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Mechanical stimulation prevents impairment of axon growth and overcompensates microtubule destabilization in cellular models of Alzheimer's disease and related Tau pathologies

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Alzheimer's disease (AD) and related tauopathies such as frontotemporal dementia (FTD) or traumatic brain injury (TBI) are neurodegenerative disorders characterized by progressive loss of memory and cognitive function. The main histopathological features of AD are amyloid- β plaques and Tau neurofibrillary tangles, suggested to interfere with neuronal function and to cause microtubule (MT) destabilization. We recently demonstrated that low mechanical forces promote MT stabilization, which in turn promotes axon growth and neuronal maturation. As neurites may become dystrophic due to MT destabilization in tauopathies, we hypothesized that force-induced MT stabilization is neuroprotective in cell models subjected to tauopathy-like stress. We set up two different pathological cellular models subjecting them to AD-related Tau pathology stressors. We found that exposure of mouse primary neurons to Tau oligomers and neurons derived from human induced pluripotent stem cell (hiPSC) to amyloid- β oligomers resulted in neurotoxic effects such as axonal shortening, reduction in dendrite number, and MT destabilization. Mechanical stimulation (i) prevented delays in axonal extensions and dendrite sprouting, restoring axon outgrowth to physiological levels, and (ii) compensated for axonal MT destabilization by increasing MT stability to levels higher than in control conditions. In summary, we here demonstrate that low mechanical force can be used as a neuroprotective extrinsic factor to prevent MT destabilization and axon degeneration caused by AD-like or tauopathy-like stressors.

KEYWORDS

nano-pulling, mechanical stimulation, microtubule stabilization, Alzheimer's disease, Tau pathology

1 Introduction

Alzheimer's disease (AD) and related tauopathies such as frontotemporal dementia (FTD) or traumatic brain injury (TBI) are neurodegenerative disorders characterized by progressive loss of memory and cognitive function. While this is in the late stages accompanied by overt cell loss, earlier stages in AD/tauopathy are associated with more subtle effects such as synapse, neurite, and cytoskeleton dysfunction. The main histopathological features of AD are

extracellular amyloid- β (A β) plaques and intracellular microtubule-associated protein (MAP) Tau neurofibrillary tangles (NFT) (1). A β plaques are aggregates made up of incorrectly folded peptides that derive from aberrant cleavage of the transmembrane Amyloid Precursor Protein (APP), a cell surface receptor with physiological functions relevant to neurite growth, neuronal adhesion, axonogenesis, and synaptogenesis (2). Tau pathology is the hallmark of several neurodegenerative disorders belonging to the category of tauopathies, of which AD is the most common. In AD, according to the amyloid cascade hypothesis, the presence of A β aggregates triggers the changes that lead to Tau pathogenic cascade resulting in Tau misorting and aggregation, neuronal dysfunction, neurite retraction, and eventually cell death (2, 3). Despite recent genetic evidence demonstrated the existence of a protective APP variant (4, 5) and partially effective anti-amyloid treatments are emerging (6, 7), the amyloid cascade hypothesis is disputed: Braak and Braak, renowned for their staging of AD based on Tau pathology, have presented findings that challenge the centrality of the amyloid cascade hypothesis. Their work demonstrated that Tau pathology often appears first in the entorhinal cortex, frequently without the presence of senile plaques (SPs), which are associated with (A β) deposition (8, 9). This observation suggests that tau-related NFT may precede amyloid deposition in the pathological sequence of AD. In the overwhelming majority of tauopathies, the initiator of disease is anyway not amyloid- β , but either physical trauma resulting most likely in axonal disruption due to shearing forces such as in TBI (10), a range of genetic disorders leading to Tau pathology as a secondary event, or Tau itself [e.g., Tau pathogenic mutations resulting in a subset of FTD (11). Tau protein has the physiological functions of binding microtubules (MTs), controlling their assembly and stabilizing them, therefore regulating axonal transport, neurons growth, and synapse establishment (12). Alterations in post-translational modifications, including hyperphosphorylation, cause a conformational change in Tau that underlies the pathogenesis of AD and related tauopathies, leading to the formation of intracellular NFT (13). Tau dissociates from MTs and is sequestered into these insoluble aggregates, causing MT destabilization and loss from axons. These pathological conditions disrupt axonal transport and synapses and ultimately lead to neuronal death and loss of cognitive function (14–16). Since MTs play a key role in neuron function, being involved in axonal transport, research in the area of new therapeutic strategies for AD and related tauopathies included also MT-stabilizing agents in recent years (17, 18). Recently, mechanical force has emerged as an extrinsic factor that stabilizes MTs (19). It has been suggested that MTs are inherently mechano-sensitive, and computational studies indicate that pulling forces (in the range of a few piconewtons) stimulate MT assembly by promoting the formation of lateral non-covalent bonds between GTP-tubulin dimers, thereby aiding the closure of the protofilament wall (20). Supporting this prediction, increased traction forces have been shown to slow down MT depolymerization (21). Evidence supporting the role of MTs as mechanosensors comes from classical micromanipulation experiments, which showed that traction forces ranging from 0.5 to 2 piconewtons (pN) decrease the likelihood of growing MTs of undergoing shrinkage, while increasing the probability of short MTs of resuming growth (22). Moreover, force can indirectly affect MT stability by modulating the activity of MAPs. For instance, studies on interactions between MTs and molecular motors have already shown

that force influences the probability of MT/MAP association (23, 24). Similarly, it has been found that force can modulate interactions between MTs and other MAPs, such as proteins that associate with the plus end. XMAP215, an enzyme that catalyzes MT growth by adding tubulin dimers to the MT plus end, was found to increase the rate of MT growth under a 1 pN force application (25), possibly by enhancing enzyme activity. Interestingly, compressive forces also seem to affect MTs by causing the re-localization of plus-tip proteins [EB1 and CLASP2] from the microtubule end to the microtubule shaft (26). Recently, our group set up a method named nano-pulling (based on the use of magnetic nanoparticles (MNPs) in presence of magnetic fields) to mechanically stimulate neurons *in vitro* chronically by generation of a pulling force in the order of 10 pN (27–33). We used nano-pulling to test the effect of extremely low forces applied chronically in neural cells. We found, in both mature neurons (hippocampal neurons and dorsal root ganglion neurons) and neural progenitor cells (NPCs) undergoing neural differentiation, that force induces axonal MT stabilization (29, 32–34). When axonal MTs become more stable, their turnover rate declines, leading to their accumulation. Since MTs serve as the primary cytoskeletal “tracks” for axonal transport, this accumulation results in a local build-up of vesicles and organelles within the axon, which are components of translation platforms. This, in turn, raises the likelihood of forming translational platforms (35). Such positive regulation of axonal transport and local translation helps facilitate the addition of new mass required for axon growth (19, 32).

Since MT destabilization is one of the key features of AD and suggested for other tauopathies (36, 37), findings regarding MT stabilization in response to force could be very relevant to tauopathies and possibly for AD. In this study, we explored the hypothesis that mechanical stimulation of neurons could be exploited as a novel neuroprotective strategy to prevent or decrease MT instability and its neurotoxic effects in AD and related tauopathies. The present study represents a proof of concept, aimed at investigating the neuroprotective effect of mechanical force in *in vitro* models. We set up two different pathological cellular models using mature and immature neurons and subjecting them to AD-related Tau pathology stressors. Specifically, we treated primary mouse hippocampal neurons (HNs) with Tau protein oligomers (oTau), and NPCs derived from human induced pluripotent stem cells (hiPSCs) undergoing neuronal differentiation in cortical neurons with amyloid- β oligomers (oA β) and tested the presumptive neuroprotective effect of mechanical force.

2 Materials and methods

2.1 Ethical statement

Animal procedures were performed in strict compliance with protocols approved by the Italian Ministry of Public Health and the local Ethics Committee of the University of Pisa, in accordance with the European Directive for the Care and Use of Animals 2010/63/EU (project license no. 39E1C.N.5Q7 approved on 30/10/2021). C57BL/6 J mice were used. Animals were maintained in a regulated environment ($23 \pm 1^\circ\text{C}$, $50\% \pm 5\%$ humidity) with a 12-h light–dark cycle and food and water *ad libitum*. The here described hiPSCs are commercially

available,¹ in several modifications, and are listed in several registries (e.g., <https://hpscereg.eu/cell-line/UCSFi001-A>). It is thus an established and commercially available cell line.

2.2 Cell culture

For primary mouse HNs culture, P0/P1 (postnatal day 0/1) stage mice were sacrificed and both hippocampi were explanted and dissected in a solution of ice-cold 6.5 mg/mL D-glucose in Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, #14190-144). Tissue was digested for 10 min in 0.25% Trypsin (Gibco, #15050-065) at 37°C and for 5 min in 0.25% Trypsin added with 1% DNase 10 mg/mL (Sigma-Aldrich, #DN25) at 37°C. After centrifugation at 1,200 rpm for 1 min, mechanical dissociation was carried out by passing the cells through a Pasteur glass pipette (VWR, #612-1702) 30 to 40 times. Cells were seeded at cell density of 50,000 cells/cm² in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, #21063-029) modified with 10% fetal bovine serum (FBS, Gibco, #10270-106), 100 IU/mL penicillin, 100 µg/mL streptomycin (Gibco, #15140-122), and GlutaMAX supplement (Gibco, #35050-038). Cells were seeded on 13 mm circular plastic coverslips (Sarstedt, #83.1840.002) pre-coated with 100 µg/mL Poly-L-lysine (PLL, Sigma-Aldrich, #P4707) and incubated at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂. The medium was replaced at day *in vitro* 0 (DIV0) 4 h after seeding with Neurobasal-A medium (Gibco, #12349-015) modified with B27 supplement (Gibco, #17504-044), GlutaMAX, 50 IU/mL penicillin, 50 µg/mL streptomycin, and 5 µg/mL MNPs.

Human cortical neurons were derived from hiPSC line Ngn2-WTC11 as generated in (38). hiPSCs were routinely cultured in StemMACS iPS-Brew-XF medium (Miltenyi Biotec, #130-104-368) supplemented with Antibiotic-Antimycotic Solution (Merck, #A5955) on plates pre-coated with 200 µg/mL Geltrex (Thermo Fisher Scientific, #A1413302) in KnockOut DMEM (KO-DMEM, Thermo Fisher Scientific, #10829018). Cells were incubated at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO₂ and regularly passaged when 80% confluent in a 1:10 ratio, using Versene (Thermo Fisher Scientific, #15040066) and StemMACS iPS-Brew-XF supplemented with 2 µM thiazovivin (Axon Medchem, #Axon1535) for the first 24 h. For pre-differentiation, on the first day (DIV0), cells were incubated in Accutase (Sigma-Aldrich, #A6964) for 5 min at 37°C; then, DPBS was added, and cells were centrifuged at 400 g for 5 min. Supernatant was discarded, and the pellet was re-suspended in StemMACS iPS-Brew-XF. Cells were seeded onto Geltrex-coated plates in pre-differentiation medium consisting of KO-DMEM/F12 (Thermo Fisher Scientific, #12660012) containing N2 supplement (Pan-Biotech, #P07-11010), non-essential amino acids (NEAA, Thermo Fisher Scientific, #11140035), 1.5 µg/mL mouse laminin (Sigma, #L2020), 10 ng/mL brain-derived neurotrophic factor (BDNF, Peprotech, #450-02), 10 ng/mL neurotrophin-3 (NT3, Peprotech, #450-03), 2 µM thiazovivin, 2 µg/mL doxycycline (Merck, #D9891), and Antibiotic-Antimycotic solution. The medium was changed daily for 2 days to fresh pre-differentiation medium without thiazovivin.

The duration of the pre-differentiation protocol is 3 days (from DIV0 to DIV2). Hereafter, we refer to these cells as induced neurons (iN) when the precursors have completed the pre-differentiation protocol and are at any stage of terminal differentiation ($24 \geq \text{DIV} \geq 3$). For terminal differentiation (DIV3), cells were dissociated as described for pre-differentiation and seeded onto 13 mm circular plastic coverslips pre-coated with 20 µg/mL Poly-D-Lysine (Sigma-Aldrich, #P7886) and 20 µg/mL Cultrex 3-D Laminin I (Biotechne, #3446-005-01) in maturation medium consisting of 50% DMEM/F12 (Thermo Fisher, #11320033), 50% Neurobasal-A, B27 supplement, N2 supplement, GlutaMAX, NEAA, 1.5 µL/mL mouse laminin, 10 ng/mL BDNF, 10 ng/mL NT3, 2 µg/mL doxycycline, and Antibiotic-Antimycotic solution at a seeding density of 50,000 cells/cm². The medium was replaced 4 h after seeding by maturation medium supplemented with 5 µg/mL MNPs.

For both cell cultures, the coverslips were placed inside 35 mm Petri dishes (VWR, #734-2317) that are compatible with the insertion inside the magnetic applicator.

2.3 Magnetic nanoparticles

The MNPs used in this study are the FluidMAG-ARA (Chemicell, #4115). They are characterized by an organic iron oxide core with a diameter of 75 ± 10 nm and a magnetic saturation of 59 Am²/Kg, as stated from the supplier. They are coated with a polysaccharide shell composed of glucuronic acid, making the hydrodynamic diameter approximately 100 nm. MNPs were added to the cell growth medium at a concentration of 5 µg/mL.

2.4 Magnetic field and application of mechanical force

Twenty-four hours after seeding, an external static magnetic field was applied to the cell cultures by placing the 35 mm Petri dishes within a toroidal magnetic applicator Halbach-type that provides a constant magnetic field gradient (46.5 T/m) in the radial centrifugal direction (27, 39). Cells were maintained inside the magnet for 48 h of stimulation.

2.5 Neurotoxic oligomer preparation and treatment

For oTau preparation, 1 µM recombinant human hT40 protein (TAU441, Abcam, #ab191460) was incubated with 18.75 µM arachidonic acid (Cayman Chemicals, #90010.1) for 15 min at room temperature (RT) in a polymerization buffer consisting of 10 mM HEPES pH 7.6, 100 mM NaCl, 5 mM DTT, and 0.1 mM EGTA. The solution was stored at −80°C. prior to treatment, the solution was centrifuged at 1,400g for 5 min; then, oTau were administered to primary mouse HNs in conditioned growth medium at a final concentration of 50 nM, 24 h after seeding (DIV1).

For oAβ preparation, Aβ40 and Aβ42 powder (21st Century Biochemicals, #AB40-0010 and #AB42-0010) were dissolved in Hexafluoro-2-propanol (HFIP, 100%) to 1 mM concentration. Then, the HFIP was completely evaporated overnight (ON) in a

¹ https://www.coriell.org/0/Sections/Search/Sample_Detail.aspx?Ref=GM25256

Speedvac centrifugational evaporator, and the lyophilized powder aliquots were stored at -80°C . For reconstitution, directly before use, dried aliquots were dissolved in 50 mM NaOH; then, A β 40 and A β 42 were mixed in a 7:3 ratio and added with DPBS and 50 mM HCl obtaining a final A β concentration of 100 μM . To induce oA β formation, the A β mixture was incubated at 37°C for 1 h; then, oA β were administered to hiPSC-derived cortical iNs in conditioned maturation medium at a final concentration of 1 μM , 24 h and 48 h after seeding (Day 1 and Day 2 of terminal differentiation).

2.6 Immunostaining

At DIV3 for HN and at DIV6 for iNs, cells were fixed, immunostained, and imaged.

For HN, after two washes with DPBS, cells were fixed in 2% w/v paraformaldehyde (PFA) and 7.5% w/v sucrose (Sigma-Aldrich, #S0389) in DPBS at RT for 20 min. Samples were washed three times with DPBS and permeabilized in 0.5% v/v Triton X-100 (Sigma-Aldrich, #X100) in DPBS at RT for 10 min. After three 3-min washes in 0.1% v/v Triton X-100 in DPBS, cells were blocked in 5% v/v goat serum (Gibco, #16210-064) and 0.3% v/v Triton X-100 in DPBS for 1 h at RT and incubated with the primary antibodies ON at 4°C in 3% v/v goat serum and 0.2% v/v Triton X-100 in DPBS. After ON incubation, samples were washed and incubated with secondary antibodies and Hoechst 33342 (Invitrogen, #H3570, dilution 1:1,000) in 3% v/v goat serum and 0.2% v/v Triton X-100 in DPBS and then washed three times in DPBS.

For iNs, after one wash in DPBS, cells were fixed in 3.7% w/v PFA and 4% sucrose in DPBS at RT for 1 h. After three washes in PBS, cells were permeabilized and blocked in 0.5% v/v Triton X-100 and 5% w/v BSA (Carl Roth, #8076.4) in DPBS at RT for 5 min. Cells were washed once in DPBS and incubated with the primary antibodies ON at 4°C in DPBS. After ON incubation, samples were washed three times in DPBS and incubated with secondary antibodies in DPBS for 2 h at RT. After two washes in DPBS, samples were incubated with NucBlue (Thermo Fisher Scientific, #R37605, dilution 1:1,000) in DPBS for 10 min at RT and washed once in deionized water.

The primary antibody used for axon length analysis is Anti- β -Tubulin III (TUBBIII, Sigma-Aldrich, #T8578, dilution 1:500). The primary antibodies used for MT stability analysis are Anti-acetylated α -tubulin (Sigma-Aldrich, #T7451, dilution 1:400) and Anti-tyrosinated α -tubulin [YL1/2] (Abcam, #ab6160, dilution 1:400). The secondary antibodies used are Thermo Fisher Scientific #A11029, #R6393, and #A11077 (dilution 1:1000).

Coverslips were mounted onto glass microscope slides (Eppredia, #AA00000102E01MNZ10) using Aqua-Poly/Mount (Polysciences, #18606-20) and left to dry ON at RT protected from light.

2.7 Imaging

In HN, cell imaging for analysis of axon and dendrite length and pyknotic nuclei was performed at 10X magnification using an inverted fluorescence microscope (Nikon Eclipse Ti) equipped with Nikon DS-Ri2 camera and the help of NIS-Elements AR software version 5.11; cell imaging for analysis of MT stability was performed at 60X

magnification with a laser scanning confocal microscope (Nikon AX) and the help of NIS-Elements software version 5.42.

In iNs, cell imaging for analysis of neural processes length and pyknotic nuclei was performed at 20X magnification using a fluorescence microscope (Zeiss AxioScope 5) and the ZenBlue Pro imaging software version 2.5; cell imaging for analysis of MT stability was performed using the same microscope and software, at 40X magnification.

2.8 Images analysis

Images were analyzed with Fiji software version 1.54i.

Length of TUBBIII-positive axons and dendrites was measured, and the number of dendrites per cell was counted manually using the NeuronJ plugin. A total of 30 non-interconnected axons per replicate were traced using the tracing tool, and their length data were collected. A total of 30 cells per replicate were used for dendrites analysis, and the average length and number of dendrites per cell data were collected.

For oligomer neurotoxicity testing, cell death was calculated as the percentage of pyknotic nuclei per replicate, analyzing at least 2,000 total nuclei or six pictures per replicate. The status (pyknotic/non-pyknotic) of nuclei was evaluated by the operator considering the level of brightness and compactness. Pyknotic and total nuclei in a picture were counted with Fiji's CellCounter tool.

For MT stability analysis, the intensities of fluorescent signals of acetylated and tyrosinated α -tubulin were quantified as mean fluorescence ($\bar{f}$). Mean fluorescence ($\bar{f}$) of a specific channel in a specific region of interest (ROI) was calculated as follows:

$$\bar{f} = \frac{\text{IntDen} - (\bar{f}_{\text{back}} \cdot A)}{A}$$

where IntDen is the integrated density (the sum of the intensities of all the pixels in the ROI), $\bar{f}_{\text{back}}$ is the mean fluorescence of background readings, and A is the area of the ROI. All these parameters are measured by Fiji software. As a ROI, non-interconnected axons, dendrites, or somas were selected. For background readings, three circular ROIs were selected in close proximity to the analyzed axons/dendrites/somas and their fluorescence values were averaged. At least 12 cells per replicate were analyzed. Stability was calculated as the ratio between acetylated versus tyrosinated α -tubulin mean fluorescence.

2.9 Statistical analysis

For elongation and MT stability, *a priori* power analysis for required sample size computation was performed with GPower 3.1 software (input parameters: $\alpha = 0.05$, $\beta = 0.20$, two-tailed hypothesis, effect size calculated from pilot study data). Data were plotted, and statistical analysis was performed using GraphPad Prism software, version 8.0.2. Values are reported as mean $\pm$ standard error of the mean (SEM) from four replicates. Data distribution was checked through different normality tests: Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov. Specifically, when data did not show a

Gaussian distribution, Mann–Whitney test was used for comparing two groups and Kruskal–Wallis test followed by Dunn’s multiple comparisons test was used for comparing three groups. When data showed a Gaussian distribution, *t*-test was used for comparing two groups and one-way analysis of variance (ANOVA) followed by Holm–Sidak’s multiple comparisons test was used when comparing three groups. For the comparison of the frequency distribution of dendrites number per cell, chi-squared test was applied. *p*-values of ≤ 0.05 were considered statistically significant.

The positive control (nano-pulling) is not plotted, but it is reported for the dataset related to iNs in [Supplementary Figure S2](#).

3 Results

3.1 AD-related Tau pathology stressors cause axonal shortening in *in vitro* models

Although various forms of A β and Tau aggregates have been shown to exhibit some level of toxicity, oligomers are currently considered to be the most harmful (40, 41). For this reason, we used oligomeric species of Tau and A β as stressors in our models. For Tau oligomerization, we modified the protocol from Caneus et al. (41), optimizing the concentration, 50 nM, that is able to induce a 30% shortening of axon length in mouse HNs ([Supplementary Figure S1](#)). For A β oligomerization, we used an oligomeric preparation of a 7:3 ratio of A β 40/A β 42 at 1 μ M concentration since we had previously reported that it vigorously induces hallmarks of AD (17). As this oligomers mix shows reversion of toxicity after 24 h (17), treatment was repeated for 2 consecutive days.

HNs were treated with 50 nM oTau at DIV1, and analysis was performed at DIV3 ([Figure 1A](#)). To evaluate the detrimental effect of the neurotoxic oligomers, we measured the axonal length, already used in literature as an indicator of neuron functionality (42). A statistically significant delay of outgrowth of axons in the treated

samples compared to control samples was observed. The axon length reduction obtained by administering 50 nM oTau to mouse HNs is 28.6% [average axon length was $127.8 \pm 5.8 \mu\text{m}$ in controls and $91.3 \pm 3.6 \mu\text{m}$ in oTau-treated samples, $p < 0.0001$ ([Figure 1B](#))]. The average percentage of cell death was $5.3 \pm 0.14\%$ in controls and $5.4 \pm 0.14\%$ in treated samples ($p = 0.32$, [Figure 1C](#)).

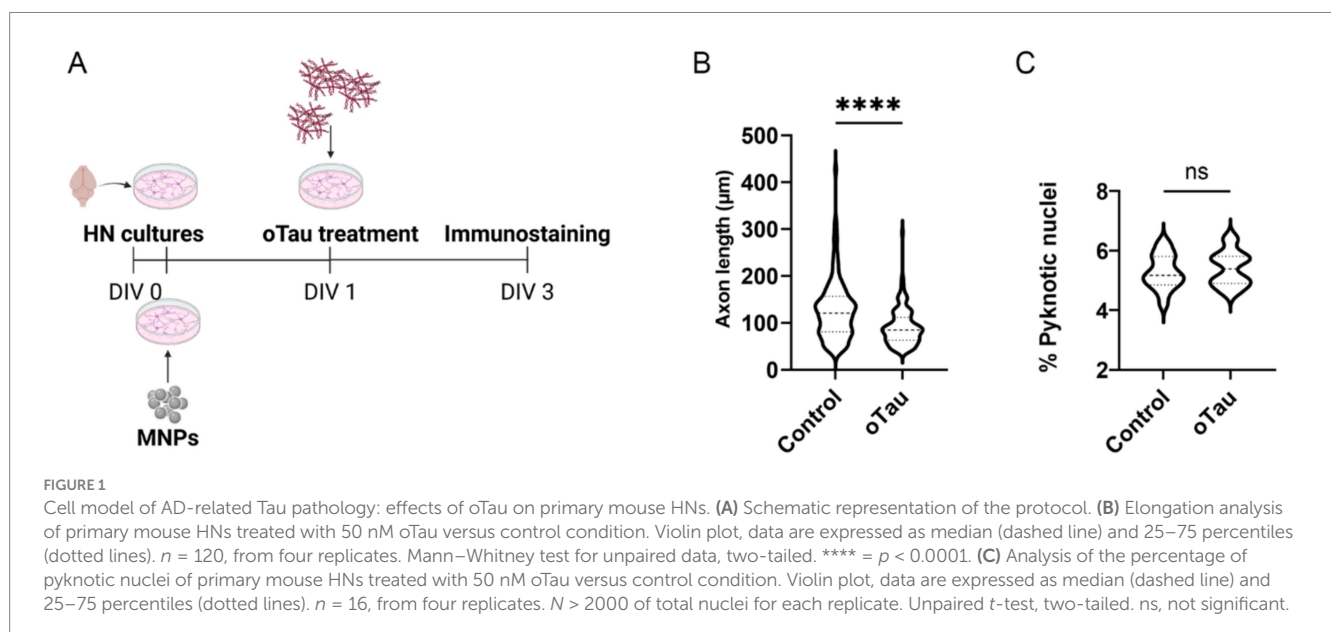
iNs were treated with 1 μ M oA β at DIV4, and analysis was performed at DIV6 ([Figure 2A](#)). Similarly to the previous model, a reduction in the length of the neural processes was obtained. The reduction obtained was 24.6% [the average length of neural processes was $155.3 \pm 8.2 \mu\text{m}$ in controls and $113.4 \pm 5.3 \mu\text{m}$ in oA β -treated samples, $p = 0.0002$ ([Figure 2B](#))], and the average percentage of cell death was $8.7 \pm 0.4\%$ in controls and $8.9 \pm 0.4\%$ in treated samples ($p = 0.65$, [Figure 2C](#)).

These results show that our treatments impact process elongation but not cell viability, mimicking the first stage of pathology.

3.2 Nano-pulling compensates for axonal shortening in cell models subjected to AD-related Tau pathology stressors

To assess the neuroprotective effect of mechanical stimulation, three experimental groups were established: a control group that provides the baseline and reference values of the analyzed parameters, a group treated with oligomers from which we expect a decline in these values, and a group treated with oligomers and subjected to mechanical stimulation, from which we expect a recovery of the control values if the nano-pulling exerts a neuroprotective effect.

For HNs, MNPs were applied 4 h after cell isolation and seeding, oligomer treatment was performed at DIV1, and the mechanical stimulation was performed from DIV1 to DIV3 ([Figure 3A](#)). The analysis of axon length at DIV3 showed that the reduction in the axon length due to oTau treatment was recovered when the cells were subjected to nano-pulling ([Figures 3B1, 3B2, 3B3](#)). Specifically, the average axon length in the control group



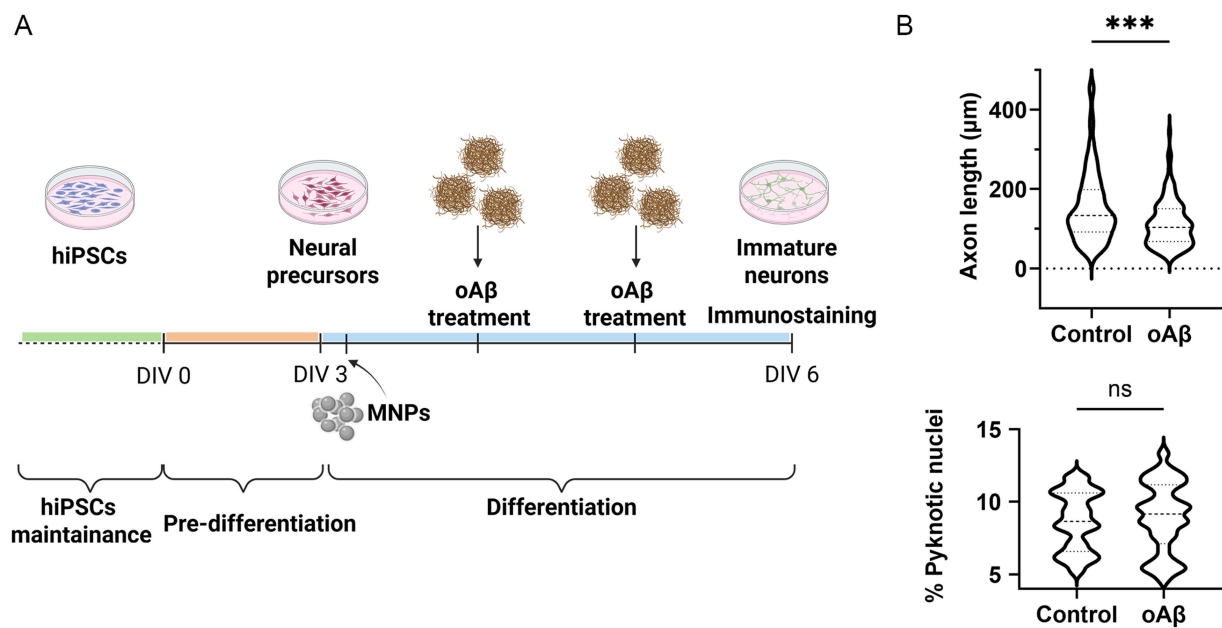


FIGURE 2

Cell model of AD: effects of oAβ on hiPSC-derived cortical iNs. **(A)** Schematic representation of the protocol. **(B)** Elongation analysis of hiPSC-derived cortical iNs treated with 1 μM oAβ versus control condition. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 120$, from four replicates. Mann–Whitney test for unpaired data, two-tailed. *** = $p < 0.001$. **(C)** Analysis of the percentage of pyknotic nuclei of hiPSC-derived cortical iNs treated with 1 μM oAβ versus control condition. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 24$, from four replicates. $N > 1,000$ of total nuclei or at least six pictures analyzed for each replicate. Unpaired t -test, two-tailed. ns, not significant.

was $134.6 \pm 5.4 \mu\text{m}$, in the pathological group was $92.3 \pm 3.6 \mu\text{m}$, and in the pathological group subjected to mechanical stimulation was $133.8 \pm 4.9 \mu\text{m}$ (Figure 3B4) ($p < 0.0001$ between the pathological and control groups and $p = 0.18$ between the pathological group subjected to nano-pulling and the control group).

Given that Tau pathology primarily affects dendrites, we also evaluated the effects of oTau and nano-pulling in this compartment. We found that dendrite length is not influenced by oTau nor oTau in combination with nano-pulling (specifically, the average dendrite length in the control group was $22.93 \pm 0.74 \mu\text{m}$, in the pathological group was $25.65 \pm 0.90 \mu\text{m}$, and in the pathological group subjected to mechanical stimulation was $22.91 \pm 0.85 \mu\text{m}$, $p = 0.1$ between the pathological and control groups, and $p = 1$ between the pathological group subjected to nano-pulling and the control group) (Figure 3B5). However, the study of the frequency distribution of the dendrite number per cell shows a reduction by oTau treatment with respect to the control ($p = 0.005$) that is compensated when cells are simultaneously subjected to nano-pulling ($p = 0.39$ versus control) (Figure 3B6).

For iNs, MNPs were applied 4 h after the beginning of terminal differentiation (DIV3), oligomer treatment was performed at DIV4, and the mechanical stimulation was performed from DIV4 to DIV6 (Figure 4A). A similar effect was observed on the neural processes of iNs: The average length in the control group was $147.4 \pm 7.6 \mu\text{m}$, in the pathological group was $113.7 \pm 4.9 \mu\text{m}$, and in the pathological group subjected to mechanical stimulation was $175.9 \pm 9.9 \mu\text{m}$ (Figure 4B) ($p = 0.009$ between the pathological and control groups

and $p = 0.18$ between the pathological group subjected to nano-pulling and the control group).

These data show that mechanical stimulation with piconewton forces prevents axonal shortening induced by oTau and oAβ, restoring outgrowth to normal physiological levels.

3.3 Nano-pulling overcompensates for MT destabilization in cell models subjected to AD-related Tau pathology stressors

Using the same experimental design, to assess whether the neuroprotective effect of mechanical stimulation is mediated by force-induced MT stabilization, we evaluated the stability of axonal MTs in the two models. Cells were co-immunostained for acetylated and tyrosinated α -tubulin, routinely used markers of stable and dynamic MTs, respectively (43–45), and the ratio between the two fluorescent signals was used as a measure of MT stability (Figure 3C). Analysis was performed at DIV3 for HNs and at DIV6 for iNs (Figure 4C).

Data analysis showed that in HNs treated with oTau in the absence of mechanical stimulation, there is a statistically significant destabilization of MTs in the axons compared to the control group (2.02 ± 0.05 and 1.74 ± 0.06 for control and oTau, respectively, $p = 0.0065$), while in presence of mechanical stimulation, there is a statistically significant increase of the stability compared to the control group (2.02 ± 0.05 and 2.55 ± 0.1 for control and oTau+nano-pulling, respectively, $p < 0.0001$) (Figure 3D). Similarly, in iNs treated with the oAβ in absence of mechanical stimulation, there is a statistically significant destabilization of MTs in the neural

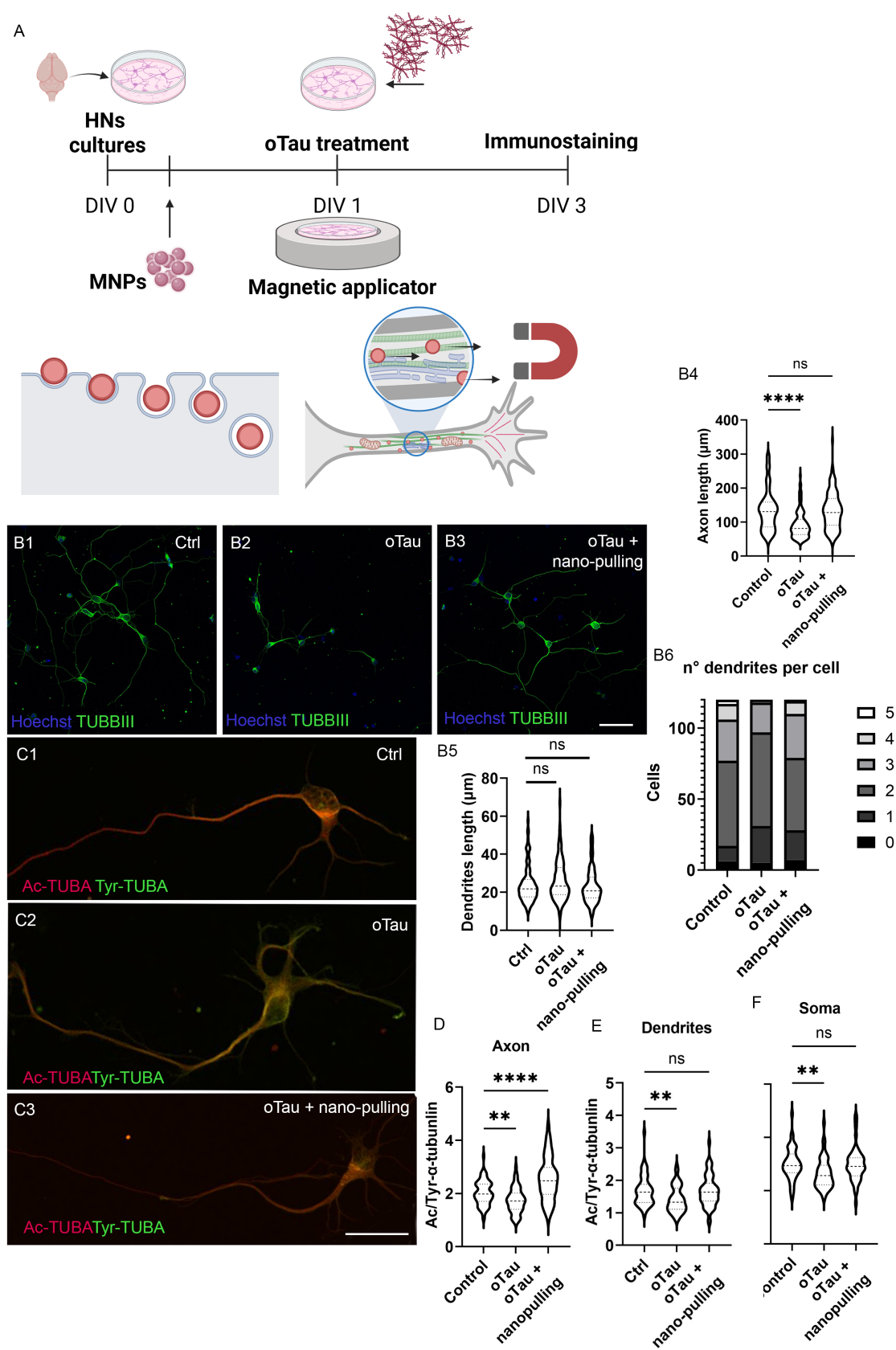


FIGURE 3
Effect of nano-pulling on primary mouse HNs treated with oTau. **(A)** Schematic representation of the protocol. Nano-pulling is a technique that consists in adding MNPs to neurons (DIV0). The particles are internalized and accumulate in cell cytoplasm, including the axoplasm, where they interact with organelles and cytoskeleton. When external magnetic applicator is applied (DIV1), a stretching force inside the axon is generated. **(B1–B3)** (Continued)

FIGURE 3 (Continued)

Representative pictures of mouse HNs subjected to the different experimental conditions and stained for TUBBIII (green) and Hoechst (blue). Ctrl = control group, oTau = pathological group, oTau + nano-pulling = pathological group simultaneously subjected to nano-pulling. Scale bar corresponds to 50 μm . **(B4)** Axon elongation analysis of primary mouse HNs treated with 50 nM oTau and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 120$, from four replicates. Kruskal–Wallis test for unpaired data followed by Dunn’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of control group. **** $p < 0.0001$, ns, not significant. **(B5)** Dendrite elongation analysis of primary mouse HNs treated with 50 nM oTau and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 120$, from four replicates. Kruskal–Wallis test for unpaired data followed by Dunn’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of control group. ns, not significant. **(B6)** Dendrite number per cell in primary mouse HNs treated with 50 nM oTau and simultaneously “stretched” with mechanical stimulation. Contingency plot. $n = 120$ cells, from four replicates. Chi-squared test. Control vs. oTau: Chi-square = 16.97, $df = 5$, $p = 0.005$. Control vs. oTau+nano-pulling: Chi-square = 5.198, $df = 5$, $p = 0.39$. **(C1–C3)** Representative pictures of mouse HNs subjected to the different experimental conditions and stained for Ac- α -tubb (red) and Tyr- α -tubb (green). Ctrl = control group, oTau = pathological group, oTau + nano-pulling = pathological group simultaneously subjected to nano-pulling. Scale bar corresponds to 25 μm . **(D)** Axonal MT stability analysis of HNs treated with 50 nM oTau and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 70$ from four replicates. One-way ANOVA test for unpaired data followed by Holm–Sidak’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of control group. **** $p < 0.0001$ ** $p < 0.01$. **(E)** MT stability analysis in dendrites of HNs treated with 50 nM oTau and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 60$ from four replicates. Kruskal–Wallis test for unpaired data followed by Dunn’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of control group. ** $p < 0.01$, ns, not significant. **(F)** Somatic MT stability analysis of HNs treated with 50 nM oTau and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 50$ from four replicates. Kruskal–Wallis test for unpaired data followed by Dunn’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of control group. ** $p < 0.01$. ns, not significant.

projections compared to the control group (2.13 ± 0.11 and 0.98 ± 0.05 for control and oA β , respectively), while in presence of mechanical stimulation, there is a statistically significant increase of the stability compared to the control group (2.13 ± 0.11 and 3.93 ± 0.21 for control and oA β + nano-pulling, respectively, $p < 0.0001$) (Figure 4D). This result suggests that oTau and oA β are able to induce MT destabilization at the axonal level and nano-pulling not only compensates this loss but also increases MT stability over physiological levels, acting as a neuroprotective factor.

We also evaluated whether the stabilizing effect propagates to MT in the cell soma, using the same experimental design. Data analysis showed that in cells treated with the neurotoxic oligomers in the absence of mechanical stimulation, there is a statistically significant destabilization of MTs in the somas compared to the control group. In particular, in the HNs model, the ratio between the mean fluorescence of acetylated and tyrosinated α -tubulin found in somas of controls was 1.50 ± 0.04 , while in samples treated with 50 nM oTau it was 1.32 ± 0.04 ($p = 0.0017$) (Figure 3F). In the iNs model, the ratio was 2.05 ± 0.13 in controls and 1.06 ± 0.07 in samples treated with 1 μM oA β ($p < 0.0001$). Mechanical stimulation was able to restore the soma MT stability levels of control condition in the HNs model (ratio 1.49 ± 0.04 , $p = 1$ compared to the control group) (Figure 3F). In the soma of oA β -treated iNs, nano-pulling was able to increase the level of MT stability beyond that of the control group (ratio 2.71 ± 0.17 , $p = 0.02$ compared to the control group) (Figure 4E). This result suggests that oTau and oA β also induce MT destabilization at the soma level and nano-pulling is able to compensate for induced destabilization, restoring or overcompensating MT stability levels in soma, depending on the cell model.

Similarly in dendrites of HNs, MT stability is reduced by oTau treatment and compensated in the group simultaneously subjected to nano-pulling: the ratio between the mean fluorescence of acetylated and tyrosinated α -tubulin found in dendrites of controls was 1.68 ± 0.06 , in samples treated with 50 nM oTau was 1.42 ± 0.05 ($p = 0.0029$ versus control), and in treated samples simultaneously subjected to nano-pulling was 1.68 ± 0.48 ($p = 1$ versus control)

(Figure 3E). These results suggest that oTau also induce damages at the dendrites level (consisting in reduction in number and MT destabilization) and nano-pulling is able to compensate, restoring dendrites number and MT stability to a physiological level.

4 Discussion

In tauopathies, Tau sequestration into neurofibrillary tangles induces MT destabilization, causing alterations in axonal transport and eventually leads to neurite collapse (46). In light of this, researchers have proposed MT stabilization in neurons through exogenous factors as a potential therapeutic strategy for these pathologies, based on a functional substitution of Tau (47). Pharmacological approaches have already been tested, both *in vivo* and *in vitro*, using MT-stabilizing compounds that in most cases are already used as anti-cancer drugs (48). In *in vitro* models, these drugs have been found to rescue degenerating primary neurons in the presence of neurotoxicity induced by amyloid- β (49) or Tau (50). In transgenic animal models of tauopathy, they were able to reverse axonal transport deficits (51), improve cognitive deficits (52, 53), reduce neurons loss (54), and reverse spine changes (55–57). More recently, these drugs showed reduction in molecular markers of pathology, cognitive improvement, and mitigation of synaptic pathology also in transgenic animal models of AD (58–60). These findings prove that MT stabilization can compensate for the loss of Tau function and its detrimental effects in AD-related Tau pathology, demonstrating the therapeutic potential of this approach. However, MT-stabilizing agents are widely used in clinics as chemotherapeutic drugs, and there are many side effects associated with their use (61), limiting their translational application to neurodegenerative disorders. In this study, we explored the use of mechanical force as an alternative extrinsic factor to stabilize MTs (11). Specifically, the nano-pulling technique developed by our group for chronic mechanical stimulation of neurons *in vitro* (39) has been shown to stimulate MT stabilization (27, 29, 32, 34) and induce differential expression of genes associated with MT cytoskeleton organization, MT-binding proteins,

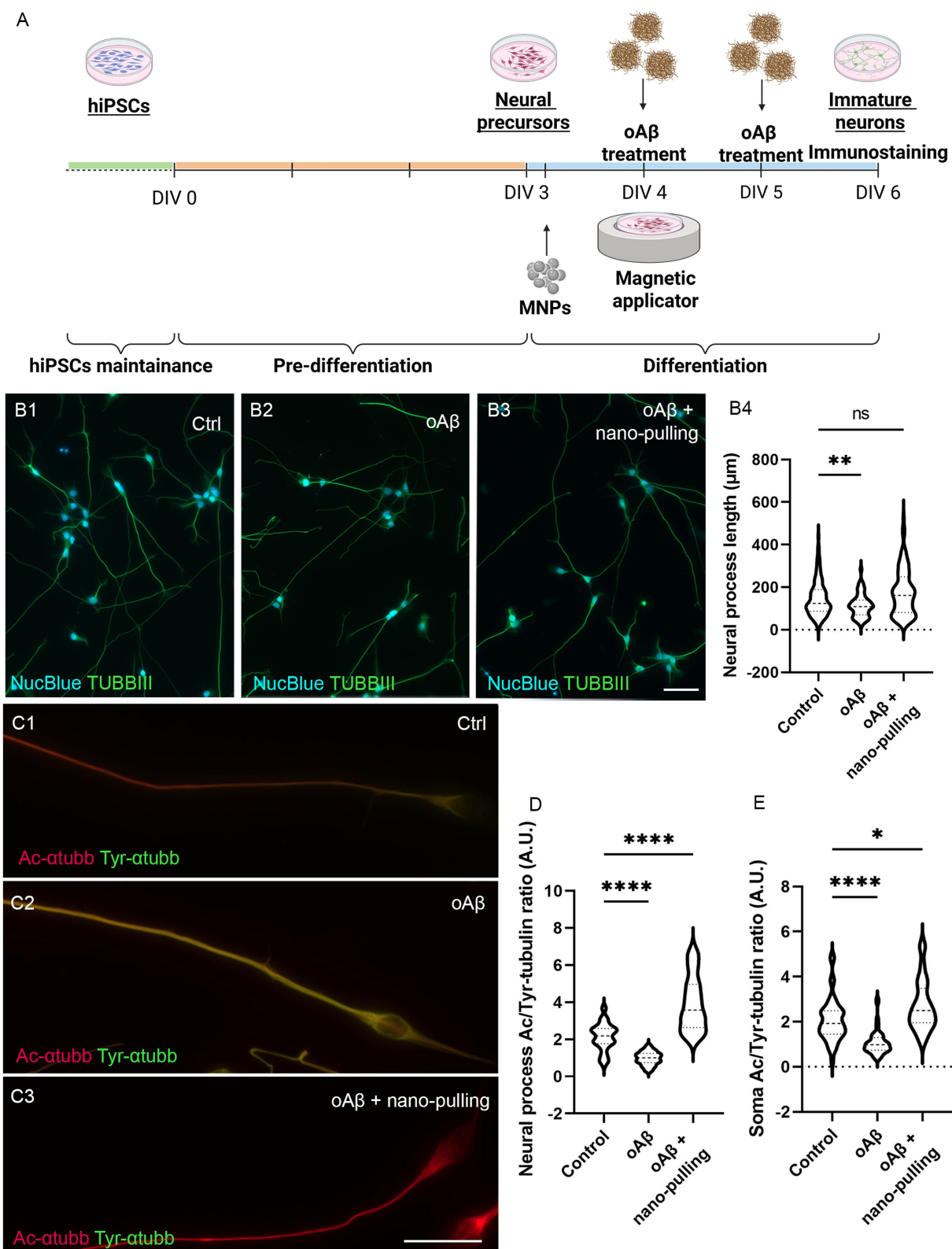


FIGURE 4

Effect of nano-pulling on hiPSC-derived cortical iNs treated with oAβ. **(A)** Schematic representation of the protocol. **(B1–B3)** Representative pictures of hiPSC-derived cortical iNs subjected to the different experimental conditions and stained for TUBBIII (green) and NucBlue (cyan). Ctrl = control group, oAβ = pathological group, oAβ + nano-pulling = pathological group simultaneously subjected to nano-pulling. Scale bar corresponds to 50 μm. **(B4)** Elongation analysis of hiPSC-derived cortical iNs treated with 1 μM oAβ and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 120$, from four replicates. Kruskal–Wallis test for unpaired data followed by Dunn’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of the control group. ** $p < 0.01$, ns, not

(Continued)

FIGURE 4 (Continued)

significant. **(C1–C3)** Representative pictures of hiPSC-derived cortical iNs subjected to the different experimental conditions and stained for Ac- α -tubb (red) and Tyr- α -tubb (green). Ctrl = control group, oA β = pathological group, oA β + nano-pulling = pathological group simultaneously subjected to nano-pulling. Scale bar corresponds to 25 μ m. **(D)** Analysis of MT stability in neural processes of iNs treated with 1 μ M oA β and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 48$ from four replicates. Kruskal–Wallis test for unpaired data followed by Dunn’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of the control group. **** $p < 0.0001$. **(E)** Somatic MT stability analysis of iNs treated with 1 μ M oA β and simultaneously “stretched” with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 50$ from four replicates. Kruskal–Wallis test for unpaired data followed by Dunn’s multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of the control group. **** $p < 0.0001$ * $p < 0.05$.

and MT motor protein. More importantly, we extensively tested nano-pulling in primary neurons (HNs, dorsal root ganglion neurons) (29, 32, 33), NPCs (34), and spinal cord tissue (34), and no signs of toxicity have ever been reported.

In this context, we hypothesized that nano-pulling could be exploited as a novel neuroprotective strategy for the prevention or reduction in MT instability and its neurotoxic consequences in paradigms of tauopathy. We here aimed to test its effects in *in vitro* models of amyloid- β or Tau-oligomers induced neurotoxicity. Several studies have shown that oligomers can be internalized and that this is likely an important contributor to the neurotoxic effects. The process typically happens through receptor-mediated endocytosis or clathrin-mediated pathways (62, 63). Despite this, not all oligomers are immediately internalized: Some interact with membrane receptors, such as metabotropic glutamate (mGluR) receptors or N-methyl-D-aspartate receptor (NMDAR), altering signaling at the cell surface before endocytosis (64, 65). Specifically, we decided to treat iNs with oA β because, in our previous studies, we found that iPSC-derived human neurons exhibited profound changes when exposed to oligomeric A β (e.g., Tau missorting, loss of microtubule stability, decreased neurotransmission as assessed by calcium imaging, and reduced synaptic density) but not in response to the aggregation/oligomerization-prone P301L-TAU (66, 67). On the other hand, considering that the splice-isoform conversion of murine Tau toward more aggregation-prone 4R isoforms occurs at a later developmental stage and earlier in rodents than in humans (68), we opted to test oTau on postnatal murine neurons. Our data support the hypothesis that nano-pulling prevents axonal shortening by acting on MT stability (Figures 3D, 4D). These results are consistent with those coming from pharmacological approaches using MT-stabilizing drugs (48). More interestingly, MT stabilization is an ubiquitous effect that was observed not only in the axons but also in the soma (Figures 3F, 4E) and dendrites (Figure 3E). More specifically, we found that oTau treatment induces a decrease in dendrite sprouting, measured as the number of dendrites per cell, which is prevented when HNs undergo nano-pulling (Figure 3B5). However, neither oTau nor nano-pulling affects dendrite length. This observation is consistent with our proposed model, in which nano-pulling-induced elongation depends on MT stabilization (69). This process occurs in axons—where MTs are preferentially oriented with the + end directed toward the growth cone—when the force is applied from the soma to the tip, but not when it is applied from the tip to the soma (27). Conversely, dendrites—which lack a preferential MT orientation—do not undergo nano-pulling-mediated elongation.

To the best of our knowledge, it is the first time that nano-pulling technique has been applied as a neuroprotective strategy to neurodegeneration models. A limitation of this study is certainly

related to the models. In fact oligomers are able to induce detrimental effects such as MT destabilization and processes shortening, being thus useful for modeling certain aspects of the pathology, but are obviously not able to fully mimic all the aspects of the disease and the complexity of the pathological environment characterized by oxidative stress and neuroinflammation. Speculating on a future pre-clinical testing of the methodology in a neurodegeneration setting, nano-pulling could be applied as a minimally invasive strategy consisting in the administration of MNPs to the animal model and the application of a wearable magnetic device to activate the strategy in the area of interest (70). In addition, several approaches involving MNPs have recently been described for crossing the blood–brain barrier and even targeting specific neuronal populations (71–73), making this approach potentially translatable to the treatment of brain neurodegenerative disorders.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: DOI: [10.5281/zenodo.13987314](https://doi.org/10.5281/zenodo.13987314).

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Ministry of Public Health and the local Ethics Committee of the University of Pisa. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AG: Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing, Investigation. LS: Investigation, Writing – review & editing. JK: Investigation, Formal analysis, Methodology, Writing – review & editing. MK: Formal analysis, Investigation, Methodology, Writing – review & editing. HZ: Formal analysis, Methodology, Conceptualization, Validation, Writing – review & editing. VR: Conceptualization, Formal analysis, Methodology, Validation, Writing – review & editing, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmed.2025.1519628/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

Effects of different concentrations of α Tau on primary mouse HNs. Elongation analysis of primary mouse HNs treated with [Tau] = 25 nM, [Tau] = 50 nM, and [Tau] = 75 nM versus control condition. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 60$, from two replicates. Kruskal-Wallis test for unpaired data followed by Dunn's multiple comparisons test, two-tailed. Mean rank of each group is compared with mean rank of control group. **** = $p < 0.0001$. A shortening of axons in the treated samples compared to control samples was observed. α Tau concentration of 50 nM was chosen, as the lower concentration (25 nM) had no statistically significant effect compared to control neurons, and the higher concentration (75 nM) showed no dose-dependent effect compared to 50 nM.

SUPPLEMENTARY FIGURE S2

Effects of α Tau and nano-pulling on iNs—all controls. (A) Elongation analysis of hiPSC-derived cortical iNs treated with 1 μ M α A β and subjected to nano-pulling. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 120$, from four replicates. Kruskal-Wallis test for unpaired data followed by Dunn's multiple comparisons test, two-tailed. Interaction: $F(1, 476) = 0.3023$, $p = 0.58$. Row Factor (oligomer treatment): $F(1, 476) = 16.98$, $p < 0.0001$. Column Factor (nano-pulling): $F(1, 476) = 47.44$, $p < 0.0001$. (B) Analysis of MT stability in neural processes of iNs treated with 1 μ M α A β and "stretched" with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 48$ from four replicates. Kruskal-Wallis test for unpaired data followed by Dunn's multiple comparisons test, two-tailed. Interaction: $F(1, 188) = 5.381$, $p = 0.02$. Row Factor (oligomer treatment): $F(1, 188) = 25.42$, $p < 0.0001$. Column Factor (nano-pulling): $F(1, 188) = 275$, $p < 0.0001$. (C) Somatic MT stability analysis of iNs treated with 1 μ M α A β and "stretched" with mechanical stimulation. Violin plot, data are expressed as median (dashed line) and 25–75 percentiles (dotted lines). $n = 50$ from four replicates. Kruskal-Wallis test for unpaired data followed by Dunn's multiple comparisons test, two-tailed. Interaction: $F(1, 202) = 8.963$, $p = 0.031$. Row Factor (oligomer treatment): $F(1, 202) = 19.80$, $p < 0.0001$. Column Factor (nano-pulling): $F(1, 202) = 89.08$, $p < 0.0001$.

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Glossary

AD - Alzheimer's disease

DIV - day *in vitro*

DMEM - Dulbecco's Modified Eagle Medium

DPBS - Dulbecco's Phosphate-Buffered Saline

FBS - fetal bovine serum

iN - iPSC-derived neural progenitor cells undergoing neuronal differentiation in cortical neurons

h - human

HFIP - hexafluoro-2-propanol

HN - hippocampal neuron

iPSC - induced pluripotent stem cell

MAP - microtubule-associated protein

MNP - magnetic nanoparticle

MT - microtubule

NFT - neurofibrillary tangles

ns - non-significant

oA β - A β oligomers

ON - overnight

oTau - Tau oligomers

P - postnatal

PFA - paraformaldehyde

pN - piconewton

ROI - region of interest

RT - room temperature

PLL - poly-L-lysine

TUBBIII - β -tubulin III