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Phenotyping iPSC-derived neurons from Angelman patients and partial rescue via JNK pathway inactivation

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Abstract

Background Angelman Syndrome (AS, MIM #105,830) is a rare neurodevelopmental disorder caused by imprinting defects, and it is marked by motor impairment, intellectual disability, speech limitations, and seizures. The condition arises due to the loss of function in neurons of the maternally-inherited *UBE3A* gene, mapping in the imprinted chromosomal region 15q11.2-q13, through a variety of genetic and epigenetic mechanisms. *UBE3A* encodes a ubiquitin E3 ligase, which is essential for synaptic plasticity. Most studies of AS have been performed on animal models, while induced pluripotent stem cells (iPSCs), mainly generated from patients with deletion, remain limited in number.

Methods Here we report on the generation and characterization of human cortical neurons derived from iPSCs obtained from children representative of the main AS genotypes: 3 with 15q11–13 deletion, 3 with *UBE3A* mutations, 3 with uniparental disomy, and 2 with imprinting defects.

Results During early neuronal differentiation, morphological analyses highlighted a reduced dendritic branching and an increased number of thin filopodia, while migration assays revealed reduced neurite mobility in AS human neurons with the most severe genetic defect, namely the 15q11–13 deletion. At a later differentiation time point, electrophysiological analyses revealed impaired neuronal activity in AS patient-derived neurons. To investigate pathways potentially involved in synaptic dysfunction, we examined the JNK (c-Jun N-terminal kinase) signalling pathway, which plays key roles in neuroinflammation and synaptic development. We found increased JNK activation and, importantly, treatment with a JNK-inhibitor, D-JNKI1, partially rescued the neuronal morphological defects, significantly reducing the number of immature protrusions, as well as defects in electrophysiological properties.

Conclusions Our patient-specific models contribute elucidate how loss of *UBE3A* leads to aberrant neuronal maturation and function in the precocious stages of neuronal development and how treatment with D-JNKI1 may pave the way for new therapeutic possibilities.

Keywords E6AP, i-neurons, Patch-clamp recordings, Thin spines, Neurite mobility, JNK inhibitor

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Background

Angelman syndrome (AS; MIM #105,830) is a rare (1/12,000–1/20,000 individuals) neurodevelopmental disorder, characterized by moderate to severe intellectual disability [1], absence of speech, ataxic or broad-based gait, happy demeanor, and distinctive pathognomonic EEG patterns. Additional traits such as facial gestalt, distinguished by prognathism and spaced teeth, seizures, and sleep impairment, are often reported and included in the updated AS consensus criteria [2, 3].

AS molecular basis includes four classes of 15q11.2-q13 alterations disrupting the neuronal monoallelic expression of the *UBE3A* gene, which encodes an enzyme that catalyzes the ubiquitination of specific substrates and their degradation via the 26S proteasome [4]. In neuronal cells, *UBE3A* expression is regulated by a tissue-specific imprinting mechanism, leading to monoallelic expression of the maternal *UBE3A* allele [5]. Progressive silencing of the paternal allele occurs during embryogenesis [6, 7]. Approximately 50–75% of AS children carry a de novo 5–7Mb deletion on the maternal allele, defined by a common distal breakpoint, BP3, and two different centromeric breakpoints, BP1 or BP2. Both BP1-BP3 and BP2-BP3 deletions encompass the *UBE3A* gene and the *GABRB3* gene, while BP1-BP3 deletion also includes the *NIPA1*, *NIPA2*, *CYFIP1* and *MKRN3* genes. Loss of function of *GABRB3* gene is associated with developmental and epileptic encephalopathy 43 (#617,113), while genes within BP1-BP2 interval have been implicated in patients with neurodevelopmental delay and autism spectrum disorder [8]. Less frequent genetic alterations include paternal uniparental disomy of chromosome 15, UPD(15) pat (3–7% of cases) and imprinting defects (ID) (3%), whereas maternally inherited or de novo *UBE3A* point mutations are reported in 11% of AS patients. Studies on large cohorts have confirmed a robust genotype–phenotype correlation with better cognitive performance, communication skills, earlier walking age and later onset or absence of seizures for UPD(15)pat and ID cases, as well as for specific *UBE3A* missense and late truncating variants [9, 10]. A limited number of induced pluripotent stem cells (iPSCs) from AS patients have been generated, including lines from 12 individuals carrying deletions [11–17], four with UPD(15)pat [18, 19], one with ID [20] and four with *UBE3A* point mutations [7, 14, 21, 22] as well as CRISPR-CAS9 engineered human embryonic stem cells [23]. These models have been used to dissect the mechanism underlying the syndrome in a human cellular context and to test the potential therapeutic efficacy of candidate drugs. However, the phenotype and functional alterations in neurons or organoids derived from human AS iPSCs have not been extensively studied.

In mouse model of AS, synaptic dysfunctions [24, 25], increased oxidative stress, metabolomic alterations and

apoptosis in neural precursors cells [26, 27] had all been reported.

The JNK3 signaling pathway is particularly relevant in this framework, and its activation following oxidative stress has been reported to contribute to neuronal death [28]. Notably, JNK3 hyperactivation has been observed in the *Ube3a* m-/p+ mouse model and a specific JNK3 inhibitor, the D-JNKI1 peptide, is available and has been shown to rescue several AS-related phenotypes in mice [29, 30]. D-JNKI1 (D-retro-inverso form of c-Jun N-terminal kinase-inhibitor) is a chemically-designed form of JNKI1 peptide [31], and it may cross the blood–brain-barrier (BBB) [32, 33]. This peptide combines a 19-amino acid effector domain derived from the scaffold protein IB1/JIP1 with a 10-amino acid active transporter TAT (transactivator of transcription) and two proline spacers positioned between them; it is cell penetrating protease-resistant, specifically inhibits JNK3 binding to its substrates and upon being activated acts almost exclusively in the pathological processes [34, 35].

By interacting with its synaptic targets, D-JNKI1 attenuates the damage triggered by pathway hyperactivation at the neuronal level [34]. Therapeutic effect of D-JNKI1 have been carried out on retinal exotoxicity, neuropathic pain, protection of Purkinje cells, auditory disorders, and Rett syndrome [36–38]. Moreover, a study on AS mice indicated that inhibiting the JNK pathway with D-JNKI1 can prevent synaptic impairment and improve cognitive function [30].

Here, we generated and characterized the morphology and functional properties of a panel of cortical neurons derived from iPSCs of 11 individuals representative of the AS genetic heterogeneity. Based on the evidence in AS mouse models, we set out to explore JNK pathway, a pathway related to oxidative stress and apoptosis, and testing DJNKI1 efficacy, in AS iPSC-derived cortical neurons (cortical i-neurons).

Materials and methods

Selection of AS patients for iPSC reprogramming

A cohort of 11 Italian patients with a genetic diagnosis of Angelman syndrome participated in our project, funded by Angelman Syndrome Alliance (ASA, <https://angelmanalliance.org>). Seven patients received their molecular diagnosis in our laboratory, while the remaining 5 replicated the test before being included in the study. Samples from healthy controls, 3 females (CTRL 1,4,6: two patients' sisters in pediatric age and a healthy unrelated adult) and 2 males (CTRL5, CTRL8: a patient's brother and a young healthy adult) were included in the study. For 7 patients genotypes, the acronyms used in this manuscript and clinical data are reported in Table 1, while for the remaining 4 cases the clinical feature were not available in detail. For the rarest and clinically mildest

Table 1 Consistent clinical features in AS patients selected for establishment of iPSC-derived neurons

PATIENT DETAILS	Molecular alteration	DEL 1	DEL 2	DEL 3	UBE3A N-TER	UBE3A in frame DEL	UBE3A 3C-TER	UPD(15)pat
		BP1-BP3	BP2-BP3	BP1-BP3	N-ter truncating UBE3A variant NM_130838.4:c.1039dupG, p.Ala347GlyfsTer25 (de novo)	In frame deletion UBE3A variant NM_130838.4:c.510_515del p.Glu171Lys172del (maternal inheritance)	C-ter truncating UBE3A variant NM_130838.4:c.2507_2510del, p.(Lys836ArgfsTer4) (de novo)	UPD(15)pat
CONSISTENT CRITERIA (100%)	Sex	M	F	M	M	M	F	M
	Age at diagnosis	5–6 months	3y		4,5y	5y	3y	2y3m
	Age at blood collection to obtain iPSCs	29y	9y	7y	11y	6y	9y	8y
	Developmental mental delay*	severe	severe	severe	severe	moderate	moderate	moderate
	Speech impairment: no or minimal use of words	no words	no words	no words	two words	no word, but comprehension is good	some words, use gesture, comprehension	5–6 words, good comprehension
	Movement or balance disorder (ataxia)	+	+	++	+	+	very mild	+
	Age of walking	4y	4–5y	he does not walk autonomously	na	na	23 month	1,5y
	AS behavior: frequent laughter, hyperactivity	+	+	+	++	++	present in the childhood, improved in adolescence	±
	Microcephaly	+	+	+	-	+	-	-
	Abnormal EEG	+	+	+	+	+	+	+
FREQUENT (> 80%)	Seizure and age of onset	5–6 m in hyperpyrexia, 18 month myoclonies, focal seizures to generalized, absences, non-convulsive status epilepticus (NCSE) drug resistance	34 m in hyperpyrexia, 1–2 seizures/year	14 m in hyperpyrexia, absences and tonic–clonic seizures since 3 y	4y focal semiology, ocular re-vulsion, oral rim deviation, right hemiclony, at 7y plurymonthly seizures, which disappeared raising valproid acid dosage	infantile spasm since 4 m. Treated with Topamax, free from seizures between 13 months and 7,5 y when seizures appeared again	-	-

AS defects, two patients affected with ID, and three with UPD(15)pat, iPSCs were obtained and two clones each were differentiated to cortical neurons. However, in this study, morphological analyses, motility study, and electrophysiological recording were focused on the first subset of samples, representative of most classical AS conditions. The selection was based on both disease severity and the prevalence of the respective genetic variant. All the AS children's and controls' parents signed the informed consent and were reprogrammed to iPSCs and differentiated to cortical neurons using the same protocol.

iPSCs generation and characterization

Peripheral Blood Mononuclear Cells (PBMCs) from controls and AS patients were isolated with SEPIMATE[™] tubes (STEMCELL), in accordance with the protocol. The PBMCs obtained were cultured in SFEM Medium (STEMCELL) for 10 days to enrich the erythroblast population and transduced with Cytotune 2.0 Sendai Reprogramming Kit (Life Technologies). After three weeks, iPSC colonies were observed and maintained on the Mouse Embryonic Fibroblast (MEF) layer. Single clones were expanded and characterized for stemness and genomic stability. The expression of specific pluripotency markers was confirmed by Immunofluorescence, using antibodies against OCT3/4 and TRA-1-60, and RT-PCR with primers for OCT3/4, SOX2, and NANOG [39]. (Supplementary Fig. 1 A,B).

Karyotype analysis

Karyotype analysis on at least 20 metaphases allowed the visualisation of chromosomal rearrangement, which occurred during reprogramming. Chromosomal preparations were obtained from each iPSC line. To arrest cells in metaphase, cultures were treated overnight with colcemid (10 µg/mL) at 37 °C under a humidified atmosphere of 5% CO₂. Subsequently, the iPSCs were subjected to hypotonic shock using 0.56% KCl for 6 min at room temperature, followed by a 3-min wash with 5% acetic acid. The cellular pellet was then fixed in a methanol/acetic acid solution (3:1). Q-banded metaphase spreads were visualized and imaged at 100X magnification using a Leica microscope equipped with a digital camera. Karyotypic analysis was performed on more than 30 mitoses utilizing the CytoVision software (Leica) (Supplementary Fig. 1 C).

Mutation sequencing

Genomic DNA was extracted from iPSC clones to confirm the original monoallelic *UBE3A* pathogenic variant (Supplementary Fig. 2).

UBE3A was amplified with GoTaq DNA Polymerase (PROMEGA), using the following primers:

Variant NM_130838.4:c.1039dupG (exon 3).

Forward 5' CAAAGCGATGAGCAAGCTA 3'

Reverse 5' AAGTGGTTTTTCGACAATCCA 3'

Variant NM_130838.4: c.510_515del (exon3).

Forward 5' GCCTAGAATGTTTGGCTGTTT 3'

Reverse 5' CCTTGAGGAAGATGCTTCTGA 3'

Variant NM_130838.4:c.2507_2510del (exon 10).

Forward 5' TTCTAAGTGTATTTCCCATGACT 3'

Reverse 5' GAAGGGAGGCACAGACATAG 3'

The PCR fragments were purified with Illustra[™] ExoProStar[™] enzyme (GE Healthcare) and sequenced by Sanger method, using the Big Dye Terminator (Applied Biosystems). The capillary electrophoresis was performed on the ABI PRISM 3500 Genetic Analyzer (Applied Biosystem), and the electropherograms were analyzed with Sequencing Analysis Software 6 (Applied Biosystem).

SNP Array [39]

Infinium HD Assay Ultra with Illumina multi-sample DNA Analysis BeadChips was performed to detect CNVs (copy number variations), duplications, deletions, and loss of heterozygosity, comparing DNA from iPSC clones versus the blood of each case. The data were imported from iScan Control Software into GenomeStudio 2.0 Genotyping Module Software provided by Illumina (Supplementary Fig. 1D, E AND Supplementary Fig. 3 A,C).

MS-MLPA

The MRC-Holland kit (MRC Holland, Amsterdam, Netherlands) SALSA MLPA Probemix ME028 Prader-Willi/Angelman was used to confirm ID alteration, following the manufacturer's instructions. Raw data were analyzed by using Coffalyser.Net software (MRC Holland) (Supplementary Fig. 3 B,D,E).

Generation of Cortical i-neurons

At least two characterized clones for each patient/control were differentiated into cortical neurons according to the monolayer protocol, as described [39, 40].

In brief, human induced pluripotent stem cells (iPSCs) were manually passaged and transitioned to N2B27 medium (Neurobasal medium, 2% B27 supplement, 1% Insulin Transferrin selenium (ITS), 1% N2 supplement, 2mM L-glut, 0.5% Pen/Strep) supplemented with 500 ng/mL Noggin to initiate neural induction. Following 10–14 days of culture, emergent neural rosettes were manually isolated and expanded as clusters on poly-ornithine/laminin-coated surfaces. Upon reaching confluence, these neural stem cells were dissociated using TrypLE and replated at high density in N2B27 supplemented with 10 µM Y-27632 to facilitate survival. At week 4, the medium was transitioned to Neural Differentiation

Medium (NDM) (Neurobasal medium, 2% B27 supplement, 1% NEAA, 2mM L-Glut) enriched with 10ng/mL BDNF, 10ng/mL GDNF, 1μM ascorbic acid, and 200μM cAMP to promote neuronal specification. Finally, at week 5, neural progenitors were seeded at low density ($1.0\text{--}1.5 \times 10^5$ cells/well) for terminal differentiation. Long-term maturation was supported by bi-weekly half-media changes with NDM+GFs and supplemental laminin (1 μg/mL) to maintain neurite attachment and structural integrity.

Real-Time PCR

RNA was isolated from blood and iPSCs with Quick-RNA™ Mini Prep (Zymo Research). 1μg was reverse transcribed into cDNA using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher) in accordance with the manufacturer's instruction manual. The cDNAs were analyzed by Real-time PCR using QuantStudio 12k Flex Applied Biosystems (Life Technologies). We used SYBR® Green (Thermo Fisher) with the *UBE3A* primer pair reported below. Each sample was run in triplicate, and three independent experiments were performed. The results were calculated using the $2^{-\Delta\Delta C_t}$ method to allow the normalization of each sample to GAPDH. The high reproducibility of the *GAPDH* expression allowed us to use only one reference gene.

UBE3A primers for cDNA analysis

UBE3A Forward 5' CCCAACAACTCCTGCTCT 3'

UBE3A Reverse5' CAAGAATTTTCATATACCTTCTC TTCTG 3'

GAPDH Forward5' ATGTTTCGTCATGGGTGTGAA 3'

GAPDH Reverse5' GGTGCTAAGCAGTTGGTGGT3'

Protein extraction and western blot

Total proteins from neurons were extracted using RIPA Buffer (Tris-HCl 50 mM pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sódio deossicolato, 0.1% SDS), supplemented with protease and phosphatase inhibitors (Roche). The protein concentration was assessed using the DC Protein Assay Kit (Biorad). 15μg of total extracts were separated on a 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (iBlot Gel Transfer Stacks Nitrocellulose, ThermoFisher). After blocking with 5% non-fat dry milk, TS1X Buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl), and 0.3% Tween-20 for 1 h, membranes were incubated with primary antibodies overnight at 4°C, and then with secondary antibodies for 2 h at room temperature. The Clarity™ Western ECL Substrate (Biorad) was used for the detection of the HRP-conjugated secondary antibody. The list of antibodies is shown in Supplementary Table 1.

Immunofluorescence

I-neurons were fixed in 4% paraformaldehyde (20 min, 37°C) at 42 culture days for morphological analysis and at 100 days for cortical neuron characterization with specific mature markers and *UBE3A*. IPSC-derived neurons (i-neurons) from AS patients and controls, cultured without and with DJNK11, were double-stained with MAP2 and TUJ1 neuron-specific markers. Primary antibodies in gelatin dilution buffer (0.2% gelatin, 0.3% Triton-X-100, 20mM Sodium Phosphate Buffer pH 7.4, 0.45M NaCl, all by Sigma) were incubated overnight at 4°C. The appropriate secondary antibodies were then incubated for 2 h at room temperature. Nuclei were stained with DAPI. 1024×1024-pixel images were acquired with a Nikon Ti Eclipse-C2-SH confocal microscope with a 40X magnification for arborization and neuronal length measurement, and with a 60X objective for the analysis of protrusions along neurites. The list of antibodies is provided in Supplementary Table 1.

Analysis

Morphological analyses, carried out on 42-day i-neurons (young neurons) from AS patients and controls, were performed on two clones for each patient and matched with a set of controls. This analysis was performed at two different magnifications, 40x and 60x, on isolated neurons.

We used ImageJ software (NIH) and its Skeletonize Plugin (<https://imagej.net/plugins/analyze-skeleton>) for the analysis of about 50 cells for clone and two independent experiments were performed. The images of each neuron were binarized, skeletonized, and analysed by the software to calculate the average length of branches, the number of end points per cell, and the neuronal length (Supplementary Fig. 4). The analyses were performed by three distinct operators, all of whom produced identical outcomes. The number of primary neurites (i.e. derived from the soma) was manually counted to categorize uni/bipolar versus multipolar (≥ 3 primary neurites per cell) neurons; these results are expressed in pie charts and reported in histograms with the percentages of multipolar and unipolar/bipolar cells. For the protrusions' analysis, at least 50 sections (obj 60x, with 50μm size) were examined for every clone.

Single-cell motility assay

Progenitors at 35 days were seeded on Willco-dish glass-bottom dishes (GWST-3512, WillCo Wells BV, Amsterdam, NL), at a density of $25\text{--}50 \times 10^3$ cells/cm² and cultured in a standard neuronal-differentiation medium. At a range of time points, after 3–7–15 days in vitro (D3, D7, D15; ± 1 day), living-cell imaging was performed

using an inverted Nikon-TiPSF wide-field microscope (Nikon, Japan), equipped with an incubated chamber coupled to the microscope (Okolab, Italy) and an air 20x (NA 0.45, Plan-Fluor) objective, for 5–6 h, sampling every minute. Movies were analyzed with the ImageJ manual tracking plugin MTrack, similarly to our previous work [41, 42]. We tracked soma (i.e. the center of cell soma) or neurite tip movements every minute. We measured:—the soma *speed* (the length of the track from the start point to the last point divided by time, in $\mu\text{m}/\text{min}$);—the soma *displacement* (i.e. the displacement from the start point of the track to the last point, here reported in μm). We analysed all the cells with a neuronal-like phenotype visible in each image (not in clusters); cells moving on an underlying layer of glial cells were discarded. For neurite tips, we measured:—the neurite *speed* (in $\mu\text{m}/\text{min}$);—the neurite *displacement* (in μm ; the mean displacement with respect to the initial position). We analysed all the visible neurites of cells with a neuronal-like phenotype in each image.

We tested i-neurons derived from 9 different clones: 5 CTRLs versus DEL1 (2 clones), DEL2 and DEL3, always run in couples (one CTRL and one AS, but blindly for their genotype), analysing at least 10 neurons/sample, in a blind. Data are reported as the average value $\pm$ the standard error of the mean (mean $\pm$ SEM), with $n \geq 3$ independent samples for each condition (at least 50 soma or 59 neurites per condition in total); the mean values obtained in each repeated experiment were assumed to be normally distributed about the true mean. Data were statistically analyzed by two-Way ANOVA to compare more than 2 conditions and groups. Statistical significance refers to results where $P < 0.05$ was obtained.

Electrophysiological in vitro recordings

Patch-clamp recordings were performed on i-neurons obtained from control subjects and AS patients DEL1, DEL2, DEL3, and UBE3 N-ter (Table 1). Recordings were carried out serially at differentiation weeks 14, 15, and 16. Recordings were acquired using an Axopatch 200B amplifier and pClamp-10 software (Axon Instruments). Recording pipettes were fabricated from borosilicate glass capillary (World Precision Instrument) using a two-stage puller (Narishige) and had a tip resistance of 3–5 M Ω . Recordings were conducted in Krebs'-Ringer's-HEPES (KRH) external solution (NaCl 125 mM, KCl 5 mM, MgSO₄ 1.2 mM, KH₂PO₄ 1.2 mM, CaCl₂ 2 mM, glucose 6 mM, HEPES-NaOH pH 7.4 25 mM); pipettes were filled with the potassium gluconate-based intracellular solution (KGluc 130 mM, KCl 10 mM, EGTA 1 mM, HEPES 10 mM, MgCl₂ 2 mM, MgATP 4 mM, GTP 0.3 mM).

In order to identify electrophysiological events (spontaneous excitatory synaptic events and neuronal spiking activity), cells were held at -70 mV and patch-clamp recordings were performed in whole-cell or cell-attached configuration (injected current = 0), respectively (as in Pizzamiglio et al., 2021) [43].

Membrane properties, including input resistance and membrane capacitance (for whole-cell recordings), were monitored throughout the experiments. Series resistance was regularly assessed, and only recordings with stable access were included. Cells were excluded from analysis if series resistance exceeded 20 M Ω or varied by more than 20% during the recording. Cells displaying unstable holding current, large changes in input resistance, or excessive leak currents were also excluded from analysis.

The recorded traces were analyzed offline using Clapfit-pClamp 10 software, applying a manually-defined threshold for event detection.

For pooled analyses, frequency, amplitude, and charge data from each patient were normalized to the mean value obtained from the control patient pool, which was set to 1. For developmental-stage analyses (weeks 14, 15, and 16), all measurements were normalized to the mean value of the control patient at week 14, which was used as the common reference condition. Raw data are reported in the Supplementary Table 3.

DJNKI1 treatment

DJNKI (AM-111 MedChemExpress) was diluted in distilled water at 5 μM concentration and added to cortical neurons at each media change for 1 week. For morphological analysis and the study of early biomarkers, it was administered from 35 to 42 differentiation days, while to carry out electrophysiological recordings and Western blot experiments, the drug was administered at a greater degree of maturation, from day 93 to day 100 of the same neuronal cultures.

Statistical analysis

Datasets were used for the statistical analysis, and data were expressed as average value $\pm$ the standard error (mean $\pm$ SD), except where differently stated. Statistical analysis was performed using Graph Pad Prism 7 program. For non-parametric data (after Shapiro-Wilk normality test), One-way ANOVA, followed by Kruskal-Wallis and Dunn's post hoc test, was used, if not otherwise stated. Sidak correction test was used for the analysis of the effect of DJNKI on the JNK pathway (Fig. 6 E). Conversely, for multipolarity studies (comparison between uni/bipolar versus multipolar cells), we used the Mann-Whitney test (Fig. 3 G). The specific P values (considered significant for $P < 0.05$) are shown on the respective graphs.

Results

iPSC-derived cortical neurons model the four pathological mechanisms of AS

To represent AS genetic heterogeneity, we reprogrammed erythroblasts into iPSCs from a cohort of 11 patients, carrying the different genetic alterations known to cause AS (Table 1 and specific session in methods). iPSC were obtained from all the patients and characterized for stemness by immunostaining of the pluripotency markers OCT3/4 and Tra-1-60 (Fig. 1A and Supplementary Fig. 1A) and by assessing the expression of OCT3/4, SOX2, and NANOG by RT-PCR (Supplementary Fig. 1B). To exclude cytogenetic and submicroscopic rearrangements, which might occur during in vitro culture, genomic stability was verified by karyotyping and SNP-array analyses (Supplementary Fig. 1C, D). The whole panel of karyotypes and SNP arrays of each clone involved have been uploaded in the raw data. No chromosomal anomalies were identified, while some CNVs were detected by SNP Array, but the clones carrying anomalies were discarded. The presence of the expected genetic alteration in each clone was confirmed by DNA sequencing for *UBE3A* pathogenic variants, while deletion, uniparental disomy, and imprinting defects were assessed by MS-MLPA and SNP array as described in methods.

iPSCs were then differentiated into cortical neurons, in accordance with the monolayer protocol well-established in our laboratory [44, 45]. The i-neurons obtained properly expressed neuronal markers (Fig. 1B).

Clinical features of AS patients

We selected a restricted cohort of AS subjects, whose i-neurons are the main focus of this study. The cohort includes two boys harboring a BP1-BP3 deletion (DEL1 and DEL3), a girl with BP2-BP3 deletion (DEL2), a boy carrier of a heterozygous variant leading to an N-terminal stop codon (*UBE3A* N-ter), a boy with an in-frame deletion (*UBE3A* in frame DEL) and a girl with a late truncating variant (*UBE3A* C-ter). Only one individual carrying UPD(15)pat was selected. The clinical features of the patients and their specific pathogenic variants in *UBE3A* cases are described in detail in Table 1, and in Supplementary Table 2 the additional signs are reported. The three patients with deletion and the boy carrier of the *UBE3A* N-ter stop codon displayed a severe intellectual disability; as expected, patients with the in-frame deletion, *UBE3A* C-ter variant, and UPD(15)pat achieved better cognitive and motor performance.

Multi-level characterization of iPSC-derived cortical neurons

As first, the amount of *UBE3A* mRNA and protein were measured in i-neurons. mRNA expression levels were analyzed in mature cortical neurons from one clone of DEL1, DEL2, DEL3, *UBE3A* N-ter, *UBE3A* C-ter, in-frame and UPD(15)pat by qRT-PCR experiments (Supplementary Fig. 5A). A highly significant reduction in mRNA expression of *UBE3A* gene was confirmed for all AS samples, except for *UBE3A* C-ter case, which showed expression levels comparable to those of healthy controls.

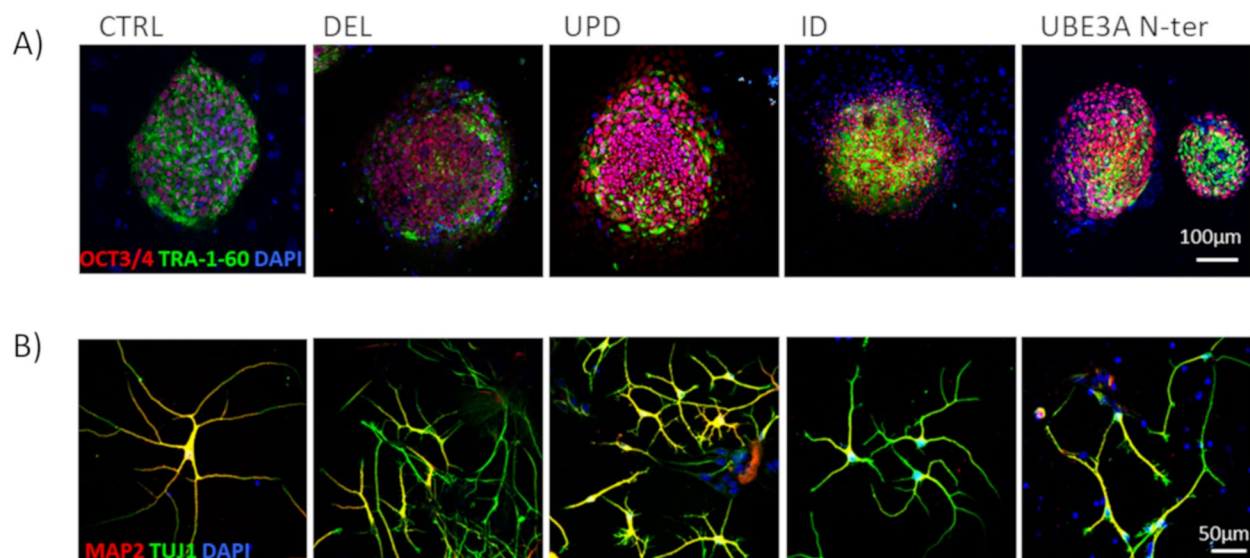


Fig. 1 Cohort of AS patients and derived in vitro models. **A** iPSC clones derived from AS patients, representative of the four known genetic mechanisms causing Angelman syndrome and stained with the pluripotency markers OCT3/4 (red) and TRA-1-60 (green). Nuclei were counterstained with DAPI (blue). Scale bar: 100 μm. Images acquired with a 20× objective. **B** Cortical neurons differentiated from iPSCs, showing the expression of the neuronal markers MAP2 (red) and TUJ1 (green), with nuclei labelled with DAPI (blue). Scale bar: 50 μm. Images were acquired using a 40× objective.

Immunofluorescence and Western blot results revealed a significant decrease in UBE3A protein levels in AS patients-derived i-neurons compared with healthy control i-neurons (Supplementary Fig. 5B, 5C and Supplementary Fig. 6A, 6B).

As shown in Supplementary Fig. 5D–5F, mature i-neurons (100 days) showed the presence of neuronal markers as CUX1 (specific of cortical neurons), VGLUT1 (specific of excitatory neurons), and synaptophysin (specific for a synaptic protein), together with an almost null presence of UBE3A. These results demonstrate that we successfully generated cortical, excitatory, and mature neurons, representative of AS human neuronal models.

Morphological analyses of human cortical neurons

As a deficit in dendritic arborization and synaptic deficit have been reported in adult mice [46], we investigated the neuronal morphology at a precocious timepoint (42 days of differentiation, one week after the neural progenitor stage), when the i-neurons were still cultured at low density and could be easily analyzed as isolated cells. Morphological analyses were carried out on young i-neurons from DEL1, DEL2, DEL3, and UBE3A N-ter.

In Fig. 2A, representative images of i-neurons is shown for each genotype. Data from i-neurons of DEL1, DEL2 and DEL3 clones pooled together (pool DEL) and from i-neurons of two clones of UBE3A N-Ter (pool UBE3A N-ter) are represented in Fig. 2B–C–D. A marked increase in the number of filopodia-like protrusions (expressed as End Points Number) (Fig. 2B), a significant reduction in average branch length, without distinction between dendrites and axons, (Fig. 2C), and neuronal length (Fig. 2D) were observed in i-neurons from DEL1, DEL2, DEL3 and UBE3A N-ter patients.

At this early developmental stage, dendritic arborization exhibited a markedly reduced complexity in AS patient-derived i-neurons compared to controls. By comparing the frequency distribution of different neuronal morphologies (i.e. unipolar, bipolar, multipolar) across i-neurons, we found that i-neurons from patients displayed an higher proportion of uni- and bipolar cells ($66.01\% \pm 1.9$ SEM pool DEL and $69.19\% \pm 4.7$ SEM pool UBE3A N-terT respectively). In contrast, control cells demonstrated the opposite distribution, with a predominance of multipolar cells (70.35%), defined by the presence of three or more neurites projecting from the soma (Fig. 2E).

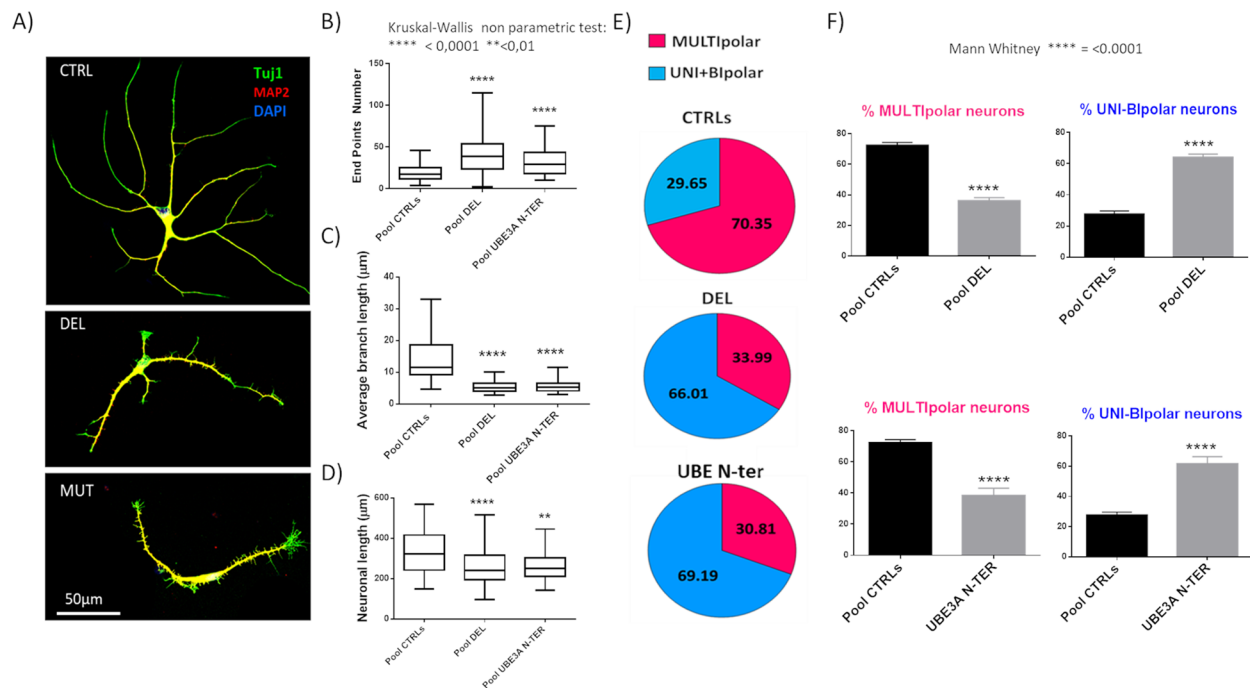


Fig. 2 Morphological analyses of young cortical neurons **A** Confocal images of young cortical neurons (42 days of differentiation) from a healthy control (CTRL), a patient with a deletion (DEL), and a patient with a UBE3A mutation (UBE3A N-ter). The cells were stained with the neuronal markers MAP2 (in red) and Tuj1 (in green), and the nuclei were counterstained with DAPI (in blue). **B–D** Quantitative analysis of neuronal morphology: **B** number of neurite terminations (End Points), **C** average branch length/cell, and **D** neuronal length/cell (μm). Data were obtained from at least 50 cells per condition, analysing three deletion cases, two clones for each line (Pool DEL) and one UBE3A mutation case, but two different iPSC clones (Pool UBE3A N-ter) were used. **E** Pie charts depicting the distribution of multipolar versus uni-/bipolar neurons in the control and different AS i-neurons. **F** Bar graphs comparing the percentage of multipolar (left panels) and uni/bipolar i-neurons (right panels) between data pooled from control samples and from AS patients—either deleted (top graphs) or mutated (bottom graphs). Statistical analyses were performed via unpaired t tests and Mann–Whitney nonparametric tests (GraphPad Prism); p values are indicated in the graphs

These findings suggest a significant delay in the early stages of neuronal maturation and branching complexity associated with the pathological condition, when compared to the control group (Fig. 2F). While this phenotype likely reflects a slower rate of neurite morphogenesis rather than a complete failure in establishing polarity, it highlights a precocious developmental defect that aligns with the long-term arborization deficits observed in animal models.

Morphological analysis of filopodia-like protrusions

At the same timepoint, morphological analyses were replicated at higher magnification to identify details and quantify the number of neurite protrusions with a filopodia-like shape, which may represent an indicator of neuronal maturation.

Two clones for each sample (DEL1, DEL2, DEL3 and UBE3A N-ter) were analyzed and matched with a set of healthy controls (Fig. 3A). Only cells positive for both MAP2 and TUJ1 pan-neuronal markers were included in the analysis to exclude glial cells. We confirmed a significantly increased density of immature protrusions in neurons from deleted and UBE3A N-ter patients compared with control cells ($P < 0.0001$, Fig. 3B). The quantifications were performed on i-neurons stained by MAP2 and TUJ1.

As the cytoskeleton of dendritic spines is primarily composed of filamentous (F)-actin, the same neurons were stained with two additional markers expressed in dendritic protrusions: DREBRIN, an actin-binding protein, highly enriched in dendritic postsynaptic spines and involved in early synaptogenesis (Fig. 3C) and Phalloidin (Fig. 3D). An increased number of protrusions in AS patients-derived neurons was confirmed with all the three markers.

Neuronal motility

We investigated the migration behavior of our i-neurons, cultured on standard glass dishes and imaged by time-lapse bright-field microscopy for several hours. Their movements were tracked at different stages of differentiation in vitro (D3, D7, D15) (Fig. 4A). Neurons were more mobile at the early stage, and their mobility decreased with advancing differentiation. The speed of neuronal soma migration decreased after D3, then remaining stable at D7 and D15, for both CTRL and DEL1, DEL2 and DEL3 samples ($P = 0.0176$ for time in vitro, 2-way Anova; Fig. 4B). The mean net soma displacement with respect to the initial position (i.e. the distance reached from the $t = 0$ position) was not influenced by either time or genotype, and was equal between the two populations over time (Fig. 4C).

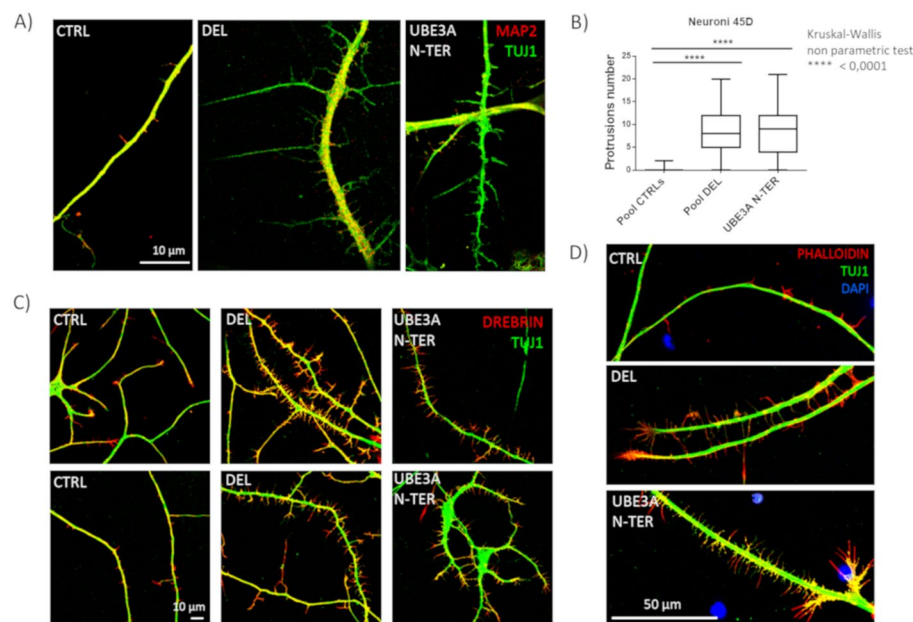


Fig. 3 Protusion analyses. We strictly selected fields of view that exhibited a comparable and uniform cell density across all experimental groups and conditions. All images were acquired using a 60x objective to ensure high-resolution visualization. The quantification was performed by analyzing standardized 50 μm sections to ensure consistency. The Number of 50 μm sections analyzed was: Pool CTRLs 106 sections, Pool CTRLs + DJNKI 106, Pool DEL 135, Pool DEL + DJNKI 135, Pool Mut N-TER 51, and Pool Mut N-TER + DJNKI 45. **A** Confocal images of 42-day differentiated neurons immunostained with MAP2 (in red) and TUJ1 (in green) and analysed for protrusion quantification; scale bar = 10 μm . **B** The graph summarizes the quantification results of the number of protrusions. Statistical analysis was performed via GraphPad Prism; the test used and corresponding p values are reported in the figure. **C-D** Visualization of neuritic protrusions in AS neurons via two independent markers: **C** Drebrin immunostaining (red) and **D** phalloidin labelling (red). TUJ1 is shown in green, and nuclei are counterstained with DAPI (blue). Images in **C** were acquired with a 20x objective; **D** was acquired with a 60x objective. Scale bars are indicated in the images

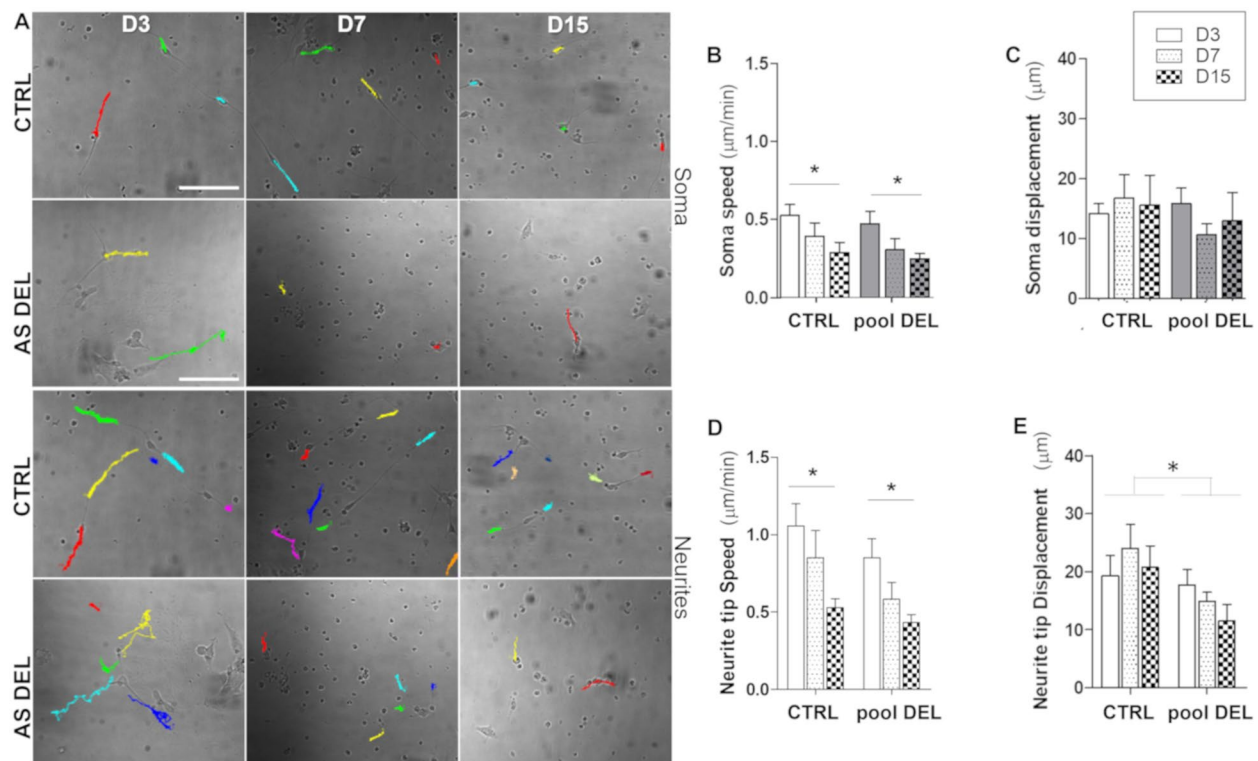


Fig. 4 IPCS-derived neuron Motility. **A** Representative bright-field images of CTRL and DELs from cortical IPCS-derived neurons and their soma and neuritic tip migration patterns (colored tracks) at different time points, D3, D7 and D15 (scale bars = 100 μm). **B–C** Soma migration of CTRL (white) and pooled DEL (gray) neurons at different times in vitro, D3, D7 and D15: b) Soma speed; * $P = 0.0176$; two-way ANOVA for time in vitro; c) Soma displacement (from the $t = 0$ position). Data are presented as the means $\pm$ SEMs, $n \geq 4$; at least 50 neurons were measured for each condition. **D–E** Neuritic tip mobility for CTRL (white) and pool DEL (gray) neuritic tips at D3–7–15: d) neurite speed; * $P = 0.0114$ two-way ANOVA for time in vitro; e) neurite displacement (from the $t = 0$ position); * $P = 0.0289$, two-way ANOVA for genotype. Data are presented as the means $\pm$ SEMs, $n \geq 3$; at least 59 neurites were measured for each condition

We then quantified the mobility of neurites, by tracking the protrusion/retraction of tips (Fig. 4A). Neuritic tips were more mobile than the soma displaying a migration speed that was nearly twice as high, although they exhibited a similar temporal pattern. Neurites from CTRLs and the three cases explored their surroundings at higher speed during early time in vitro, then slowed down their velocity with the differentiation time ($P = 0.0114$ for time in vitro, 2-way Anova; Fig. 4D). Neurite displacement relative to the initial position remained similar in CTRL neurons at all times, while neurons from DEL1, DEL2 and DEL3 showed a progressive reduction in displacement with the increase of the differentiation time in vitro (Fig. 4E). In this analysis, neurite displacement was tuned by genotype ($P = 0.0289$ for genotype, 2-way Anova; Fig. 4E).

Overall, the mobility of i-neurons decreases with the differentiation time in vitro. At the soma level, the migration abilities were similar between CTRL and Angelman samples, at all time points. Neurites were more mobile than soma but still showed a decrease in neurite migration speed with the increase of time in culture. Specifically, DEL1, DEL2 and DEL3 exhibited migration speed

comparable to those of CTRL neurites but showed a lower final displacement, thus suggesting more frequent back and forth movements and less efficient directional exploration.

Analysis of synaptic activity indicates electrophysiological changes in AS i-neurons

Investigation of morphological features revealed severe defects in i-neurons, as indicated by the higher number of filopodia-like (immature) protrusions and reduced length of neuronal branches. Thus, we tested their ability to generate functional synaptic activity as hallmarks of cellular maturation and proper developmental progression. To this end, patch-clamp recordings were conducted on eight iPSC lines derived from four patients and three control subjects: DEL1 (two clones), DEL2 and DEL3 patients, two clones from one patient with UBE3A-N-ter mutation and individual clones from three control subjects. Based on visual inspection, all recorded cells were identified as sparse bipolar neurons. Voltage clamp were then performed to monitor spontaneous excitatory post-synaptic currents (sEPSCs; synaptic events, Fig. 5A) and cellular spiking activity (recordings

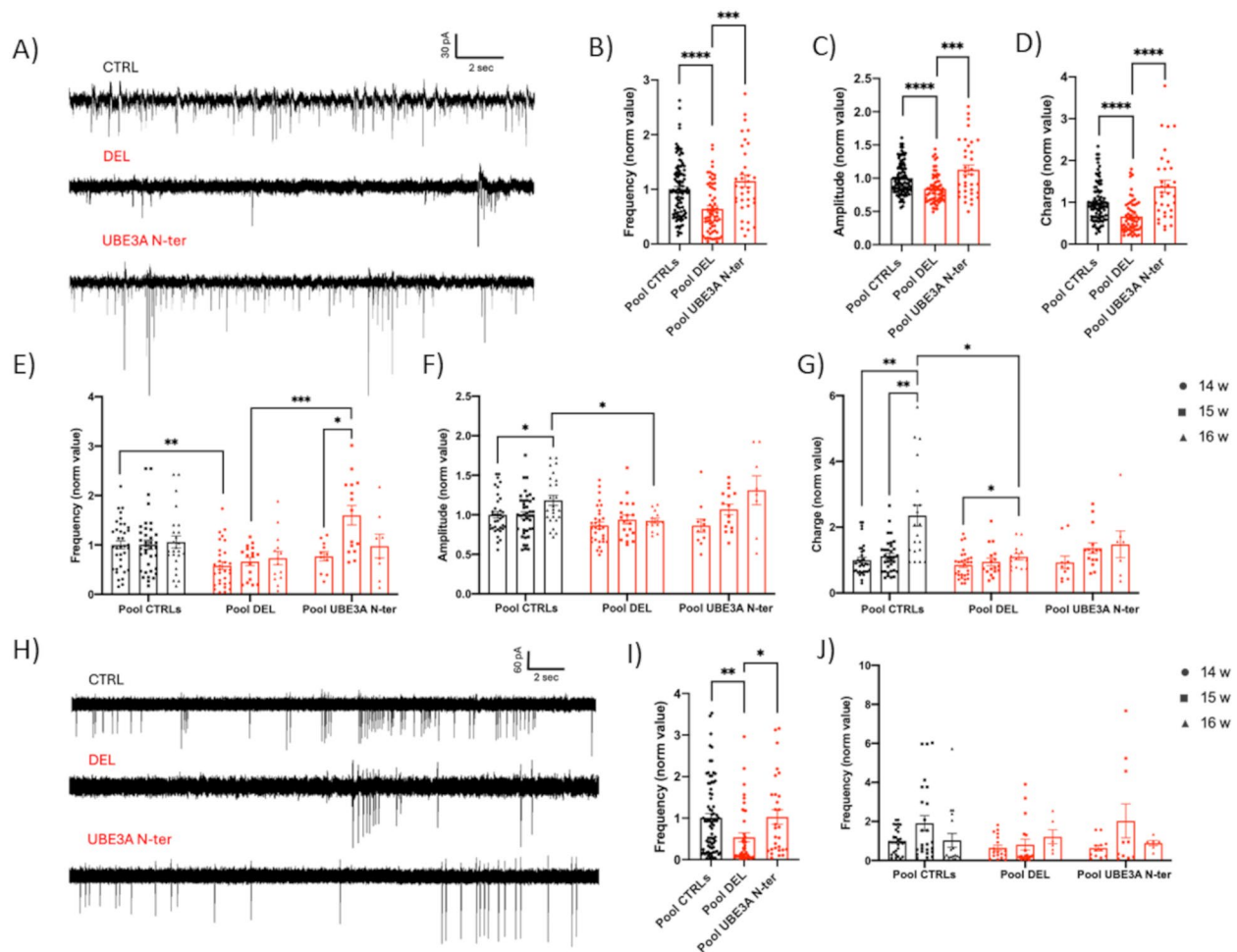


Fig. 5 Electrophysiological recordings. **A** Representative electrophysiological traces of sEPSC events recorded in 16-week-old i-neurons established from controls (CTRL) and patients with UBE3A gene deletion (DEL) or UBE3A gene truncation (UBE3A N-Ter). **B, C, D** Histogram plots of sEPSC frequency, amplitude and charge obtained by pooling data from different i-neurons recorded at 14, 15, and 16 weeks. Each dot represents the value of the corresponding parameter estimated from a single neuron. (Frequency: number of recorded cells, CTRLs = 103, DEL = 64, UBE3A N-Ter = 35; Kruskal–Wallis test, followed by Dunn’s multiple comparisons test; Amplitude: Kruskal–Wallis test, followed by Dunn’s multiple comparisons test; Charge: Kruskal–Wallis test, followed by Dunn’s multiple comparisons test). **E, F, G** Histogram plots of sEPSC frequency, amplitude and charge recorded in i-neurons from CTRL and AS patient lines at 14, 15 and 16 weeks (i.e., over time) (frequency: two-way ANOVA with genotype and time as factors, followed by Tukey’s multiple comparisons test). Amplitude: Two-way ANOVA with genotype and time as factors, followed by Tukey’s multiple comparisons test. Charge: Two-way ANOVA with genotype and time as factors, followed by Tukey’s multiple comparisons test. **H** Representative electrophysiological traces of MU (Multi Unit) events recorded in 16-week-old i-neurons. **I** Quantitative analysis of MU frequency, where each dot represents the frequency value from a single neuron at 14, 15, and 16 weeks. (n. of recorded cells: CTRLs = 77, DEL = 41, UBE3A N-ter = 29; Kruskal–Wallis test, followed by Dunn’s multiple comparisons test). **J** Histogram plot of MU frequency over time (14, 15, and 16 weeks) (two-way ANOVA with genotype and time as factors, followed by Tukey’s multiple comparisons test)

in loose cell-attached configuration, Fig. 5H). Indeed, the limited mechanical stability of less differentiated cells and the low abundance of more mature multipolar neurons in these cultures were restricted to the subset of neurons that could be reliably maintained during patch-clamp experiments. Results in Fig. 5B, C, D indicate reduced frequency, amplitude and charge of sEPSCs recorded in pools of 14–16 weeks DEL i-neurons compared with controls. In contrast, no significant changes were observed in the context of UBE3A gene truncation (UBE3A N-ter), indicating that the immature structural phenotype correlates with functional defects in the

context of UBE3A deletion but not in the case of UBE3A N-ter truncation, where near-normal synaptic activity was unexpectedly observed. Also, analyzing sEPSCs frequency, amplitude and charge against time in CTRL and AS-derived neurons, we found specific differences. In CTRL and UBE3A N-Ter, sEPSCs frequency remained constant and similar between the two groups over time, suggesting that the maximal efficiency of synaptic vesicles recycling has already been reached by week 14 or that an appropriate abundance of presynaptic terminals is mainly established at this stage (Fig. 5E). Interestingly, sEPSCs frequency in the UBE3A N-ter i-neurons was

higher compared to the CTRL during development (div 107). Furthermore, regarding sEPSCs amplitude (Fig. 5F), in CTRL i-neurons we measured increasing values over time, consistent with network maturation and developmental insertion of AMPA receptors. This phenomenon is still evident in UBE3A N-ter i-neurons but is totally prevented in those established from DEL patients, where sEPSCs amplitude remains similar across 14, 15 and 16 week-old cultures (Fig. 5F). The data on charge further support the notion of a significant block in maturation in this genetic context (Fig. 5G). Finally, to assess the ability of AS i-neurons to establish spontaneous spiking activity, we measured single-neuron spiking activity in voltage-clamp recordings performed in the cell-attached configuration (current injected = 0; Fig. 5I). The analysis revealed a significant reduction in terms of spiking frequency (Fig. 5I), suggesting that neurons with an *UBE3A* gene deletion fire less frequently within the network compared to CTRL cells, possibly due to a lower integration of excitatory currents at the synapse. Once again, no changes occurred in the case of *UBE3A* gene truncation (*UBE3A* N-ter). When plotting spiking frequency over time (Fig. 5J), we observed that CTRL and *UBE3A* N-ter i-neurons followed a comparable developmental trajectory, whereas *UBE3A* DEL cultures exhibited a distinct pattern, with consistently lower values and only a slight increase at later time points, further supporting the presence of impaired functional maturation in this genetic context. Overall, electrophysiological analysis of glutamatergic transmission displays marked defects in i-neurons with BP1-BP3 and BP2-BP3 deletion and a fairly normal behaviour in *UBE3A* N-ter mutations, suggesting the possibility of compensatory mechanisms in the glutamatergic system, specifically in *UBE3A* N-ter truncation.

c-Jun N-terminal Kinase (JNK) pathway activation in Angelman Syndrome i-neurons

The analyses performed in this study highlighted significant synaptic dysfunction in AS i-neurons, suggesting that these abnormalities might be amenable to pharmacological modulation through JNK inhibition. Moreover, a previous study [29] demonstrated JNK activation and the therapeutic efficacy of DJNKI1 in AS mouse models. Before exploring the therapeutic potential of JNK inhibition in ameliorating Angelman syndrome phenotype, we first investigated the JNK pathway dysfunction in the patient-specific i-neurons, focusing on cultures derived from patients with the most severe clinical features.

JNK3 activation can be measured using the p-JNK/JNK ratio, which increases in the presence of stress due to JNK3 autophosphorylation [47]. Thus, the p-JNK/JNK ratio was studied by WB in 100-day-old neurons derived from DEL1 (two clones), DEL2, and DEL3 patients together with four healthy controls (Fig. 6A). Data

from the ratio in individual clones are shown in Fig. 6B, whereas the comparison between p-JNK/JNK ratio in the pool of deleted neurons versus that of controls is reported in Fig. 6C. The results highlight the activation of the JNK pathway in AS neurons, as proved by a significantly increased ratio between pJNK/JNK (Supplementary Fig. 7).

DJNKI1 delivery rescues morphological and functional changes of AS i-neurons

D-JNKI1 (AM111- MedChem Express, distributed by DBA Italy) was diluted to final concentrations of 2 μ M and 5 μ M and administered to i-neurons from DEL1 and DEL2 and one healthy control at 93–100 days of differentiation. The most effective concentration was 5 μ M D-JNKI1. Western blot experiments were performed on mature cortical neurons (100 differentiation days). Analysis of individual clones revealed a significant rescue of JNK pathway activation levels in deleted neurons following D-JNKI1 treatment, whereas no significant effect was observed in control cultures (Fig. 6D, 6E and Supplementary Fig. 8).

Recovery of morphological biomarkers

To examine whether or not D-JNKI1 could ameliorate the cellular abnormalities previously observed in AS i-neurons, 5 μ M D-JNKI1 was added to cultures derived from DEL1, DEL2, DEL3, and *UBE3A*N-ter patients at each medium change.

As shown in Fig. 7A, the acute DJNKI1 treatment significantly rescued the morphological phenotype in all analysed clones, with an impressive reduction of immature protrusions. Figure 7B summarizes the quantitative analysis performed in three different pooled controls, three pooled deletion cases (including two DEL1 clones), and two clones derived from the *UBE3A* N-ter patient.

The graphs with all the individual test fields are in the Supplementary Fig. 8.

The comprehensive analysis of morphological parameters demonstrated that DJNKI1 significantly reduced the number of neuritic terminal points (protrusions) (Fig. 7C), and increased average branch length (Fig. 7D), whereas neuronal length was not significantly improved (Fig. 7E).

Impact of DJNKI1 in the recovery of electrophysiological parameters

Furthermore, electrophysiological recordings of DJNKI1-treated i-neurons revealed a significant rescue of sEPSCs frequency (Fig. 7F–G) with a slight increment in the sEPSCs amplitude (Fig. 7H), suggesting that JNK pathway dysregulation contributes, at least in part, to AS neuronal dysfunction. Following treatment, sEPSC charge remained impaired in AS neurons, reflecting AMPA

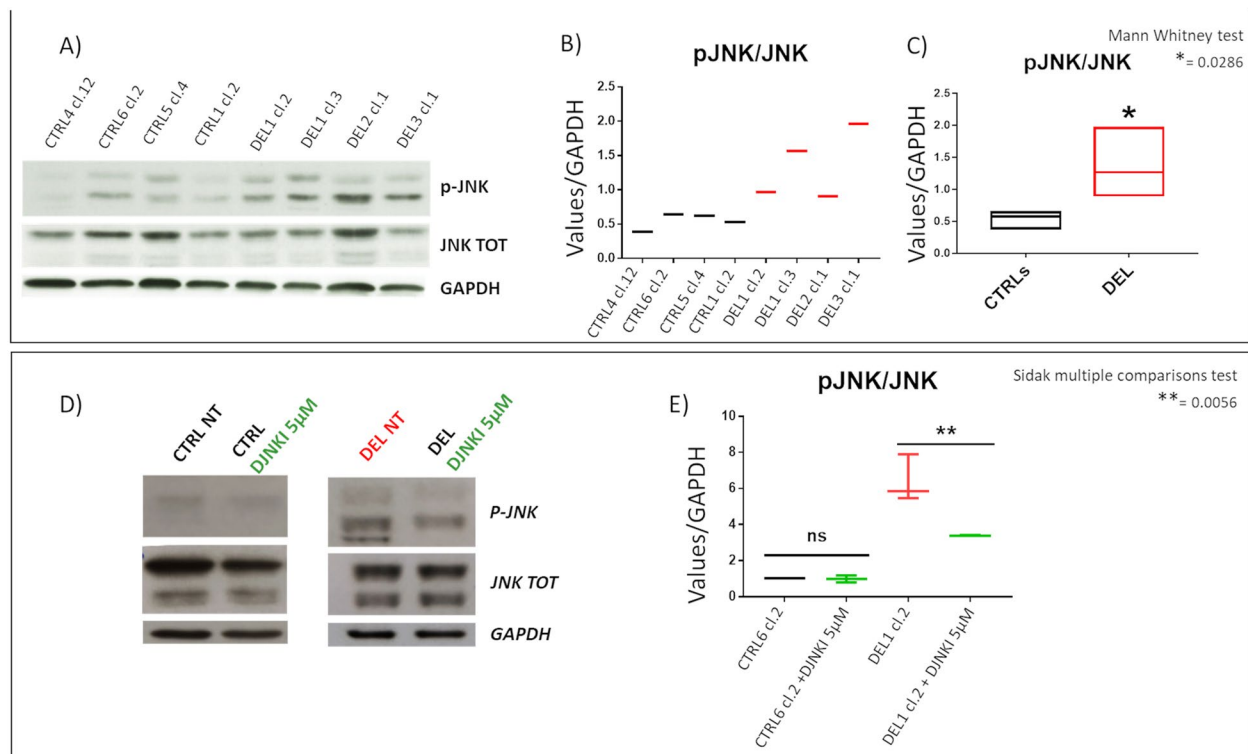


Fig. 6 Analysis of JNK pathway activation in mature cortical neurons from AS patients **A** Representative Western blot showing the expression of the two phosphorylated bands (52–54 kDa) of JNK (pJNK), total JNK (52–54 kDa), and GAPDH (38 kDa) in mature i-neurons (> 100 days) derived from four control individuals and three Angelman syndrome (AS) patients with deletions (including two distinct clones from patient DEL1). **B** Quantification of JNK pathway activation, expressed as the pJNK/JNK ratio, in neurons from four controls and four AS patients with deletions. Densitometric analysis was performed via ImageJ software on the basis of the blot in panel **A**. **C** Comparison of the pJNK/JNK ratio between pooled control and deleted AS patient samples. Quantification was performed via ImageJ, and statistical analysis was conducted via GraphPad Prism. The used tests and p-values are indicated in the figure. **D–E** Evaluation of the effect of a 7-day treatment with DJNKI1 (5 μ M) on the JNK pathway in mature cortical neurons derived from AS patients and controls. A significant reduction in pathway activation after treatment is shown via Western blotting (**D**), with the corresponding quantification in panel (**E**). Statistical analysis was performed via one-way ANOVA followed by Sidak's multiple comparisons test; the p-value is reported in the graph

receptor kinetics and indicating no improvement in AMPA receptor gating (Fig. 7I).

Discussion

Several studies on AS and its underpinning mechanisms have been conducted using animal models carrying Ube3a-maternal deletion in neurons [48–50], which recapitulate the clinical phenotype and the monoallelic gene expression. However, given the differences and the complexity of human neurons, iPSCs and IPCS-derived-neurons have recently emerged as valuable tools for studying the disorder [51, 52]. Patient-specific models provide a unique opportunity to explore the basis of genotype–phenotype correlations. Indeed, children affected by deletion of the 15q11.2-q13 region or by specific truncating UBE3A variants suffer from more severe epilepsy, cognitive and communication deficits than children with UPD(15)pat or imprinting disorder. Several studies have reported iPSCs derived from patients carrying the most common AS genetic defects (i.e. BP1-BP2 and BP2-BP3 deletion), but only a limited number of reports describe

iPSCs from patients with UPD(15)pat and ID. To fill this gap, and based on the availability of a large cohort of nearly 200 AS probands diagnosed in our lab, we generated a collection representative of the major pathogenic mechanisms of AS, including three UBE3A pathogenic variants, differently affecting the protein, three UPD(15)pat and 2 ID. These human neuronal cell models enable the exploration and comparison of the consequences of AS genetic heterogeneity. Our data confirmed the loss of UBE3A protein in mature i-neurons. However, consistent with previous reports [5, 7], we observed a gradual decrease in UBE3A expression over time, further supporting the progressive silencing of the paternal allele during neuronal maturation [6]. Comparative analysis of the UBE3A protein expression revealed the absence of UBE3A transcript not only in neurons carrying maternal deletion or UPD(15)pat neurons, but also in UBE3A N-ter samples, likely as a consequence of RNA-mediated decay. In contrast, UBE3A transcript levels in the UBE3A C-ter sample were comparable to those observed in controls.

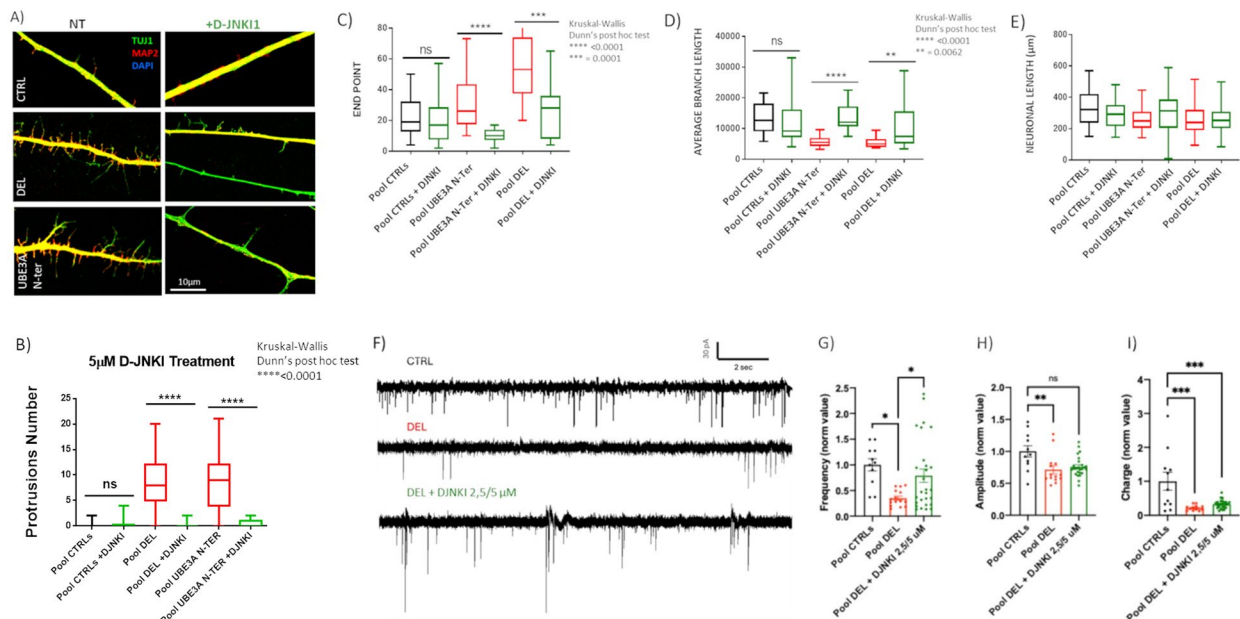


Fig. 7 Effect of DJNK1 treatment on morphological alterations and electrophysiological recordings after DJNK1 treatment. **A** The panel shows images of i-neurons derived from controls and patients, first (left column) and after 7 days of treatment with 5 μM DJNK1 (right column). Each image represents a section of 50 μm, acquired at 100X magnification, to determine the number of protrusions precisely. Neurons were immunolabelled with MAP2, TUJ1, and DAPI. The scale bar is indicated in the figure. **B** The graph shows the number of protrusions before and after 7 days of treatment with 5 μM DJNK1. The test was carried out on a pool of three controls, a pool of three patients with deletions, and two clones from a mutated patient. At least 50 50-μm sections were analysed for each clone of every sample. The counting procedure was carried out by three different operators. The data were analysed via GraphPad Prism, and the statistical tests selected for the study, along with their corresponding p values, are shown in the figure. The box plots presented in Fig. 7C, D, and E depict the impact of treatment with DJNK1 on early biomarkers identified through morphological studies and include endpoints in C, average branch length in D, and neuronal length in E. The statistical analyses and corresponding p values were computed via GraphPad Prism and are presented in the respective graphs. **F** Representative electrophysiological traces of sEPSC events recorded in 16-week-old i-neurons established from control (CTRL) and AS patients with UBE3A gene deletion (DEL) treated with or without DJNK1 at a concentration of 2,5/5 μM. **G, H, I** Histogram plots of sEPSC frequency, amplitude and charge recorded in each neuronal group (frequency: number of recorded cells, CTRLs=11, DEL=15, DEL + DJNK1=28; ordinary one-way ANOVA test, followed by Tukey's multiple comparisons test; amplitude: Kruskal–Wallis followed by Dunn's multiple comparisons test; charge: ordinary one-way ANOVA, followed by Holm–Sidak's multiple comparisons test)

For the first time, we demonstrate significantly altered neuronal morphology in AS i-neurons at early stage of in vitro development. At this developmental stage, connections among cells are at low density, facilitating accurate morphological measurements, as previously suggested [53]. i-neurons from patients with the most severe phenotype (Deletion and UBE3A early truncating variants) revealed a markedly immature phenotype compared with control neurons, showing reduced neuritic arborization and shorter neurites. Interestingly, we observed an increased number of terminal end-points in all the clones derived from the three biological replicates of the deleted patients and also N-ter truncation of *UBE3A*. Upon high-magnification analysis and immunostaining with βIII-tubulin for neuronal cytoskeleton, we were able to visualize in AS i-neurons an abnormal abundance of thin, needle-like protrusions, resembling dendritic filopodia. The staining with phalloidin, marker specific for filopodia, and drebrin, specific for synaptic spines also at immature stage, supported the nature of dendritic filopodia [54]. Future studies will be required to determine

whether an overexpression of drebrin contributes to the formation of these aberrant filopodia protrusions. These data are consistent with previous studies on AS animal models. For example, Furusawa [25] demonstrated in *Drosophila* class IV dendrite arborization sensory (C4da) neurons that Ube3a actively promoted presynaptic elimination, whereas Ube3a inhibition results in defective dendrite pruning. Similarly, in a study on the primary visual cortex of Ube3a m⁻/p⁺ mice, a longitudinal in vivo two-photon microscopy analysis revealed an increased proportion of thin spines versus other more stable types when compared with WT mice [46]. Moreover, several studies have reported reduced dendritic arborization in murine AS models [46, 55, 56]. Taken together, our results suggest that the loss of UBE3A promotes an early and aberrant accumulation of thin filopodia, which may subsequently interfere with proper dendritic branching and synapse formation. These findings support a role for *UBE3A* gene in dendritic growth and synapse remodeling processes, and may partially explain the neurodevelopment impairment.

Neuronal cells are generally characterized by limited mobility cells in vitro, and our i-neurons confirmed this tendency. We found that a progressive decrease in both soma and neurite motility with increasing differentiation time in both CTRL and AS populations. Our results confirm a general neuronal behaviour for i-neurons in vitro, and in fact, as neurons adhere to the substrate and establish connections, their migration tends to slow down. This reduction can be attributed to several factors (substrate properties, adhesion coating, and intrinsic programs).

At the level of soma, the migration abilities of i-neurons are comparable between control and AS neurons. In contrast, AS neurites exhibited reduced net displacement from their initial position, thus suggesting that they, although moving, may change direction more frequently, move less efficiently, or oscillate without making effective forward progress. These data suggest that AS neurites are less mobile with respect to the CTRL ones, and so may be less capable of exploring their environment, reaching appropriate targets and establishing connections. These results are consistent with previous findings in UBE3A-silenced SH-SY5Y cells, which displayed altered focal adhesion dynamics, delayed adhesion, and defective contact guidance in response to directional topographical stimuli, despite an overall preserved migration activity [42].

A previous study reported severe impairments in intrinsic electrical properties, synaptic transmission, and synaptic plasticity in iPSC-derived neurons established from AS patients with complete *UBE3A* deletion [21].

Here, we confirm these findings in neurons derived from patients carrying the same genetic deletion and extend the functional characterization to neurons harboring a truncating *UBE3A* variant. In this distinct genetic background, neurons exhibit only mild functional defects, highlighting the possibility of a genotype–phenotype variability in electrophysiological phenotype. Our data indicate that in neurons, while the *UBE3A* deletion cause both structural and functional deficits, probably due to a brake in the neuronal development and maturation, the *UBE3A* N-ter truncation primarily affect neuronal morphology while largely preserving glutamatergic transmission. Of note, in the present study we focused on voltage-clamp analyses and did not include investigation on passive properties already described in previous studies. Based on synaptic dysfunction observed, we evaluated the therapeutic potential of D-JNKI1, a molecule previously shown to be effective in animal models [29].

In this study, we observed that in cortical i-neurons derived from deletion patients exhibited the activation of the JNK3 pathway, as demonstrated by increased pJNK/JNK ratios. Importantly, treatment with the JNK3 inhibitor D-JNKI1 restored JNK pathway activity to

near-control levels. Moreover, the aberrant morphology, enriched with thin filopodia-like protusions, reverted with D-JNKI1 supplementation. Furthermore, functional experiments conducted in the presence of the inhibitor revealed the selective ability of the drug to rescue molecular alterations responsible for the reduced sEPSCs frequency detected. This effect may result from increased release of glutamatergic vesicles, a greater number of newly-formed excitatory presynaptic terminals, or enhanced survival of the existing excitatory presynaptic terminals. However, the lack of recovery in sEPSC amplitude indicates D-JNKI1 has limited effects on AMPA receptor trafficking or synaptic insertion. Together, these observations indicate that distinct pre- and post-synaptic compartments contribute to specific morphological and functional alterations in AS neurons.

Using patient-derived neuronal models, our study contributes to the elucidation of how the lack of *UBE3A* leads to aberrant neuronal maturation at a precocious time of neuron development, and highlights migration and functional deficits. Nonetheless, we acknowledge that a limitation of the present work is the lack of isogenic, CRISPR-corrected control lines to complement our patient-derived cellular panel. Although our study successfully models the distinct molecular classes of AS using a broad patient repository, subtle variations in individual genetic backgrounds could potentially influence or mask specific phenotypic traits. Future efforts will benefit from utilizing these established lines to engineer precise isogenic pairs via CRISPR/Cas9 technologies. This will include the correction of patient clones carrying *UBE3A* missense and terminating mutations, as well as the introduction of targeted chromosomal microdeletions into healthy control iPSC backgrounds to faithfully reproduce the classical structural BP1–BP3 and BP2–BP3 variants. While engineering such large deletions remains technically challenging, similar ambitious genome editing strategies have been successfully applied to create isogenic controls for Dup15q patient lines [57], significantly maximizing the experimental utility, specificity, and robustness of our human AS modeling platform. In parallel to addressing these genetic background variables, we investigated the deregulation of the JNK pathway in AS and the efficacy of D-JNKI1, one of the small molecules functioning as JNK3 inhibitors capable of preventing synaptic degeneration or dysfunction.

These data may pave the way for new therapeutic possibilities [58].

Abbreviations

AS	Angelman Syndrome
IPSCs	Induced Pluripotent Stem Cells
JNK	c-Jun N-terminal kinase
D-JNKI1	D-retro-inverso form of c-Jun N-terminal kinase-inhibitor
EEG	Electroencephalogram
UPD(15)pat	Paternal uniparental disomy of chromosome 15

ID	Imprinting Defect
BBB	Blood brain barrier
TAT	Transactivator of transcription
i-neurons	IPSC-derived cortical neurons
ASA	Angelman Syndrome Alliance
MEF	Mouse Embryonic Fibroblast
CNVs	Copy number variations
mEPSCs	Spontaneous excitatory post-synaptic currents

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s11689-026-09728-7>.

Supplementary Material 1. Supplementary Figure 2. Sanger sequencing analysis of iPSC clones from UBE3A-mutated patients. Sanger sequencing was performed on iPSC clones to confirm the presence of the original UBE3A gene mutations using mutation-specific primers. A) NM_130838.4:c.1039dup, resulting in p.(Ala348fs). B) NM_130838.4:c.2506_2509delAAAG, resulting in p.(Lys836ArgfsTer4). C) NM_130838.4:c.510_515delGGAAAA, resulting in p.(Glu171Lys172del).

Supplementary Material 2. Supplementary Figure 3. Confirmation of the genetic defect in iPSCs from patients with UPD, ID and UBE3A deletions. A) SNP array analysis of peripheral blood and iPSC clone 1 from a patient with uniparental disomy (UPD), using a healthy control as reference. The UPD is confirmed by a Log R Ratio centered around zero and a complete loss of heterozygosity (LOH) in the chromosomal region 15q11.2, demonstrating the maintenance of the genetic defect in the reprogrammed cells. B) Representative MS-MLPA analysis comparing peripheral blood and an iPSC clone from a patient with UPD, confirming the methylation defect. The black circle highlights the typical hypomethylation pattern in presence of normal copy number observed in UPD patients. C) SNP array analysis of peripheral blood and a corresponding iPSC clone from an Imprinting Defect (ID) patient showing a pattern similar to that of a healthy control, indicating no detectable copy number or allelic imbalance. D) MS-MLPA analysis performed on both peripheral blood and the same iPSC clone to verify the presence of SNRPN hypomethylation. The black circle highlights the hypomethylation pattern, confirming the preservation of the epigenetic defect in the iPSCs. The absence of CNVs as well as UPD confirmed the diagnosis of ID. E) Representative methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) analysis comparing peripheral blood and iPSCs from a patient with a BP1-BP3 deletion (DEL1), revealed by CNVs and hypomethylation in each sample.

Supplementary Material 3. Supplementary Figure 4. Workflow for semi-automated morphological analysis of cultured neurons using ImageJ. Representative pipeline illustrating the extraction of neurite morphometric parameters. (A) Merged confocal image (40x magnification) of a single neuron immunolabeled for TUJ1 (green) and MAP2 (red). (B) Isolated TUJ1 (green) channel selected as the target for morphological tracing. (C) Binary (black and white) image obtained after intensity threshold setting. (D) Skeletonized representation of the neuron. The yellow overlay highlights an individual branch, while fuchsia dots indicate terminal endpoints. (E) Representative output data table generated by the ImageJ Analyze Skeleton plugin. Red arrows emphasize quantified parameters for the selected skeleton (row 8), including total branch number, and average branch length.

Supplementary Material 4. Supplementary Figure 5. UBE3A expression and neuronal identity in AS-derived cortical neurons. A) UBE3A transcript levels in cortical neurons derived from a control subject, three patients with deletions, two with point mutations, and one with uniparental disomy (UPD). Statistical analyses were conducted using GraphPad Prism; tests and corresponding p-values are indicated in the figure. B) Immunofluorescence images of neurons derived from a control, a deletion case, and a patient carrying a UBE3A mutation: UBE3A in red, TUJ1 in green, and nuclei stained with DAPI (blue). Images were acquired with a 60x objective. Scale bar: 50 μ m. C) Western blot analysis of mature cortical neurons from AS patients compared to a pooled control sample. E6AP (102 kDa) expression was quantified relative to GAPDH (38 kDa) using ImageJ software. The graph below presents the quantification results. D–F) Immunostaining

analysis for neuronal subtype markers: (D) CUX1 (cortical marker, red), (E) VGLUT1 (excitatory marker, green), and (F) Synaptophysin (SYP; synaptic marker, red), in neurons derived from both a deleted and a mutated AS patient compared to control. Scale bars are included in each panel.

Supplementary Material 5. Supplementary Figure 6. Evaluation of Ube3A protein expression across different Angelman Syndrome genotypes. (A) Representative western blot analysis of Ube3A protein levels in neurons differentiated for 100 days. Protein extracts were obtained from a healthy control line (CTRL4) and Angelman Syndrome (AS) patient-derived lines: C-terminal mutation (UBE3A C-TER) associated with a mild phenotype, imprinting defect (ID), N-terminal mutation (UBE3A N-TER) associated with a severe phenotype, and paternal uniparental disomy (UPD). GAPDH was used as an internal loading control to ensure equal protein loading. (B) Densitometric quantification of Ube3A protein levels. Band intensities were quantified using ImageJ software and normalized to GAPDH. Data are expressed as relative protein expression (fold change) compared to the control group, which was set to 1.0. Bar graphs represent the mean $\pm$ SEM (or SD) of three independent biological replicates (n = 3). All AS-derived neurons show a marked reduction in Ube3A levels, with the lowest residual expression observed in the N-TER mutant line. Statistical significance was determined by one-way ANOVA followed by Dunnett's post-hoc test; *p < 0.05, **p < 0.01.

Supplementary Material 6. Supplementary Figure 7. Activation of the JNK signaling pathway in neurons of Angelman patients. A) Western blot analysis and B) corresponding densitometric quantification of phosphorylated JNK (p-JNK) relative to total JNK (JNK tot). Analysis was performed on cell lysates obtained from a healthy control and Angelman syndrome patients harboring distinct genetic alterations in the UBE3A locus. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Supplementary Material 7. Supplementary Figure 8. Morphological impairment and rescue in the single clones. Box Plot on the left illustrate the number of neurite protrusions quantified in three independent control samples (A), three patients with UBE3A gene deletions (B)—including two distinct clones from patient DEL1—and two clones from a patient carrying an N-terminal UBE3A mutation (C). Graphs on the right show protrusion counts before and after a 7-day treatment with DJNKI1 (5 μ M), aimed at evaluating the potential rescue of the morphological defect. All analyses were independently conducted by three operators. Data were analyzed using GraphPad Prism; the statistical tests and corresponding p-values are indicated in the graphs.

Supplementary Material 8. Supplementary Figure 9: number of cells analysed. This page contains the exact number of cells analysed to generate the data shown in each figure of this manuscript.

Supplementary Material 9. Supplementary table 1 : Primary And Secondary Antibodies Used.

Supplementary Material 10. Supplementary Table 2: Additional Clinical Features.

Supplementary Material 11. sEPSCs raw data – outliers excluded.

Supplementary Material 12.

Supplementary Material 13.

Supplementary Material 14.

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Authors' contributions

VA, AV, SP and SR generated iPSCs, VA, AV, SP and SR differentiated in i-neurons and characterized the i-neurons, MC performed the karyotype analyses of stem cell clones, VA and SP performed morphological analyses and D-JNKI1 administration; CC and FA recorded by patch-clamp the cells; CDC and IT

carried on the migration assays; SR recruited the patients, conceived and coordinated the study; VA, SR drafted the manuscript, FA and IT wrote the chapters relative to their experiments, LL contributed to the revision of the manuscript. All the Authors read and approved the text.

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Data availability

The data supporting the findings of this study and the supplementary tables and figure are openly available in Zenodo at <https://doi.org/10.5281/zenodo.17224410>

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Istituto Auxologico Italiano, IRCCS (protocol code 2019_09_24_03).

Consent for publication

All the parents of the AS children and controls signed informed consent forms.

Competing interests

The authors declare no competing interests.

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References

1. Dagli AI, Mathews J, Williams CA. Angelman Syndrome. In: Adam MP, Feldman J, Mirzaa GM, Pagon RA, Wallace SE, Amemiya A, eds. GeneReviews®. University of Washington, Seattle; 1993. Accessed August 13, 2025. <http://www.ncbi.nlm.nih.gov/books/NBK1144/>
2. Williams CA, Beaudet AL, Clayton-Smith J, et al. Angelman syndrome 2005: updated consensus for diagnostic criteria. *Am J Med Genet A*. 2005;140(5):413–8. <https://doi.org/10.1002/ajmg.a.31074>.
3. Cerrato F, Sparago A, Ariani F, et al. DNA methylation in the diagnosis of monogenic diseases. *Genes (Basel)*. 2020;11(4):355. <https://doi.org/10.3390/genes11040355>.
4. Krzeski JC, Judson MC, Philpot BD. Neuronal UBE3A substrates hold therapeutic potential for Angelman syndrome. *Curr Opin Neurobiol*. 2024;88:102899. <https://doi.org/10.1016/j.conb.2024.102899>.
5. Hsiao JS, Germain ND, Wilderman A, et al. A bipartite boundary element restricts UBE3A imprinting to mature neurons. *Proc Natl Acad Sci U S A*. 2019;116(6):2181–6. <https://doi.org/10.1073/pnas.1815279116>.
6. Judson MC, Sosa-Pagan JO, Del Cid WA, Han JE, Philpot BD. Allelic specificity of Ube3a expression in the mouse brain during postnatal development. *J Comp Neurol*. 2014;522(8):1874–96. <https://doi.org/10.1002/cne.23507>.
7. Stanurova J, Neureiter A, Hiber M, et al. Angelman syndrome-derived neurons display late onset of paternal UBE3A silencing. *Sci Rep*. 2016;6:30792. <https://doi.org/10.1038/srep30792>.
8. Baldwin I, Shafer RL, Hossain WA, et al. Genomic, clinical, and behavioral characterization of 15q11.2 BP1-BP2 deletion (Burnside-Butler) syndrome in five families. *Int J Mol Sci*. 2021;22(4):1660. <https://doi.org/10.3390/ijms22041660>.
9. Keute M, Miller MT, Krishnan ML, et al. Angelman syndrome genotypes manifest varying degrees of clinical severity and developmental impairment. *Mol Psychiatry*. 2021;26(7):3625–33. <https://doi.org/10.1038/s41380-020-0858-6>.
10. Fujimoto M, Nakamura Y, Hosoki K, et al. Genotype-phenotype correlation over time in Angelman syndrome: researching 134 patients. *Hum Genet Genomics Adv*. 2024;5(4):100342. <https://doi.org/10.1016/j.xhgg.2024.100342>.
11. Niki T, Imamura K, Enami T, Kinoshita M, Inoue H. Establishment of human induced pluripotent stem cell line from a patient with Angelman syndrome carrying the deletion of maternal chromosome 15q.112–q13. *Stem Cell Res*. 2019;34:101363. <https://doi.org/10.1016/j.scr.2018.101363>.
12. Chamberlain SJ, Chen PF, Ng KY, et al. Induced pluripotent stem cell models of the genomic imprinting disorders Angelman and Prader-Willi syndromes. *Proc Natl Acad Sci U S A*. 2010;107(41):17668–73. <https://doi.org/10.1073/pnas.1004487107>.
13. Pólora-Brandão D, Joaquim M, Godinho I, et al. Loss of hierarchical imprinting regulation at the Prader-Willi/Angelman syndrome locus in human iPSCs. *Hum Mol Genet*. 2018;27(23):3999–4011. <https://doi.org/10.1093/hmg/ddy274>.
14. Pandya NJ, Wang C, Costa V, et al. Secreted retrovirus-like GAG-domain-containing protein PEG10 is regulated by UBE3A and is involved in Angelman syndrome pathophysiology. *Cell Rep Med*. 2021;2(8):100360. <https://doi.org/10.1016/j.xcrm.2021.100360>.
15. Maranga C, Pereira C, Raposo AC, et al. Generation and characterization of induced pluripotent stem cell line (IBISTI004-A) from an Angelman syndrome patient carrying a class II deletion of the maternal chromosome 15q11.2–q13. *Stem Cell Res*. 2022;61:102757. <https://doi.org/10.1016/j.scr.2022.102757>.
16. Camões Dos Santos J, Cazaux Mateus F, Arez M, Bekman EP, da Rocha ST. Generation of two pairs of induced pluripotent stem cells from Angelman syndrome patients with class I 15q11.2–q13.1 deletions and their unaffected familial controls. *Stem Cell Res*. 2025;86:103741. <https://doi.org/10.1016/j.scr.2025.103741>.
17. Das DK, Tapias V, D'Aiuto L, et al. Genetic and morphological features of human iPSC-derived neurons with chromosome 15q11.2 (BP1-BP2) deletions. *Mol Neuropsychiatry*. 2015;1(2):116–23. <https://doi.org/10.1159/000430916>.
18. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126(4):663–76. <https://doi.org/10.1016/j.cell.2006.07.024>.
19. Cazaux Mateus F, Camões Dos Santos J, Arez M, Bekman EP, da Rocha ST. A stem cell-based toolkit to model Angelman syndrome caused by paternal uniparental disomy of chromosome 15. *Hum Cell*. 2025;38(6):161. <https://doi.org/10.1007/s13577-025-01287-8>.
20. Neureiter A, Brändl B, Hiber M, Tandon R, Müller FJ, Steenpass L. Generation of an iPSC line of a patient with Angelman syndrome due to an imprinting defect. *Stem Cell Res*. 2018;33:20–4. <https://doi.org/10.1016/j.scr.2018.09.015>.
21. Fink JJ, Robinson TM, Germain ND, et al. Disrupted neuronal maturation in Angelman syndrome-derived induced pluripotent stem cells. *Nat Commun*. 2017;8:15038. <https://doi.org/10.1038/ncomms15038>.
22. Li S, Zhu Q, Cai Y, Yang Q. Generation of an induced pluripotent stem cell line from a patient with Angelman syndrome carrying UBE3A mutation. *Stem Cell Res*. 2022;62:102791. <https://doi.org/10.1016/j.scr.2022.102791>.
23. Gilmore RB, Gorka D, Stoddard CE, Sonawane P, Cotney J, Chamberlain SJ. Generation of isogenic models of Angelman syndrome and Prader-Willi syndrome in CRISPR/Cas9-engineered human embryonic stem cells. *PLoS ONE*. 2024;19(11):e0311565. <https://doi.org/10.1371/journal.pone.0311565>.
24. Dindot SV, Antalffy BA, Bhattacharjee MB, Beaudet AL. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. *Hum Mol Genet*. 2008;17(11):111–8. <https://doi.org/10.1093/hmg/ddm288>.
25. Furusawa K, Ishii K, Tsuji M, Tokumitsu N, Hasegawa E, Emoto K. Presynaptic Ube3a E3 ligase promotes synapse elimination through down-regulation of BMP signaling. *Science*. 2023;381(6663):1197–205. <https://doi.org/10.1126/science.ade8978>.
26. Simchi L, Gupta PK, Feuermann Y, Kaphzan H. Elevated ROS levels during the early development of Angelman syndrome alter the apoptotic capacity of the developing neural precursor cells. *Mol Psychiatry*. 2023;28(6):2382–97. <https://doi.org/10.1038/s41380-023-02038-7>.
27. Gupta PK, Barak S, Feuermann Y, Goobes G, Kaphzan H. 1H-NMR-based metabolomics reveals metabolic alterations in early development of a mouse model of Angelman syndrome. *Mol Autism*. 2024;15(1):31. 10.1186/s13229-024-00608-2.
28. Bruckner SR, Tammariello SP, Kuan CY, Flavell RA, Rakic P, Estus S. JNK3 contributes to c-Jun activation and apoptosis but not oxidative stress in nerve growth factor-deprived sympathetic neurons. *J Neurochem*. 2001;78(2):298–303. <https://doi.org/10.1046/j.1471-4159.2001.00400.x>.
29. Musi CA, Marchini G, Giani A, et al. Colocalization and Interaction Study of Neuronal JNK3, JIP1, and β -Arrestin2 Together with PSD95. *Int J Mol Sci*. 2022;23(8):4113. <https://doi.org/10.3390/ijms23084113>.

30. Musi CA, Agrò G, Buccarello L, Camuso S, Borsello T. JNK signaling activation in the Ube3a maternal deficient mouse model: its specific inhibition prevents post-synaptic protein-enriched fraction alterations and cognitive deficits in Angelman syndrome model. *Neurobiol Dis.* 2020;140:104812. <https://doi.org/10.1016/j.nbd.2020.104812>.
31. Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF. Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes.* 2001;50(1):77–82. <https://doi.org/10.2337/diabetes.50.1.77>.
32. Davoli E, Sclip A, Cecchi M, et al. Determination of tissue levels of a neuroprotectant drug: the cell permeable JNK inhibitor peptide. *J Pharmacol Toxicol Methods.* 2014;70(1):55–61. <https://doi.org/10.1016/j.vascn.2014.04.001>.
33. Stefanoska K, Bertz J, Volkerling AM, van der Hoven J, Ittner LM, Ittner A. Neuronal MAP kinase p38 α inhibits c-Jun N-terminal kinase to modulate anxiety-related behaviour. *Sci Rep.* 2018;8(1):14296. <https://doi.org/10.1038/s41598-018-32592-y>.
34. Repici M, Mare L, Colombo A, et al. C-Jun N-terminal kinase binding domain-dependent phosphorylation of mitogen-activated protein kinase kinase 4 and mitogen-activated protein kinase kinase 7 and balancing cross-talk between c-Jun N-terminal kinase and extracellular signal-regulated kinase pathways in cortical neurons. *Neuroscience.* 2009;159(1):94–103. <https://doi.org/10.1016/j.neuroscience.2008.11.049>.
35. Zhao Y, Spigolon G, Bonny C, Culman J, Vercelli A, Herdegen T. The JNK inhibitor D-JNKI-1 blocks apoptotic JNK signaling in brain mitochondria. *Mol Cell Neurosci.* 2012;49(3):300–10. <https://doi.org/10.1016/j.mcn.2011.12.005>.
36. Hirt L, Badaut J, Thevenet J, et al. D-JNKI1, a cell-penetrating c-Jun-N-terminal kinase inhibitor, protects against cell death in severe cerebral ischemia. *Stroke.* 2004;35(7):1738–43. <https://doi.org/10.1161/01.STR.0000131480.03994.b1>.
37. Eshraghi AA, Aranke M, Salvi R, et al. Preclinical and clinical otoprotective applications of cell-penetrating peptide D-JNKI-1 (AM-111). *Hear Res.* 2018;368:86–91. <https://doi.org/10.1016/j.heares.2018.03.003>.
38. Musi CA, Castaldo AM, Valsecchi AE, et al. JNK signaling provides a novel therapeutic target for Rett syndrome. *BMC Biol.* 2021;19(1):256. <https://doi.org/10.1186/s12915-021-01190-2>.
39. Alari V, Russo S, Rovina D, et al. Generation of three iPSC lines (IAli002, IAli004, IAli003) from Rubinstein-Taybi syndrome 1 patients carrying CREBBP non sense c.4435G>T, p.(Gly1479*) and c.3474G>A, p.(Trp1158*) and missense c.4627G>T, p.(Asp1543Tyr) mutations. *Stem Cell Research.* 2019;40:101553. <https://doi.org/10.1016/j.scr.2019.101553>.
40. Alari V, Scalmani P, Ajmone PF, et al. Histone deacetylase inhibitors ameliorate morphological defects and hypoexcitability of iPSC-neurons from Rubinstein-Taybi patients. *Int J Mol Sci.* 2021;22(11):5777. <https://doi.org/10.3390/ijms22115777>.
41. Tonazzini I, Jacchetti E, Meucci S, Beltram F, Cecchini M. Schwann cell contact guidance versus boundary-interaction in functional wound healing along nano and microstructured membranes. *Adv Healthc Mater.* 2015;4(12):1849–60. <https://doi.org/10.1002/adhm.201500268>.
42. Mezzena R, Masciullo C, Antonini S, et al. Study of adhesion and migration dynamics in ubiquitin E3A ligase (UBE3A)-silenced SY5H5Y neuroblastoma cells by micro-structured surfaces. *Nanotechnology.* 2021;32(2):025708. <https://doi.org/10.1088/1361-6528/abbb03>.
43. Pizzamiglio L, Focchi E, Cambria C, et al. The DNA repair protein ATM as a target in autism spectrum disorder. *JCI Insight.* 2021;6(3):e133654. <https://doi.org/10.1172/jci.insight.133654>.
44. Mertens J, Marchetto MC, Bardy C, Gage FH. Evaluating cell reprogramming, differentiation and conversion technologies in neuroscience. *Nat Rev Neurosci.* 2016;17(7):424–37. <https://doi.org/10.1038/nrn.2016.46>.
45. Alari V, Russo S, Terragni B, et al. iPSC-derived neurons of CREBBP - and EP300 -mutated Rubinstein-Taybi syndrome patients show morphological alterations and hypoexcitability. *Stem Cell Res.* 2018;30:130–40. <https://doi.org/10.1016/j.scr.2018.05.019>.
46. Kim H, Kunz PA, Mooney R, Philpot BD, Smith SL. Maternal loss of Ube3a impairs experience-driven dendritic spine maintenance in the developing visual cortex. *J Neurosci.* 2016;36(17):4888–94. <https://doi.org/10.1523/JNEUROSCI.4204-15.2016>.
47. Nakano R, Nakayama T, Sugiyama H. Biological Properties of JNK3 and Its Function in Neurons, Astrocytes, Pancreatic β -Cells and Cardiovascular Cells. *Cells.* 2020;9(8):1802. <https://doi.org/10.3390/cells9081802>.
48. Mabb AM, Judson MC, Zylka MJ, Philpot BD. Angelman syndrome: insights into genomic imprinting and neurodevelopmental phenotypes. *Trends Neurosci.* 2011;34(6):293–303. <https://doi.org/10.1016/j.tins.2011.04.001>.
49. Kaphzan H, Buffington SA, Jung JI, Rasband MN, Klann E. Alterations in intrinsic membrane properties and the axon initial segment in a mouse model of Angelman syndrome. *J Neurosci.* 2011;31(48):17637–48. <https://doi.org/10.1523/JNEUROSCI.4162-11.2011>.
50. Miao S, Chen R, Ye J, et al. The Angelman syndrome protein Ube3a is required for polarized dendrite morphogenesis in pyramidal neurons. *J Neurosci.* 2013;33(1):327–33. <https://doi.org/10.1523/JNEUROSCI.2509-12.2013>.
51. Saiga R, Shiga K, Maruta Y, et al. Structural differences between human and mouse neurons and their implementation in generative AIs. *Sci Rep.* 2025;15(1):25091. <https://doi.org/10.1038/s41598-025-10912-3>.
52. Wong HHW, Chou CYC, Watt AJ, Sjöström PJ. Comparing mouse and human brains. *Elife.* 2023;12:e90017. <https://doi.org/10.7554/eLife.90017>.
53. Dimitrion P, Zhi Y, Clayton D, et al. Low-Density Neuronal Cultures from Human Induced Pluripotent Stem Cells. *Mol Neuropsychiatry.* 2017;3(1):28–36. <https://doi.org/10.1159/000476034>.
54. Mizui T, Takahashi H, Sekino Y, Shirao T. Overexpression of drebrin A in immature neurons induces the accumulation of F-actin and PSD-95 into dendritic filopodia, and the formation of large abnormal protrusions. *Mol Cell Neurosci.* 2005;30(4):630–8.
55. Margolis SS, Salogiannis J, Lipton DM, et al. EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. *Cell.* 2010;143(3):442–55. <https://doi.org/10.1016/j.cell.2010.09.038>.
56. Khatri N, Gilbert JP, Huo Y, et al. The autism protein Ube3A/E6AP remodels neuronal dendritic arborization via caspase-dependent microtubule destabilization. *J Neurosci.* 2018;38(2):363–78. <https://doi.org/10.1523/JNEUROSCI.1511-17.2017>.
57. Elamin M, Dumarchey A, Stoddard C, et al. The role of UBE3A in the autism and epilepsy-related Dup15q syndrome using patient-derived, CRISPR-corrected neurons. *Stem Cell Reports.* 2023;18(4):884–98. <https://doi.org/10.1016/j.stemcr.2023.02.002>.
58. Wu Y, Zhao Y, Guan Z, Esmaeili S, Xiao Z, Kuriakose D. JNK3 inhibitors as promising pharmaceuticals with neuroprotective properties. *Cell Adh Migr.* 2024;18(1):1–11. <https://doi.org/10.1080/19336918.2024.2316576>.

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