

# Structural and functional alterations of neurons derived from sporadic Alzheimer's disease hiPSCs are associated with downregulation of the LIMK1-coflin axis

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# **Abstract**

**Background** Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by the accumulation of pathological proteins and synaptic dysfunction. This study aims to investigate the molecular and functional diferences between human induced pluripotent stem cells (hiPSCs) derived from patients with sporadic AD (sAD) and agematched controls (healthy subjects, HS), focusing on their neuronal diferentiation and synaptic properties in order to better understand the cellular and molecular mechanisms underlying AD pathology.

**Methods** Skin fbroblasts from sAD patients (*n*=5) and HS subjects (*n*=5) were reprogrammed into hiPSCs using non-integrating Sendai virus vectors. Through karyotyping, we assessed pluripotency markers (OCT4, SOX2, TRA-1–60) and genomic integrity. Neuronal diferentiation was evaluated by immunostaining for MAP2 and NEUN. Electrophysiological properties were measured using whole-cell patch-clamp, while protein expression of Aβ, phosphorylated tau, Synapsin-1, Synaptophysin, PSD95, and GluA1 was quantifed by western blot. We then focused on PAK1-LIMK1-Coflin signaling, which plays a key role in regulating synaptic structure and function, both of which are disrupted in neurodegenerative diseases such as AD.

**Results** sAD and HS hiPSCs displayed similar stemness features and genomic stability. However, they difered in neuronal diferentiation and function. sAD-derived neurons (sAD-hNs) displayed increased levels of AD-related proteins, including Aβ and phosphorylated tau. Electrophysiological analyses revealed that while both sAD- and HShNs generated action potentials, sAD-hNs exhibited decreased spontaneous synaptic activity. Signifcant reductions in the expression of synaptic proteins such as Synapsin-1, Synaptophysin, PSD95, and GluA1 were found in sADhNs, which are also characterized by reduced neurite length, indicating impaired diferentiation. Notably, sAD-hNs demonstrated a marked reduction in LIMK1 phosphorylation, which could be the underlying cause for the changes in cytoskeletal dynamics that we found, leading to the morphological and functional modifcations observed in sADhNs. To further investigate the involvement of the LIMK1 pathway in the morphological and functional changes observed in sAD neurons, we conducted perturbation experiments using the specifc LIMK1 inhibitor, BMS-5.

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Neurons obtained from healthy subjects treated with the inhibitor showed similar morphological changes to those observed in sAD neurons, confrming that LIMK1 activity is crucial for maintaining normal neuronal structure. Furthermore, administration of the inhibitor to sAD neurons did not exacerbate the morphological alterations, suggesting that LIMK1 activity is already compromised in these cells.

**Conclusion** Our fndings demonstrate that although sAD- and HS-hiPSCs are similar in their stemness and genomic stability, sAD-hNs exhibit distinct functional and structural anomalies mirroring AD pathology. These anomalies include synaptic dysfunction, altered cytoskeletal organization, and accumulation of AD-related proteins. Our study underscores the usefulness of hiPSCs in modeling AD and provides insights into the disease's molecular underpinnings, thus highlighting potential therapeutic targets.

**Keywords** hiPSCs, Alzheimer's disease, Human neurons, LIMK1, Neurites, Synaptic function

# **Background**

Alzheimer's disease (AD) is a complex neurodegenerative disorder marked by the accumulation of pathological proteins, such as amyloid-β (Aβ) and hyperphosphorylated tau (pTau), along with synaptic dysfunction. While transgenic animal models have contributed signifcantly to our understanding of AD, they primarily represent familial AD, which accounts for only 10% of cases [\[1](#page-18-0)]. The more common form of AD, comprising the majority of cases, is sporadic (sAD) and is infuenced by a range of environmental and genetic factors that are not fully replicated in the animal AD models  $[1-7]$  $[1-7]$ .

Emerging evidence highlights the potential of human induced pluripotent stem cells (hiPSCs) as a more representative model for studying sAD [\[8](#page-18-2)[–11](#page-18-3)]. hiPSCs derived from sAD patients allow researchers to investigate the disease at a molecular and cellular level, providing unique insights into the pathophysiology of sporadic cases. These models retain disease-specific features such as Aβ accumulation and tau hyperphosphorylation  $[8-$ [11\]](#page-18-3), making them a valuable tool for studying sAD in a way that animal models cannot fully capture.

A key feature of AD pathology is synaptic dysfunction, particularly the loss of dendritic spines, which are critical for synaptic plasticity  $[12-25]$  $[12-25]$ . Dysregulation of the actin cytoskeleton, essential for maintaining dendritic spine structure, is implicated in this process  $[12-25]$  $[12-25]$  $[12-25]$ . One pathway of interest involves LIM domain-containing protein kinase 1 (LIMK1), which regulates actin dynamics through phosphorylation of coflin, an actin-depolymerizing factor [[26\]](#page-18-6). In AD, LIMK1-mediated pathways are disrupted, leading to cytoskeletal abnormalities and synaptic degeneration [[27–](#page-18-7)[30](#page-18-8)]. Previous studies have shown that pharmacological inhibition of LIMK1 can rescue dendritic spine loss caused by Aβ, suggesting that this pathway could be a potential therapeutic target [\[31\]](#page-19-0).

Activation of LIMK1 through its phosphorylation on Thr508 induces phosphorylation and inactivation of actin-depolymerizing factor (ADF)/coflin, leading to actin polymerization and structural plasticity of dendritic spines  $[32-35]$  $[32-35]$  $[32-35]$ . Interestingly, both increased and decreased activity of the LIMK1 substrate coflin are associated with AD  $[30, 36-42]$  $[30, 36-42]$  $[30, 36-42]$  $[30, 36-42]$ . Indeed, blocking cofilin activity or expressing an inactive, non-phosphorylable form of coflin can mitigate Aβ-induced spine loss [[43,](#page-19-5) [44\]](#page-19-6). Thus, hyperactivation of LIMK1 in AD brains may refect a compensatory mechanism to counteract neuronal changes induced by coflin on the actin cytoskeleton.

In this study, we investigated the morphological, electrophysiological, and biochemical characteristics of human neurons derived from sAD-hiPSCs in comparison with those of their neurotypical controls. Our fndings revealed an accumulation of Aβ and tau hyperphosphorylation in sAD-hNs, accompanied by decreased neurite length, increased neuronal excitability and diminished synaptic function. Furthermore, our research proposes a potential mechanism for the observed alterations in neuronal morphology, excitability, and synaptic function in these sAD-hNs, focusing on the LIMK1-coflin axis, which is pivotal for cytoskeletal reorganization and neurite outgrowth and is impaired in sAD-hNs. This approach provides a novel perspective on AD pathology and may reveal new therapeutic avenues for targeting cytoskeletal dysfunction in sAD.

# **Methods**

# **Fibroblasts cultures**

A piece of skin over the ankle bone was obtained from fve sAD patients and fve HS using a 4 mm biopsy punch (HBP40, HS, Italy). Patients were enrolled after providing informed consent in accordance with good clinical practice and local regulations. Patients enrolled in the study met the clinical criteria for sAD as defned by the National Institute on Aging-Alzheimer's Association workgroups  $[45, 46]$  $[45, 46]$  $[45, 46]$ . This included cognitive impairment confrmed through a thorough neuropsychological assessment and positive AD biomarkers, as shown by PET amyloid imaging or cerebrospinal fuid analysis. Age-matched neurotypical control participants were

enrolled after providing written informed consent. The biopsy was rinsed three times in DPBS (#14040117, ThermoFisher Scientifc, Waltham, MA, USA) supplemented with 1% PSN (#15640055, ThermoFisher Scientific, Waltham, MA, USA) and 1% gentamicin (#15710064, ThermoFisher Scientific, Waltham, MA, USA) and maintained in "fbroblast medium" composed of DMEM high glucose (#31053044, ThermoFisher Scientific, Waltham, MA, USA) medium with 10% FBS, heat-inactivated (#A3160501, ThermoFisher Scientific, Waltham, MA, USA). Under sterile conditions, biopsies were minced and cultured in 10 cm<sup>2</sup> Petri dishes previously coated with 0.2% gelatin-2% (G1393, Sigma-Aldrich, USA) in DPBS (#14040117, ThermoFisher Scientific, Waltham, MA, USA). Fibroblast medium was added after 8 h to favor the adhesion of the pieces to the plastic bottom. Petri dishes were maintained in a humidifed incubator at 37 °C with  $5\%$  CO<sub>2</sub> and fibroblast medium was changed every other day until 90% confuency was reached. Confuent fbroblasts were dissociated using  $TrypLE^{m}$  Express Enzyme (1X) (#12605036, ThermoFisher Scientific, Waltham, MA, USA) and amplifed to be reprogrammed.

# **Reprogramming fbroblasts to hiPSCs**

Human fbroblasts were reprogrammed into hiPSCs using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (#A16517, ThermoFisher Scientific, Waltham, MA, USA). Briefy, following the manufacturer's protocol, 500.000 cells were split and plated in two wells of a 6-well plate. Two days after plating, the cells, cultured in fbroblast medium, were transduced using pre-established MOI (Multiplicity Of Infection): KOS MOI=5, hc-Myc  $MOI = 5$ , hKlf4  $MOI = 3$ . The day after transduction, the medium was completely replaced with fresh fbroblast medium and replaced every other day. On day 7 post-transduction, cells were trypsinized, counted, and plated on Matrigel-coated 6-multiwell at a density of  $2 \times 10^4$ – $1 \times 10^5$  cells/well. Twenty-four hours later, the fibroblast medium was switched to StemFlex<sup>™</sup> Medium (#A3349401, ThermoFisher Scientific, Waltham, MA, USA) and replaced every other day. Three to four weeks after transduction, hiPSC colonies emerged and were manually picked and transferred onto 12-well culture plates for expansion and analyses.

# **hiPSCs maintenance and immunocytochemistry**

hiPSCs were cultured on Matrigel<sup>®</sup> (#354277, Corning Inc., USA)-coated 6-well dishes in StemFlex™ Medium, which was replaced every other day. Once 70–80% confluence was reached, hiPSCs were split using ReLeSR<sup>™</sup> Passaging Reagent (#100–0483, STEMCELL Technologies Inc., Vancouver, Canada). The pluripotency of hiPSCs was confrmed by an immunocytochemical assay using the StemLight<sup>™</sup> Pluripotency Antibody Kit (#9656, Cell Signaling Technology, Danvers, MA). Briefy, 100.000 cells, obtained by dissociation with  $StemPro<sup>π</sup>$ Accutase<sup>™</sup> Cell Dissociation Reagent (#A1110501, ThermoFisher Scientifc, Waltham, MA, USA) were plated on a 2 Well Glass Chamber Slide, fxed with 10% formalin solution (pH 7.4 with NaOH) for 10 min at room temperature (RT), and then permeabilized with 0.3% Triton X-100 (#X-100, Sigma-Aldrich, USA) in PBS for 15 min. Cells were incubated for 20 min at RT in a blocking solution composed of 0.3% BSA (#A2153, Sigma-Aldrich, USA) in PBS. Cells were incubated overnight at 4 °C with an appropriate combination of the following antibodies diluted in the same blocking solution: mouse anti-Oct4A (C30A3) (#2840, 1:200, Cell Signaling Technology, Danvers, MA), rabbit anti-SOX2 (D6D9) (#3579, 1:200, Cell Signaling Technology, Danvers, MA), rabbit anti-Nanog (D73G4) XP® (#5232 1:200, Cell Signaling Technology, Danvers, MA), mouse anti-SSEA4 (MC813) (#4755 1:200, Cell Signaling Technology, Danvers, MA), mouse anti-TRA-1–60(S) (#4746 1:200, Cell Signaling Technology, Danvers, MA), mouse anti-TRA-1–81 (#4745 1:200, Cell Signaling Technology, Danvers, MA), rabbit anti-MAP2 (M9942 1:200, Sigma-Aldrich, USA), mouse anti-NeuN (#MAB377 1:200, Sigma-Aldrich, USA) rabbit anti-Synapsin-1 (#5297 1:200, Cell Signaling Technology, Danvers, MA), mouse anti-Synaptophysin (ab8049 1:200, Abcam, Cambridge, UK), rabbit anti-GluA1 (Ab-849 1:200, SAB, Baltimore, MD, USA), rabbit anti-PSD95 (#3550 1:200, Cell Signaling Technology, Danvers, MA). After primary antibody removal, cells were rinsed in PBS and then incubated for 90 min at RT with the proper secondary antibodies, diluted in the blocking solution: Alexa Fluor 546 donkey anti-mouse, Alexa Fluor 546 donkey anti-rabbit, Alexa Fluor 488 donkey anti-rabbit (1:1000, #A10036, # A10040, #A-21206, ThermoFisher Scientifc, Waltham, MA, USA). Finally, cells were incubated with 0.5 mg/mL 4,6-diamidino-2-phenylindole (DAPI, #D1306, ThermoFisher Scientific, Waltham MA, USA) in PBS for 10 min at RT in order to counterstain their nuclei. Cells were then coverslipped with ProLong Gold antifade reagent (#36982, ThermoFisher Scientific, Waltham, MA, USA) and studied by confocal microscopy. Confocal images (1.024 $\times$ 1.024 pixels) were acquired at 20 $\times$ magnifcation with a confocal laser scanning system (Nikon-Ti Eclipse). MAP2, NEUN, Synapsin-1, Synaptophysin, GluA1, PSD95, Aβ 6E10 fuorescence was recorded by a confocal laser scanning microscope (Nikon-Ti Eclipse,  $60 \times$  magnification) and 3D images of cells, deriving from a reconstruction of z series, were used for the analysis performed with Image J software. Signals from single ROIs (90 cells for each subject) were used to calculate the average values (mean) for fuorescence intensity (MFI).

We used no-primary antibody controls to ensure the specificity of the staining.

#### **Karyotyping**

DNA was extracted and purifed from hiPSCs using the Quick-DNA Miniprep kit (#D3024, Zymo Research Corp. Irvine, CA, USA). 300 ng of DNA was used to perform karyotyping using hPSC Genetic Analysis Kit (#07550, STEMCELL Technologies Inc., Vancouver, Canada) according to the manufacturer's instructions. Briefy, genomic DNA was mixed with a ROX reference dye and double-quenched probes tagged with 5-FAM. The probes represented eight common karyotypic abnormalities that have been reported to arise in hiPSCs: chr 1q, chr 8q, chr 10p, chr 12p, chr 17q, chr 18q, chr 20q, and chr Xp. Sample-probe mixes were analyzed using a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientifc, Waltham, MA, USA). Copy numbers were analyzed using the ∆∆Ct method. The results were normalized to the copy number of a control region in chr 4p, as explained in [[47](#page-19-9)].

Karyotype analysis was also performed using the conventional G (GTG) banding procedure. Briefy, cells at  $\approx$ 50% confluence were incubated O/N with 0.01  $\mu$ g/ ml of colcemide (#J63900.MF, ThermoFisher Scientific, Waltham, MA, USA). The following day, cells were detached by trypsinization and pelleted by centrifugation. The pellet was resuspended in a hypotonic solution composed of 15% FBS; 6% MgCl2 (#208337 Sigma-Aldrich, USA); 4% Hyaluronidase (#H3506-1G, Sigma-Aldrich, USA), and incubated in a water bath at 37 °C for 10'. Subsequently, cells were fxed in 3:1 methanol: glacial acetic acid solution (#32213-M Sigma-Aldrich, USA, #1.00063 Sigma-Aldrich, USA) 30' on ice, fnally were washed twice in 1:1 methanol:glacial acetic acid solution. Metaphases were acquired by the MetaSystems instrument (Altlussheim, Germany). At least 10 cells were counted for each sample, and 3 karyotypes were mounted.

# **APOE genotyping**

For APOE genotyping, a 219 bp region of exon 4, fanking the rs429358 and rs7412 SNPs, that permit to distinguish ɛ2, -3 or -4 alleles (Fig. [S1](#page-17-0)C) for the schematic representation), was amplifed. Briefy, gDNA was extracted using the standard salting out procedure. DNA was quantifed and qualifed by spectrophotometric analysis (230, 260, and 280 nm absorbance and ratios; Multiskan GO, Thermo Scientific). Fifty ng of gDNA was amplified in PCR using fuorophore labelled primers: APOE\_ex4F: FAM 5'-AGGCGGAGCAGGCCCGGCTGGGCACGG ACAT-3'; APOE\_ex4R: HEX 5'-CGGAGCCCTCGC AGGCCCCGGCCTGGTACA-3'.

Two mismatches per primer were introduced (underlined) in order to abolish inappropriate HhaI restriction sites. PCR was performed in a fnal volume of 12.5 μl using 1X DreamTaq Hot Start PCR Master Mix (#K9011, ThermoFisher Scientific, Waltham, MA, USA), 0.4 μM of each primer and 5% DMSO (#D5879, Sigma-Aldrich, USA). The amplification cycle was: 95 °C 5'; (95 °C 45''; 68 °C 45''; 72 °C 30'') $\times$ 30; 72 °C 5'; 4 °C. The whole PCR reaction was digested in a fnal volume of 20 μl, with 10U of HhaI (#R0139S, New England Biolabs), 1X Cut-Smart bufer (#B6004S, New England Biolabs) and incubated O/N at 37 °C. Fragment analysis was performed using capillary electrophoresis using SeqStudio 8Flex Genetic Analyser (Thermo Scientific) and analysed using the GeneMapper 6 software (Thermo Scientific). The electropherograms showing the genotype of the 10 cell cultures are reported in (Fig. S[1](#page-17-0)D).

#### **In vitro EB formation and tri‑lineage diferentiation**

To generate embryoid bodies (EBs), hiPSCs at 80–90% confuency were dissociated into small clumps and cultured on low-attachment tissue plates using STEMdiff™ Cerebral Organoid Kit, following the manufacturer's instructions (STEMCELL Technologies Inc., Vancouver, Canada). EBs were subjected to diferentiation into the three germ layers: ectoderm, mesoderm, and endoderm for 5 days, and then collected for real-time analyses.

#### **RNA Isolation, cDNA Synthesis, and qPCR Analysis**

Total RNA was isolated from fbroblasts (at passage 2), hiPSCs (at passage 10), and embryoid bodies (EBs) using TRIzol reagent (#15596026, Thermo Fisher Scientifc, Waltham, MA, USA). cDNA was synthesized from 1 μg of total RNA with a high-capacity cDNA reverse transcription kit (#4368814, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Quantitative real-time PCR amplifcations were conducted as described in [[48](#page-19-10)], utilizing specifc primers (SeV primers: FW 5'- GGATCACTAGGTGAT ATCGAGC-3', RV 5'- ACCAGACAAGAGTTTAAG AGATATGTATC-3'; *KLF4* primers: FW 5'-TTCCTG CATGCCAGAGGAGCCC-3'; RV 5'-AATGTATCGAAG GTGCTCAA-3'; *MSX1* primers: FW 5'- CGAGAGGAC CCCGTGGATGCAGAG-3', RV 5'- GGCGGCCATCTT CAGCTTCTCCAG-3'; *PAX6* primers: FW 5'- GTCCAT CTTTGCTTGGGAAA-3', RV 5'- TAGCCAGGTTGC GAAGAACT-3'; *GATA6* primers: FW 5'- ACCACCTTA TGGCGCAGAAA-3', RV 5'- ATAGCAAGTGGTCTG GGCAC-3') and the Power SYBR™ Green PCR Master Mix (#4367659, Thermo Fisher Scientific, Waltham, MA, USA) on an AB7500 instrument. The relative quantification of target genes was determined using the  $2^{\wedge -\Delta\Delta Ct}$ method, with GAPDH as the housekeeping gene.

### **Lentiviral constructs**

The lentiviral constructs used for neuronal induction of hIPSCs included: pLV-TetO-hNGN2-eGFP-Puro (a gift from Kristen Brennand; Addgene plasmid #79823), FUW-M2rtTA (a gift from Rudolf Jaenisch; Addgene plasmid #20342), pRSV-Rev, pMD2.G, pMDLg/pRRE (gifts from Didier Trono; Addgene plasmid #12253, Addgene plasmid #12259, Addgene plasmid #12251, respectively).

# **Lentiviral production**

All lentiviral vectors were handled in a class II biosafety laboratory. Three helper plasmids (pRSV-REV, pMDLg/ pRRE, PMD2.G, respectively 2.5  $\mu$ g, 5  $\mu$ g, 2.5  $\mu$ g) and alternately pLV-TetO-hNGN2-eGFP-Puro and FUW-M2rtTA (10 μg each one) were co-transfected with polyethyleneimine (#00618–25, Polysciences, Inc., Warrington, USA) and Opti-MEM<sup>™</sup> medium (#31985062, ThermoFisher Scientific, Waltham, MA, USA) into 70–80% confluent HEK 293 T cells plated on 10  $\text{cm}^2$ Petri Dishes as described previously in [\[49\]](#page-19-11). At 24 h post-transfection, the culture medium was exchanged completely, and the supernatant containing lentivirus particles was collected after 48 h and 72 h. The supernatant was spun at  $800 \times g$  for 10 min. To concentrate the lentivirus, PEG 8000 (#P5413, Sigma-Aldrich, USA) was added to the virus supernatant until a fnal concentration of 25% v/v was reached. The supernatant was left to stand overnight at 4 °C and centrifuged the next day at  $1600 \times g$ for 60 min. The pellet was then resuspended in 300  $\mu$ l, aliquoted and stored at -80 °C. Only virus preparations capable of infecting more than 90% of cells were used for the experiments. The infection capacity was assessed by evaluating the expression of EGFP.

# **Excitatory neuron diferentiation**

The protocol used for neuronal induction is based on the overexpression of *Neurogenin2 (NGN2)*, as reported in [[50\]](#page-19-12), but with slight modifications. Briefly, on day 0, sADand HS- hiPSCs (at passage 10) were plated at a density of 80.000 cells/cm<sup>2</sup> in a 6-well plate and in a 24-well plate, respectively, for western blot (WB) and immunocytochemistry/electrophysiology/Incucyte® experiments in StemFlex<sup>™</sup> Medium supplemented with 10  $\mu$ M Y27632 (#Y0503, Sigma-Aldrich, USA). On day 1, cells were infected with 1 μl of two lentiviruses in 24 well-plates: i) TetO-hNGN2-eGFP-Puro in which *NGN2-eGFP-Puro* are under the control of a tetracycline-responsive element (TRE3G) and ii) FUW-M2rtTA which carries a tetracycline responsive transcriptional activator (Tet3G) that binds TRE3G promoter to activate gene transcription only in the presence of doxycycline, in StemFlex™ Medium added with 10 μM Y27632 and Polybrene (8 μg/

μl, #TR-1003, Sigma-Aldrich, USA). Plates were placed in the incubator for 10 min and then spun for 1 h at  $1000 \times g$ at RT. After spin infection, the plates were placed in an incubator overnight. The next day, the medium was completely replaced with fresh StemFlex<sup>™</sup> Medium supplemented with doxycycline (#D9891, 2 μg/ml, Sigma-Aldrich, USA), which was retained in the medium until the end of the experiment. On day 3, the medium was completely replaced with "diferentiation medium" containing N2/DMEM/F12/NEAA/Glutamax (#17502048, #11330032, #35050061, #11140050, Thermo Fisher Scientifc, Waltham, MA, USA), human BDNF (#450–02-10 µg, 10 μg/l, PeproTech Inc., USA), human NT-3 (#450– 03-10 µg, 10 μg/l, PeproTech Inc., USA), doxycycline, added with puromycin (#P8833, 2 μg/ml, Sigma-Aldrich, USA) for antibiotic-resistant cell selection. On day 4, the diferentiation medium was replaced; mouse glia and Ara-C (#251010, 2 g/l, Sigma-Aldrich, USA) were added to the medium to inhibit astrocyte proliferation. 50% of the medium was replaced every other day until the end of the experiments. Cells that underwent treatment with the LIMK1 inhibitor, BMS-5 (1 μM, #4745 Tocris Bioscience, Bristol UK) and vehicle (Veh:DMSO) were treated starting from the seventh-day post-infection with lentivirus, with 1  $μ$ m of BMS-5, which The inhibitor was maintained in the culture medium until the end of the experiments.

# **Patch‑clamp**

Neuronal excitability and basal synaptic transmission were investigated using the whole-cell patch-clamp technique at room temperature (23–25 °C), as previously described [\[51–](#page-19-13)[53\]](#page-19-14). Data acquisition was performed using a MultiClamp 700B amplifer (Molecular Devices) and digitized at 10 kHz with the Digidata 1440A data acquisition system (Molecular Devices). Data were analyzed using pClamp 11 software (Molecular Devices). The patch-clamp electrodes, with resistances ranging from 3–5 MΩ, were fabricated from borosilicate glass capillaries using a micropipette puller (PC-10; Narishige) and flled with an internal solution containing the following components (in mM): 130 K-gluconate, 10 KCl (#P3911, Sigma-Aldrich, USA), 1 EGTA (#E6511,Sigma-Aldrich, USA), 10 HEPES (#H3375,Sigma-Aldrich, USA), 2 MgCl<sub>2</sub> (#M1028,Sigma-Aldrich, USA), 4 MgATP (#A9187,Sigma-Aldrich, USA), and 0.3 Tris-GTP (#G9002, Sigma-Aldrich, USA). Throughout the recordings, the cells were continuously perfused with Tyrode's solution, which consisted of (in mM): 140 NaCl (#31,434,Sigma-Aldrich, USA), 2 KCl, 10 HEPES, 10 glucose, 1 MgCI<sub>2</sub>, and 2 CaCl<sub>2</sub> (#223506 Sigma-Aldrich, USA), with a pH of 7.4 and an osmolarity of 312 mOsm. Resting membrane potential, input resistance and evoked fring were recorded in current-clamp mode. Evoked

fring was measured by a series of 800 ms current pulses (every 5 s) from  $-20$  to 340 pA, 20 pA steps. The membrane input resistance was measured by a series of 600 ms hyperpolarizing current steps from−50 to 0 pA, 10 pA steps with 1 s interval. Amplitude and frequency of spontaneous miniature postsynaptic currents were recorded in voltage-clamp mode for over 60 s at−70 mV membrane potential.

# **Morphological analyses**

Incucyte® SX5 Live-Cell Analysis System (Sartorius AG, Göttingen, Germany) equipped with NeuroTrack (NT) processing module was used to analyze the neurite outgrowth properties of hNs. sAD- and HS-hiPSCs are plated at density of 80.000 cells/cm<sup>2</sup> in 24-well plate and monitored for 30 days after induction of diferentiation (DIV30). The plate was scanned every  $4 h$  for a total of 30 days using a 10X objective. Four images per well were captured and the cell body cluster area (sum of areas of cell body clusters/area of image) and neurite length (sum of lengths of all neurites/area of image) were analyzed. The parameters used for fluorescence-based Neurotrack analysis were as follows: Channel (Green), Acquisition Time (200 ms), Color Neurites (Green), Cell-Body Cluster Segmentation (Adaptive), Threshold Adjustment (10.000 GCU); Cleanup: Hole fill (0.0000  $\mu$ m<sup>2</sup>), Adjust Size (0 pixels), Min Cell Width (7.0000 μm); Cell-Body Cluster Filters: Area in  $\mu$ m<sup>2</sup> (Not selected); Neurite Parameters: Neurite Coarse Sensitivity (8), Neurite Fine Sensitivity (0.6), Neurite Width (1  $\mu$ m). Furthermore, to obtain a more detailed view of neuronal structure we evaluated branching complexity in single post-fxed cells expressing EGFP and stained with MAP2. Samples were analysed by using a Zeiss microscope with a motorized stage, connected to Neurolucida 7.5 software (MBF), and Sholl analysis was applyed [[54,](#page-19-15) [55\]](#page-19-16). The structural parameters taken into consideration were: process length (in μm), number of bifurcating nodes, and total number of intersections between process and a shell of the Sholl.

# **Western blot**

sAD- and HS-hNs cultured in 6-well plates were lysed as described previously [[56,](#page-19-17) [57](#page-19-18)], underwent two PBS washes and were subsequently scraped in cold RIPA bufer containing 1 mM phenylmethylsulfonyl fuoride, phosphatase and protease inhibitor mixtures (#52332, Sigma-Aldrich, USA), and 0.1% sodium dodecyl sulfate (#436143, Sigma-Aldrich, USA). Following a 30 min incubation on ice, the cellular suspensions were centrifuged at  $10.000 \times g$  for 30 min at 4 °C. The resulting supernatants were collected and assessed for protein concentration using the Micro BCA protein assay (#23235, Thermo Fisher Scientific, Waltham, MA). Equal amounts

of proteins (30 μg) were loaded onto either 8% or 15% Tris–glycine polyacrylamide gels for electrophoretic separation. Subsequently, the proteins were electroblotted onto nitrocellulose membranes for WB analysis. The membranes were blocked with 5% nonfat dry milk in Tris-bufered saline containing 0.1% Tween-20 (#P1379, Sigma-Aldrich, USA) for 1 h at room temperature, and then they were incubated with primary antibodies at a final concentration of 1  $\mu$ g/mL. The primary antibodies included rabbit anti-MAP2 (M9942, Sigma-Aldrich, USA), mouse anti-NeuN (#MAB377, Sigma-Aldrich, USA) rabbit anti-Synapsin-1 (#5297, Cell Signaling Technology, Danvers, MA), mouse anti-Synaptophysin (ab8049, Abcam, Cambridge, UK), rabbit anti-GluA1 (Ab-849, SAB, Baltimore, MD, USA), rabbit anti-PSD95 (#3550, Cell Signaling Technology, Danvers, MA), rabbit anti-Phospho-Tau Ser199 (#44-734G, Thermo Fisher Scientific, Waltham, MA), rabbit anti-Phospho-Tau Thr181 (#12885, Cell Signaling Technology, Danvers, MA), rabbit anti-Phospho-Tau Thr205 (#49561, Cell Signaling Technology, Danvers, MA), rabbit anti-Phospho-Tau Thr231 (ab151559, Abcam, Cambridge, UK), rabbit anti-Phospho-Tau Thr217 (ab291080, Abcam, Cambridge, UK), mouse anti-HT7 (#MN1000, Thermo Fisher Scientific, Waltham, MA), rabbit anti-coflin (#5175, Cell Signaling Technology, Danvers, MA), rabbit anti-phosphorylated S3 (pS3) coflin (#3313, Cell Signaling Technology, Danvers, MA), rabbit anti-phosphorylated T508 LIMK1 (#3841, Cell Signaling Technology, Danvers, MA), mouse anti-LIMK1 (#611748, BD Biosciences, USA), mouse anti-GAPDH (#97166, Cell Signaling Technology, Danvers, MA). After incubation with the appropriate secondary horseradish peroxidase-conjugated antibodies (1:2500; Cell Signaling Technology, Danvers, MA), visualization was performed with WESTAR ECL (#XLS3, Cyanagen, Bologna, Italy), using UVItec Cambridge Alliance. Molecular weights for immunoblot analysis were determined through Precision Plus Protein™ Standards (#1610374, BioRad, Hercules, CA, USA). Densitometric analysis was carried out using UVItec software. Experiments were repeated at least three times. Dot blot experiments were conducted to assess Aβ oligomers and were performed by using 15 μg of protein lysate directly applied to the membrane. Following blocking in 5% nonfat dry milk in tris-bufered saline containing 0.1% Tween-20 for 1 h at room temperature, the membranes were subjected to overnight probing with anti-Aβ antibody (1:1000, 803014-6E10, BioLegend, San Diego, CA, USA). Subsequently, the appropriate secondary horseradish peroxidase-conjugated antibodies (1:2500; Cell Signaling Technology, Danvers, MA) were used, and visualization was performed with WESTAR ECL using UVItec Cambridge Alliance. To ensure accurate

quantifcation, optical density values of the Aβ signal were normalized against red ponceau staining, serving as a measure of total protein loading. The results are presented as a fold change relative to control samples, which were set at a value of 1.

# **Statistical analysis**

The statistical tests used are indicated in the corresponding fgure legends for each experiment. All statistical tests were two-tailed, they are indicated in the main text and in the corresponding fgure legends for each experiment. The level of significance was set at  $< 0.05$ . Results are shown as the mean  $\pm$  SEM. The number of repetitions (n) for each experimental condition is reported in the fgure legends. Statistical comparisons and analyses were carried out with GraphPad Prism.

# **Results**

# **Stemness of hiPSCs derived from sAD**

First, we characterized hiPSC clones originating from fbroblasts isolated from skin biopsies of fve sAD patients and fve age-matched controls. We selected patients fulflling "core" clinical criteria for AD according to the National Institute on Aging-Alzheimer's Association workgroups [[58](#page-19-19)], with cognitive impairment documented with a formal and extensive neuropsychological evaluation and positive biomarkers  $[45, 46, 59]$  $[45, 46, 59]$  $[45, 46, 59]$  $[45, 46, 59]$  $[45, 46, 59]$  $[45, 46, 59]$ . The HS group includes a mix of male and female subjects aged 52–74, while the sAD group consists of individuals aged 59–68, with CDR scores ranging from 0.5 to 1, indicating mild cognitive impairment or early dementia (Fig. [1](#page-7-0)A, B). hiPSCs from HS and sAD groups carrying diferent APOE genotype as shown in Fig. [S1D](#page-17-0).

At passage 1, fbroblasts were reprogrammed with nonintegrating Sendai virus vectors carrying the Yamanaka factors OCT4, SOX2, KLF4 and c-MYC [[60\]](#page-19-21). On days 18–25, HS- and sAD-hiPSCs observed by optical microscopy showed the typical hiPSCs-like morphology with a round-shape and a clearly defned border (Fig. [1C](#page-7-0)). Notably, the morphological assessment of hiPSC colonies, the timing of colony formation starting from the third-week post-infection, and the expression levels of pluripotency markers, including SOX2, NANOG, SSEA-4, OCT3/4, and TRA-1-60, evaluated by immunocytochemistry, revealed remarkable homogeneity across HS- and sAD-hiPSC lines (Fig. [1C](#page-7-0)). To exclude the possibility that hiPSCs accumulated genomic alterations and mutations through reprogramming processes and long-term culture, we used a qPCR-based loci analysis kit to detect minimal critical hotspot regions within the genome that are frequently mutated during the reprogramming process and extended cell passaging [[61–](#page-19-22)[63](#page-19-23)]. When testing our newly generated hiPSCs, we found no increases or decreases in copy number outside the confdence interval of 1.8 to 2.2. No abnormalities were detected in the 9 most common hotspot zones on chr 1q, chr 4p, chr 8q, chr 10p, chr 12p, chr 17q, chr 18q, chr 20q, and chr Xp, which are the genomic regions covering the majority of reported abnormality areas (Fig. S[1](#page-17-0)). Additionally, karyotyping using G-banding was conducted on all ten cell populations derived from both healthy subjects (HS) and sAD patients; each batch of cells analyzed exhibited a normal karyotype.

To ensure that the expression of pluripotency markers originates from the cellular genome, we confrmed the elimination of the Sendai virus by performing RT-PCR using virus-specific primers. The results confirmed that the virus was successfully cleared from the hiPSCs, as compared to cells just after infection served as a positive control (Fig. [S2A](#page-17-0)). Pluripotency was further validated by assessing the mRNA expression of Klf4. Real-time PCR data confrmed its expression in all samples, compared to negative controls, represented by fbroblasts (Fig. [S2](#page-17-0)B).

An EBs formation assay was conducted to assess the diferentiation potential of hiPSCs into derivatives of the three germ layers. RT-PCR analysis revealed that all tested hiPSCs were capable of diferentiating into the three germ layers, as indicated by the increased expression levels of the ectoderm marker PAX6, the mesoderm marker MSX, and the endoderm marker GATA6 (Fig. S[2C](#page-17-0)).

#### **sAD‑hNs exhibit functional and structural alterations**

The next step was to differentiate sAD- and HS-hiPSCs into glutamatergic neurons by using a rapid and efficient method through the overexpression of *NGN2* induced by doxycycline treatment (Fig. [2](#page-8-0)A-C). The maturation of sAD- and HS-hNs was assessed at DIV30 by evaluating the expression levels of the mature neuronal markers NEUN and MAP2 by immunofuorescent (Fig. [2D](#page-8-0)-F) and WB analyses (Fig.  $2G$  $2G$ , H). This finding indicates that the employed protocol allowed us to generate viable human neurons from hiPSCs of both HS and sAD subjects. When examining neurons derived from 5 HS and 5 sAD patients, no diferences in NEUN and MAP2 expression were observed at 30 DIV, as assessed by both immunofuorescence and WB analyses (Fig. [2](#page-8-0)F, H).

Of note, hNs derived from sAD hiPSCs recapitulated the typical molecular hallmarks of the disease. Specifcally, they exhibited signifcant accumulation of Aβ, as assessed by immunofuorescent analyses (11.7 fold increase in hNs derived from sAD compared to those from HS,  $n=5/\text{group}, p=0.008$ ; Fig. [3A](#page-9-0), B) and dot-blot experiments (+19%; *n*=5/group, *p*=0.008; Fig. [3C](#page-9-0),D). Additionally, these neurons showed signifcantly enhanced tau phosphorylation at Thr181  $(+460\%; n=5/$ 



<span id="page-7-0"></span>**Fig. 1** Generation and characterization of hiPSCs from HS and sAD patients. **A** Schematic representation of the reprogramming process used to generate hiPSCs from HS and sAD fbroblasts. Human somatic cells from both HS (*n*=5) and sAD (*n*=5) donors were reprogrammed into hiPSCs using Sendai virus. **B** Table summarizing demographic data and clinical information of the donors used for generating HS and sAD hiPSCs. The table includes information on age, gender, ethnicity, APOE genotype, and Clinical Dementia Rating (CDR). (C) Representative of hiPSCs exhibiting pluripotent stem cell morphology (in contrast phase) and immunofuorescence images showing the expression of pluripotency markers in HS- and sAD-derived hiPSCs. Cells were stained for SOX2 (green), NANOG (green), OCT3/4 (green), SSEA-4 (red), and TRA-1–60 (red). DAPI (blue) was used to label nuclei. Both HS and sAD hiPSCs are positive for these pluripotency markers, confrming their stem cell identity. Scale bars: 50 µm

group, *p*=0.008), Ser199 (+14%; *n*=5/group, *p*=0.031), and Thr217 (+126%;  $n = 5$ /group,  $p = 0.008$ ) (Fig. [3E](#page-9-0),F). No diferences were found in tau phosphorylation at Thr205 and Thr231 between HS-hNs and sAD-hNs (Fig.  $3E$  $3E$ ,F). These findings further validate the ability of sAD-derived hNs to model crucial neuropathological features of sAD.

Morphological analysis of sAD- and HS-hNs was performed at DIV1, DIV14, DIV21 and DIV30 through livecell imaging, taking advantage of the EGFP expression in our cell cultures and the IncuCyte® SX5 Live-Cell Analysis systems, as well as in post-fxed cells expressing EGFP and stained with MAP2 antibody by using Neurolucida and Sholl analysis (MBF) (Fig. [4\)](#page-10-0). We found a signifcant

decrease in neurite length in sAD-hNs compared to control groups (Fig. [4A](#page-10-0),B) starting from DIV14 (*n*=15/ group; DIV1: *p*>0.999; DIV14: -27.9%, *p*<0.0001; DIV21: -27.2%, *p*<0.0001; DIV30: -15.9%, *p*<0.0001). No signifcant changes in cell body clusters were observed in sADhNs compared to HS-hNs ( $n=15$ /group; DIV1,  $p=0.833$ ; DIV14, *p*=0.582; DIV21, *p*=0.797; DIV30, *p*=0.618, Fig. [4C](#page-10-0)). Additionally, cell viability, evaluated through the MTT assay, did not signifcantly decline from DIV1 to DIV30 (*n*=15/group; DIV1, *p*=0.999; DIV14, *p*=0.951; DIV21, *p*>0.999; DIV30, *p*>0.999, Fig. [4D](#page-10-0)). This finding suggests that the observed efects do not depend on changes in cell number in the sAD condition. The complexity of dendritic branching in both HS and sAD



<span id="page-8-0"></span>**Fig. 2** Diferentiation of hiPSCs into neurons and analysis of neuronal markers in HS and sAD neurons. **A** Schematic representation of the Ngn2-mediated diferentiation process used to convert hiPSCs from HS and sAD donors into neurons. Neurons were induced using doxycycline (Doxy) and selected with puromycin (Puro). **B** Diagram of the lentiviral constructs used in the diferentiation process, including Ngn2, EGFP, and Puromycin resistance genes. **C** Timeline of neuronal diferentiation showing key steps from hiPSC plating (DIV0), doxycycline induction (DIV1), puromycin selection (DIV3), and fnal analysis at DIV30. **D** Immunofuorescence staining of neuronal markers in HS and sAD-derived neurons at DIV30. DAPI (blue) stains nuclei, EGFP (green) marks neurons, and NEUN (red) marks mature neurons. Merged images confrm co-localization of neuronal markers. Scale bars: 50 µm. **E** Immunofuorescence staining of the neuronal marker MAP2 (red) in HS and sAD-derived neurons, with DAPI and EGFP labeling. **F** Quantifcation of MAP2 and NEUN median fuorescence intensity (in arbitrary units, a.u.) in HS and sAD neurons, showing no signifcant diference (n.s.) between groups (*n*=5/group). **G** Representative Western blot images of MAP2 and NEUN in HS and sAD neurons, with GAPDH as the loading control. **H** Densitometric analysis of MAP2 and NEUN expression in HS and sAD neurons showing no signifcant differences in fold change( $n=5$ /group). Each dot represents the average of three independent experiments. Data are presented as mean±SEM. n.s. *p*>0.05, assessed by Mann Whitney Rank Sum test

neurons was also assessed using the Sholl analysis. sAD-hNs exhibited a signifcantly marked reduction in dendritic length compared to HS-hNs (−46.2%, *n*=34 neurons from 5 samples of HS and *n*=36 neurons from 5 samples of sAD,  $p < 0.0001$ ), with a significantly lower number of bifurcating nodes (-52.7%, *n*=34 neurons from 5 samples of HS and *n*=36 neurons from 5 samples of sAD, *p* < 0.0001) and fewer intersections (−45.3%, *n*=34 neurons from 5 samples of HS and *n*=36 neurons from 5 samples of sAD, *p*<0.0001; Fig. [4](#page-10-0)E-H).



<span id="page-9-0"></span>**Fig. 3** Neurons obtained from sAD-hiPSCs recapitulate the typical hallmarks of AD. **A** Immunofuorescence staining for Aβ using 6E10 antibody (red), DAPI (nuclei, blue), and EGFP (neurons, green) in HS and sAD hiPSC-derived neurons. sAD neurons show strong 6E10 staining, indicating the presence of Aβ pathology. Scale bars: 50 μm. **B** Quantification of 6E10 median fluorescence intensity (in arbitrary units, a.u.) showing a signifcant increase in Aβ accumulation in sAD-hNs neurons compared to HS-hNs; (*n*=5/group). **C** Representative dot blot images of Aβ levels in DIV30 HS- and AD-hNs lysates; (**D)** Bar graphs showing the band intensity analysis of Aβ in HS- and sAD-hNs. Red Ponceau, RP, was used as loading index and used to normalize sample; synthetic oligomeric Aβ42 (200 nM) was used as positive control; (**E**) Representative WB images of p-TAU (Thr181, Ser199, Thr205, Thr217 and Thr231) levels in DIV30 HS- and sAD-hNs lysates; (**F**) Densitometric analysis of p-TAU (Thr181, Ser199, Thr205, Thr217 and Thr231), normalized to total human TAU (Ht7) protein levels; *n*=5/group. GAPDH was used as a loading control;; norm. normalized protein levels. Each dot represents the average of three independent experiments conducted on *n*=5/group. Data are presented as mean±SEM, n.s. *p*>0.05, \*\* *p*<0.01 vs HS-hNs, assessed by Mann Whitney Rank Sum test

These data indicate that sAD-hNs undergo substantial neurite degeneration, as evidenced by reduced neurite length and decreased dendritic branching, without any decrease in cell viability. These findings are consistent with the known pathological features of AD, where changes in the length of neurites are indicative of disease progression [\[16](#page-18-9), [17](#page-18-10), [64,](#page-19-24) [65](#page-19-25)].

Next, we performed electrophysiological experiments to investigate the functional properties of HS- and sADhNs. No signifcant diferences in the resting membrane potential  $(n=40$  for HS;  $n=44$  for sAD;  $p=0.28$ ) and input resistance (*n*=18 for HS and *n*=19 for sAD,  $p=0.36$ ; Fig.  $5A,B$  $5A,B$ ) were detected between the two groups of neurons. Both the HS- and sAD-hNs generated action potentials in response to depolarizing currents (Fig. [5](#page-12-0)C,D). Most of the studied neurons exhibited either a single action potential or a few action potentials upon depolarization. Remarkably, the number of neurons fring multiple action potentials in response to depolarizing pulses was 3 of 26 in the control group and 7 of 27 in the sAD group (Fig. S[3\)](#page-17-0). When the fring rate of the latter was analysed, a noticeable increase in evoked activity was observed in sAD-hNs compared to HS-hNs (+175.4%;  $n=3$  and  $n=7$  for HS- and sAD-hNs,  $p=0.034$ ; Fig. [S3](#page-17-0)). We also studied synaptic function by measuring spontaneous activity in voltage-clamp experiments that revealed a signifcant decrease in the frequency of spontaneous postsynaptic currents in sAD-hNs (-22.0%, *n*=13 for HS,  $n=16$  for sAD,  $p=0.045$ ), with no significant changes in their amplitude  $(p=0.74;$  Fig. [5](#page-12-0)E-G) and kinetics (rise time,  $n=11$  for HS;  $n=12$  for sAD,  $p=0.41$ ; decay time:  $n=11$  for HS;  $n=12$  for sAD,  $p=0.14$ , Fig. [5](#page-12-0)H,I). The reduction in spontaneous frequency could indicate a decrease in either synaptic contacts (e.g., presynaptic terminals or dendritic spines) or synaptic proteins.

#### **Altered expression of synaptic proteins in sAD‑hNs**

Looking for the molecular determinants of morphological and functional alterations we found in sAD-hNs, we analyzed the expression of a number of proteins that are critically involved in synaptic function. By WB experiments, performed at DIV30, sAD-derived human neurons exhibited marked decreases in the levels of Synapsin-1 (-50%, *n*=5/group, *p*=0.008; Fig. [6](#page-13-0)A,B) and Synaptophysin (-19%, *n*=5/group, *p*=0.016; Fig. [6](#page-13-0)A,B), two key synaptic proteins involved in the release and fusion of neurotransmitter-containing synaptic vesicles [[66](#page-20-0), [67\]](#page-20-1), compared to HS-hNs. We also analyzed the expression of the postsynaptic protein PSD95, which regulates the trafficking and localization of glutamate receptors [[68\]](#page-20-2) and the expression of the GluA1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor [[69\]](#page-20-3). In sAD-hNs we observed a signifcant protein expression decrease of both PSD95 (-18%, *n*=5/ group, *p*=0.016; Fig. [6A](#page-13-0),B) and GLUA1 (-40%, *n*=5/ group,  $p = 0.008$ ; Fig.  $6A,B$  $6A,B$ ) compared to HS-hNs. Consistent with our WB results, immunofuorescence analysis revealed a signifcant reduction in the fuorescence intensity for SYN-1, SYP, PSD95 and GluA1 in sAD-hNs, indicating lower levels of these pre- and postsynaptic markers compared to HS-hNs (SYN-1: -47.1%, *n*=5/ group, *p*=0.008; SYP: -20.3%, *n*=5/group, *p*=0.008; PSD95: -36%, *n*=5/group, *p*=0.008.; GluA1: -48.3%,  $n = 5$ /group,  $p = 0.008$ ; Fig. [6](#page-13-0)C,D).

These results highlight a substantial decrease in the expression of critical synaptic proteins in sAD-hNs, likely contributing to the observed morphological and functional deficits in these neurons. This synaptic protein downregulation may underlie the impaired synaptic connectivity and reduced dendritic complexity that characterizes sAD-hNs.

# **sAD‑hNs show decreased expression of LIMK1**

In neurons, the formation and stabilization of developing neurites require the interaction between cytoskeletal proteins such as actin and microtubules [[70\]](#page-20-4). In particular, the actin cytoskeleton undergoes reorganization in actin flaments, facilitating the creation of neurite outgrowth

<sup>(</sup>See fgure on next page.)

<span id="page-10-0"></span>**Fig. 4** sAD-hNs show altered neurite morphology compared to HS-hNs. **A** Cell body morphology and neurite length of HS- and sAD-hNs from DIV1 to DIV30 were measured by performing fluorescence-based NeuroTrack analysis (NT) in IncuCyte® time-lapse microscopy system by exploiting EGFP-expression of hNs; Cell-body cluster area (in yellow), Neurites length (in red). **B** Dot plot graph showing cell body cluster area quantifcation of neuronal cells during 30 days of diferentiation (*n*=5/group); (B) Quantifcation of neurite length per neuron across diferent time points (DIV1, DIV14, DIV21, DIV30) shows a signifcant increase in neurite complexity in sAD neurons at DIV14, DIV21, and DIV30, while there is no signifcant diference at DIV1 (*n*=5/group). **C** Analysis of cell bodies shows no signifcant diferences between HS and sAD neurons across time points (*n*=5/ group). **D** Quantifcation of the cell viability at diferent time points, showing no signifcant diference between HS and sAD neurons (*n*=5/group). **E** Representative camera lucida drawings from HS and sAD neurons at DIV30 with Sholl analysis. **F**–**H** Bar graphs showing diferences in dendritic length (F), total number of bifurcating nodes (G) and total number of dendritic intersections (H) indicating decreased branching complexity in sAD neurons compared to HS neurons. Each dot in Figs B,C,D represents a triplicate of an experiment conducted on *n*=5/group. Each dot in Figs F–H represents a single cell analysis conducted on *n*=5/group. Data are presented as mean±SEM, n.s. *p*>0.05, \*\*\* *p*<0.001, \*\*\*\* *p*<0.0001 vs HS-hNs, assessed by assessed by 2way ANOVA-Sidak's multiple comparisons



**Fig. 4** (See legend on previous page.)

and microtubules, adjust their alignment into bundles, providing stability to the extending neurite [[71,](#page-20-5) [72\]](#page-20-6). A critical molecular mechanism for cytoskeletal reorganization during the initiation and elongation phases of neurites involves the Rho family of small G proteins [[73](#page-20-7), [74\]](#page-20-8).

Crucial for cytoskeletal reorganization is the LIMK1 coflin axis [[75,](#page-20-9) [76](#page-20-10)]. PAK inhibition, but not ROCK



<span id="page-12-0"></span>**Fig. 5** Electrophysiological properties of neurons derived from HS and sAD patients. **A** Summary graphs of membrane potential values and (**B**) input resistance values recorded from HS- and sAD-hNs. **C** Representative traces showing current-evoked action potentials. **D** Frequency of action-potentials plotted against current pulse values. **E** Representative traces of spontaneous excitatory postsynaptic currents (sEPSCs) in HS (gray) and sAD (orange) neurons. **F, G** Quantifcation of sEPSC amplitude (F) shows no signifcant diferences (n.s., *p*>0.05), while frequency (G) is significantly decreased in sAD neurons compared to HS neurons ( $p < 0.01$ ,  $n = 12$  cells per group). **H, I** Analysis of rise time (H) and decay time (I) reveals no signifcant diferences between HS and sAD neurons; *n*=12 cells per group. Data are presented as mean±SEM, n.s. *p*>0.05, \* *p*<0.05 assessed by two tailed Student's t test

inhibition, reduced LIMK1 phosphorylation and activation in hippocampal neurons, suggesting that PAK is predominant for LIMK1 activation in important neuronal compartments, like dendritic spines [[77\]](#page-20-11). Once phosphorylated, LIMK1 promotes rapid polymerization of actin by inhibiting (ADF)/coflin and promoting cytoskeleton reorganization [\[76](#page-20-10), [77](#page-20-11)]. Indeed, LIMK1 has been proposed to play a critical role in neurite outgrowth  $[26, 35, 76]$  $[26, 35, 76]$  $[26, 35, 76]$  $[26, 35, 76]$  $[26, 35, 76]$  $[26, 35, 76]$  $[26, 35, 76]$ . Thus, we wondered if the downregulation of the PAK1-LIMK1-coflin axis could be involved in the morphological and functional alterations we observed in sAD-hNs. To determine the levels of PAK1, LIMK1 and coflin activation in human neurons, we analyzed PAK1 phosphorylation on serine 204 [\[78](#page-20-12)], LIMK1 phosphorylation on threonine 508 [\[79\]](#page-20-13) and coflin phosphorylation on serine 3 [[80\]](#page-20-14) (Fig. [7](#page-14-0)). Immunoblotting with antibodies specifc for phospho-PAK1 (Ser204) and total PAK1 revealed no signifcant diferences in the ratio of phospho-PAK1 to total PAK1 in sAD-hNs compared to HS-hNs (*n*=5/group, *p*=0.222, Fig. [7](#page-14-0)A,B). However, we found a signifcant decrease in the ratio of phospho-LIMK1 to total LIMK1 (-46.0%,  $n = 5/\text{group}, p = 0.008$ , Fig. [7A](#page-14-0), B) in sAD-hNs compared to HS-hNs. In line with these results, we found a signifcant decrease in the ratio of phospho-coflin to total coflin (-13.0%, *n*=5/group,  $p=0.031$ , Fig.  $7A,B$  $7A,B$ ) in sAD-hNs compared to HS-hNs. These findings suggest that activation of LIMK1 and cofilin is impaired in neurons originating from AD patients. This altered signalling could play a critical role in the morphological and functional alterations we observed in sAD-hNs.

# **Pharmacological LIMK1 inhibition recapitulates sAD neuronal pathology in healthy neurons without exacerbating morphological defcits in sAD neurons**

To further examine the involvement of the LIMK1 pathway in the morpho-functional alterations observed in sAD neurons, we conducted perturbation experiments using the specifc LIMK1 inhibitor, BMS-5. HS neurons



<span id="page-13-0"></span>**Fig. 6** Neurons obtained from sAD-hiPSCs show impairments in synaptic protein expression. **A** Representative WB images of pre- and postsynaptic proteins in DIV30 HS- and sAD-hNs lysates; Densitometric analyses of presynaptic proteins: SYN-1, SYP and postsynaptic proteins: PSD95,GluA1; GAPDH was used as loading control; *n*=5/group. **C** Immunofuorescence staining of synaptic markers SYN-1, SYP, PSD95, and GluA1 in HS and sAD neurons. Co-localization with EGFP (neuronal marker) is shown. sAD neurons display decreased synaptic marker expression. Scale bars: 50 µm. **D** Quantifcation of median fuorescence intensity (MFI) in HS and sAD neurons shows a signifcant reduction in SYN-1, SYP, PSD95, and GluA1 in sAD neurons compared to HS neurons; *n*=5/group. Each dot represents the average of three independent experiments conducted on *n*=5/group. Data are presented as mean ± SEM, \*  $p$  < 0.05, \*\*  $p$  < 0.01 vs HS-hNs, assessed by Mann Whitney Rank Sum test

treated with BMS-5 exhibited morphological changes similar to those observed in veh-treated sAD neurons, including reduced neurite length (-16.1%, *n*=15/group, *p* < 0.0001) measured in live-imaging experiments at DIV30 (Fig. [8](#page-15-0)A,B). BMS-5 induced a signifcant reduction in neurite length in HS-hNs starting from DIV14 (Fig. [S4](#page-17-0)). Interestingly, in the same experimental conditions, treating sAD neurons with BMS-5 did not worsen their already compromised morphology observed in veh-treated sAD-hNs. Neurite length in BMS-5-treated sAD neurons were comparable to vehicle-treated sAD neurons  $(n = 15/$ group,  $p = 0.174$ ; Figs. [8](#page-15-0)A,B, and S[4\)](#page-17-0), indicating that LIMK1 activity may already be impaired in sAD neurons. No signifcant changes in cell body clusters or cell viability were observed in each hNs condition (Figs. [8C](#page-15-0),D, and S[4](#page-17-0)).

The Sholl analysis ( $n = 17$  neurons from  $n = 5$  HS + Veh;  $n=29$  neurons from  $n=5$  HS+BMS-5;  $n=17$  neurons from  $n=5$  sAD+Veh;  $n=15$  neurons from  $n=5$ sAD+BMS-5) demonstrated decreased neurite length and dendritic complexity in BMS-5-treated HS-hNs, similar to sAD neurons. Specifcally, BMS-5 treatment in HS-hNs led to a signifcant reduction in neurite length  $(-32.6\%, p=0.0009)$  without affecting the length of neurites in sAD-hNs ( $p=0.352$ ; Fig. [8E](#page-15-0),F). Similarly, dendritic complexity was signifcantly reduced in HS-hNs following BMS-5 treatment (bifurcating nodes: -28.6%, *p*=0.031; intersections: -35.5%, *p*=0.0009), while no signifcant changes were observed in sAD-hNs (bifurcating nodes,  $p=0.364$ , intersections  $p=0.763$ , Fig. [8G](#page-15-0),H), indicating that LIMK1 pathway is crucial for maintaining dendritic architecture in healthy neurons but it is already compromised in sAD neurons.



<span id="page-14-0"></span>**Fig. 7** sAD-hNs display reduced phosphorylation levels of actin-modulating proteins LIMK1 and coflin. **A** Representative WB images of p-PAK1 Ser204, p-LIMK1 Thr508 and p-Cof Ser3 levels in HS- and AD-hNs lysates at DIV30; (**B**) Bar graphs showing the densitometric analysis of p-PAK1 Ser204, p-LIMK1 Thr508 and p-Cof Ser3 levels in HS- and AD-hNs (*n*=5/group). GAPDH was used as a loading control. norm. normalized protein levels. Each dot represents the average of three independent experiments conducted on *n*=5/group. Data are presented as mean±SEM, n.s. *p*>0.05, \* *p*<0.05, \*\* *p*<0.01 vs HS hNs, assessed by Mann Whitney Rank Sum test

These results suggest that LIMK1 activity is crucial for maintaining normal neuronal structure, and its inhibition in healthy neurons mimics the pathological features seen in sAD neurons. The absence of further morphological deterioration in sAD neurons treated with the inhibitor supports the notion that LIMK1 dysregulation is already contributing to the structural deficits in these cells  $[33]$  $[33]$ . Overall, these fndings emphasize the critical role of LIMK1 in neuronal integrity and point to LIMK1 dysfunction as a key driver of neurite degeneration in sAD neurons.

# **Discussion**

hiPSCs have emerged as a valuable tool for modeling neurodegenerative diseases such as AD [\[81,](#page-20-15) [82\]](#page-20-16). Specifcally, hiPSCs have been used to model both familial and sporadic forms of AD, providing insights into the pathological phenotypes associated with these conditions [[8\]](#page-18-2).

Here we reprogrammed skin biopsies from individuals diagnosed with sAD to generate and characterize human hiPSC lines and their derived excitatory neurons. The hiPSC clones from sAD patients and neurotypical control individuals exhibited typical hiPSC-like morphology and expressed pluripotency markers homogeneously (Fig. [1A](#page-7-0)-C). Additionally, G-banding karyotype and chromosomal stability analysis revealed no signifcant copy number alterations, indicating that no remarkable mutations occurred in critical genomic regions (Fig [S1A](#page-17-0),B). The absence of significant morphological and genetic diferences between sAD- and HS-hiPSCs allowed us

to exclude the possibility that the changes we subsequently observed in hiPSC-derived neurons depend on diferences due to the reprogramming process of human fbroblasts into hiPSCs. In addition, analyses of EBs originating from HS and sAD-hiPSCs revealed the expression of the neural progenitor marker MSX1, PAX6 and GATA6, confrming that three geminal layers development occurred in both experimental groups (Fig. [S2C](#page-17-0)).

Analysis of protein expression in sAD-hNs revealed several alterations. First, we detected Aβ accumulation and signifcant increases in Tau phosphorylation at Thr181, Ser199 and Thr217 (Fig. [3](#page-9-0)A-E). Moreover, at DIV30, we found a marked decrease in the expression of key synaptic proteins, including Synapsin-1, Synaptophysin, PSD-95, and GluA1 (Fig. [6](#page-13-0)A-D). The above-mentioned synaptic proteins play crucial roles in various neuronal functions, particularly those related to synaptic activity  $[66-69]$  $[66-69]$  $[66-69]$ . The reduced levels of synaptic proteins in sAD-hNs underline and confrm the signifcant role of the altered structure and function of synapses in the pathophysiology of the disease, while the increased levels of the AD hallmarks, Aβ and pTau, demonstrate, as previously reported [[9\]](#page-18-11), how neurons derived from hiPSCs of sAD patients retain the molecular signature of the disease. In agreement with previous reports [[8](#page-18-2)], we found that both sAD and control neurons produced action potentials when depolarized, with no signifcant diferences observed in resting membrane potential and input resistance between the two groups (Fig. [5A](#page-12-0),B). However, the percentage of



<span id="page-15-0"></span>**Fig. 8** Efect of BMS-5 treatment on neurite outgrowth and morphology in neurons derived from HS and sAD patients. **A** Representative images of neurons treated with vehicle (Veh) or BMS-5, an inhibitor of LIMK1, in HS and sAD neurons at DIV30. EGFP (green) shows the neuronal processes, and yellow indicates neurite tracing. Neuronal complexity appears reduced in sAD neurons compared to HS, and BMS-5 treatment does not rescue neurite complexity in sAD neurons. Scale bars: 50  $\mu$ m. **B** Quantification of neurite length per neuron shows no significant difference between BMS-5-treated HS-hNs and sAD-hNs treated with veh or BMS-5 (*n*=5/group). BMS-5-treated HS-hNs showed signifcantly reduced neurite length than Veh-treated HS-hNs. Quantifcation of cell bodies (**C**) and cell viability (**D**) shows no signifcant diference between vehicle and BMS-5-treated neurons in both HS and sAD groups (*n*=5/group). **E** Representative images of camera lucida drawings obtained from HS and sAD neurons treated with vehicle or BMS-5. **F–H** Results of Sholl analysis showing the quantifcation of neurite length, number of bifurcating nodes, and total number intersections. No signifcant diferences were found between BMS-5-treated HS-hNs and sAD-hNs treated with vehicle (*n*=5/groups) or BMS-5 (*n*=5/groups). BMS-5-treated HS-hNs showed signifcantly reduced neurite length than Veh-treated HS-hNs. Each dot in Figs B,C,D represents a triplicate of an experiment conducted on *n*=5/group. Each dot in Figs F,G,H represents a single cell analysis conducted on *n*=17 neurons from *n*=5 for HS+Veh; *n*=29 neurons from *n*=5 for HS+BMS-5; *n*=17 neurons from *n*=5 for sAD+Veh; *n*=15 neurons from *n*=5 for sAD+BMS-5). Data are presented as mean±SEM, n.s. *p*>0.05, \*\* *p*<0.01, \*\*\*\**p*<0.0001 vs HS hNs, assessed by assessed by 2way ANOVA-Sidak's multiple comparisons

neurons showing repetitive fring activity upon membrane depolarization and their discharge rate were signifcantly higher in sAD-hNs (Fig. [S3](#page-17-0)), indicating a signifcant increase in neuronal excitability, as we previously observed in animal models of AD [[83\]](#page-20-17). Functional alterations in sAD-hNs also included a signifcant decrease in the frequency of spontaneous postsynaptic currents, which was not accompanied by signifcant changes in their amplitude or kinetics ([Fig](#page-12-0).  $5E-I$  $5E-I$ ). This alteration could be directly linked to the alteration we documented in synaptic compartments in sAD-hNs. One possible explanation is that the hyperexcitability in sAD neurons could refect an early compensatory mechanism in response to synaptic dysfunction, as observed in early AD pathology [[83](#page-20-17), [84](#page-20-18)]. Increased evoked responses might indicate that neurons require stronger or more coordinated stimuli to elicit a response, a sign of altered synaptic connectivity or network dysfunction [[85\]](#page-20-19). Studies suggest that calcium dysregulation, driven by Aβ and tau pathologies, plays a critical role in the progression of AD by impairing synaptic function and exacerbating neuronal excitability [[84](#page-20-18)]. Similarly, changes in ion channels, such as  $K_v4.2$ depletion, have been shown to increase dendritic excitability in early AD stages, further contributing to network dysfunction [[83,](#page-20-17) [86\]](#page-20-20). Importantly, the imbalance between evoked and spontaneous neuronal activity may serve as an early indicator of neural network disruptions that precede overt neurodegeneration, which is a defning characteristic of AD progression. Evidence from animal models has demonstrated that early-stage network hyperexcitability and compensatory mechanisms can contribute to cognitive defcits and neuronal loss observed later in the disease course [[28](#page-18-12)].

Morphological analyses conducted on sAD-hNs revealed a reduction in neurite length and dendritic complexity compared to their relative controls (Fig. [4\)](#page-10-0). These morphological changes, accompanied by no signifcant changes in neuronal viability or soma size, manifesting as early as DIV14, resonate with previous neuropathological studies highlighting neuritic atrophy as a hallmark of AD pathology [[87](#page-20-21)]. This finding suggests that neuritic impairment in sAD might be a consequence of altered cytoskeletal dynamics rather than cell death.

The interaction between cytoskeletal proteins, specifically actin and microtubules, is crucial for the formation and stabilization of developing neurites in neurons [[71–](#page-20-5) [73\]](#page-20-7). Neurite outgrowth is facilitated by the reorganization of the actin cytoskeleton, whereas microtubules contribute to the stability of the extending neurite  $[71-73]$  $[71-73]$ . The observed decrease in the ratio of phospho-LIMK1 to total LIMK1 and phospho-coflin to total coflin in sADhN compared to HS-hNs (Fig. [7\)](#page-14-0) suggests an impairment in the activation of LIMK1 and coflin in neurons afected by sAD. These findings are particularly significant in the context of neuronal cytoskeletal reorganization, as the LIMK1-coflin axis plays a critical role in the initiation and elongation phases of neurite outgrowth by inhibiting coflin and facilitating the reorganization of the cytoskeleton [[88](#page-20-22)].

LIMK1 activation enhances actin polymerization, which is critical for maintaining dendritic spine stability and strengthening synaptic connections. Under normal physiological conditions, this activation supports synaptic plasticity [\[21](#page-18-13), [88\]](#page-20-22). However, in pathological contexts such as AD,  $\mathbf{A}\beta$  toxicity can lead to excessive activation of the LIMK1/coflin pathway, disrupting normal actin dynamics and causing abnormal spine morphology. Pharmacological inhibition of LIMK1 has been shown to mitigate the overactive pathway, restoring actin turnover and preventing further synaptic loss induced by  $\mathbf{A}\beta$  [\[31](#page-19-0)]. Similarly, abnormal increase in phosphorylated LIMK1 has been linked to neuronal dystrophy observed in brain regions of patients afected by AD [[29](#page-18-14)].

Aβ oligomers also promote coflin dephosphorylation, leading to synaptic protein loss and long-term potentiation (LTP) deficits in AD models, such as APP/PS1 mice [[89\]](#page-20-23). Additionally, expression of inactive coflin can block Aβ42-induced spine loss  $[43, 44]$  $[43, 44]$  $[43, 44]$  $[43, 44]$  $[43, 44]$ . In the human brain, signifcantly reduced levels of phosphorylated coflin were observed in AD patients, indicating increased coflin activity  $[90]$  $[90]$  $[90]$ . This suggests that elevated LIMK1 activity in AD brains may act as a compensatory mechanism to counteract the increased coflin activity triggered by phosphatases such as Slingshot (SSH). The reduction of phosphorylated LIMK1 (p-LIMK1) in hiPSC-derived neurons refects potential early-stage changes in sAD that are distinct from postmortem fndings [\[90\]](#page-20-24).

Moreover, upstream regulators of LIMK1, such as ROCK (Rho-associated kinase), have also been implicated in actin dynamics and synaptic stability, with dysregulation potentially contributing to synaptic dysfunction in AD [[31\]](#page-19-0). Furthermore, phosphatases like PP1 and PP2A are involved in dephosphorylating LIMK1, and their dysregulation is associated with neurodegenerative diseases  $[39]$  $[39]$ . These disruptions could destabilize synaptic connections, contributing to the synaptic protein loss observed in AD models. Furthermore, the presence of Hirano bodies, which are actin-rich inclusions commonly found in the brains of AD patients, may refect broader disruptions in actin dynamics and contribute to cytoskeletal disorganization and the loss of synaptic proteins [[91](#page-20-25), [92\]](#page-20-26).

The presence of sAD-related stressors, such as increased Aβ accumulation or tau pathology, could further exacerbate these disruptions by infuencing calcium homeostasis and mitochondrial function, both of which are key to maintaining synaptic integrity. This combination of factors could destabilize synaptic connections and result in the reduction of synaptic protein levels observed in our model system.

Finally, our perturbation experiments using the LIMK1 inhibitor BMS-5 have provided key insights into the role of the LIMK1 pathway in the morphological and functional changes of sAD neurons (Fig. [8\)](#page-15-0). Treatment of neurons from HS with the inhibitor resulted in morphological changes similar to those observed in sAD neurons, highlighting the critical role of LIMK1 in maintaining normal neuronal structure. Moreover, inhibition of LIMK1 in sAD neurons did not worsen the morphological abnormalities, suggesting that LIMK1 activity may already be impaired in these cells (Fig. [8](#page-15-0)).

# **Conclusions**

Our fndings support the use of hiPSCs as a viable model to study sAD, allowing for controlled in vitro investigations of disease mechanisms. Indeed, hiPSC-derived hNs recapitulate numerous molecular, morphological and functional alterations that are typical of AD. Among the diferent molecular alterations identifed in our in vitro sAD model the impaired activation of LIMK1 and coflin highlights the potential implication of the LIMK1-coflin axis in the structural and functional defcits observed in sAD-hNs.

Further studies on the LIMK1-coflin axis and its involvement in the pathophysiology of sAD, extending on hNs derived from female sAD patients, could advance our understanding of synaptic failure in AD and ofer potential targets for therapeutic intervention, also using engineered LIMK1 analogs [\[88](#page-20-22)], aimed at preserving cytoskeletal dynamics and neuronal health.

sdic Alzheimer's disease patients derived human induced

# **Abbreviations**



# **Supplementary Information**

The online version contains supplementary material available at [https://doi.](https://doi.org/10.1186/s13195-024-01632-3) [org/10.1186/s13195-024-01632-3](https://doi.org/10.1186/s13195-024-01632-3).

<span id="page-17-0"></span>Supplementary Material 1: Fig. S1. q-PCR genetic analysis to detect small chromosome abnormalities in hiPSC lines. (A) The copy numbers of chr1q, chr8q, chr10p, chr12p, chr17q, chr18q, chr20q and chrXp were normalized to chr4p expression which was set to be 2. (*n*=5 HSl, *n*=5 sAD). (B) Boxes indicate a karyotype analysis of HS (left) and sAD (right). Both karyotype are 46,XY and no showing genetic aberration. (C) The fgure shows the amplifcation of the exon 4 of APOE. The PCR was performed using both

fuorophore-marked primers fanking the rs429358 and rs7412 SNPs. This region (219 bp) has four HhaI restriction sites (GCG^C) that allow to obtain diferent length fragments corresponding to the ɛ2, -3 or -4 alleles (blue and green arrows). For genotyping, the size of the fragments are: 85-110 bp for  $\varepsilon$ 2-/ $\varepsilon$ 2; 35-110 bp for  $\varepsilon$ 3/ $\varepsilon$ 3 and both at 35 bp for  $\varepsilon$ 4/ $\varepsilon$ 4. In (D) are shown the genotype of the HS (left) and sAD (right) cell cultures. Fig. S2. Verifcation of Sendai virus clearance and diferentiation potential of hiPSCs derived from HS and sAD patients. (A) RT-PCR analysis confrms the successful elimination of the Sendai virus from hiPSCs derived from HS and sAD patients. Infected fbroblasts were used as positive controls 5 days after SeV transduction.. (**B**) mRNA expression levels of Klf4 to futher validate the pluripotency. Human skin fbroblasts were used as negative controls for pluripotency. **(**hiPSCs (**C**) qPCR for mRNA expression of three germ layer genes. Quantifcation of expression of the ectoderm (PAX6), mesoderm (MSX1), and endoderm (GATA6) markers compared to hiPSCs. Matched hiPSCs, from which the diferentiated germ layer was derived, were utilized as negative controls. Fig. S3. Action potential fring properties in neurons derived from HS and sAD patients. (A) Representative traces showing current-evoked fring of HS-hNs (grey) and sAD-hNs (orange). (B) Bar graph depicting quantifcation of evoked fring in the experimental conditions shown in (E) (*n*=4 HS, *n*=6 sAD). Data are expressed as mean±SEM. \*\**P*<0.05; statistics by two-tailed Student's t test. Fig. S4. Efect of BMS-5 treatment on neuronal morphology and neurite outgrowth in neurons derived from HS and sAD patients at diferent time points. **(A)** Representative images showing neuronal morphology over time under vehicle (Veh) or BMS-5 treatment in HS and sAD neurons. EGFP (green) labels neuronal processes, while yellow and red indicate neurite tracing and soma, respectively. Neurons were analyzed at DIV1, DIV14, and DIV21 (B) Neurite length appears reduced in HS BMS-5 hNS, sAD Veh hNs, sAD BMS-5 hNs compared to HS Veh hNs at DIV14 and DIV21. At DIV14, neurite length decreased by 28.0% in sAD Veh (*p*<0.0001), 30.4% in HS BMS-5 (*p*<0.0001), and 33.7% in sAD BMS-5 (*p*<0.0001) compared to HS Veh. At DIV21, neurite length was reduced by 23.6% in sAD Veh (*p*<0.0001) and 19.4% in sAD BMS-5 (*p*<0.0001) relative to HS Veh. (C) No signifcant changes in cell body clusters or cell viability were observed in each hNs condition at DIV1, DIV14 and DIV21. (D) No signifcant changes in cell viability were observed in each hNs condition at DIV1, DIV14 and DIV21. Scale bars: 50 µm. Each dot in Figs B-D represents a triplicate of an experiment conducted on *n*=5/group. Data are presented as mean±SEM, n.s. *p*>0.05, \*\*\*\**p*<0.0001, assessed by 2way ANOVA-Sidak's multiple comparisons

#### **Acknowledgements**

We would like to acknowledge the contribution of the Core Facility G-STeP "Electrophysiology", Fondazione Policlinico Universitario "A. Gemelli" IRCCS.

#### **Authors' contributions**

C.R. and C.G. conceived and coordinated the study. R.S. carried out and analyzed all research not otherwise attributed. M.G.V., C.M. and G.M.G. enrolled AD patients and neurotypical controls. D.D.L.P., R.S. and C.C. generated hIPSCs and hNs. R.S. and F.P. performed Western blot experiments. G.A. and C.R. performed and analysed electrophysiological experiments. D.D.L.P. generated EB and performed qPCR analysis. F.P. performed Sholl analysis. A.C. and J.C. provided the resources, including hIPSCs. A.N., S.M. and F.D.T. perfomed genetic analysis and G-banding karyotyping. R.S., C.R., and C.G. wrote the manuscript with input from all authors. All authors approved the fnal version of the manuscript.

#### **Funding**

This work was supported by the Alzheimer's Association Research Grant (AARG-21–847593 to C.R.) from the Alzheimer's Association and by the Italian Ministry of Health (GR-2021–12373657 to C.R. and Ricerca Corrente to C.G.).

#### **Data availability**

No datasets were generated or analysed during the current study.

#### **Declarations**

#### **Ethics approval and consent to participate**

Isolation and culture of skin fbroblasts from patients and hiPSC generation were performed in accordance with the international standard of GCP (Legislative Decree D.M. 15 July 1997) with the ethical permit granted by the Fondazione Policlinico Gemelli Ethics Committee (protocol #0005057 dated 16/02/2023).

#### **Competing interests**

The authors declare no competing interests.

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#### Received: 11 April 2024 Accepted: 26 November 2024 Published online: 19 December 2024

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