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Leila Elyasi

Mossakowski Medical Research Institute, Polish Academy of Sciences

Anna Chołoniewska

Mossakowski Medical Research Institute, Polish Academy of Sciences

Eliza Lutostańska

Mossakowski Medical Research Institute, Polish Academy of Sciences

Natalia Mierzwa

Mossakowski Medical Research Institute, Polish Academy of Sciences

Dorota Sulejczak

Mossakowski Medical Research Institute, Polish Academy of Sciences

Matylda Macias

International Institute of Molecular and Cell Biology

Aleksandra Szybińska

International Institute of Molecular and Cell Biology

Sylwia Gawlik-Zawiślak

Institute of Psychiatry and Neurology

Marta Woźniak

Institute of Psychiatry and Neurology

Anna Filipek-Gliszczyńska

National Medical Institute of the Ministry of the Interior and Administration

Małgorzata Bednarska-Makaruk

Institute of Psychiatry and Neurology

Jacek Zaremba

Institute of Psychiatry and Neurology

Cezary Żekanowski

Mossakowski Medical Research Institute, Polish Academy of Sciences

Michalina Wężyk

mwezyk@imdik.pan.pl

Mossakowski Medical Research Institute, Polish Academy of Sciences

Article

Keywords:

Posted Date: October 29th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11225065/v1>

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Additional Declarations: No competing interests reported.

SHED-Derived Exosomes Attenuate Alzheimer's Disease Pathology in iPSC-Derived Cerebral Organoid Model

Running title: SHED-Derived Exosomes in AD Pathology

Authors: Leila Elyasi^{#1}, Anna Chołoniewska^{#1}, Eliza Lutostańska¹, Natalia Mierzwa¹, Dorota Sulejczak², Matylda Macias³, Aleksandra Szybińska³, Sylwia Gawlik-Zawiślak⁴, Marta Woźniak⁴, Anna Filipek-Gliszczyńska⁵, Małgorzata Bednarska-Makaruk⁴, Jacek Zaremba⁴, Cezary Żekanowski¹, Michalina Wężyk^{1*}✉

Affiliations

¹ Department of Neurogenetics and Functional Genomics, Mossakowski Medical Research Institute, Polish Academy of Sciences, 5 Pawińskiego Street, 02-106 Warsaw, Poland

² Department of Experimental Pharmacology, Mossakowski Medical Research Institute, Polish Academy of Sciences, 5 Pawińskiego Street, 02-106 Warsaw, Poland

³ Microscopy and Cytometry Facility, International Institute of Molecular and Cell Biology, Ks. Trojdena 4 Street, 02-109 Warsaw

⁴ Department of Genetics, Institute of Psychiatry and Neurology, Sobieskiego 9, 02-957 Warsaw, Poland;

⁵ Department of Neurology, National Medical Institute of the Ministry of the Interior and Administration, Wołoska Street Warsaw, Poland

✉ Correspondence to Michalina Wężyk, mwezyk@imdik.pan.pl

Abstract

Introduction: Developing disease-modifying therapies for Alzheimer’s disease (AD) that go beyond single-target monoclonal antibodies is a pressing translational priority. Stem cells from human exfoliated deciduous teeth (SHED) and their exosomal cargo represent a promising biological therapy. To bridge the gap between basic exosome research and clinical application, we evaluated SHED-derived exosomes in human-relevant 2D and 3D patient-derived models.

Methods: Induced pluripotent stem cell (iPSC)-derived 2D cortical neurons and 3D cerebral organoid models were generated from patients representing both familial AD (FAD; *PSEN1*) and sporadic AD (SAD). Models were treated with SHED-derived exosomes. Pathological markers including p181-tau, A β 42, and A β 40 were quantified, functional excitotoxicity was assessed via *N*-methyl-D-aspartate (NMDA)-induced calcium dynamics, and mitochondrial reactive oxygen species (ROS) were measured. High-throughput microRNA (miRNA) profiling was performed to identify functional exosomal cargo.

Results: SHED exosome treatment demonstrated genotype-specific efficacy, selectively reducing p181-tau levels and restoring the p181-tau/A β 42 ratio in FAD lines while elevating the protective A β 42/A β 40 ratio across both FAD and SAD backgrounds. Functionally,

treatment prevented NMDA-induced calcium overload, accelerated intracellular calcium clearance, and suppressed stress-induced mitochondrial ROS. High-throughput profiling identified enriched therapeutic miRNAs (hsa-miR-23a-3p, hsa-miR-16-5p, and hsa-miR-191-5p) targeting key networks in tau hyperphosphorylation and amyloid processing.

Discussion: SHED-derived exosomes attenuate key AD pathologies and functional excitotoxicity in human 2D and 3D organoid models. These findings provide translational validation for exosome-based therapeutics, helping close the knowledge gap in multi-target biological treatments for dementia.

Keywords: Alzheimer's disease, stem cell therapy, SHED-derived exosomes, human brain organoids, familial Alzheimer's disease, sporadic Alzheimer's disease, disease-modifying therapy, microRNA exosomes profiling.

Graphical Abstract

Introduction

Alzheimer's disease (AD) is one of the most pressing neurological challenges facing ageing populations, accounting for roughly 70% of dementia cases worldwide. Progressive neuronal loss culminates in total cognitive decline and death over decades, or, in about 35% of cases, within just a few years ¹. Conventional pharmacological approaches primarily manage symptoms rather than halting or reversing disease progression ².

Recently approved disease-modifying therapies ³ target a hallmark of AD: overproduction and extracellular aggregation of amyloid-beta (A β) into plaques, which drive intracellular accumulation of hyperphosphorylated tau into neurofibrillary tangles ⁴. However, these therapies show limited efficacy for full clinical recovery, partly due to AD's complex genetic etiology: less than 1% of cases are monogenic, autosomal-dominant early-onset familial AD (FAD), while most are polygenic, late-onset sporadic AD (SAD) ⁵. Clinical utility is further hindered by adverse effects such as amyloid-related imaging abnormalities (ARIA), including cerebral edema and microbleeds ⁶. There is thus a clear need for alternative therapies directly targeting neuronal loss itself.

Stem cell-based interventions offer regenerative potential, protecting endangered neurons and stimulating endogenous neurogenesis ⁷. Transplantation of human neural stem cells in AD models reduces neuronal apoptosis, restores synaptic function, upregulates learning- and memory-related proteins, and decreases amyloid burden ^{8, 9}. Among candidate sources, dental pulp stem cells (DPSCs) from children's permanent teeth have emerged as viable for anti-neurodegenerative therapy ¹⁰ by inhibiting apoptosis and supporting tissue repair.

Stem cells from human exfoliated deciduous teeth (SHED)—a highly proliferative, neural crest-derived subgroup of mesenchymal stem cells ¹¹—show faster doubling rates, superior donor availability, and stronger multipotency than adult DPSCs ¹². Their capacity to repair neuroectodermal tissue, along with evidence that transplantation upregulates neuronal markers in murine brains, underscores their promise for neurodegenerative disease ^{13, 14}.

In preclinical AD models, intranasal delivery of SHED-derived conditioned medium improved cognition and provided neuroprotection¹⁵. SHED transplantation in SAMP8 mice alleviated cognitive deficits and pathological markers¹⁶, and enhanced spatial memory via PPAR γ signaling¹⁷. Despite these findings, evidence on SHED's direct effects on core AD pathology—calcium dyshomeostasis, mitochondrial dysfunction, oxidative stress, and excitotoxic synaptic decay—remains incomplete¹⁸.

Further work is needed to validate translational efficacy and clarify underlying mechanisms, which may extend beyond A β clearance to encompass calcium dyshomeostasis, mitochondrial dysfunction, chronic neuroinflammation, oxidative stress, and excitotoxic synaptic decay^{19, 20, 21, 22}. Exosomes—nanosized extracellular vesicles (30–150 nm) released via fusion of multivesicular endosomes with the plasma membrane—are key mediators of such signaling, transferring proteins, lipids, nucleic acids, and metabolites between cells²³. This cargo can exert epigenetic effects in recipient cells, modulating genes relevant to neural development, synaptic plasticity, neuroinflammation, and neuroprotection²⁴. In neurodegeneration, exosomes play a dual role: propagating pathological A β and tau, or acting as neuroprotective vehicles when derived from healthy or therapeutically active cells^{23, 24}. Given the scope of this study, particular focus is placed on the SHED-derived exosomal miRNA signature as a functionally active cargo component, whose regulatory and therapeutic potential in AD remains largely unelucidated.

To address these gaps, this study investigated the therapeutic impact of SHED and SHED-derived exosomes using patient-specific models. We generated iPSC-derived neurons and 3D cerebral organoids from skin biopsies of healthy controls, FAD patients carrying PSEN1

mutations (P117L and I213F, associated with Very Early Onset AD), and SAD patients, evaluating cellular responses across antioxidant, calcium-handling, excitotoxic, and apoptotic assays. Our findings reveal possible novel cytoprotective pathways activated by SHED-based interventions, supporting their development as early-stage AD therapeutic strategies.

Materials and Methods

Ethics of the Study Involving Human Subjects

This study was conducted in accordance with the ethical standards of the Declaration of Helsinki. Ethical approval for human subject recruitment and sample collection was granted by the Local Ethical Committee of the Central Clinical Hospital of the Ministry of Internal Affairs in Warsaw, Poland (decision numbers: 31/2013, 108/2017, and 203/2020). Human subjects were recruited at the Institute of Psychiatry and Neurology (Warsaw, Poland) and the National Medical Institute of the Ministry of the Interior and Administration (Warsaw, Poland). Written informed consent was obtained from all study participants (or their legal representatives) prior to study inclusion.

Study Cohort and Material Availability

Primary fibroblasts were obtained from **three** patients with familial Alzheimer's disease (FAD) carrying pathogenic *PSEN1* variants (two P117L and one I213F), **three** patients with sporadic Alzheimer's disease (SAD), and **five** cognitively healthy controls. Participants with

FAD_ *PSEN1*_P117L and selected controls were recruited at the Institute of Psychiatry and Neurology in Warsaw; the participant with FAD_ *PSEN1*_I213F, the SAD group, and additional controls were recruited at the National Medical Institute of the Ministry of the Interior and Administration in Warsaw. The fibroblasts were reprogrammed into iPSCs and are available from the Department of Neurogenetics and Functional Genomics, Mossakowski Medical Research Institute.

The study also included **two** iPSC lines from FAD patients carrying *APP* V717L and V717I variants ²⁵, obtained under a material transfer agreement from Kyoto University and the Center for iPS Cell Research and Application (CiRA). Detailed clinical and demographic characteristics are provided in Supplementary Table 1. The mean age was 34 ± 5 years in the FAD group and 62.3 ± 5.8 years in the SAD group; the mean age of the control group was not available in the source text.

All participants underwent comprehensive neuropsychological evaluation and genetic screening by Sanger sequencing of genomic DNA. The FAD cases carried established pathogenic *PSEN1* variants (P117L or I213F). The FAD_ *PSEN1*_P117L and SAD cases, together with family controls, also underwent whole-exome sequencing. Raw data are available through NCBI's Sequence Read Archive under BioProject PRJNA1498779. The SAD cases had no history or clinical evidence of stroke or other neurological disease.

Clinical Description

The patients and control individuals who served as the source of biological material for neuronal cultures and cerebral organoid derivation in this study presented the below-described clinical backgrounds.

Familial Alzheimer's Disease (FAD) Lines

Two patients carrying the PSEN1 P117L mutation were included — one male donor who was presymptomatic at the time of biopsy (age 27, line P117L_presymptomatic) and one male donor at an advanced disease stage (age 34, line P117L_advanced), both followed at the genetic counselling at the Institute of Psychiatry and Neurology in Warsaw. Both belonged to the Polish kindred in which this mutation was first identified and described in a landmark publication by Wiśniewski et al.²⁶ (New York University Medical Center and Institute for Basic Research in Developmental Disabilities, New York) in collaboration with the team of Prof. Jerzy Kulczycki (Neurological Department of the Institute of Psychiatry and Neurology in Warsaw), and reported it as causing one of the earliest average ages of onset and death than known in any presenilin-linked familial AD kindred. The three-generation pedigree of this family demonstrated a clear autosomal dominant inheritance pattern: the proband's father (generation I) was genetically non-tested but demented, dying at age 38; generation II included two affected sisters — carrying the P117L mutation — who developed MCI at ages 32 and 33 and died at 36 and 35 years respectively, as well as a third affected individual who died at 35 years and was likewise genetically non-tested; generation III comprised multiple mutation carriers across different branches of the family, with documented onset of MCI between ages 30 and 36, one individual remaining asymptomatic at age 27 at the time of

assessment, and one case of fully advanced dementia at age 43, alongside confirmed non-carriers who remained healthy — with fibroblast lines available from several generation III individuals, as indicated in the pedigree (Supplementary Figure 1A). The mutation was confirmed by Sanger sequencing across all tested family members. The mutation was associated with an exceptionally aggressive form of early-onset familial Alzheimer's disease, with symptom onset typically occurring between ages 29–35 (average ~31 years), beginning with memory disturbances and disorientation, followed rapidly by myoclonus, extrapyramidal rigidity, and complete loss of function within months, leading to death on average around age 37 — with some cases dying as early as 28–35 years of age²⁸. Neuropathologically, affected family members showed an exceptionally high amyloid burden — 2 to 6-fold greater than in sporadic Alzheimer's disease and Down syndrome — with dense senile plaques of various morphologies throughout the cortex, diffuse brain atrophy most prominent in the temporal lobes, and relatively sparse neurofibrillary tangles, as documented in detailed neuropathological studies performed at the Institute of Psychiatry and Neurology in Warsaw^{28, 26}.

One female patient carrying the PSEN1 I213F mutation was included at an advanced disease stage (age 38, line I213F_advanced). She presented with early-onset familial Alzheimer's disease consistent with the clinical profile described by Żekanowski et al.²⁹ in a Polish kindred in which multiple family members (sister, brother, mother, and aunt) had died before the age of 40, with onset as early as 33 years. Initial symptoms consisted of progressive memory impairment and disorientation, followed by mood lability, bradykinesia, and rigidity, with rapid cognitive decline reaching an MMSE score of 10 within approximately 5 years of

onset. The mutation affected a highly conserved residue in transmembrane domain IV of presenilin-1 — the same codon as two other known pathogenic mutations (I213T and I213L) — and was neuropathologically characterized by severe amyloid plaque burden, including distinctive "cotton wool plaques," pronounced cortical atrophy, and in some cases co-occurring Lewy body pathology, as described in the Italian kindred reported by Bugiani et al.³⁰. The pedigree of the I213F family is provided in Supplementary Figure 1B.

Two female iPSC lines carrying **APP** mutations at codon 717 were obtained from the CiRA cell bank²⁵: one carrying the **V717I** mutation (line ADP2/CiRA00002, biopsy age ~50) and one carrying the **V717L** mutation (line ADP3/CiRA00003, biopsy age ~50). Both mutations affect the same codon in exon 17 of the amyloid precursor protein gene and cause autosomal dominant early-onset familial Alzheimer's disease through the same molecular mechanism — shifting APP processing toward increased A β 42 production and raising the A β 42/A β 40 ratio. The V717I mutation (known as the "London mutation") was the first pathogenic APP mutation ever identified, described by Goate et al.³¹ in a landmark discovery that directly established the causal role of amyloid metabolism in Alzheimer's disease, with affected individuals presenting with progressive memory loss and cognitive decline with onset typically between ages 45–55, and neuropathology characterized by abundant A β 42-rich plaques, widespread neurofibrillary tangles, and frequent cerebral amyloid angiopathy. The V717L mutation, first described by Murrell et al.³², showed considerably greater phenotypic variability across kindreds — with age of onset ranging broadly from the late 30s to the mid-50s and clinical features that, beyond progressive memory impairment, included psychiatric symptoms (depression, irritability), seizures, myoclonus, and pyramidal signs in some

207 families — while sharing the same fundamental neuropathological substrate of dense
208 amyloid plaques and neurofibrillary tangles³³.

209 Sporadic Alzheimer's Disease (SAD) Lines

210 Three patients with sporadic Alzheimer's disease were included, all at early disease stages.
211 Two females presenting at ages 60 (line ANG_C32_SAD_MCI) and 69 (line
212 KABA_C28_SAD_mild), and one male presenting at age 58 (line TAPLA_C12B_SAD_mild).
213 Whole exome sequencing (WES) was performed in all three cases. Their clinical picture was
214 dominated by a subtle but progressive impairment of episodic memory, most notably
215 difficulty in encoding and retaining new information, while other cognitive domains and
216 general functional independence remained largely preserved. On formal
217 neuropsychological testing, deficits were detectable — particularly on delayed recall tasks
218 — and were accompanied in some cases by subtle impairments in attention, executive
219 function, or visuospatial abilities, while neuroimaging (MRI) revealed early hippocampal and
220 entorhinal cortex atrophy, and biomarker brain and cerebrospinal fluid (CSF) studies
221 demonstrated reduced A β 42, elevated tau and phospho-tau levels, and amyloid deposition,
222 reflecting the underlying Alzheimer's pathological process that typically precedes overt
223 clinical symptoms by many years.

224 Control (CTRL) Lines

225 Five control iPSC lines from healthy donors were included. The commercially available
226 Gibco™ hiPSC line (catalogue number A18945) was obtained from a healthy male donor. The

227 CTL07-II line was derived from a healthy male donor (age 19) and described by Kele et al.³⁴.
228 The IIMCBi002 (ELE002) line was derived from a healthy female donor (age 30) and described
229 by Liszewska et al.³⁵. Additionally, two healthy female donors from the P117L family —
230 confirmed as non-carriers of the mutation — were included as genetically matched controls
231 (lines CTRL_1_OLMA_P117L_family, age 33, and CTRL_2_WAMO_P117L_family, age 32),
232 providing a particularly valuable within-family control background for comparison with the
233 P117L mutation carriers.

234 1. Cell Culture and Characterization

235 A) Culture and Phenotypic Validation of SHED

236 Human exfoliated deciduous teeth stem cells (SHED, SKU: 10HU-229; IX CELL
237 Biotechnology, San Diego, CA, USA) were cultured in Exfoliated Deciduous Teeth Stem Cell
238 Growth Medium (Cat #MD-0096, IX CELL Biotechnology) at 37°C in a humidified atmosphere
239 containing 5% CO₂. Frozen aliquots were rapidly thawed in a 37°C water bath, centrifuged
240 at 1,000 rpm for 5 min, and resuspended in complete growth medium. Media changes were
241 performed every 2–3 days, and cells were enzymatically passaged at 80–90% confluence
242 using 0.25% Trypsin-EDTA (Gibco, USA) at a seeding density of 5×10^3 cells/cm². For long-
243 term cryopreservation, cells were suspended in freezing media consisting of 90% fetal
244 bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO), cooled progressively to -80°C, and
245 stored in liquid nitrogen.

To confirm compliance with established mesenchymal stem cell (MSC) criteria, surface marker expression was analyzed up to the fifth passage ³⁷. Immunocytochemical (ICC) staining was conducted using primary antibodies against CD105 (BD Biosciences, Cat #560839; PE-conjugated), CD44 (BD Biosciences, Cat #555478; FITC-conjugated), and CD90 (BD Biosciences, Cat #561557; PerCP-Cy5-conjugated). Fixed cells were visualized via fluorescence microscopy to evaluate spatial expression and co-localization. Quantitative validation of the cell population was achieved through flow cytometry. Cells were analyzed based on side scatter area (SSC-A) versus fluorescence intensity, applying specific gating strategies (P3, P4, and P5 gates) to determine the precise percentages of parent and total populations positive for CD90, CD105, and CD44.

B) Reprogramming, Cell Culture, and Validation of iPSCs

Primary skin fibroblast lines were established from healthy controls, FAD patients (carrying mutations in PSEN1, P117L and I213F), and SAD patients according to optimized clinical protocols ³⁸. Fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% antibiotics (Invitrogen). Reprogramming into iPSCs was executed utilizing Sendai viral vectors delivery systems encoding the Yamanaka pluripotency factors (Oct4, Sox2, and Klf4) ³⁸. One week post-transduction, cells were transitioned onto Laminin-521 (BioLamina)-coated substrates and maintained in Essential 8 (E8) medium (Gibco) for a minimum of 14 passages to ensure stable line establishment.

Pluripotency was rigorously confirmed via reverse transcription-quantitative PCR (RT-qPCR) tracking the activation of endogenous pluripotency markers (OCT3/4, MYC, KLF4, NANOG),

simultaneous downregulation of fibroblast-specific markers (S100A4), and complete clearance of the Sendai viral transgene (SeV). Additionally, alkaline phosphatase enzymatic activity assays and ICC profiling for Oct4, Nanog, and TRA-1-60 were performed to confirm the pluripotency status of each line before neural differentiation.

Karyotyping of iPSC lines

Chromosomal analysis was performed in the Institute of Psychiatry and Neurology in Warsaw using G-banding staining. Over twenty metaphases were analyzed for each sample. Cytogenetic analysis and karyotyping were performed with a light microscope Nikon Eclipse 50i (Nikon Corporation, Tokyo, Japan) integrated with LUCIA Cytogenetics Imaging Software ver. 3.1. A detailed description of the procedure together is provided elsewhere³⁹.

C) Induction of iPSC-Derived Neuroepithelial-like Stem Cells

Human induced pluripotent stem cells (iPSCs) were cultured under feeder-free conditions using a defined medium (e.g., mTeSR1) on Matrigel-coated plates. Upon reaching approximately 80% confluency, cells were harvested and seeded at an optimal density to initiate neural induction.

Neuroepithelial-like stem cells (NESCs) were induced by dual inhibition of SMAD signaling pathways. Cells were treated with small-molecule inhibitors Noggin (or recombinant BMP inhibitor) and SB431542, a TGF- β receptor inhibitor, to suppress BMP and activin/nodal pathways, respectively. This treatment was maintained for the first 7–10 days in a neural

induction medium composed of DMEM/F12, Neurobasal medium, N2 and B27 supplements (without vitamin A), L-glutamine, and penicillin/streptomycin. Medium was changed daily.

During this period, the cells underwent morphological changes indicative of neuroepithelial identity, forming polarized epithelium with rosette structures characteristic of neuroepithelial-like stem cells. The cells were monitored by immunostaining for early neural markers including PAX6 and SOX1^{40, 41}.

D) Neural Differentiation following Dual SMAD Inhibition Protocol

Following initial induction, NESC were further cultured with continued dual SMAD inhibition using Noggin and SB431542 for an additional 7–14 days to reinforce dorsal forebrain neural fate. This established a population of neural progenitors committed to the cortical lineage. The medium was supplemented and replaced daily, maintaining cells in a defined neural differentiation medium. Cells were passaged using gentle enzymatic or mechanical methods to maintain neuroepithelial morphology and prevent spontaneous differentiation. Verification of differentiation stages included assessment of marker expression such as SOX2, FOXG1, and other forebrain-specific transcription factors by immunocytochemistry and qRT-PCR analysis^{40, 41}.

E) Generation of Cerebral Brain Organoids

To generate cerebral organoids, human iPSCs from CTRL, FAD, or SAD subjects were dissociated into single-cell suspensions and seeded into AggreWell™ plates to ensure uniform embryoid bodies formation and size standardization^{42, 38, 43}. To initiate neural

induction and promote spontaneous regional patterning, the aggregated cells were cultured in neural induction medium supplemented with the dual-SMAD inhibitors: 2 μ M Dorsomorphin and 10 μ M SB431542. After 5–7 days, the uniform spheroids were transferred to orbital shakers to enhance nutrient and oxygen distribution, and the medium was transitioned to an expansion/proliferation medium supplemented with epidermal growth factor (10 ng/ml EGF) and fibroblast growth factor (FGF 10 ng/ml).

To promote long-term cortical maturation, the growth factors were subsequently removed, and the organoids were maintained in maturation medium supplemented with brain-derived neurotrophic factor (BDNF), with medium changes performed every 2–3 days. During this period, cerebral cortical-like structures developed, including discrete ventricular zone-like regions and cortical plate-like neurons. Periodic characterization of the organoids was performed through immunocytochemistry/immunohistochemistry for neural markers such as TUJ1 (neuronal), GFAP (glial), and cortical layer-specific markers, alongside functional validation including GCaMP-mediated live calcium imaging^{44, 45}.

F) SHED and Organoids Co-Culture and Optimization Rationale

Preliminary one-month co-culture experiments showed that prolonged exposure to Neurobasal/B27 organoid maturation medium induced unwanted neural differentiation of SHED and progressively reduced exosome secretion. We therefore adopted a dynamic replacement strategy to maintain an undifferentiated SHED phenotype and a consistent secretome.

Paracrine co-cultures were established using Transwell supports with 0.4 μm pores, allowing soluble factors to pass while preventing direct cell-to-cell contact. Cerebral organoids were placed in the lower compartment, and freshly prepared SHED were seeded on the upper membrane at approximately 50,000 cells/cm². Co-cultures were maintained for 6 weeks in Neurobasal A medium supplemented with B27. To sustain exosome output and prevent medium-induced differentiation, SHED-containing inserts were replaced with freshly seeded inserts every 48 hours throughout the experiment.

2. Isolation and Characterization of SHED-Derived Exosomes

A) Differential Ultracentrifugation

Exosomes were isolated from SHED conditioned media using differential ultracentrifugation. Media collected from cultures under standard conditions were first centrifuged at 300 x g for 10 min at 4°C to sediment intact cells, followed by a 2,000 x g spin for 15 min to clear cellular debris. Supernatants were subsequently centrifuged at 10,000 x g for 45 min at 4°C to remove apoptotic bodies and large microvesicles. The remaining supernatant was passed through a 0.22 μm polyethersulfone syringe filter to eliminate any residual macro-contaminants. Vesicles were pelleted by high-speed ultracentrifugation at 100,000 x g for 90 min at 4°C using an ultracentrifuge. The exosomal pellet was resuspended in sterile phosphate-buffered saline (PBS), washed via an additional ultracentrifugation step at 100,000 x g for 70 min, and finally resuspended in PBS before being aliquoted and stored at -80°C. This structural preservation approach was selected over chemical precipitation methods (which tie up water molecules to force vesicles out of solution) to maintain

exosome morphology and physiological function. The methodology was adapted based on Coughlan et al.⁴⁶.

B) Structural and Molecular Validation

Physical characterization of the isolated vesicles was achieved using Transmission Electron Microscopy (TEM) to assess vesicle size and morphology. Aliquots from four distinct experimental designs—isolated SHED in a single culture and SHED co-cultured with cerebral brain organoids from control subjects (CTRL_SHED), FAD patients (FAD_SHED), and SAD patients (SAD_SHED)—were negative-stained and visualized. Nanoparticle Tracking Analysis (NTA) was utilized to determine absolute size distributions and hydrodynamic vesicle concentrations. Molecular concordance with exosome identity was verified by transcript profiling of exosome and microvesicle markers by RNA sequencing of total SHED exosomal fraction, as described below. Exosomal phenotype of the surface protein expression profile of the isolated vesicles was finally confirmed by an enzyme-linked immunosorbent assay kit (ExoELISA-Ultra; System Biosciences, USA) according to the manufacturer's instructions. SHED-derived exosome samples were normalized and loaded at an absolute density of 3.45×10^9 particles/mL per well in a final volume of 50 μ L. The specific pre-coating was against canonical exosomal tetraspanins (CD9, CD63, and CD81) alongside a negative control matrix protein (cis-Golgi marker GM130) to evaluate cellular contamination in the SHED-derived exosome fractions.

3. RNA Sequencing, miRNA Profiling, and Bioinformatic Network Analysis

368 Total RNA, ensuring complete retention of small RNA fractions, was extracted from purified
369 exosomes using the miRNeasy Micro Kit (Qiagen, Germany) per the manufacturer's
370 directions.

371 High-throughput next-generation sequencing (NGS) was executed to establish
372 comprehensive small RNA expression profiles. Small RNA sequencing was performed on an
373 Illumina NovaSeq X instrument using the NovaSeq X Series 10B Reagent Kit (300 cycles,
374 Illumina, cat. no. 20085594) in paired-end 2×150 bp mode, following the manufacturer's
375 standard protocol with a 1% PhiX control library spike-in (Illumina, cat. no. FC-110-3001).
376 Raw reads were trimmed for adapters and low-quality bases using Cutadapt v3.7 ⁴⁷ with
377 parameters --minimum-length 12 and --nextseq-trim=12. Trimmed reads were aligned to the
378 human reference genome (GRCh38) using Bowtie2 v2.4.4 ⁴⁸ with default parameters. Aligned
379 reads were assigned to miRNA transcripts annotated in miRBase using HTSeq-count v0.11.2
380 ⁴⁹ with the -a 0 flag. To validate transcriptomic identity, the global sequencing profiles were
381 benchmarked against historical SHED data obtained from the NCBI Sequence Read Archive
382 (SRA) database. Differential expression analysis of genes (DEGs) and exosomal miRNAs was
383 completed using DESeq2. Sample-to-sample relationships were assessed via a Euclidean
384 distance matrix calculated from regularized log-transformed (rlog) count data,
385 accompanied by hierarchical clustering. Statistical thresholds for identifying valid DEGs
386 were visualized using Volcano plots plotting statistical significance against log2 fold-change
387 values. RNA-seq data quality and distribution profiles were verified with probability density
388 plots mapping normalized log2 counts. The presence of the core GGAG exosome-specific
389 sequencing motif—responsible for directing selective RNA sorting into extracellular

vesicles—was cross-referenced and quantified within the read counts. To investigate downstream functional signaling networks, identified exosomal miRNAs were analyzed using bioinformatics platforms, including miRWalk, miRNet, Cytoscape, and TAM 2 functional annotation tool, to determine tissue enrichment and target-gene signaling pathways. The Raw miR and RNAseq data are available as BioProject PRJNA1497052 at SRA NCBI.

4. Pathological and Physiological Phenotyping Assays

A) Quantification of Amyloid Beta and Tau Proteins by MILLIPLEX® Magnetic Bead-Based Multiplex Assay

Levels of Amyloid Beta 1-40 (A β 40), Amyloid Beta 1-42 (A β 42), total Tau, and phosphorylated Tau (pTau) were quantified in conditioned media using the MILLIPLEX® Human Amyloid Beta & Tau Magnetic Bead Panel (HNABTMAG-68K; MilliporeSigma, Burlington, MA, USA), in accordance with the manufacturer's protocol. This assay is based on Luminex® xMAP® technology, enabling simultaneous multiplexed detection of multiple analytes within a single sample volume. All reagents were equilibrated to room temperature (20–25°C) prior to use. Lyophilized standards were reconstituted in the provided assay buffer and serially diluted to generate a seven-point standard curve. Quality controls (QC) were prepared as instructed and included in each assay run to ensure inter-assay consistency.

Conditioned Media Collection and Preparation

Conditioned media were collected from cerebral organoid cultures at the designated experimental timepoints. To remove cellular debris and vesicular contamination that could interfere with bead-based detection, samples were centrifuged at $300 \times g$ for 5 minutes, followed by a second centrifugation step at $2,000 \times g$ for 10 minutes at 4°C . Clarified supernatants were aliquoted and stored at -80°C until analysis. Prior to the assay, samples were thawed on ice and placed in assay plate along with the standard curve. Each sample was assayed in duplicate.

Assay Procedure

A 96-well flat-bottom plate was pre-wetted with wash buffer and subsequently aspirated. Antibody-coupled magnetic beads — specific to each target analyte — were vortexed and diluted in assay buffer, and 25 μL of the bead mixture was added to each well. Standards, quality controls, and conditioned media samples (25 μL each) were then added to the appropriate wells. To maximize assay sensitivity, which is particularly critical for the detection of low-abundance neurodegeneration biomarkers such as $\text{A}\beta_{42}$ in conditioned media, the plate was sealed and incubated overnight (approximately 16 hours) at $2-8^{\circ}\text{C}$ on an orbital plate shaker set at 600 rpm.

Washing and Detection

Following overnight incubation, the plate was placed on a handheld magnetic separator to immobilize the beads, and the supernatant was carefully aspirated. Wells were washed three times with wash buffer to remove unbound material. Subsequently, 25 μL of

biotinylated detection antibody mixture was added to each well, and the plate was incubated for 30 minutes at room temperature on a shaker (600 rpm), protected from light. After three additional washes, 25 μ L of Streptavidin-Phycoerythrin (Streptavidin-PE) was added to each well and incubated for a further 30 minutes at room temperature, with shaking, protected from light.

Data Acquisition and Analysis

Following a final three-step wash, beads were resuspended in 150 μ L of sheath fluid and the plate was read on a MAGPIX® Luminex analyzer (Luminex Corporation, Austin, TX, USA). A minimum of 50 bead events per analyte per well were acquired. Median fluorescence intensity (MFI) values were converted to analyte concentrations using a five-parameter logistic (5-PL) curve-fitting algorithm applied to the standard curve, using MILLIPLEX Analyst software (MilliporeSigma).

B) Calcium Homeostasis and Synaptic Excitotoxicity

Functional changes in intracellular calcium (Ca^{2+}) handling were recorded in iPSC-derived neurons using ratio-metric fluorescent calcium indicators. Basal Ca^{2+} levels were recorded, followed by the induction of acute excitotoxic stress via exposure to N-methyl-D-aspartate (NMDA). Peak calcium influx (overload magnitude) and subsequent kinetics of clearance back to baseline resting levels were tracked and compared between untreated and SHED co-cultured neurons.

C) Mitochondrial Oxidative Stress Profiling

Mitochondrial reactive oxygen species (ROS) accumulation was measured using cell-permeable fluorogenic probes specific for mitochondrial superoxide. Neurons were evaluated under basal conditions and following chemical induction of oxidative stress via treatment with doxorubicin (doxo). Fluorescence microscopy was used to capture multi-channel signals: neuronal networks (immunostained for structural markers), active mitochondrial areas, and localized red/yellow ROS fluorescent signals. Quantitative signal intensity analysis was performed across all groups (CTRL, FAD, SAD) in both the presence and absence of SHED co-culture to assess antioxidant capacity.

5. Statistical Analysis

All statistical analyses were performed using OriginPro 8.0 software (OriginLab Corporation, Northampton, MA, USA). Data are presented as mean $\pm$ SD or mean $\pm$ SEM, as indicated in the figure legends. For experiments involving matched organoid-derived or paired experimental conditions, paired statistical tests were applied. Comparisons between two paired groups were analyzed using a two-tailed paired t-test for normally distributed data or the Wilcoxon matched-pairs signed rank test for non-normally distributed data. A p value < 0.05 was considered statistically significant.

For RNA sequencing and miRNA profiling analyses, differential expression was determined using DESeq2, with significance defined according to the thresholds described in the corresponding bioinformatic analysis workflow. For multiplex protein quantification and functional phenotyping assays, statistical comparisons were performed across experimental groups as specified above.

470 Results

471 1. Phenotypic Confirmation of Isolated SHED Lines

472 Before functional co-culture, the identity and purity of the SHED population were confirmed.
473 Immunocytochemical analysis demonstrated a highly interconnected, spindle-shaped
474 morphology characteristic of mesenchymal stem cells. The cultured cells exhibited strong,
475 uniform fluorescence for core MSC surface markers: CD105 (PE channel) showed a
476 structured cellular network; CD44 (FITC channel) delineated the plasma membrane and
477 cytoskeleton; and CD90 (PerCP-Cy5 channel) was expressed throughout the cytoplasm. A
478 merged image confirmed that these markers were co-expressed within the same cell
479 population (Fig. 1A). To quantify population purity, flow cytometry analysis was performed.
480 Side-scatter versus fluorescence intensity plots showed a distinct, uniform shift into gated
481 regions (P3, P4, and P5), indicating high expression levels (Fig. 1B). Quantitative analysis of
482 the cell population confirmed high purity of marker distributions (Fig. 1C). These combined
483 morphological and automated sorting data confirm that the SHED isolates represent a highly
484 pure population of mesenchymal stem cells matching standard defining criteria.

485 2. Physical Metrics, Characterization of Secreted Exosomes, and Parameters of Co-cultures 486 with Cerebral Organoids

487 Ultracentrifugation of SHED-conditioned media yielded a consistent population of small
488 extracellular vesicles. Transmission Electron Microscopy (TEM) confirmed the presence of
489 classic cup-shaped, membrane-bound spherical structures (Fig. 2A, panel SHED).

Nanoparticle Tracking Analysis (NTA) confirmed that the diameters of the isolated vesicles fell within the expected range for exosomes (50 nm to 200 nm). The size distribution profile revealed a distinct peak at approximately 165 nm, with a maximum concentration of approximately 11×10^8 exosomes/mL (Fig. 2B). The sequence profile revealed predominance of exosome-specific motif GGAG (Fig. 2C), also provided as Supplementary Table 2. At the transcript level, the expression of typical exosome and micro-vesicles markers was also confirmed (Fig. 2D, E), as well as listed in the Supplementary Table 3. Finally, the expression of exosomal vesicular markers at the transcript level was confirmed by immunoassay for canonical tetraspanins and a non-exosomal cellular markers, with CD9 yielding the highest signal (OD₄₅₀ = 0.95), followed by CD63 (OD₄₅₀= 0.81) and CD81 (OD₄₅₀ = 0.65), as well as GM130, nearly undetectable in the SHED-derived exosome isolates (OD₄₅₀ = 0.06) (Fig. 2F).

The ability of SHED to secrete fully competent exosomes was tested before studies employing their co-cultures with cerebral brain organoids derived from healthy controls, FAD, and SAD patients. All co-cultures were conducted for 6 weeks, starting from ~120-day-old cerebral organoids and ending with ~165-day-old cerebral organoids. While all groups (SHED, CTRL_SHED, FAD_SHED, SAD_SHED) successfully secreted these vesicles, spatial differences were observed: the CTRL_SHED group displayed a higher structural density per field of view, whereas the FAD_SHED group contained larger, more distinct vesicular structures under high magnification.

3. RNA Sequencing and Exosomal miRNA Cargo Profiling

511 The global transcriptome profile of the SHED lines used in this study was compared with
512 RNA-seq samples available at the SRA database, a list of which is provided in Supplementary
513 Table 4. The number of detected genes was 13'306, and annotated was 13'098, similarly as
514 in other libraries (Fig. 3A). The library size was 29.9 M (16.5 M+/-13.8M) (Fig. 3B). The log-
515 normalized expression was similar in all datasets (Fig. 3C). The comparison revealed 1'383
516 differentially expressed genes (DEGs) in SHED used in our study versus transcriptomes
517 established for other SHED cultures (other RNA-seq files available at SRA database), top 50
518 DEG are shown on the heatmap (Fig. 3D), of which 27 neuronal-specific were revealed (Fig.
519 3E), both shown in and Supplementary Table 5A and 5B. Pairwise sample-to-sample
520 Euclidean distance matrix evaluation of global similarity between samples based on
521 expression profiles (from rlog-transformed data); the color gradient represents the distance
522 metric: darker blue cells indicate shorter distances (higher similarity and closer
523 relationship), while lighter blue cells reflect larger distances (greater dissimilarity); rows and
524 columns are clustered hierarchically (Fig. 3F). Volcano plots displaying statistical
525 significance versus fold change for a dataset of annotated genes differentially expressed in
526 SHED compared with various SRA samples from other studies, based on the use of SHED
527 cells (Fig. 3G). Density plot of DESeq2-normalized $\log_2$ counts for RNA-seq data; x-axis
528 represents the normalized expression levels ($\log_2$ counts), and the y-axis shows the
529 estimated probability density; the curve displays the distribution for samples and exhibits a
530 characteristic RNA-seq profile, with the primary peak representing moderately-to-highly
531 expressed genes, and a lower-end tail or secondary peak corresponding to lowly expressed
532 or non-coding transcripts (Fig. 3H). Finally, the used in this study SHED compared versus

other SHED (based on available FASTQ files from the RNA-seq experiments available in the SRA database) revealed particular enrichment in neuronal-specific processes based on the differentially expressed transcript dataset, pointing to their particular potential for neuronal plasticity changes.

Among the 13,306 mapped genes in the total RNA of SHED used in our study (based on the whole-transcriptome analysis of SHED cells, BioSample SAMN61768645 and SAMN61768646 at SRA database, the “S16038 sample” in our record database), there were 735 hits for mature microRNAs (miRNAs) found in total RNA extract from SHED cells (Supplementary Table 6A). Of these, 468 miRNA hits were found in the exosome preparation from SHED cells cultured under standard conditions, i.e., with media and supplements dedicated to SHED (Supplementary Table 6B, BioSample SAMN61768647, the “S16044” sample in our record database). Finally, 37 miRNAs were found to be produced by SHED when maintained in neurobasal medium in co-culture with cerebral organoids (derived from control, FAD, or SAD subjects) (Supplementary Table 6C, BioSamples SAMN61768641-SAMN61768644 at SRA data base and Fig. 4A). These 37 miRNAs are listed in the diagram, with the highest percentages attributed to hsa-miR-23a-3p, 16-5p, 191-5p, 486-5p, and 25-3p (Fig. 4B, and Supplementary Table 6C). Of these 37 miRNAs, 10 miRNAs were from exosomes uniquely produced under such co-culture conditions (Fig. 4A). Of note, organoids alone did not produce exosomes, nor did they produce any miRNAs of such origin. All 37 exosomal miRNAs were tested for enrichment based on 15’255 targeted genes, and the 10 unique miRNAs were tested against 11’918 targets (acc. mirWalk) (Supplementary Table 7A and 7B). The targets of the 10 miRNAs were 100% common with the 37-target pool (Fig. 4C,

Supplementary Table 7A and 7B). The target gene enrichment analysis for the 10 miRNAs revealed several GO terms and KEGG pathways related to neuronal specificity and synapses (Fig. 4D, E, F), as well as exosomal and vesicular-related pathways (Fig. 4G).

In detail, enrichment of selected KEGG pathways among predicted miRNA targets is shown in Supplementary Figure 2, including axon guidance (hsa04360), synaptic vesicle cycle (hsa04721), glutamatergic synapse (hsa04724), GABAergic synapse (hsa04727), Alzheimer disease (hsa05010), long-term potentiation (hsa04720), long-term depression (hsa04730), neurotrophins signaling pathway (hsa04722), pathways of neurodegeneration—multiple diseases (hsa05022), cholinergic synapse (hsa04725), neuroactive ligand–receptor interaction (hsa04080), and neuroactive ligand signaling (hsa04082). These pathways collectively suggest that exosomal miRNAs may substantially modulate neuronal competence, synaptic plasticity, and cognitive function, potentially influencing processes associated with cognition, learning, and memory-related phenotypes.

Quantitative abundance tracking showed that a small subset of miRNAs dominated the exosomal profile, including hsa-miR-23a-3p 13,086 counts, accounting for 19% of total reads, hsa-miR-16-5p 7,703 counts, accounting for 11% of total reads, hsa-miR-191-5p 7,411 counts, accounting for 11% of total reads, hsa-miR-486-5p 5,637 counts, accounting for 8% of total reads, hsa-miR-25-3p 5,434 counts, accounting for 8% of total reads, hsa-miR-652-3p 4,178 counts, accounting for 6% of total reads. The remainder of the profile formed a long tail of low-abundance miRNAs. Overall, the functional annotation analysis based on the miRWalk tool, providing a list of target genes, either to 37 miRs specific to

exosomes overall produced by SHED, or to 10 miRs specific to exosomes produced by SHED under neuronal conditions, thus with therapeutic potential (Supplementary Table 6A and 6B) followed by enrichment in KEGG pathways, including neuronal ones, demonstrating significant tissue specificity for brain (Supplementary Table 8A, and 8B). This indicates that the exosomal cargo secreted by SHED is enriched for miRNAs relevant to neurological function. At the end, noteworthy, the 10 miRs carried in SHED exosomes that were present only with brain organoids, i.e., under neuronal conditions, represent a promising future perspective.

4. Attenuation of Alzheimer's Disease Amyloid and Tau Hallmarks

Co-culture with SHED significantly modified the biochemical hallmarks of AD pathology in patient-derived models. In familial AD (FAD) neurons and cerebral organoids, SHED treatment led to a significant reduction in tau phosphorylated at threonine 181 (p181-tau) (Fig. 5A) and decreased the pathogenic p181-tau/A β 42 ratio (Fig. 5B). Furthermore, SHED treatment increased the A β 42/A β 40 ratio in both FAD and SAD subtypes (Fig. 5C). These findings demonstrate that SHED interventions can favorably alter amyloid processing and tau phosphorylation, with a more pronounced effect observed in familial AD genetic backgrounds.

5. Metabolic Viability Controls

To ensure that the observed changes occurred in viable cultures free from vehicle or co-culture cellular toxicity, absolute cell metabolic activity was monitored across experimental contexts (Supplementary Figure. 2).

6. Restoration of Calcium Homeostasis and Protection Against Glutamatergic Excitotoxicity

SHED Co-culture Mitigates NMDA-Induced Calcium Overload and Excitotoxicity in FAD and SAD iPSC-Derived Neurons

To evaluate the impact of FAD and SAD pathology in terms of calcium homeostasis, and to investigate in this regard the potential therapeutic rescue effects of SHED, we monitored intracellular calcium ($[Ca^{2+}]_i$) dynamics following SHED co-culture using the genetically encoded calcium indicator (GECI) probe hSyn-Soma-jGCaMP8f. Successful expression and localization of the probe within the somatic regions of 8-week-old iPSC-derived human cortical neurons was confirmed via live phase-contrast and fluorescence microscopy (Fig. 6A).

SHED-exosome treatment does not alter resting intracellular calcium

To evaluate the effect of SHED-derived exosomes on calcium homeostasis, we first measured baseline intracellular calcium ($[Ca^{2+}]_i$) in neuronal cultures derived from all study subjects (FAD, SAD, CTRL). Under resting (**R**) conditions, baseline $[Ca^{2+}]_i$ did not differ significantly among FAD, SAD, and CTRL neurons and SHED-exosome exposure did not shift resting $[Ca^{2+}]_i$ in any group (Fig. 6B, “*RESTING*”). These data indicate that neither the disease

background nor the treatment produced chronic or tonic calcium dysregulation, allowing subsequent evoked responses to be interpreted independently of resting-state differences.

AD neurons display an exaggerated NMDA-evoked calcium response that is normalized by SHED-exosomes

We next challenged cultures with NMDA and quantified the evoked rise in $[Ca^{2+}]_i$. In the untreated condition, FAD and SAD neurons exhibited a significantly larger NMDA-evoked calcium transient than untreated CTRL neurons (CTRL=1030.15 v. FAD=1107.08, and SAD=1163.09; $p < 0.05$) (Fig. 6B, panel NMDA (S)), indicating a disease-associated hyper-responsiveness of the evoked glutamatergic calcium signal despite normal resting calcium. This pattern is consistent with the NMDA-receptor hyperexcitability and excitotoxic priming characteristic of Alzheimer's disease.

Following treatment with SHED-secreted exosomes, both FAD and SAD neurons showed a smaller NMDA-evoked $[Ca^{2+}]_i$ rise relative to their untreated counterparts. Critically, the treated AD responses fell to the level of untreated CTRL neurons and were not suppressed below it (Fig. 6B, panels: NMDA (S) and delta S-R). This downward correction — approaching but not overshooting the physiological control set-point — might be a sign of a trend in normalization of NMDA receptors by some SHED-derived agents.

To isolate the specific impact of the “SHED cell therapy-derived intervention”, we analyzed the *relative* (Fig. 6C) and *absolute* (Fig. 6D) changes in calcium influx directly attributable to SHED co-culture. The *relative* estimation was based on the fold change in calcium rise upon

stimulation versus resting conditions between conditions without SHED versus conditions with SHED, i.e., on the plot: -SHED($\Delta S-R$) v. +SHED($\Delta S-R$) (Fig. 6C). Both FAD and SAD neuronal cultures revealed a significantly lower response rate to such treatment, suggesting a slowing down in calcium toxic responses. The *absolute* assessment of the changes in calcium dynamics was based on a significant differential reduction in NMDA-induced calcium load, which was highly pronounced in the AD groups (Fig. 6D). This demonstrated reduced susceptibility to excitotoxicity, showing that co-culture with SHED cells actively rescues human neurons in AD.

Overall, patient-derived AD neurons exhibited baseline calcium dyshomeostasis and heightened vulnerability to excitotoxic challenge. NMDA stimulation induced a rapid, pathological influx of intracellular calcium (Ca^{2+} overload) in untreated AD neurons, paired with delayed or incomplete clearance kinetics. In contrast, neurons co-cultured with SHED demonstrated significant resilience. SHED co-culture significantly attenuated the peak magnitude of NMDA-induced calcium overload and enhanced intracellular calcium clearance mechanisms, protecting the post-synaptic architecture from excitotoxic degradation.

The normalized calcium response occurs without loss of neuronal viability

Because both excessive NMDA-evoked calcium entry and its pharmacological over-suppression can compromise neurons, we assessed viability after treatment and NMDA

challenge. Neuronal viability was fully preserved across all groups, with no significant difference between SHED-exosome-treated FAD, SAD, or CTRL cultures and their respective untreated controls (MTT assay, Supplementary Figure 3). The correction of the exaggerated calcium signal therefore proceeded without cytotoxic cost, consistent with removal of an excitotoxic liability rather than non-specific inhibition of physiological signaling.

SHED Co-culture Differentially Regulates Excitotoxic Calcium Influx in 3D AD Cerebral Organoids

To expand our findings from 2D monolayer cultures into a more physiologically relevant translation model, we assessed the therapeutic impact of SHED co-cultures on 3D human cerebral organoids representing CTRL, FAD, and SAD genetic backgrounds. Functional intracellular calcium dynamics within the complex, interconnected organoid networks were captured using the calcium sensor hSyn-Soma-jGCaMP8f (Fig. 7A). Following a localized challenge with 20 μ M NMDA, we quantified the net shift in peak intracellular calcium influx directly modulated by the presence of SHED co-cultures (Fig. 7B). While healthy control (CTRL) organoids maintained a mild positive homeostatic baseline change, and FAD organoids showed minimal alteration, SAD cerebral organoids exhibited a distinct and robust drop in post-stimulation calcium levels (Fig. 7B). Notably, the SAD cohort displayed a profound, statistically significant negative shift in peak fluorescence intensity ($p < 0.05^{**}$; Fig. 7B^{**}). This marked reduction indicates that SHED co-culture actively dampens hyper-excitotoxic calcium cascades in sporadic AD tissue architectures, suggesting that the

secretome or direct interaction with SHED provides a specialized protective barrier against NMDA-mediated calcium dysregulation in a complex 3D setting.

7. Mitigation of Baseline and Stress-Induced Mitochondrial ROS

Mitochondrial oxidative stress analysis revealed significant differences among the patient-derived lines. Under basal conditions, neurons derived from both FAD and SAD patients exhibited higher baseline levels of mitochondrial reactive oxygen species (ROS) compared to healthy control (CTRL) neurons. Introduction of the chemical stressor doxorubicin (doxo) triggered a sharp increase in bright, widespread red, yellow, and white fluorescent signals within mitochondria, indicating severe oxidative stress. This stress-induced challenge severely compromised cell morphology in the untreated SAD panel (SAD_doxo), leading to widespread cell death.

Given the intimate link between aberrant calcium homeostasis, mitochondrial dysfunction, and reactive oxygen species (ROS) in Alzheimer's disease pathology, we next investigated whether SHED co-culture could mitigate intracellular oxidative stress. We monitored ROS production and mitochondrial network alterations under both physiological baseline conditions (Fig. 8A) and under an explicit oxidative challenge triggered by doxorubicin treatment (Fig. 8B).

Quantitative analysis of ROS and mitochondrial fluorescent signals (Fig. 8C) revealed that even under basal conditions, untreated Familial (FAD_NO_SHED, ~0.72 RFI) and Sporadic (SAD_NO_SHED, ~0.74 RFI) AD neurons exhibited significantly higher baseline oxidative

695 stress compared to healthy controls (CTRL_NO_SHED, ~0.45 RFI). Remarkably, co-culture
696 with SHED successfully brought baseline ROS markers in both FAD and SAD neurons down
697 near or below control baselines (FAD_WITH_SHED and SAD_WITH_SHED, ~0.52 and ~0.42
698 RFI, respectively).

699 To test the robustness of this antioxidant defense, cells were insulted with doxorubicin. This
700 challenge forced a massive spike in oxidative stress across all groups, with the untreated
701 familial group reaching the highest levels of damage recorded (FAD_DOX_NO_SHED, ~1.2
702 RFI), followed by sporadic AD (SAD_DOX_NO_SHED, ~0.87 RFI). Strikingly, the inclusion of a
703 SHED co-culture during this chemical insult exerted a powerful protective effect. SHED
704 intervention dramatically suppressed doxorubicin-mediated ROS spikes in both FAD
705 (FAD_DOX_WITH_SHED, ~0.52 RFI) and SAD (SAD_DOX_WITH_SHED, ~0.68 RFI) neurons.
706 These results demonstrate that the therapeutic secretome or cellular interactions provided
707 by SHED bolster mitochondrial resilience, protecting human AD neural networks from heavy
708 oxidative insults.

709 Discussion

710 The present study yielded several key findings regarding dysregulated miRNAs involved in
711 AD. As shown in the results, hsa-miR-23a-3p was most abundant, followed by miR-16-5p
712 and miR-191-5p, suggesting their significance in the genetic pathophysiology of AD. These
713 results are consistent with previous research on dysregulated miRNAs in AD⁵⁰.

714 Several investigations identify miR-23a-3p as among the key microRNAs dysregulated in AD,
715 proposed as a diagnostic biomarker in serum and CSF of AD patients ⁵¹, ⁵². It is also
716 upregulated in blood-derived neurogenic exosomes of AD patients ⁵³, possibly because the
717 miR-23a/b cluster regulates Sirtuin 1 (SIRT1), a key neuroprotective protein that reduces
718 oxidative stress and inflammation. The high abundance of this miRNA in our SHED exosomes
719 suggests these vesicles may be primed to deliver a "corrective" cargo modulating miR-23a-
720 3p targets in the diseased brain, potentially offering neuroprotective effects.

721 The second most abundant miRNA, hsa-miR-16-5p, is essential for normal cell processes
722 and linked to cell survival dynamics in AD models by targeting Amyloid Precursor Protein
723 (APP) mRNA expression ⁵⁴. It can be modulated in response to A β deposition and interacts
724 with pathways governing BCL-2, a crucial anti-apoptotic protein, increasing neuronal
725 vulnerability under stress. Its presence indicates these exosomes carry powerful regulators
726 of cell viability and turnover, consistent with stem cell-derived vesicles aiming to remodel
727 damaged tissue. CSF-isolated microRNA levels can vary distinctly between late-onset and
728 early-onset AD ⁵⁵, so the physiological balance of this miRNA is a key factor when SHED
729 exosomes deliver it in the neurodegenerative context.

730 The third most abundant miRNA, sharing equal frequency with the second, was hsa-miR-
731 191-5p, a strong neuroprotection candidate. Studies show miR-191-5p can attenuate tau
732 phosphorylation and reduce A β generation, directly countering AD pathology. It
733 bioinformatically interacts with DAPK1 (Death-Associated Protein Kinase 1), implicated in
734 plaque formation and neuronal death. Other notable miRNAs included hsa-miR-486-5p, a

blood-based AD biomarker linked to synaptic plasticity, and hsa-miR-25-3p, associated with oxidative stress regulation and altered in prodromal AD, suggesting involvement in early stress-response networks.

The high abundance of these functional miRNAs is a positive finding for diagnostic and therapeutic approaches. These results align with research showing dysregulated microRNAs serve as a complementary diagnostic approach with high accuracy⁵⁶, and that monitoring exosomal microRNA changes can help evaluate the efficacy of anti-degenerative treatments given their role in cognitive decline severity and neuroinflammation⁵⁷.

To evaluate SHED's effects, we compared cellular viability, calcium homeostasis, mitochondrial oxidative stress, and AD pathology markers (A β 40, A β 42, p181-tau) in FAD and SAD models versus controls. Results showed a significant change in phosphorylated tau (p181-tau), a toxic form linked to neurofibrillary tangles. SHED treatment differentially affected familial versus sporadic AD models, most strongly favoring the FAD profile by flattening the pathological p181-tau/A β 42 ratio. A lower ratio is generally preferred, since high p-tau and low A β 42 typically correlate with extensive pathology—lower fluid A β 42 often indicates trapping within brain plaques, while therapeutic clearance or shift adjustments may alter this. A change in this ratio signifies a shift in amyloid processing; our results support SHED-derived exosomes as a promising early-stage AD therapy.

With respect to calcium dynamics, our data resolve the SHED effect into two mechanistically distinct compartments. Resting calcium homeostasis was intact and treatment-independent, with baseline [Ca²⁺]_i comparable among FAD, SAD, and control

neurons and unchanged by exosome exposure. In contrast, the NMDA-evoked calcium transient was pathologically elevated in untreated FAD and SAD neurons relative to controls—consistent with the NMDA-receptor hyperexcitability and excitotoxic priming characteristic of AD, and mirroring the clinical rationale for NMDAR-directed agents such as memantine. This pattern indicates that the relevant lesion lies in the stimulus-evoked signalling gain rather than in tonic calcium buffering. Following treatment, SHED normalized this exaggerated evoked response down to the control set-point without suppressing it below control levels, indicating a tuned recalibration of the dynamic range of NMDAR-mediated calcium signalling rather than a blunt receptor blockade. Because this correction preserved neuronal viability, it is best interpreted as the removal of an excitotoxic liability rather than non-specific inhibition of physiological calcium-dependent signalling.

Relating protein-level changes to the miRNA cargo offers a compelling molecular mechanism for SHED's observed effects, including tau suppression, amyloid modulation, and neuroprotection. Exosomes rich in miR-191-5p, which targets DAPK1 (a kinase triggering tau hyperphosphorylation), align with the significant p181-tau reduction seen in the FAD group, suggesting these exosomes suppress aberrant tau phosphorylation. A second mechanism involves amyloid processing: high miR-16-5p levels, a known regulator of APP and BACE1 (the rate-limiting enzyme cleaving APP into toxic amyloid), likely explain the shift in A β 42/A β 40 ratios by interacting with amyloid production machinery or cleavage preferences, improving the ratio in both FAD and SAD groups. Because A β is a principal driver of NMDAR hyperexcitability, and DAPK1/tau-kinase signalling feeds into excitotoxic GluN2B-linked cascades, these same amyloid- and tau-directed mechanisms provide a plausible

778 upstream route to the normalization of the exaggerated NMDA-evoked calcium response we
779 observed.

780 A third mechanism is a broader neuroprotective signaling axis. The most abundant miRNA,
781 miR-23a-3p, regulates SIRT1 and oxidative stress pathways, likely contributing to overall cell
782 survival and the distinct "rescue" phenotype seen prominently in the FAD group. This
783 subtype difference reflects that FAD models often exhibit aggressive, early-onset tau
784 pathology driven by kinase dysregulation, making them highly responsive to this "kinase-
785 braking" miRNA cargo. SAD, in contrast, is more heavily driven by polygenic, age-related
786 oxidative stress and metabolic failure; while the amyloid component (miR-16) worked
787 effectively across genotypes, tau and stress-handling components (miR-23a/miR-191) may
788 face more complex resistance in sporadic genetic backgrounds.

789 We also measured mitochondrial ROS, a key oxidative stress indicator across neuronal
790 cultures, and found SHED co-culture consistently reduced both baseline and doxorubicin-
791 induced oxidative stress. Intracellular calcium levels were measured before, during, and
792 after NMDA-induced excitotoxicity, showing SHED treatment protects neurons from
793 excessive calcium influx and improves clearance kinetics. The stability of resting calcium
794 alongside the normalization of the exaggerated evoked response indicates that this
795 protection reflects a recalibration of NMDAR-mediated signalling gain superimposed on a
796 preserved calcium-handling baseline, rather than a correction of chronic calcium overload.
797 These results align with prior data on the significant role of mitochondrial dysfunction and
798 Ca^{2+} handling defects in core AD pathology, driven through circular ROS generation and

799 oxidative damage⁵⁸. Mitochondrial damage accelerating A β production and APP processing
800 anomalies, resulting in synaptic defects⁵⁹, further connects to our findings on tau
801 phosphorylation and exosomal microRNAs linked to oxidative stress responses. The
802 convergence of reduced oxidative stress, normalized evoked calcium signalling, and
803 improved clearance kinetics is consistent with SHED acting on the shared ROS–calcium–
804 excitotoxicity axis that couples these processes.

805 Relating protein-level changes to the identified miRNA cargo represents a plausible, though
806 not yet fully proven, molecular mechanism, since exosomal cargo is complex and
807 heterogeneous—including proteins, lipids, and non-coding RNAs—with individual and
808 collective contributions still to be fully characterized. Future studies using engineered
809 exosomes loaded with specific miRNAs would help isolate and confirm individual cargo
810 contributions. The possible therapeutic mechanisms likely include:

811 **1. Anti-Tau Kinase-Brake Mechanism:** Exosomal cargo is notably rich in hsa-miR-191-5p
812 (11% of reads). This miRNA directly represses DAPK1, a major upstream kinase triggering tau
813 hyperphosphorylation, matching the significant p181-tau reduction observed specifically in
814 the FAD cohort. Delivered miR-191-5p suppresses DAPK1 expression, breaking the
815 phosphorylation cycle. FAD models, which frequently exhibit aggressive kinase
816 dysregulation, are uniquely responsive to this targeted intervention.

817 **2. Amyloid Precursor Modulator Mechanism:** The exosomal payload contains substantial
818 hsa-miR-16-5p (11% of reads), which acts as a post-transcriptional repressor of APP
819 translation and BACE1 transcript levels. This target accounts for favorable adjustments in

the A β 42/A β 40 ratio across both genetic models, operating as a broad-spectrum mechanism targeting amyloid processing directly. By lowering amyloidogenic load, this mechanism also plausibly relieves the A β -driven NMDAR hyperexcitability underlying the exaggerated evoked calcium response in both genotypes.

3. Broad Neuroprotective Mechanism: The most abundant exosomal component is hsa-miR-23a-3p (19% of total reads), which plays a complex regulatory role in managing oxidative stress responses and stabilizing cell survival pathways. While amyloid modulators functioned efficiently across genotypes, sporadic models showed distinct resistance to tau/stress signaling components compared to familial variants, likely reflecting the multifactorial oxidative and age-related etiology characteristic of sporadic AD.

The identification of 18 miRNAs uniquely expressed during co-culture with human cerebral organoids suggests dynamic bidirectional signaling, wherein stem cells alter their exosomal cargo in response to pathological signals from diseased brain tissue. These co-culture-specific miRNAs targeted networks associated with vesicular trafficking and endo-lysosomal pathways, frequently disrupted in early-stage AD.

In summary, this study demonstrates that SHED and their secreted exosomes can mitigate multiple pathological features of AD patient-specific models, with particular focus on SAD cases—selected for the typical clinical picture of mild-stage SAD—and FAD cases with pathology caused by presenilin mutations. Notably, this mitigation included normalization of a pathologically exaggerated NMDA-evoked calcium response to the physiological control set-point, achieved without disturbing resting calcium or compromising viability. These

841 findings support further development of SHED-derived exosomal therapies as non-cellular,
842 multi-target interventions for Alzheimer's disease, particularly in its early stages.

843 Limitations and future perspectives

844 While the patient-specific iPSC and 3D organoid models used in this study offer a useful
845 platform for investigating human genetic backgrounds, certain limitations remain. First, the
846 sample size of individual patient cell lines per subgroup is constrained by the availability of
847 well-characterized patient skin biopsies. Second, although 3D cerebral organoids model
848 complex multicellular architecture, they lack a fully functional vascular network, which
849 limits the evaluation of systemic or blood-brain barrier clearance mechanisms. Lastly, the
850 temporal window of 6 months, while extensive for in vitro culture, only captures initial
851 molecular and cellular phases of neurodegeneration rather than the decades of aging
852 pathology.

853 Conclusion

854 This study demonstrates that SHED co-culture and the SHED secretome exert beneficial
855 effects in both FAD and SAD models. The identified exosomal signature therefore represents
856 a potential therapeutic platform for further translational investigation. Our findings indicate
857 that SHED-derived factors can help human neurons withstand oxidative stress, calcium
858 overload, and abnormal amyloid processing, while also revealing candidate mechanisms
859 that may contribute to anti-degenerative effects in AD. Further studies should validate these

860 mechanisms and assess the therapeutic potential of SHED-derived exosomes in early-stage
861 Alzheimer's disease.

862 Declarations & Consent Statement

863 All primary cell samples and iPSC lines derived from study subjects were obtained with
864 written informed consent from all participants or their legally authorized representatives
865 prior to sample collection, in accordance with the Declaration of Helsinki and approved by
866 the institutional bioethics committee.

867 Acknowledgments

868 We thank the participants of the study and the neurologists for the diagnosis. We would like
869 to thank Dr. Ewa Liszewska from the Laboratory of Molecular and Cellular Neurobiology at
870 the International Institute of Molecular and Cell Biology in Warsaw, Poland, for kindly
871 providing the iPSC line IIMCBi002-A³⁵. We would like to thank Prof. Anna Falk and Dr. Malin
872 Kele from the Department of Neuroscience at the Karolinska Institutet, Stockholm, Sweden,
873 for kindly providing the iPSC line CTL07-02³⁴. We would like to thank Prof. Haruhisa Inoue
874 from Kyoto University and the Center for iPS Cell Research and Application (CiRA) for
875 providing the ADP2 and ADP3 lines, i.e. CiRA00002 and CiRA00003 lines, respectively²⁵. The
876 authors thank Dr. Maria Styczyńska from the National Medical Institute of the Ministry of the
877 Interior and Administration for the line with I213F mutation in PSEN1. The authors thank Prof.
878 Makarauk and Prof. Zaremba for the lines with P117L mutation in PSEN1. The authors thank
879 to Sylwia Gawlik-Zawiślak and Marta Woźniak for karyotyping.

880 Funding Declaration

881 This research was supported by the funding from National Science Centre, in a framework o
882 Polonez Bis grant (ID: UMO-2022/47/P/NZ3/03257), implemented at Department of
883 Neurogenetics and Functional Genomics, Mossakowski Medical Research Institute, Polish
884 Academy of Sciences in Warsaw, during 2024.04.26-2026.07.25), within a project entitled „
885 Study of the regenerative potential of Stem Cells from Human Exfoliated Deciduous teeth
886 (SHED) in Alzheimer’s disease”, funding: 1,208,364 PLN, Dr. Michalina Wężyk - project
887 scientific supervisor and investigator, Dr. Leila Elyasi – principal investigator.

888 **Conflicts of Interest & Financial**

889 **Disclosures**

890 The authors declare no competing financial or non-financial interests related to the research
891 presented in this manuscript.

892 Authors contribution

893 Leila Elyasi and Anna Chołoniewska contributed equally to this work (denoted by #).
894 Michalina Wężyk is the corresponding author.

895 Leila Elyasi: Conceptualization, Study Design, Experimental Contribution. Anna
896 Chołoniewska: Conceptualization, Methodology, Experimental Work (performed all
897 experiments). Eliza Lutostańska: Experimental Contribution. Natalia Mierzwa: Experimental

898 Contribution. Dorota Sulejczak: Methodology (Luminex assays). Matylda Macias:
899 Methodology (Transmission Electron Microscopy). Aleksandra Szybińska: Methodology
900 (Transmission Electron Microscopy). Sylwia Gawlik-Zawiślak: Methodology (iPSC
901 karyotyping). Marta Woźniak: Methodology (iPSC karyotyping). Anna Filipek-Gliszczyńska:
902 Resources, Clinical Data Curation (patient clinical description, SAD). Małgorzata
903 Bednarska-Makaruk: Resources, Clinical Data Curation (patient clinical description, FAD,
904 *PSEN1_P117L*). Jacek -: Resources, Clinical Data Curation (patient clinical description, FAD,
905 *PSEN1_P117L*). Maria Styczyńska: Clinical Data Curation (patient clinical description, FAD,
906 *PSEN1_I213F*). Cezary Żekanowski: Supervision, Writing – Review & Editing. Michalina
907 Wężyk: Conceptualization, Study Design, Experimental Work (performed all experiments),
908 Methodology (miRNA isolation prior to miR-RNAseq, bioinformatics of miR data, SRA
909 submission), Project Administration, Acquisition, Supervision, Writing – Original Draft,
910 Writing – Review & Editing.

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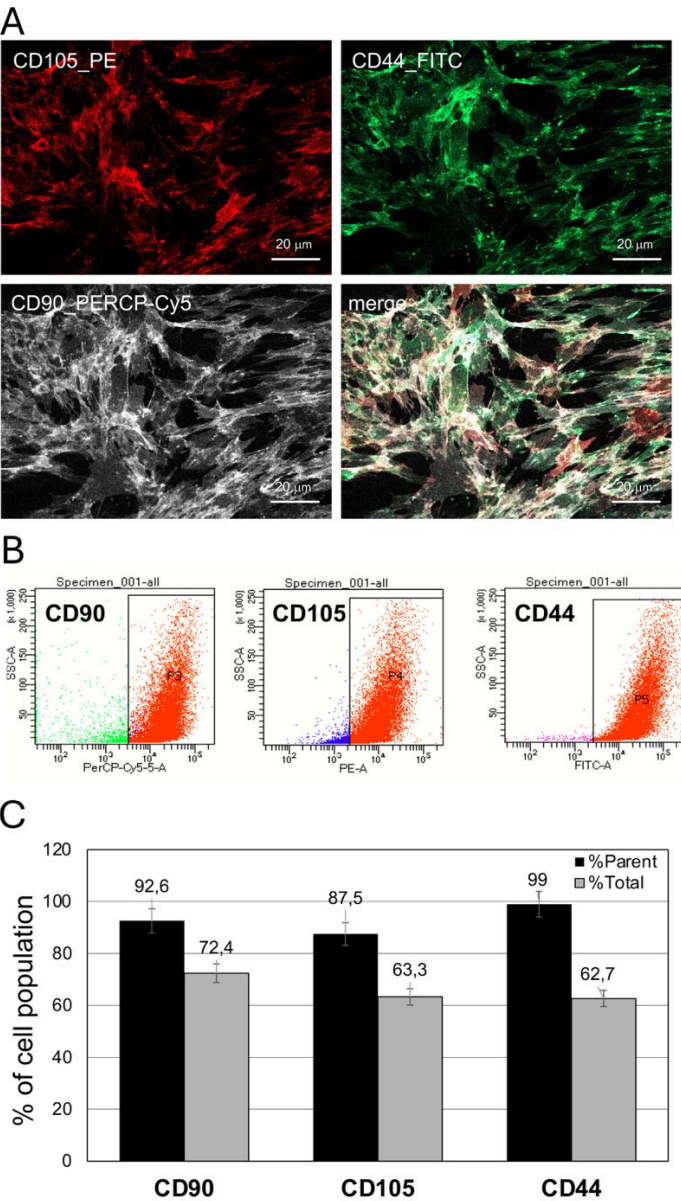
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1084

FIGURE 1



1086

1087 **Figure 1. Characterization of SHED using immunocytochemical (ICC) staining and flow**

1088 **cytometry.** A. Fluorescence microscopy images displaying CD105 (Red/PE), CD44

1089 *(Green/FITC), CD90 (White/Grayscale/PerCP-Cy5), and the merged view highlighting*
1090 *spindle-shaped mesenchymal morphology. B. Scatter plots quantifying marker expressions*
1091 *(SSC-A vs. Fluorescence Intensity) shifting into gated regions P3, P4, and P5. C. Statistical*
1092 *summary graph of marker distribution.*

1093

FIGURE 2

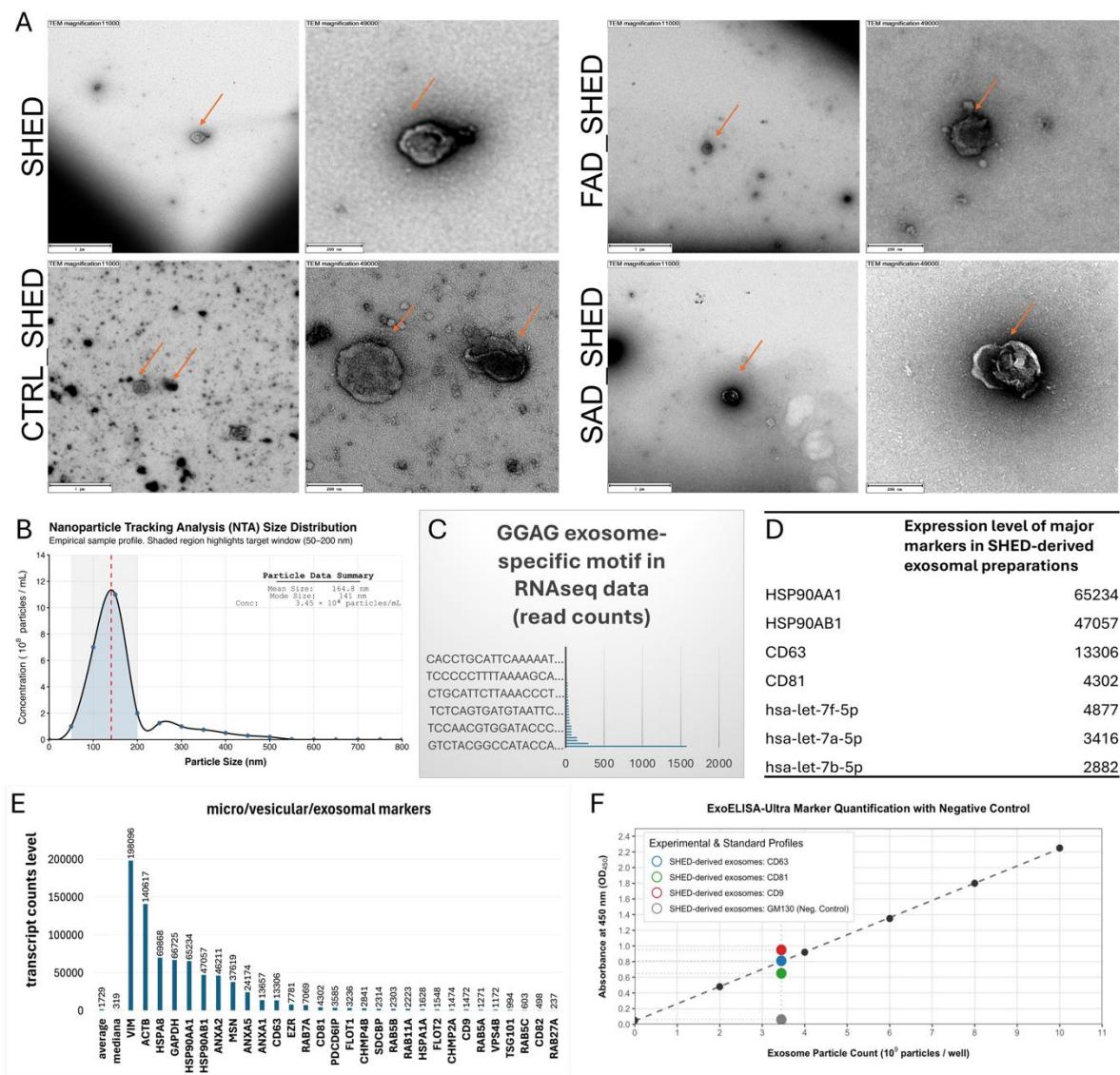


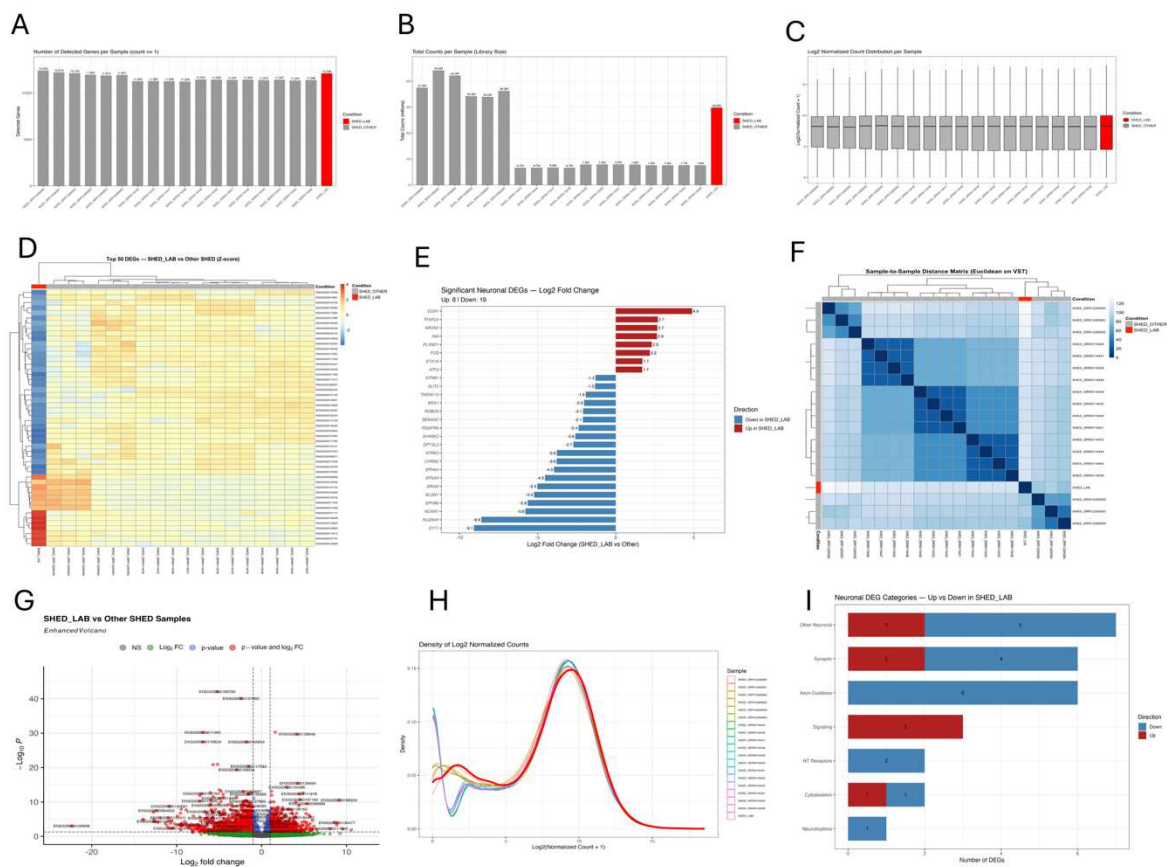
Figure 2. Physical and marker metrics of exosomal vesicles derived from SHED. A. Transmission Electron Microscopy capturing distinctive cup-shaped membrane structures across groups. B. Representative nanoparticle tracking analysis (NTA) of size-distribution of extracellular vesicles showing particle concentration versus hydrodynamic diameter, with the 50–200 nm target range shaded, a modal diameter of 141 nm, as indicated by the red

1100 dashed line, and a mean particle diameter of 164.8 nm with a total concentration of $3.45 \times$
1101 10^8 particles/mL. C. Exosomes-specific sequence motifs revealed in RNA-seq profiling of
1102 vesicular RNA fractions. D and E, Expression of exosomal/microvesicular-specific markers
1103 in the transcript profile of SHED-derived exosomal preparations. F. Quantitative analysis of
1104 SHED-derived exosomes via ExoELISA-Ultra loaded at an NTA-validated density of 3.45×10^9
1105 particles/well validating expression of canonical endosomal markers CD9 ($OD_{450} = 0.95$),
1106 CD63 ($OD_{450} = 0.81$), and CD81 ($OD_{450} = 0.65$) along a linear standard curve ($R^2 > 0.99$), while
1107 the negligible signal of cis-Golgi marker GM130 ($OD_{450} = 0.06$) confirmed high structural
1108 purity.

1109

1110

FIGURE 3



1111

1112 **Figure 3. Transcriptome profiling and comparative analysis of SHED lines against public**
1113 **datasets. A–C.** Global sequencing metrics comparing study SHED lines with SRA database
1114 samples, showing detected and annotated gene counts (~13,000) (A), library sizes (29.9 M ±
1115 13.8 M) (B), and consistent log-normalized expression levels across datasets (C). D–E.
1116 Heatmaps showing the top 50 differentially expressed genes (DEGs) out of 1,383 identified
1117 DEGs in our SHED versus other RNA-seq files from other SHED available at SRA database
1118 (D), including 27 neuronal-specific DEGs (E). F. Hierarchical clustering and pairwise sample-

1119 *to-sample euclidean distance matrix derived from rlog-transformed data, where darker blue*
1120 *cells represent higher expression profile similarity. **G.** Volcano plots displaying statistical*
1121 *significance versus fold change for annotated DEGs compared across SRA samples. **H.***
1122 *Density plot of DESeq2-normalized $\log_2$ expression counts illustrating a characteristic RNA-*
1123 *seq distribution with a primary peak for moderately-to-highly expressed genes and a*
1124 *secondary lower-end tail representing lowly expressed or non-coding transcripts. **I.***
1125 *Neuronal-specific processes enriched in the differentially expressed transcript dataset in*
1126 *our SHED versus other RNA-seq files from other SHED available at SRA database, pointing to*
1127 *their particular pro-neuronal plasticity changes potential.*

1128

FIGURE 4



Figure 4. Identification and Pathway Enrichment Analysis of SHED-Derived Exosomal miRNAs in Co-culture Conditions. A. Flow diagram outlining the multi-step filtering of

1132 mapped genes from the total RNA of SHED. Out of 13,306 mapped genes, 735 hits were
1133 identified for mature miRNAs, with 468 detected in standard exosomal preparations. A
1134 subset of 37 miRNAs was expressed during co-culture with control, FAD, or SAD cerebral
1135 organoids, 10 of which were uniquely produced under these co-culture conditions. Note that
1136 cerebral organoids alone did not yield detectable exosomal miRNAs of this signature. B.
1137 Relative abundance of the 37 identified co-culture miRNAs, highlighting the highest
1138 percentages attributed to hsa-miR-23a-3p, miR-16-5p, miR-191-5p, miR-486-5p, and miR-
1139 25-3p. C. Venn diagram/Target gene pool overlap showing the incorporation of the 11,918
1140 targets (predicted via miRWalk) from the 10 unique co-culture miRNAs into the broader pool
1141 of 15,255 targeted genes derived from all 37 miRNAs. D and E. Gene Ontology (GO) terms
1142 and KEGG pathway enrichment analysis of the target genes regulated by the 10 unique co-
1143 culture miRNAs, demonstrating significant enrichment in pathways related to neuronal
1144 specificity. F and G. Enrichment analysis mapping the 10 unique miRNA targets to neuronal
1145 and exosomal/vesicular trafficking-related pathways.

1146

FIGURE 5

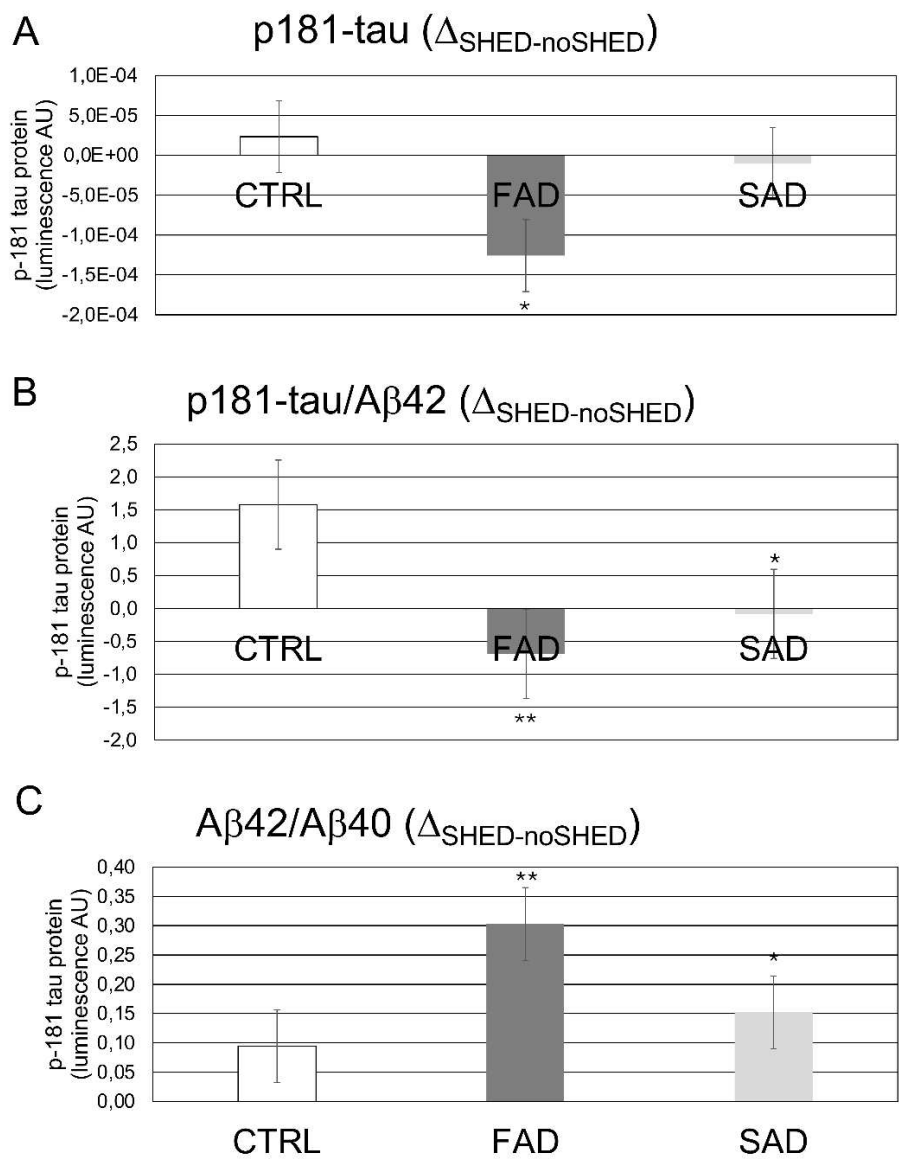


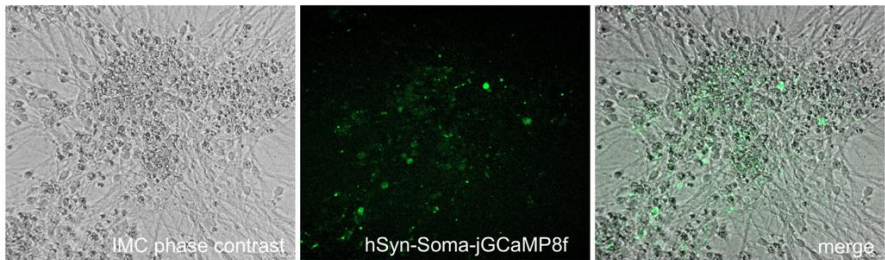
Figure 5. Luminescence-based quantification of Alzheimer’s Disease hallmarks across healthy controls, FAD, and SAD subjects in 8-week-old iPSC-derived neuronal cultures.

A. Relative changes in p181-tau protein load. B. Relative adjustments in total tau

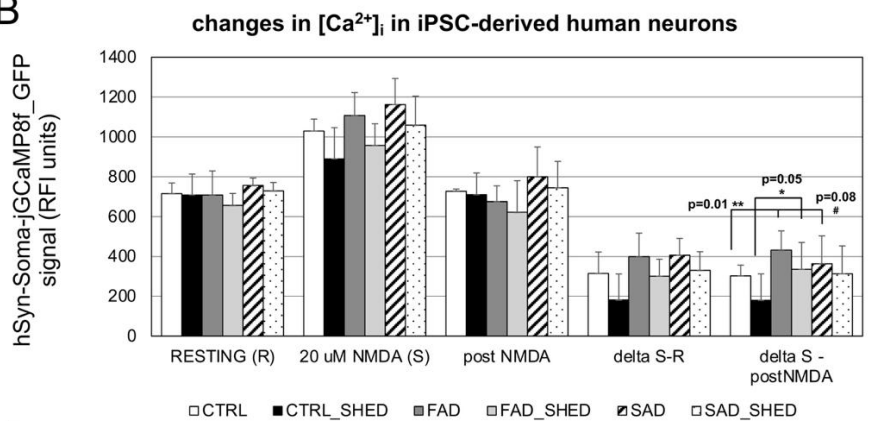
1151 concentrations. C. Shift dynamics tracking the critical $A\beta_{42}/A\beta_{40}$ cleaved peptide ratio. $N \geq$
1152 3, $*p > 0.05$.

FIGURE 6

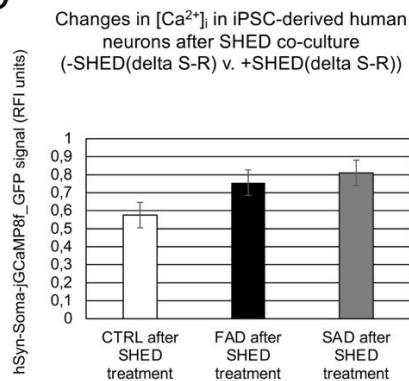
A



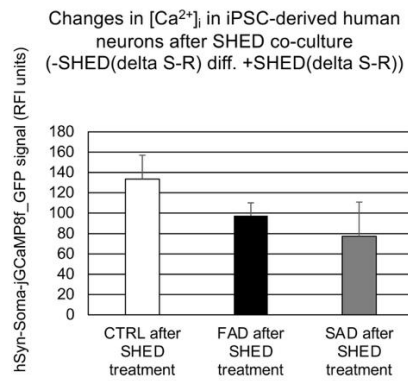
B



C



D



1153

1154 **Figure 6. Evaluation of Calcium Homeostasis and SHED Co-culture Rescue Effects in**

1155 **iPSC-Derived Neurons Following NMDA Stimulation.** A. Representative Fluorescence and

1156 Phase-Contrast Images of iPSC-Derived Neurons. Visual validation of 8-week-old human

1157 induced pluripotent stem cell (iPSC)-derived neurons expressing the genetically encoded

1158 calcium indicator (GECI) probe, hSyn-Soma-jGCaMP8f. Left: IMC phase-contrast image
1159 showing neuronal morphology. Middle: Green fluorescence channel capturing hSyn-Soma-
1160 jGCaMP8f expression. Right: Merged image demonstrating probe localization in somatic
1161 regions. B. Intracellular Calcium ($[Ca^{2+}]_i$) Dynamics Across Experimental Groups.
1162 Quantification of relative fluorescence intensity (RFI units) of the hSyn-Soma-jGCaMP8f
1163 signal across control (CTRL), Familial Alzheimer's Disease (FAD), and Sporadic Alzheimer's
1164 Disease (SAD) neurons, with or without Stem Cells from Human Exfoliated Deciduous Teeth
1165 (SHED) co-culture treatment. Measurements are shown at baseline (RESTING (R)), during 20
1166 μ M NMDA stimulation (S) to induce excitotoxicity, post-NMDA washout, the absolute
1167 response amplitude (delta S-R), and the recovery phase (delta S - postNMDA). Statistical
1168 significance is indicated by p-values (*p=0.01, p=0.05, #p=0.08, $n \geq 4$). C. Relative Changes
1169 $[Ca^{2+}]_i$ After SHED Co-culture. Comparative ratio analysis highlighting the protective effect
1170 of SHED treatment on calcium influx by contrasting non-treated versus treated conditions
1171 (-SHED(Δ S-R) vs. +SHED(Δ S-R)) across CTRL, FAD, and SAD cohorts. D. Absolute
1172 Differential Reduction of Excitotoxicity by SHED. The net difference in calcium influx
1173 amplitude brought about by SHED co-culture (calculated as -SHED(Δ S-R) diff.
1174 +SHED(Δ S-R)). This demonstrates the absolute degree to which SHED treatment managed
1175 to suppress NMDA-induced intracellular calcium overload in control, FAD, and SAD groups.

FIGURE 7

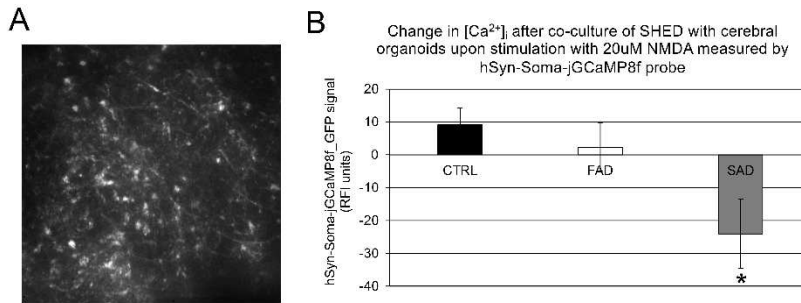


Figure 7. Modulation of NMDA-Induced Intracellular Calcium Dynamics by SHED Co-culture in Control and AD Cerebral Organoids. A. A representative fluorescence micrograph from live imaging capturing the expression of the genetically encoded calcium indicator (GECI) probe, hSyn-Soma-jGCaMP8f, within a 3D cerebral organoid network. Bright punctate fluorescence highlights somatic calcium localization across complex neuronal architectures. F. Quantitative Shift in Intracellular Calcium Responses Following SHED Intervention. The graph plots the net change in relative fluorescence intensity (RFI units) of the hSyn-Soma-jGCaMP8f signal upon stimulation with 20 uM NMDA, comparing control (CTRL), Familial Alzheimer's Disease (FAD), and Sporadic Alzheimer's Disease (SAD) cerebral organoid cohorts following co-culture with Stem Cells from Human Exfoliated Deciduous Teeth (SHED). Values reflect the modified amplitude of calcium responses attributable to the SHED therapeutic intervention. Error bars denote standard error of the mean (SEM), and the asterisk (*) indicates statistical significance relative to controls ($p < 0.05$).

FIGURE 8

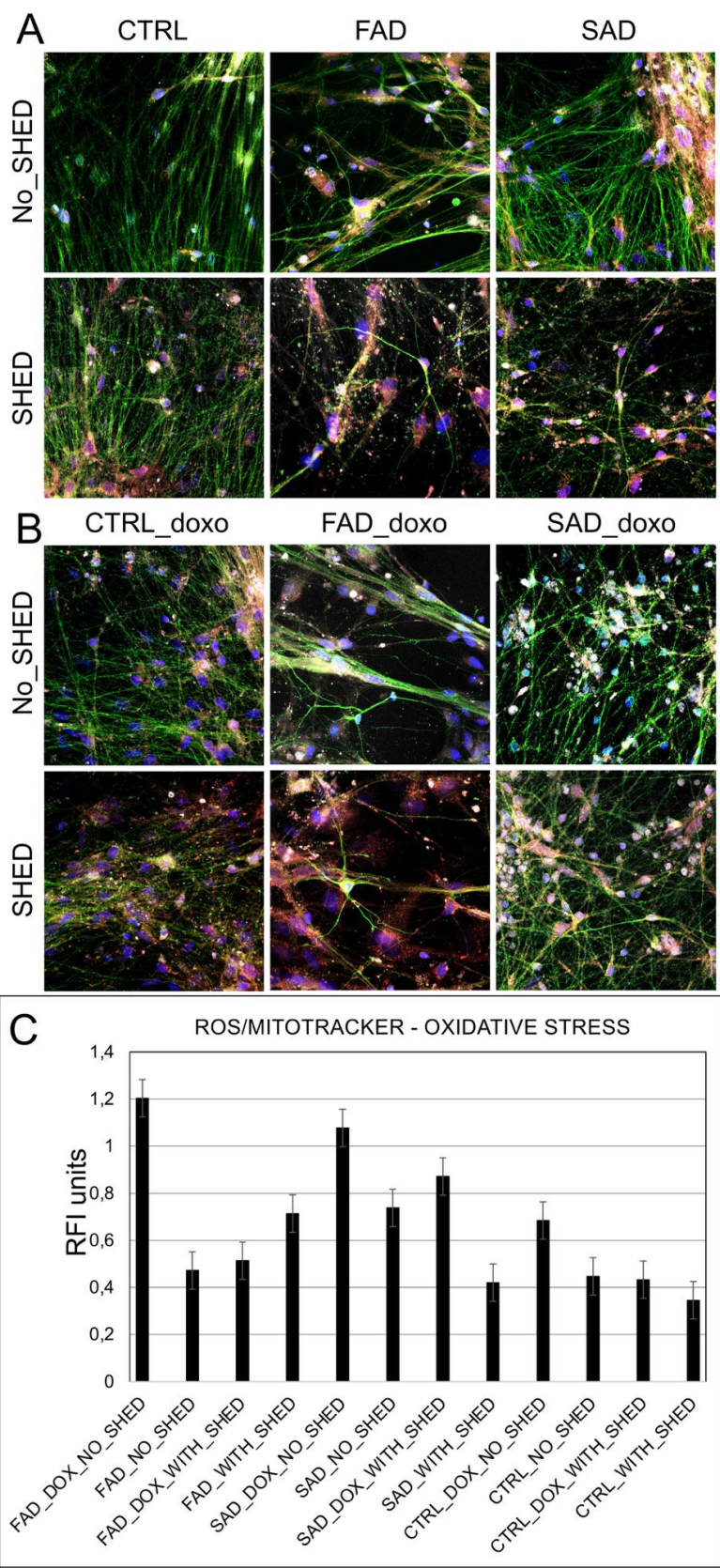
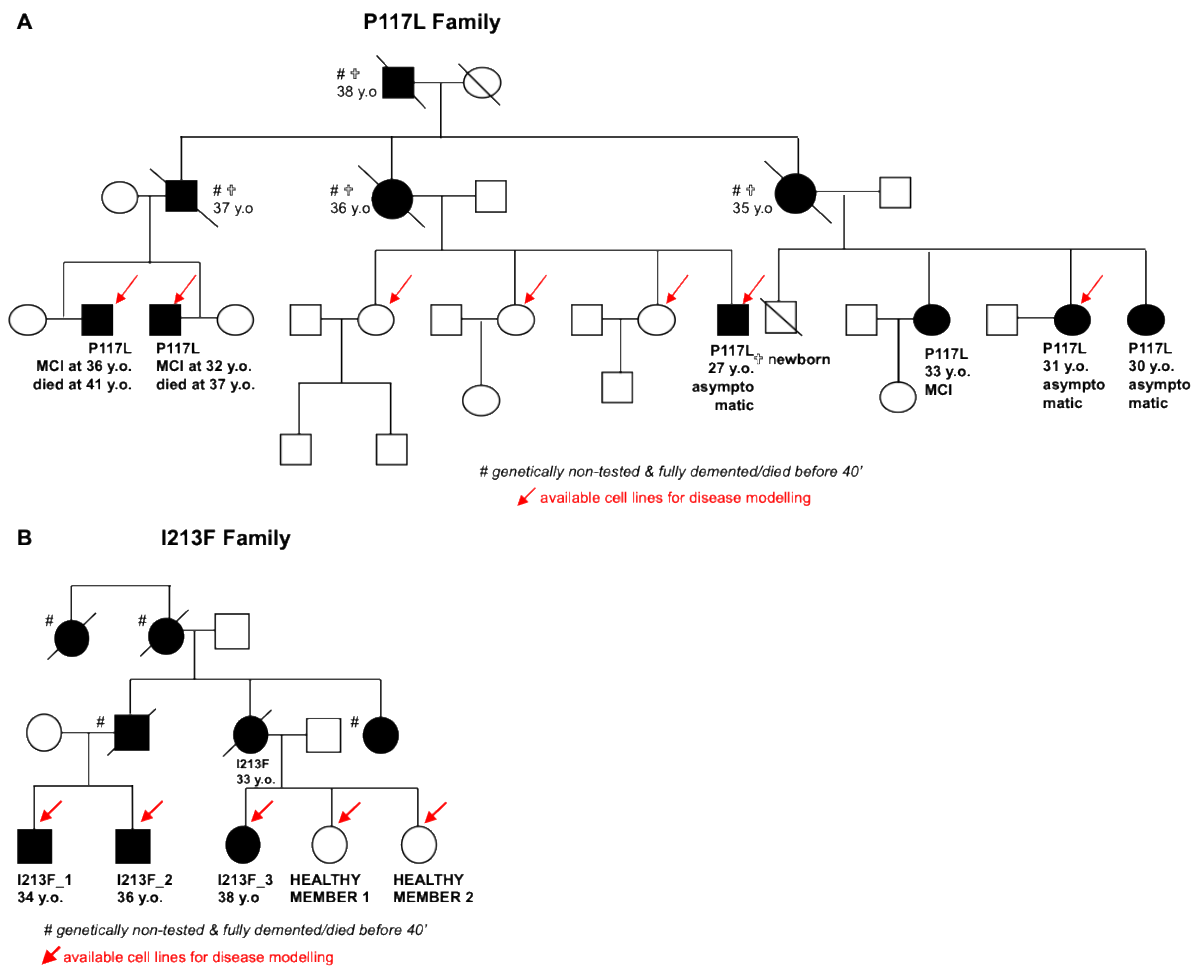


Figure 8. Attenuation of Mitochondrial Oxidative Stress by SHED Co-culture under Basal and Doxorubicin-Induced Insult. **A.** Confocal Microscopy of Basal ROS and Mitochondrial Morphology. Representative immunofluorescence images of CTRL, FAD, and SAD iPSC-derived neurons cultured in the absence (No_SHED, top row) or presence (SHED, bottom row) of SHED co-cultures under basal conditions. Staining visualizes reactive oxygen species (ROS) (WHITE) accumulation and mitochondrial networks (MitoTracker) (RED) alongside nuclear (BLUE) and beta-3 tubulin (GREEN) counterstaining. **B.** Confocal Microscopy of Oxidative Stress Following Doxorubicin Challenge. Representative immunofluorescence images of neurons subjected to doxorubicin insult (_doxo) to induce severe oxidative and mitochondrial stress. Panels contrast the structural integrity and ROS fluorescence between non-treated (no_SHED, top row) and SHED co-cultured (SHED, bottom row) groups across CTRL, FAD, and SAD cohorts. **C.** Quantification of ROS/MitoTracker-Associated Oxidative Stress. Relative fluorescence intensity (RFI units) measuring the ratio or net intensity of ROS to mitochondrial markers across all twelve experimental permutations. Data illustrate baseline stress elevations in AD backgrounds, the destructive impact of doxorubicin stress, and the systemic reduction of oxidative markers brought about by SHED therapeutic co-culture. Error bars represent the standard error of the mean (SEM).

SUPPLEMENTARY FIGURE 1



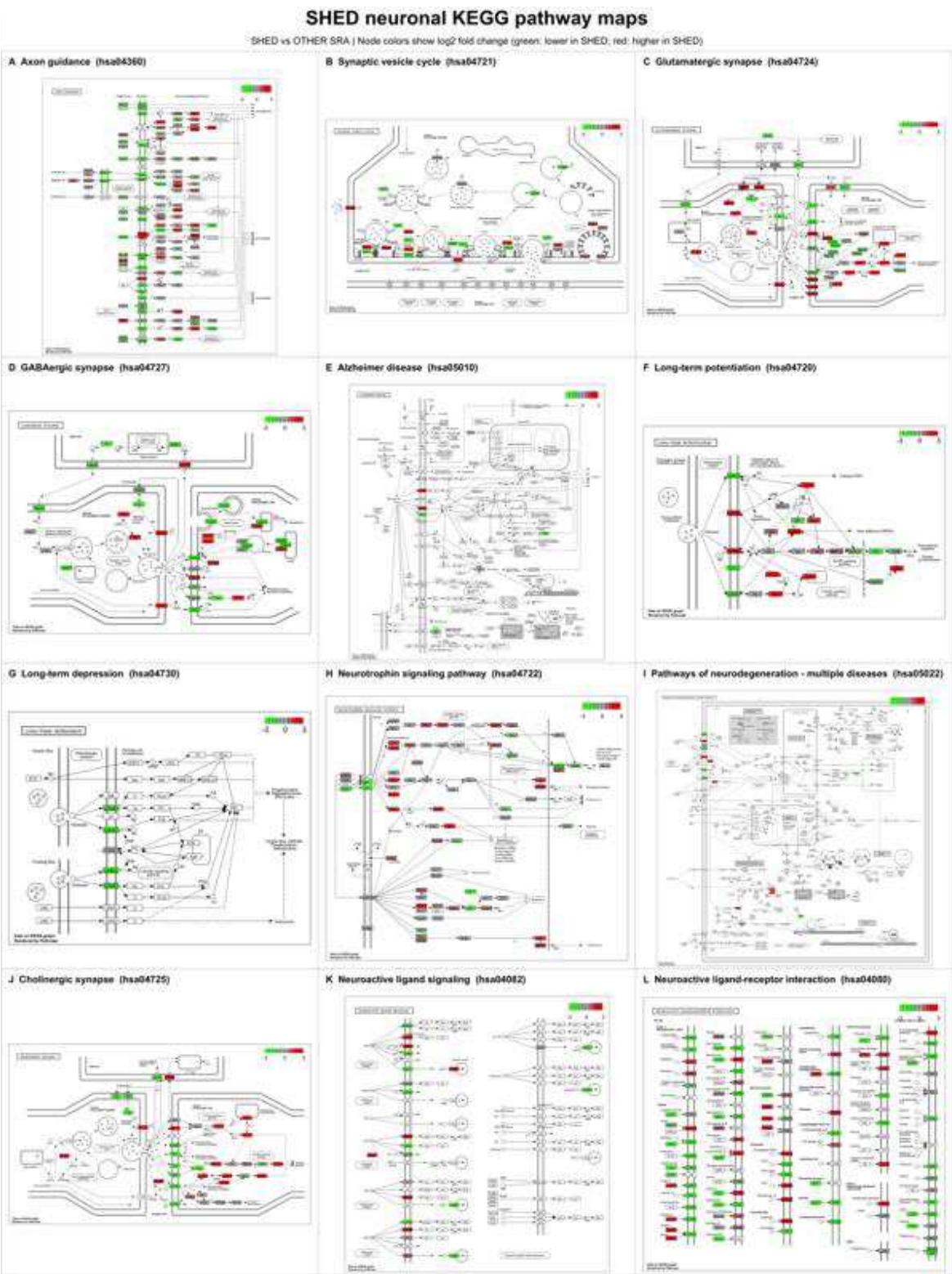
1210

1211 **Supplementary Figure 1.** Pedigree of the families of FAD cases with P117L mutation (A),

1212 and with I213F mutation (B).

1213

SUPPLEMENTARY FIGURE 2



Supplementary Figure 2. KEGG pathway enrichment analysis of predicted miRNA

targets. Enrichment of selected pathways associated with neuronal function and

neurodegeneration, including axon guidance (hsa04360), synaptic vesicle cycle (hsa04721),

glutamatergic synapse (hsa04724), GABAergic synapse (hsa04727), Alzheimer disease

(hsa05010), long-term potentiation (hsa04720), long-term depression (hsa04730),

neurotrophin signaling pathway (hsa04722), pathways of neurodegeneration—multiple

diseases (hsa05022), cholinergic synapse (hsa04725), neuroactive ligand–receptor

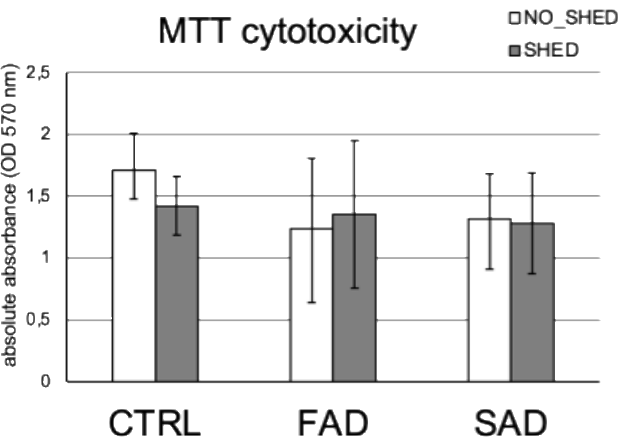
interaction (hsa04080), and neuroactive ligand signaling (hsa04082). These pathways

highlight the potential involvement of exosomal miRNA targets in neuronal function, synaptic

transmission and plasticity, and molecular processes relevant to learning, memory, and

neurodegenerative disease.

SUPPLEMENTARY FIGURE 3



Supplementary Figure 3. Potential toxicity evaluations via colorimetric MTT assays. Bar

charts illustrate absolute absorbance measurements at 570 nm mapping CTRL, FAD, and

1230 *SAD cohorts, evaluating environments with SHED (grey) versus NO_SHED (white) backdrops.*

1231 *Metabolic activity measurements remain stable within the standard 1.0 to 1.7 thresholds*

1232 *across groups.*

Supplementary Files

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- [SUPPLEMENTARYTABLESMODIFIED8alldescriptioncorrected.xlsx](#)
- [SupplementaryMaterialsSHEDedit.docx](#)