO_MF), biological processes (GO_BP) items, and KEGG pathways using default statistical parameters (threshold: count 2, ease 0.1). Functional enrichment analysis of cell

type-specific EV signatures was performed using the ToppCluster tool[11] with FDR corrected p-value < 0.05 (https://toppcluster.cchmc.org/) and replotted in Cytoscape v3.6.1.

Isolation of EVs from human brain tissues

The unfixed frozen tissue (frontal cortical grey matter, 0.5g) from deceased AD, MCI or control cases were processed for EV extraction using our published protocol[12,13]. Briefly, frozen brain tissue was chopped and dissociated in Hibernate E (Cat# A1247601, Thermo Fisher Scientific) solution containing 20 U of Papain (Cat# LK003178, Worthington-biochemical) in Earle's Balanced Salt Solution (EBSS) (Cat# 14155063, Thermo Fisher Scientific) and then incubated at 37°C for 15 min by stirring once every 5 min. After the incubation, the samples were placed on ice, and added with 6 mL of ice-cold Hibernate E solution. The dissociated brain tissue samples were gently homogenized with a glass-Teflon homogenizer (Cat# 89026-384, VWR) and filtered with 40-µm mesh filter (Cat# 22-363-547, Thermo Fisher Scientific). After filtration, the tissue samples were centrifuged at 300 × g for 10 min at 4°C to precipitate large debris, 2,000 × g for 10 min at 4°C to precipitate cellular fraction and 10,000 × g for 10 min at 4°C to precipitate plasma membrane fraction. The supernatant was filtered through a 0.22-µm polyethersulfone membrane filter (Cat# SLGP033RS, Millipore), and then ultra-centrifuged at 140,000 × *g* for 70 min using a polyallomer ultracentrifuge tube with 13.2-mL capacity (Cat# 331372, Beckman Coulter) at 4°C (Optima-XE SW41Ti, Beckman Coulter). After differential centrifugation, the pellet was resuspended in 2mL of 0.475M of sucrose solution (Cat# S5-3, Thermo Fisher Scientific). The sucrose step gradient was further created with steps starting from 2.0M to 1.5M, 1.0M, 0.825M, 0.65M, and 0.475M to facilitate capture of the EV-rich pellet by centrifuging at 200,000 × g for 20 h at 4°C. The fractions between the second (0.65M) and third (0.825M) steps corresponded to

fraction “V” (a buoyant density of 1.10 - 1.12 g/cm^3), and the third and fourth steps corresponded to fraction “VI” (a buoyant density of 1.12 - 1.15 g/cm^3) were collected separately and centrifuged at 140,000 × *g* for 70 min at 4°C. The final pellet from V and VI was combined and an equal amount of total protein was used for TMT-based proteomics. To be noted, frozen brain tissue used for immunoblotting was chopped and dissociated in Hibernate E solution containing 75 U/ml of collagenase type 3 (Cat# LS004180, Worthington-biochemical) in EBSS without further homogenizing in order to ensure the intact immune sites for antibody recognition[14]. The final pellet from V and VI was combined and an equal amount of EV proteins were loaded for immunoblotting.

TMT-labeled mass spectrometry of brain-derived EV samples

Nano LC–MS/MS/MS analysis of brain-derived EV samples was performed on an LTQ-Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific) equipped with a Proxeon EASY-nano LC 1200 liquid chromatography pump (Thermo Fisher Scientific), essentially as previously described[15]. Brain-derived EV tryptic peptides were separated on a 100 μm inner diameter microcapillary column packed with 35-cm long Accucore150 resin (2.6 μm, 150 Å, Thermo Fisher Scientific). 4 μL elution was loaded onto the column and separated using a 180 min gradient ranging from 8 to 23% acetonitrile in 0.125% formic acid at a flow rate of ~550 nL/min. An MS^3-based TMT method was used to reduce ion interference. The scan sequence began with an MS^1 spectrum using Orbitrap (resolution 120,000; mass range 400–1400 m/z; automatic gain control (AGC) target 5 × 10^5; maximum injection time 100 ms). Precursors for MS^2/MS^3 analysis were selected using a Top10 method. We used collision-induced dissociation (quadrupole ion trap; AGC 2 × 10^4; normalized collision energy (NCE) 35; maximum injection time 150 ms) for MS^2 analysis. After acquisition of each MS^2 spectrum, an MS^3 spectrum was collected by which multiple MS^2

fragment ions were captured in the MS^3 precursor population by isolation waveforms with multiple frequency notches. Finally, MS^3 precursors were fragmented by high-energy collision-induced dissociation and analyzed using Orbitrap (NCE 65; AGC 1×10^5; maximum injection time 150 ms, resolution was 50,000 at 200 Th).

TMT-MS data analysis

All raw files were converted to the mzXML format using a compendium of in-house developed software, and corrected with monoisotopic m/z measurements[16]. MS/MS spectra were searched against the *Homo sapiens* UniProt database (version October 2018). Searches were performed using a 50 ppm precursor ion tolerance for total protein level profiling and the product ion tolerance of 0.9 Da to maximize sensitivity in conjunction with SEQUEST searches and linear discriminant analysis. Fixed modifications were set for TMT tags on lysine residues and peptide N termini (+229.163 Da) and carbamidomethylation of cysteine residues (+57.021 Da), whereas a variable modification for oxidation of methionine residues (+15.995 Da). Peptide–spectrum matches (PSMs) were adjusted to a 1% false discovery rate (FDR). An in-house linear discrimination analysis (LDA) method was used to create one combined filter parameter from the following peptide ion and MS^2 spectra metrics: SEQUEST parameters XCorr and ΔCn, peptide ion mass accuracy and charge state, in-solution charge of peptide, peptide length, and mis-cleavages. Linear discrimination scores were used to assign probabilities to each MS^2 spectrum for being assigned correctly, and these probabilities were further used to filter the dataset with an MS^2 spectra assignment FDR < 1% at the protein level. After spectral assignment, peptides were assembled into proteins and were further filtered based on the combined probabilities of their constituent peptides to a final FDR of 1%. Protein assembly was guided by principles of parsimony and

proteins were quantified by summing reporter ion counts across all matching PSMs. Protein quantitation values were exported for further analysis in Microsoft Excel. Each reporter ion channel was summed across all quantified proteins.

Differential expression analysis of brain-derived EV proteins

Differentially expressed proteins (DEPs) in brain-derived EVs were conducted using one-way ANOVA ($p < 0.05$) followed by Tukey's comparison post hoc test with $p < 0.01$. To further narrow down the significant DEPs, we used a criteria of Tukey's post hoc test with $p < 0.05$ and fold change > 2. Three comparisons were considered: 1) controls versus AD, 2) controls versus MCI and 3) AD versus MCI samples. These altered proteins with corresponding p value and fold change are provided in Supplementary Tables 8 and 10.

Weighted correlation network analysis (WGCNA) of brain-derived EV proteome

WGCNA algorithm was used to define protein co-expression networks based on normalized protein abundance of brain-derived EV samples using R (version 3.6.3) as previously described approach[17,18]. The smallest power, 14, which results in the proper fit of a scale free topology model is chosen ($R^2 = 0.9$). The network was built by inputting relative protein expression values using WGCNA::blockwiseModules()function. Proteins were hierarchically clustered by calculation of topologic overlap (TO) with bicor correlation function. Module assignments were determined by the dynamic tree-cutting with the following specified parameters: soft threshold power beta = 14, deepSplit = 4, minModulesize = 20, merge cut height of 0.2, signed network with partitioning about medioids respecting the dendrogram, and a reassignment threshold of $p < 0.05$. Total 11 modules of co-expressed proteins in brain-derived EVs ranging in size from the largest module

M1 (720 proteins) to the smallest module M11 (42 proteins) were generated to define the eigenproteins (MEs; or the 1st principal component of the module), which represent the most representative abundance value for a module and explain covariance of all proteins within a module. In addition, Pearson correlations as well as the correlation significance between each protein and each module eigenprotein, and each AD trait (CDR, plaque load and Braak stage) were generated.

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