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Aβ42 Peptide Promotes Proliferation and Gliogenesis in Human Neural Stem Cells

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Abstract

Amyloid-β 42 [$A\beta_{1-42}$ (Aβ42)] is one of the main Aβ peptide isoforms found in amyloid plaques of brains with Alzheimer's disease (AD). Although Aβ42 is associated with neurotoxicity, it might mediate several normal physiological processes during embryonic brain development and in the adult brain. However, due to the controversy that exists in the field, relatively little is known about its physiological function. In the present work, we have analyzed the effects of different concentrations of monomeric Aβ42 on cell death, proliferation, and cell fate specification of human neural stem cells (hNSCs), specifically the hNS1 cell line, undergoing differentiation. Our results demonstrate that at higher concentrations (1 μM), Aβ42 increases apoptotic cell death and DNA damage, indicating that prolonged exposure of hNS1 cells to higher concentrations of Aβ42 is neurotoxic. However, at lower concentrations, Aβ42 significantly promotes cell proliferation and glial cell specification of hNS1 cells by increasing the pool of proliferating glial precursors, without affecting neuronal differentiation, in a concentration-dependent manner. At the molecular level, these effects could be mediated, at least in part, by *GSK3β*, whose expression is increased by treatment with Aβ42 and whose inhibition prevents the glial specification induced by Aβ42. Since the cellular and molecular effects are known to appear decades before the first clinical symptoms, these types of studies are important in discovering the underlying pathophysiological processes involved in the development of AD. This knowledge could then be used in diagnosing the disease at early stages and be applied to the development of new treatment options.

Keywords Aβ42 peptide · Human neural stem cells · Alzheimer's disease · Cell death · Cell fate specification · Cell proliferation

Introduction

Alzheimer's disease (AD) is the most common cause of dementia among older people. This neurodegenerative disease is characterized by a progressive deterioration of cognitive functions, memory loss, and motor alterations [1, 2]. Post mortem analysis of AD patient's brains has identified two main hallmarks of the disorder: neurofibrillary tangles and amyloid plaques. Amyloid plaques consist of extracellular fibrillary deposits of amyloid-β peptide (Aβ) [3–5]. Aβ is a 39–43 amino acid peptide with a molecular weight of approximately 4 kDa and is generated by the enzymatic processing of amyloid beta precursor protein (APP) [6, 7]. Aβ peptide is released as a monomeric peptide, but with aging, and in diseases like AD, it accumulates and aggregates into fibers that precipitate to form plaques in the cerebral parenchyma, causing neurotoxicity [8–10]. However, the presence of Aβ peptide is not always related

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to neurotoxicity, as it has been shown that at low concentrations Aβ does not tend to form oligomers [11]. Moreover, Aβ is present in normal adult brains and may mediate several physiological processes, such as controlling of synaptic activity and neuronal survival, which acts as a trophic signal [12–15] and seems to be required for normal brain development [16, 17]. There are two dominant isoforms of Aβ, Aβ1–40 (Aβ40) and Aβ1–42 (Aβ42), both present in amyloid plaques. Aβ40 is the main Aβ species produced by neurons under physiological conditions. In pathologies such as AD, Aβ40 levels are reduced, which causes an increase in the Aβ42/Aβ40 ratio. Aβ42 is more abundant than Aβ40 within amyloid plaques due to its higher rate of self-aggregation and insolubility [18].

Previous work has shown that monomeric forms of Aβ peptide can affect differentiation and proliferation of rat neural progenitor cells (NPCs) [19] and mouse neural stem cells (NSCs) [20–22]. It has also been demonstrated that freshly prepared Aβ40 stimulates neurogenesis in NPCs, whereas Aβ42 favors gliosis in the same cells [19]. Furthermore, some work has shown that exposure of human NSCs to Aβ42 stimulates their differentiation towards glial cell fates, while impeding neuronal differentiation [23]. However, other studies have shown that Aβ42 could promote neurogenesis in mouse NSCs [24]. These contradictory results are probably due to differences in the type of cells, the type of peptides, peptide aggregation state, and the doses used in different experiments.

Currently, there is no cure for AD. One of the therapies proposed is using endogenous or transplanted NSCs to compensate for the neuronal loss observed in AD. NSCs are multipotent stem cells with the potential to self-renew and to differentiate into the main cellular phenotypes (neurons, astrocytes, oligodendrocytes) of the central nervous system (CNS). These cells can be sourced from fetal, neonatal, and adult brains, or from directed differentiation of pluripotent stem cells [25, 26]. Studies in human NSCs have provided a useful tool to progress clinically in stem cell-based therapies for several neurodegenerative disorders and have facilitated a better understanding of human brain development and the molecular pathology associated with neurodegeneration [27].

In this work, we have analyzed the effects of Aβ42 on cell death, proliferation, and cell fate specification of hNS1, a human NSC line. This cell line is a clonal, multipotent stem cell line derived from the telencephalic region of the developing human brain and was immortalized with v-myc [28]. We have previously demonstrated the multipotency of hNS1 and its ability to differentiate into different cell types, such as neurons or astrocytes [28–31]. This potential can be maintained over time, making hNS1 cells a suitable model for the analysis of the effects of Aβ peptide on human NSCs.

Materials and Methods

Ethics Statement

hNS1 cells were obtained from human tissues donated for research after written informed consent, in accordance with the European Union (EU) directives and the declaration of Helsinki and in agreement with the ethical guidelines of the Network of European CNS Transplantation and Restoration (NECTAR) and Spanish Biomedical Research Law (July 2007). Ethics statements about the human fetal origin of the cells used here can be found in the original reports describing the cell line [28, 29, 32].

Cell Cultures

Isolation and immortalization of hNS1 cells has been described previously [28, 30, 32]. hNS1 cell culture conditions are based on a chemically defined HSC medium [Dulbecco's modified Eagle medium (DMEM):F12 (1:1) with GlutaMAX-I medium (Gibco) containing 1% AlbuMAX (Gibco), 50 mM HEPES (Gibco), 0.6% D-Glucose (Merck), 1% N-2 supplement (Gibco), 1% non-essential amino acids mixture (NEAA; Gibco), and 1% penicillin-streptomycin (P/S; Lonza)]. For experiments, cells were seeded at 15,000 cell/cm^2 on poly-L-lysine (10 μg/ml; Sigma)-coated plastic cultures plates. Cells were grown in HSC medium supplemented with 20 ng/ml of epidermal growth factor (EGF; PeproTech) and fibroblast growth factor 2 (FGF2; PetroTech) [28] at 37 °C in a 5% CO_2 incubator (Forma). Cell cultures were differentiated in HSC medium containing 0.5% heat-inactivated fetal bovine serum (FBS; Gibco).

Preparation and Treatment with Aβ Peptide

Lyophilized Aβ42 peptide (American Peptide Company, Sunnyvale, CA, USA) was dissolved in hexafluoro-2-propanol (Sigma) to a final concentration of 1 mM. Aliquots of 50 μg were made, allowed to dry, and stored at − 80 °C until use. Monomeric peptides were prepared by diluting the dry stock in dimethyl sulfoxide (DMSO; Sigma) to 1 mM and then further diluting to different concentrations (0.5, 1, and 5 μM) in cell differentiation medium for analysis, immediately before adding to cells. hNS1 cells were treated for the first 4.5 days of differentiation. Untreated cells and vehicle (DMSO)-treated cells were used as controls. As significant differences were not detected between untreated and vehicle-treated cells (vehicle group), the vehicle group was implemented as the control for statistical analysis in all experiments. (See scheme in Supplementary Fig. 1A).

Some cultures were treated with the GSK3β inhibitor CHIR99021 (2 μM; TOCRIS) dissolved in DMSO (vehicle) alone or in combination with Aβ42 peptide from the

beginning of differentiation to day 4.5. Schematic view is represented in Fig. 7a.

5′-Bromo-2′-Deoxyuridine (BrdU) Treatment and Detection

To detect proliferating cells, differentiation medium containing 5 μM of 5′-bromo-2′-deoxyuridine (BrdU) (Sigma) was added to the different experimental groups for 2 h, immediately washed with PBS, then fixed in 4% paraformaldehyde (PFA; Sigma) for 10 min, washed with PBS, and treated with hydrochloric acid 2 M (HCl; Merck) for 30 min at 37 °C and revealed by immunocytochemistry. BrdU is a thymidine analog and can be incorporated into newly synthesized DNA strands of mitotic cells. The incorporation of BrdU into cellular DNA can then be detected using anti-BrdU antibody, allowing assessment of cell proliferation rate [33].

Immunocytochemistry (ICC)

Cells were rinsed with PBS, fixed in 4% PFA for 10 min, washed with PBS, and blocked for 1 h at room temperature (RT) in PBS containing 0.25% Triton X-100 and 5% normal horse serum (NHS). Primary antibodies were diluted in PBS containing 0.25% Triton X-100 and 1% NHS and incubated overnight at 4 °C. The following antibodies were used: mouse monoclonal anti-GFAP (1:1000; BD Pharmigen), mouse antivimentin (1:1000; Santa Cruz), rat monoclonal anti-BrdU (1:1000; Abcam), rabbit polyclonal anti-Ki67 (1:500; Thermo Scientific), rabbit anti-β-III-Tubulin (1:500; Sigma), mouse anti-β-III-Tubulin (1:200; Biolegend), rabbit anti-activated caspase 3 (1:500; Cell Signaling), and rabbit anti-nestin (1:500; Abcam). After removal of primary antibody, cells were washed with PBS containing 0.25% Triton X-100 and incubated for 1 h at RT with one of the corresponding secondary antibodies: Alexa Fluor 555 donkey anti-mouse IgG, Alexa Fluor 555 goat anti-rat IgG, and Alexa Fluor 488 donkey anti-rabbit IgG (1:500; Life Technologies). After removal of the secondary antibody, cells were washed with PBS and nuclei were stained with Höechst 33258 (Molecular Probes) and diluted in PBS (1:1000) for 5 min at RT. Samples were analyzed under a fluorescence microscope (Leica DM IL LED). Experiments were repeated three independent times ($n = 3$) with at least three wells per marker for each condition.

RNA Isolation, cDNA Synthesis, and qRT-PCR

Total RNA was isolated with the RNeasy Mini extraction kit (Qiagen) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed at 50 °C for 60 min in a 20-μl reaction mixture using Super Script III RT (Life Technologies). Relative amounts of cDNA were analyzed by quantitative real-time PCR (qRT-PCR) using the FAST SYBR green system (Applied Biosystems). Each 15-μl reaction volume included 10 ng total cDNA and 0.3 μM of each primer. qRT-PCRs were performed using primers for the target genes included in Table 1. Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems), according to the manufacturer's protocol. The Applied Biosystems 7500 Real-Time PCR System was used to determine the amount of target mRNA in each sample, estimated by the ΔΔCt relative quantification method [34]. Gene expression levels were normalized against *TBP* levels in each sample, and the fold change was calculated by setting the expression levels of each gene in the vehicle (DMSO) control as 1. qRT-PCR analysis was done for 0.5- and 1-μM-treated groups.

Western Blot

To determine the presence of Aβ42 peptide in its monomeric form, differentiation medium with Aβ42 treatment for each condition was collected and analyzed by Western blot (WB) in each experiment. For the detection of cell death, 50 μg of protein extracts was analyzed after peptide treatment. In both cases, samples were boiled for 5 min, loaded on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked in either PBS containing 5% nonfat powdered milk with 0.05% Tween20 (Sigma) or TBS containing 3% BSA and 0.05% Tween20 (to see phosphorylated state) for 1 h at room temperature. Blots were incubated overnight at 4 °C with primary antibodies against mouse β-actin (1:1000; Sigma), mouse anti-Aβ 4G8 (1:1000; Covance), and mouse anti-phospho-histone H2A.X (γH2AFX; 1:1000; Millipore). The blots were developed using peroxidase conjugated horse anti-mouse (HAMPO; 1:3000; Vector Laboratories) and visualized using the ECL system (Millipore).

Image Analysis and Cell Counting

Analysis and photography of fluorescent cultures were done using a fluorescence microscope (Leica DM IL LED) coupled to a camera (Leica DFC 345 FX). At least eight fields per well were randomly acquired at × 40 magnification to quantify the number of positive cells for the different markers (nestin, vimentin, activated caspase 3, Ki67, BrdU, β-III-tubulin, and GFAP) compared to total number of cells (Höechst). Each marker was studied in at least three different wells of the same experiment, and each experiment was repeated three independent times ($n = 3$). Cell counting was done using the program ImageJ (National Institute of Health, http://rsb.info.nih.gov/ij) and Adobe Photoshop CS6.

Table 1 List of primers for the target and housekeeping genes used for qRT-PCR experiments

Target gene	Forward	Reverse
BIIITUB	5′-GCAACTACGTGGGCGACT-3′	5′-ATGGCTCGAGGCACGTACT-3′
GFAP	5′-GGTTGAGAGGGACAATCTGG-3′	5′AGGTTGTTCTCGGCTTCCA-3′
Ki67	5′-TGACCCTGATGAGAAAGCTCAA-3′	5′-CCCTGAGCAACACTGTCTTTT-3′
GSK-3B	5′-AAAGTATTGCAGGACAAGAG ATTT-3′	5′-CGGACTATGTTACAGTGATCTAG CTT-3′
PI3K	5′-GGCTCAAAGACAAGAACAAAGG-3′	5′-TCCAGCACATGAACGTGTAAA-3′
ERK	5′-TCTGCACCGTGACCTCAA-3′	5′-GCCAGGCCAAAGTCACAG-3′
AKT	5′-GGCTATTGTGAAGGAGGGTTG-3′	5′-TCCTTGTAGCCAATGAAGGTG-3′
GAPDH	5′-AGATCCCTCCAAAATCAAGTGG-3′	5′-GGCAGAGATGATGACCCTTTT-3′
TBP	5′-GAGCTGTGATGTGAAGTTTCC-3′	5′-TCTGGGTTTGATCATTCTGTAG-3′

Quantification of Pyknotic Nuclei

Apoptotic cells were defined as those exhibiting the morphological hallmarks of apoptosis, such as nuclear fragmentation. At least eight fields per well were randomly acquired at × 40 magnification to quantify the number of positive cells for pyknotic nuclei compared to total cells (Höechst). Cell counting was done using the program ImageJ (National Institute of Health, http://rsb.info.nih.gov/ij) and Adobe Photoshop CS6.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0. p values were calculated using one-way analysis of variance (ANOVA) and two-way ANOVA with a post hoc Tukey test. p values < 0.05 were considered to be statistically significant ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; ns = not significant). Results are presented as the mean ± SD of data from three independent experiments ($n = 3$), with at least three samples per experimental group.

Results

Characterization of hNS1 Cells

The hNS1 cell line used in this study has been widely characterized in previous works [28, 30, 31]. As shown in Fig. 1a and in line with our previous work [31], proliferating hNS1 cells present a round morphology with short extensions and are positive for the expression of NSC markers such as nestin and vimentin in almost the entire population, 95 ± 3% (Fig. 1a, b). hNS1 cells undergoing differentiation, however, acquire a branched morphology, showing long extensions and neurite outgrowth. After 7 days of differentiation, this population contains 25 ± 5% of neurons (β-III-tubulin$^+$ cells) and 45 ± 2% of astrocytes (GFAP$^+$ cells) (Fig. 1a, c) [31, 32]. These results are in agreement with our recent work, confirming the differentiation capacity and multipotency of these cells [31].

Experiments were carried out in hNS1 cells undergoing differentiation (see scheme in Supplementary Fig. 1A). hNS1 cells were cultured in differentiation medium with increasing concentrations of freshly prepared Aβ42 peptide (0.5, 1, and 5 μM). After 4.5 days of treatment, the cultures were analyzed for cell death, proliferation rate, and cell fate specification. The presence of Aβ42 peptide in its monomeric form was confirmed by WB analysis of the differentiation medium with Aβ42 treatment at lower concentrations (0.5 and 1 μM). However, at a dose of 5 μM, both monomeric and aggregated forms were detected (Supplementary Fig. 1B). As we specifically aimed our analysis towards monomeric Aβ42, the concentration of 5 μM was not further pursued.

Aβ42 Induces Apoptotic Cell Death in Differentiating hNS1 Cells

The toxic effects of aggregated forms of Aβ peptides are well known for their involvement in AD, promoting neuronal death and impairment of neural progenitor development [10, 14, 35–37]. However, the effects of the monomeric forms on human NSCs are still unknown. Cell death is not evident in cells treated with monomeric peptide (0.5 and 1 μM) as can be observed in representative phase contrast images (Fig. 2a). To further evaluate if monomeric Aβ induces cell death, we analyzed caspase 3 immunoreactivity and the number of pyknotic nuclei in the different experimental groups.

Analysis for the presence of activated caspase 3 reactivity showed the extent of induced programmed cell death in cultures treated with Aβ42 (Fig. 2b–d). As shown in Fig. 2b (upper panels), c, treatment with Aβ42 peptide provoked an increase in the percentage of caspase 3-positive cells as compared to controls. The percentage increased from 10.7 ± 0.9% in the vehicle group to 13.7 ± 1.2% in the 0.5-μM group ($p > 0.05$; $n = 3$) and 17.8 ± 0.7% in the 1-μM group ($***p < 0.001$; n = 3).

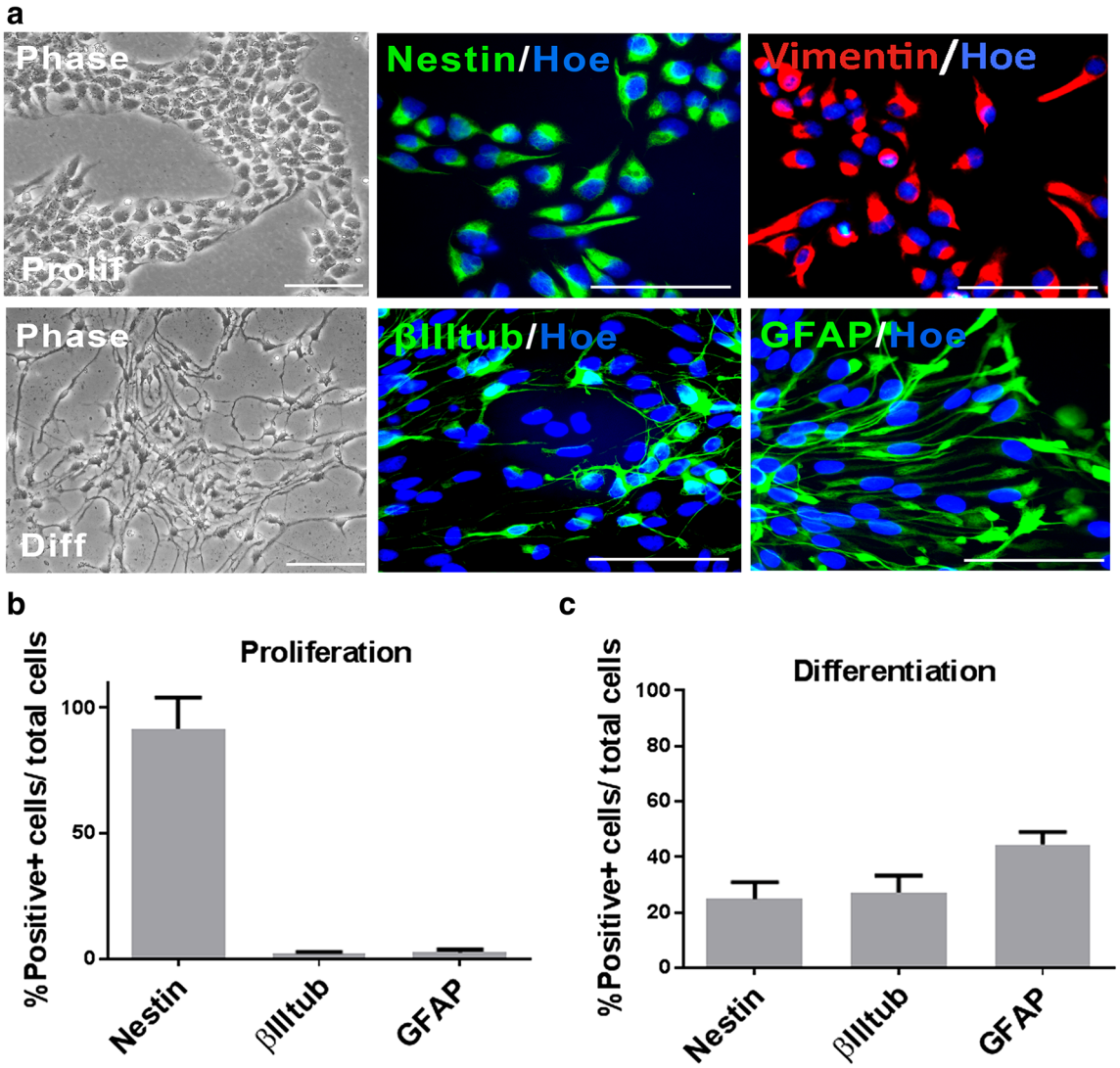

Fig. 1 hNS1 cell characterization. **a** Representative images of proliferating (Prolif) hNS1 cells in phase contrast and immunoreactivity for nestin (green) and vimentin (red) (upper panels). Representative images of differentiating (Diff) hNS1 cells at day 7 of differentiation in phase contrast and immunoreactivity for β-III-tubulin (βIIItub; green) and GFAP (green) (bottom panels). Cell nuclei appear in blue by Höechst staining. Scale bar, 100 μm (phase contrast) and 50 μm (immunofluorescence). **b**, **c** Histograms represent the percentage of positive cells for nestin, β-III-tubulin, and GFAP of proliferating (**b**) and differentiated (**c**) hNS1 cells

Pyknotic nuclei appear brighter and more fragmented than normal nuclei. The percentage of pyknotic nuclei was significantly increased after treatment with Aβ42 at 1 μM, but not at 0.5 μM (Fig. 2b (bottom panels), d). Only 2 ± 0.3% of cells in the vehicle group presented pyknotic nuclei, rising to 5 ± 1.1% at 0.5 μM ($p > 0.05$; $n = 3$) and 8.4 ± 0.3% at 1 μM (***$p < 0.001$; $n = 3$). These results confirm what we observed for caspase 3 activity (Fig. 2c).

We further analyzed the expression of γH2AFX (histone H2AFX phosphorylated at serine 139), which denotes the presence of double-strand DNA breaks. H2AX is rapidly phosphorylated upon DNA damage that occurs during normal cellular processes or as a result of external chemical or physical damaging agents. During apoptosis, however, H2AX becomes phosphorylated upon endonuclease-mediated DNA fragmentation downstream of caspase 3 activation [38]. As shown in Fig. 2e, treatment with Aβ42 induces a dose-dependent increase in γH2AFX, indicating the presence of DNA damage.

Aβ42 Increases Cell Proliferation in Differentiating hNS1 Cells

Previous reports have shown that Aβ peptides may also affect NSC proliferation, depending on cell type, peptide used, and treatment duration [19, 20]. We evaluated the proliferation of hNS1 cells treated with Aβ42 by analyzing Ki67 expression and the incorporation of the

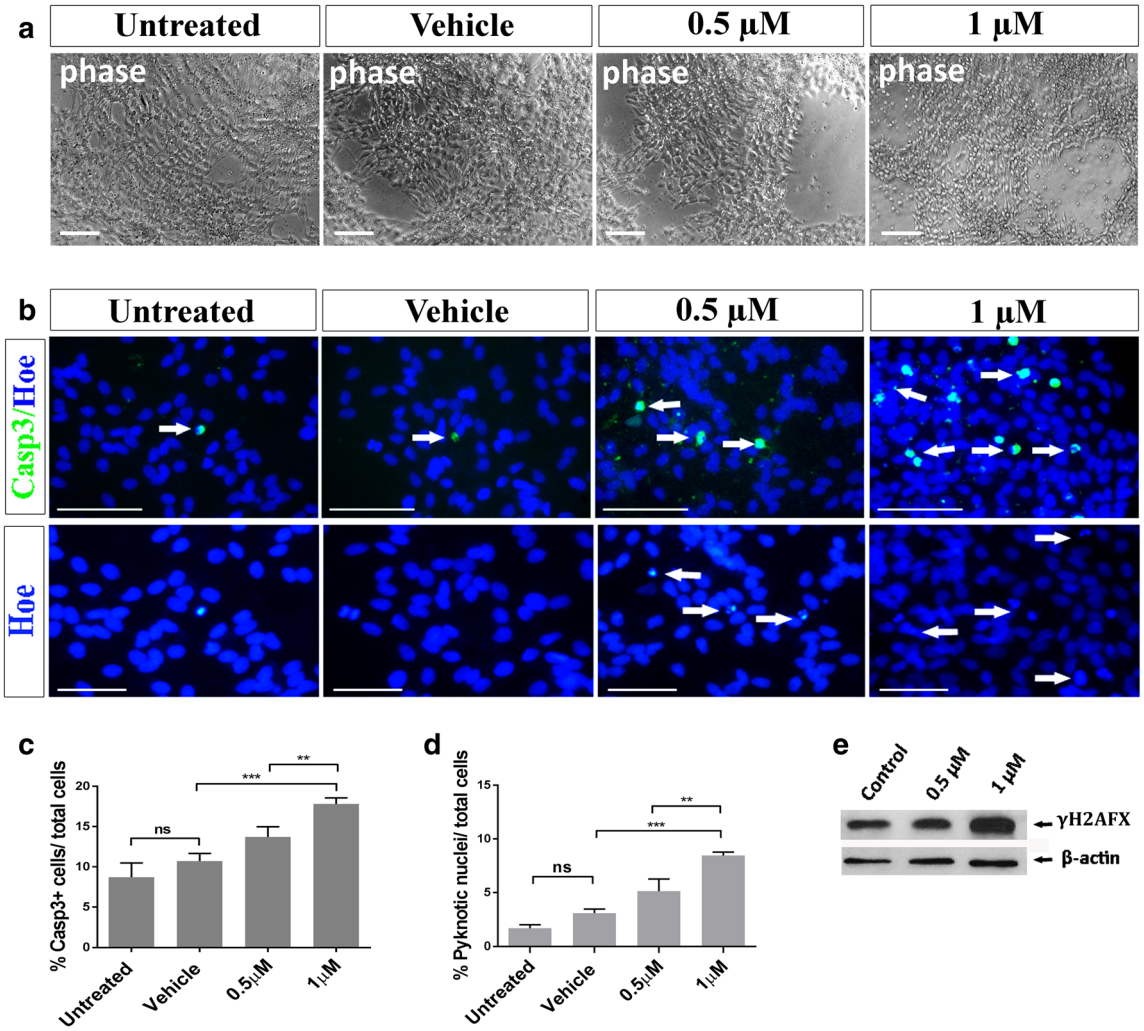

Fig. 2 Aβ42 increases cell death in differentiating hNS1 cells. **a** Representative bright field images of hNS1 cultures treated with 0.5 and 1 μM of Aβ42 peptide and controls (untreated cells and vehicle (DMSO)-treated cells) for 4.5 days. **b** Representative microphotographs of caspase 3 immunoreactivity (upper panels; Casp3; green, see arrows). Representative microphotographs of pyknotic nuclei stained with Höechst are represented in blue (bottom panels; hoe; see arrows). Scale bar, 100 μm (**a**) and 50 μm (**b**). **c** Quantification of the percentage of caspase 3+ cells in response to the specify dose of Aβ42 peptide. **d** Quantification of the percentage of pyknotic nuclei in the different experimental groups. **e** Western blot analysis of double-strand DNA breaks (using γH2AX antibody; 15 kDa) in cellular extracts after Aβ42 treatment. Actin was used as a loading control (42 kDa). Data are represented as mean ± SD of at least three different experiments ($n = 3$). Statistical significance of one-way ANOVA with the post hoc Tukey test; $^{**}p < 0.01$; $^{***}p < 0.001$; ns not significant vs control groups

thymidine analog BrdU. Ki67 protein is present in all active phases of the cell cycle, but absent in G_0 [39]. BrdU can be incorporated into newly synthesized DNA of dividing cells serving as a marker of the S phase of the cell cycle [33, 40].

Treatment with Aβ42 peptide provoked a significant increase in the percentage of Ki67-positive cells (Fig. 3a, c) at both concentrations tested, 0.5 μM (46 ± 1%) ($^{***}p < 0.001$; $n = 3$) and 1 μM (51.8 ± 1.6%) ($^{***}p < 0.001$; $n = 3$) as compared to controls (24.2 ± 1.1%). This increase in Ki67 expression was also observed at mRNA level by qRT-PCR (Fig. 3d) although only at the concentration of 1 μM ($^{***}p < 0.001$; $n = 3$), suggesting that Aβ42 promotes the proliferation of differentiating hNS1 cells.

In order to detect mitotic cells, we performed a short pulse (to avoid longer treatment that could interfere with the effects of the peptide) of BrdU for 2 h prior to the fixing and immunofluorescent staining (Fig. 3b, e). Our results confirmed that 7.3 ± 1.4% of control cells incorporated BrdU, indicating that they were proliferating. After treatment with Aβ42, a significant increase in the number of $BrdU^+$ cells was observed at the different doses tested, 13 ± 1.8% at 0.5 μM ($^{**}p < 0.01$; $n = 3$) and 21 ± 1.6% at 1 μM ($^{***}p < 0.001$; $n = 3$).

Together, these results indicate that Aβ42 treatment increases the percentage of proliferating hNS1 cells in a dose-dependent manner. This result is consistent with previous reports indicating the increase of proliferation of NPCs by Aβ42 [19].

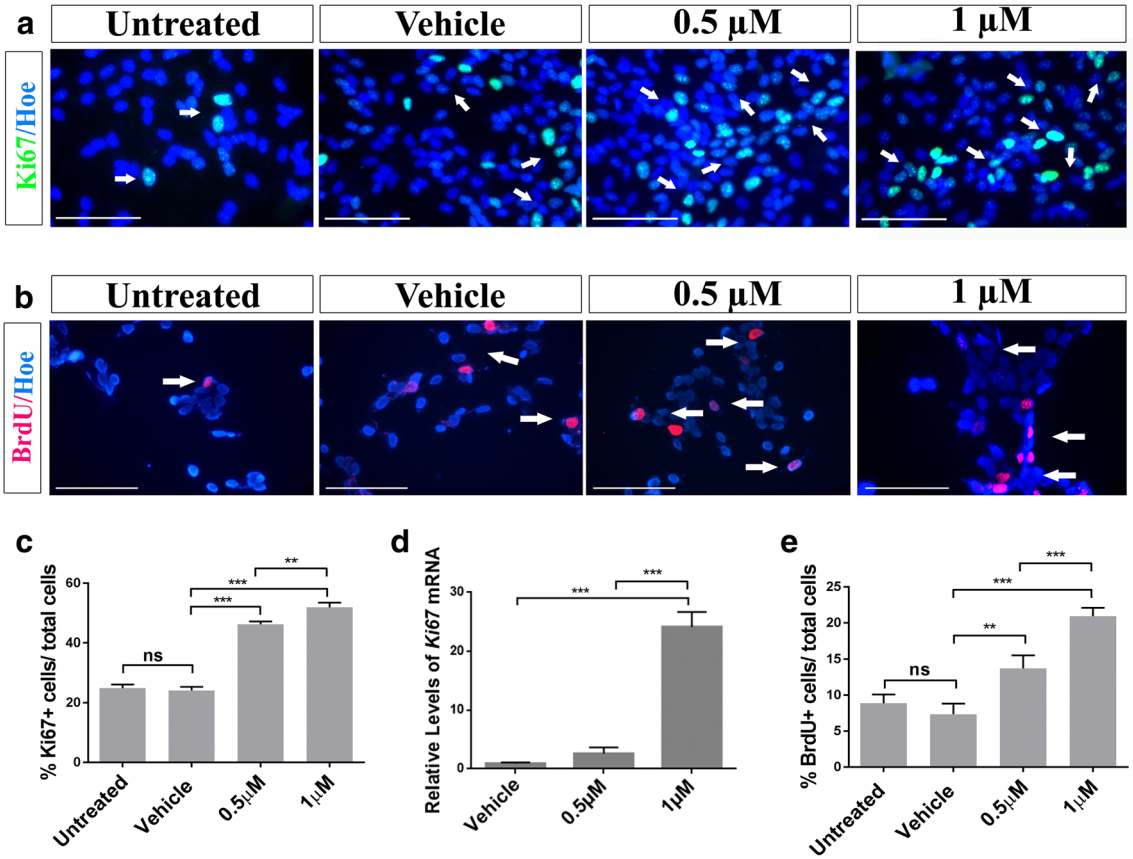

Fig. 3 Aβ42 promotes cell proliferation in differentiating hNS1 cells. **a** Representative images showing Ki67 immunoreactivity (green, arrows). **b** Microphotographs with immunoreactivity for BrdU (red, arrows). **c** Percentage of Ki67+ cells in the different experimental groups. **d** Relative expression levels of *Ki67* determined by qRT-PCR analyses. **e** Percentage of BrdU+ cells in the different cellular groups. Nuclei were counterstained in blue by Höechst. Scale bar, 50 μm. Data are represented as mean ± SD of at least three different experiments ($n = 3$). Statistical significance of one-way ANOVA with the post hoc Tukey test; $^{**}p < 0.01$; $^{***}p < 0.001$; ns not significant vs control groups

Role of Aβ42 in Cell Fate Specification of hNS1 Cells

Since Aβ peptides are involved in promoting glial cell fate specification in AD and in murine NSCs [19, 20, 23, 41], we further analyzed the induction of glial cell differentiation. Cultures were evaluated for the expression of GFAP (astrocyte marker) and as shown in Fig. 4a, b. Treatment with Aβ42 peptide significantly increased the percentage of $GFAP^+$ cells from 21.5 ± 0.9% in controls to 36.1 ± 1% at 0.5 μM ($^{***}p < 0.001$; $n = 3$) and 33.2 ± 1.7% at 1 μM ($^{***}p < 0.001$; $n = 3$). Similar results were obtained by qRT-PCR at the mRNA level (Fig. 4c). This data suggest that Aβ42 is positively regulating gliogenesis, and this regulation appears to be concentration-dependent, reaching a peak at lower concentrations (0.5 μM).

Different lines of evidence point to the role of Aβ peptides in the differentiation and phenotypic specification of NSCs. In particular, some studies have suggested the involvement of Aβ peptides in neurogenesis [10, 19, 20, 24].

To determine the effects of Aβ42 on neuronal lineage determination, we analyzed the expression of β-III-tubulin (neuronal marker) in hNS1 cells undergoing differentiation in the presence of Aβ42 peptide. No significant changes were observed between controls and Aβ42 peptide-treated groups. All groups had approximately 25 ± 2% of β-III-tubulin$^+$ cells, suggesting that this peptide does not affect neuronal lineage determination (Fig. 4d, e). This result was further confirmed by qRT-PCR (Fig. 4f) at the mRNA level.

Together, these results indicate that Aβ42 treatment of differentiating hNS1 cells favors the differentiation towards a glial phenotype, without affecting neuronal differentiation.

Aβ42 Peptide Promotes Gliogenesis in Differentiating hNS1 Cells

Earlier, we demonstrated that Aβ42 treatment increased proliferation (Fig. 3) and glial fate specification (Fig. 4) in differentiating hNS1 cells. To further investigate whether the effect of Aβ42 in enhancing lineage-specific markers was due to proliferative effects, we examined the number of cells double-positive for GFAP and Ki67 (Fig. 5a) with regard to total cells (Fig. 5b) and with regard to $GFAP^+$ cells (Fig. 5c).

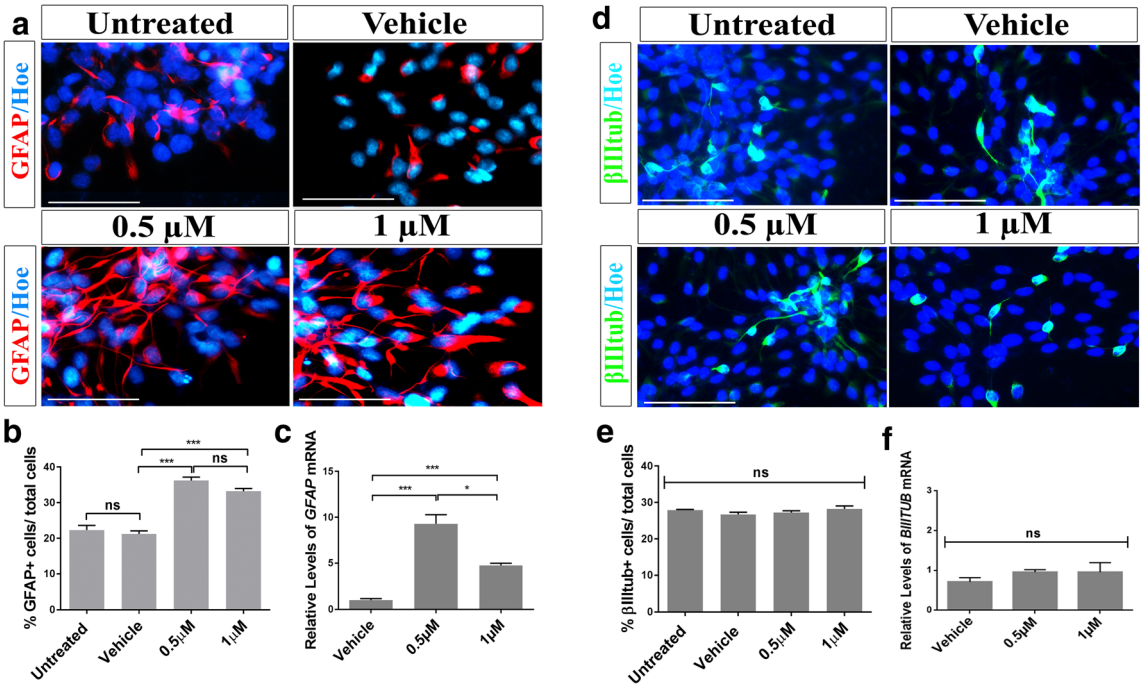

Fig. 4 Role of Aβ42 in cell fate specification of differentiating hNS1 cells. **a** Immunoreactivity for GFAP (red). Scale bar, 50 µm. **b** Analyses of the percentage of GFAP+ cells in the different groups tested after Aβ42 treatment. **c** Relative expression levels of *GFAP* mRNA obtained by qRT-PCR. **d** Representative images showing immunoreactivity for β-III-tubulin (βIIItub; green). Scale bar, 50 µm. **e** Analyses of the percentage of β-III-tubulin+ cells after Aβ42 treatment. **f** Relative expression levels of *BIIITUB* mRNA by qRT-PCR. Cell nuclei in A and D were counterstained by Höechst (blue). Data are represented as mean ± SD of at least three different experiments ($n = 3$). Statistical significance of one-way ANOVA with the post hoc Tukey test; $^{*}p < 0.05$; $^{***}p < 0.001$; ns not significant

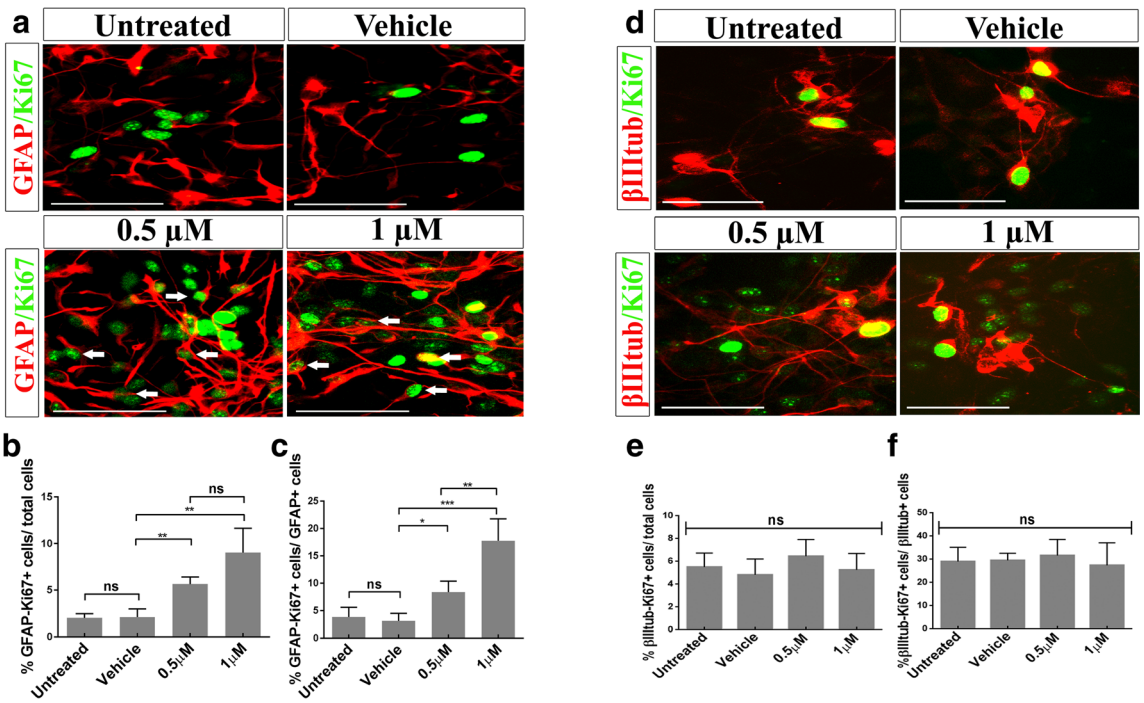

Fig. 5 Aβ42 peptide promotes gliogenesis in differentiating hNS1 cells. **a** Representative images showing immunoreactivity for GFAP (red) and Ki67 (green) (arrows). **b** Percentage of GFAP-Ki67+ cells/total cells after Aβ42 treatment. **c** Percentage of GFAP-Ki67+ cells/GFAP+ cells in the different cellular groups after Aβ42 treatment. **d** Representative images showing immunoreactivity for β-III-tubulin (βIIItub; red) and Ki67 (green). **e** Percentage of β-III-tubulin-Ki67+ cells/total cells in Aβ42 groups. **f** Percentage of β-III-tubulin-Ki67+ cells/β-III-tubulin+ cells in Aβ42 groups. Scale bar, 50 µm. Data are represented as mean ± SD of at least three different experiments ($n = 3$). Statistical significance of one-way ANOVA with the post hoc Tukey test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns not significant

In the control group, we observed that GFAP$^+$ cells were rarely double-positive for Ki67 (2.1 ± 0.8%). However, this percentage increased significantly after Aβ42 treatment, rising to 6 ± 0.5% (**$p < 0.01$; $n = 3$) in the 0.5-μM group and to 9 ± 2% (**$p < 0.01$; $n = 3$) in the 1-μM group, counting GFAP$^+$/Ki67$^+$ cells with respect to total number of cells (Fig. 5b). This increase in GFAP$^+$/Ki67$^+$ cells supports our previous results showing that Aβ42 treatment increases proliferation and favors gliogenesis in differentiating hNS1 cells. To further explain these effects, we carried out a more detailed study of GFAP$^+$/Ki67$^+$ cells compared to the population of GFAP$^+$ cells. We again observed that the groups treated with Aβ42 had a significantly increased number of GFAP$^+$/Ki67$^+$ cells, increasing from 3.5 ± 1.5% in the control group to 8 ± 2% in the 0.5-μM group (*$p < 0.05$; $n = 3$) and to 18 ± 4% (***$p < 0.001$; $n = 3$) in the 1-μM group (Fig. 5c). This suggests an increase in the number of precursors (Ki67$^+$) that are specifically differentiating to glial cells following Aβ42 treatment.

To investigate if this effect was specific to glial precursors, we performed a similar study examining the number of cells double-positive for β-III-tubulin and Ki67 (Fig. 5d) with regard to total cells (Fig. 5e) and with regard to β-III-tubulin$^+$ cells (Fig. 5f). We observed no statistically significant differences between control and Aβ42-treated groups for β-III-tubulin$^+$/Ki67$^+$ cells.

These results suggest that Aβ42 treatment increases proliferation and favors gliogenesis by increasing the pool of proliferating glial precursors, and this effect is specific to glia, without affecting neurogenesis or the levels of neuronal precursors.

Study of Molecular Pathways Involved in the Observed Effects Aβ42

The molecular pathways involved in Aβ peptide physiology are currently a popular debate in the field. Furthermore, the exact role of Aβ42 in its soluble/monomeric form remains unknown. It has been described that Aβ peptide, (mainly in its aggregated form), affects numerous intracellular signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K) pathway or Ras-MAPK signaling [42, 43]. In order to explore the possible molecular pathways involved in the effects of Aβ42 observed in this study, we analyzed the expression of some genes associated with these signaling pathways: *GSK3*β (gene for glycogen synthase kinase 3β), *PI3K* (gene for phosphatidylinositol 3-kinase), *ERK* (gene for extracellular-signal-regulated kinase) and *AKT* (gene for protein kinase B) by qRT-PCR (Fig. 6a–d). We detected that Aβ42 treatment significantly increased the expression of *GSK3*β mRNA as compared to controls (Fig. 6a). However, no significant differences were observed for the other genes analyzed (Fig. 6b–d). Together, these results indicate a possible role for *GSK3*β in the observed effects.

To determine the involvement of *GSK3β* in the effects of Aβ42 treatment on the glial specification of hNS1 cells, these

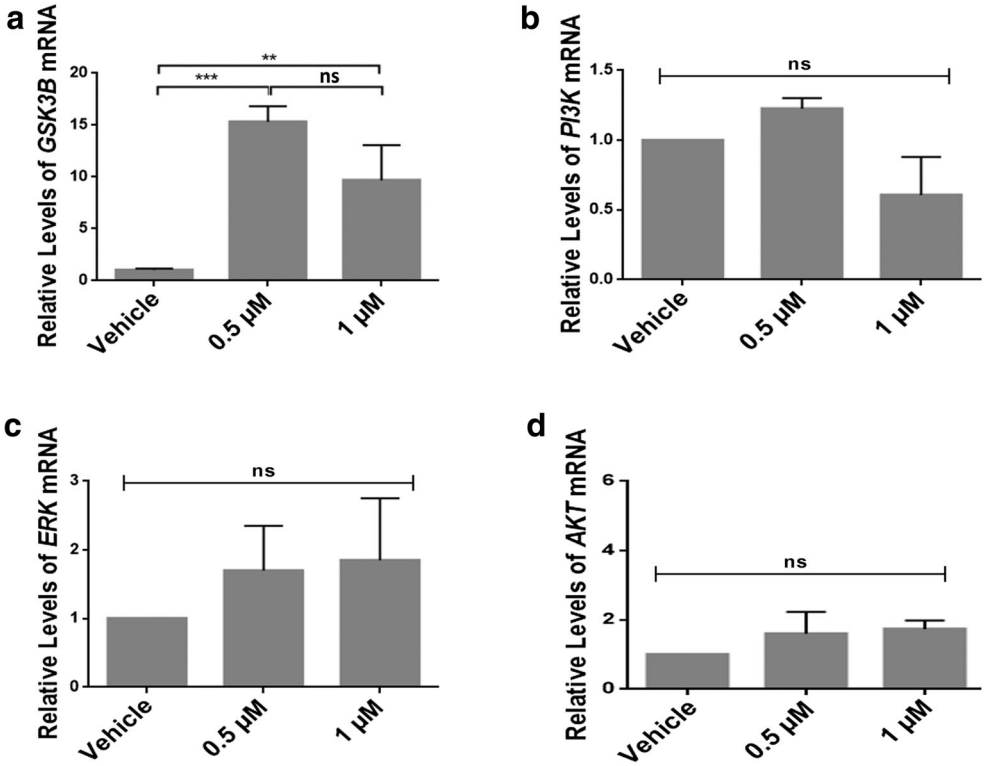

Fig. 6 Analyses of molecular pathways implicated in the effects of Aβ42 treatment. Relative expression levels of *GSK3B* mRNA (**a**), *PI3K* mRNA (**b**), *ERK* mRNA (**c**), and *AKT* mRNA (**d**) obtained by qRT-PCR after Aβ42 treatment in differentiating hNS1 cells. Data are represented as mean ± SD of at least three different experiments ($n = 3$). Statistical significance of one-way ANOVA with the post hoc Tukey test; **$p < 0.01$; ***$p < 0.001$; ns not significant

cultures were treated during differentiation (from 0 to 4.5 days) with a combination of Aβ42 (0.5 or 1 μM) + CHIR99021 (2 μM), an amino pyrimidine derivative that is a potent inhibitor of *GSK3β* (Fig. 7a). We tested different concentrations of CHIR99021 in order to find the optimal dose of this inhibitor and found that 2 μM was effective in inhibiting GSK3β activity, as CHIR99021 2-μM-treated cells showed a decreased percentage (8 ± 1.5%; $*p < 0.05$; $n = 3$) of GFAP$^+$ cells compared to vehicle (14 ± 1%), consistent with our previous work [31]. The control chosen for this experiment was Aβ42 (0.5 μM), as our results showed a peak in gliogenesis at this concentration.

Interestingly, a significant decrease of GFAP$^+$ cells was observed after treatment with Aβ42 + CHIR99021 2 μM, obtaining a percentage of 6 ± 1.5% in the Aβ42 0.5 μM + CHIR99021 2-μM group ($***p < 0.001$; $n = 3$) and 4 ± 1% in the Aβ42 1 μM + CHIR99021 2-μM group ($***p < 0.001$; $n = 3$) as compared to the Aβ42 (0.5 μM) control (28 ± 2%) (Fig. 7b, c). Moreover, these results were also confirmed at the mRNA level by qRT-PCR (Fig. 7d).

In summary, these results suggest that Aβ42 peptide may favor the proliferation of human NSCs undergoing differentiation and commit them to a glial cell fate. This cell fate specification may be, at least in part, mediated by the activation of the GSK3β pathway. However, prolonged exposure of these cells to high concentrations of peptide appears to be toxic and activates an apoptotic pathway.

Discussion

Despite the high prevalence of AD, there is no cure for this disorder. Stimulating the proliferation and differentiation of endogenous NSCs and the grafting of exogenous NSCs into the brain are possible therapeutic strategies for the treatment of AD, which are currently being evaluated. The application of human NSCs in stem cell therapy for neurodegenerative disorders depends on the ability of transplanted NSCs to survive in affected brains and to proliferate, migrate, and differentiate into different functional neural lineages.

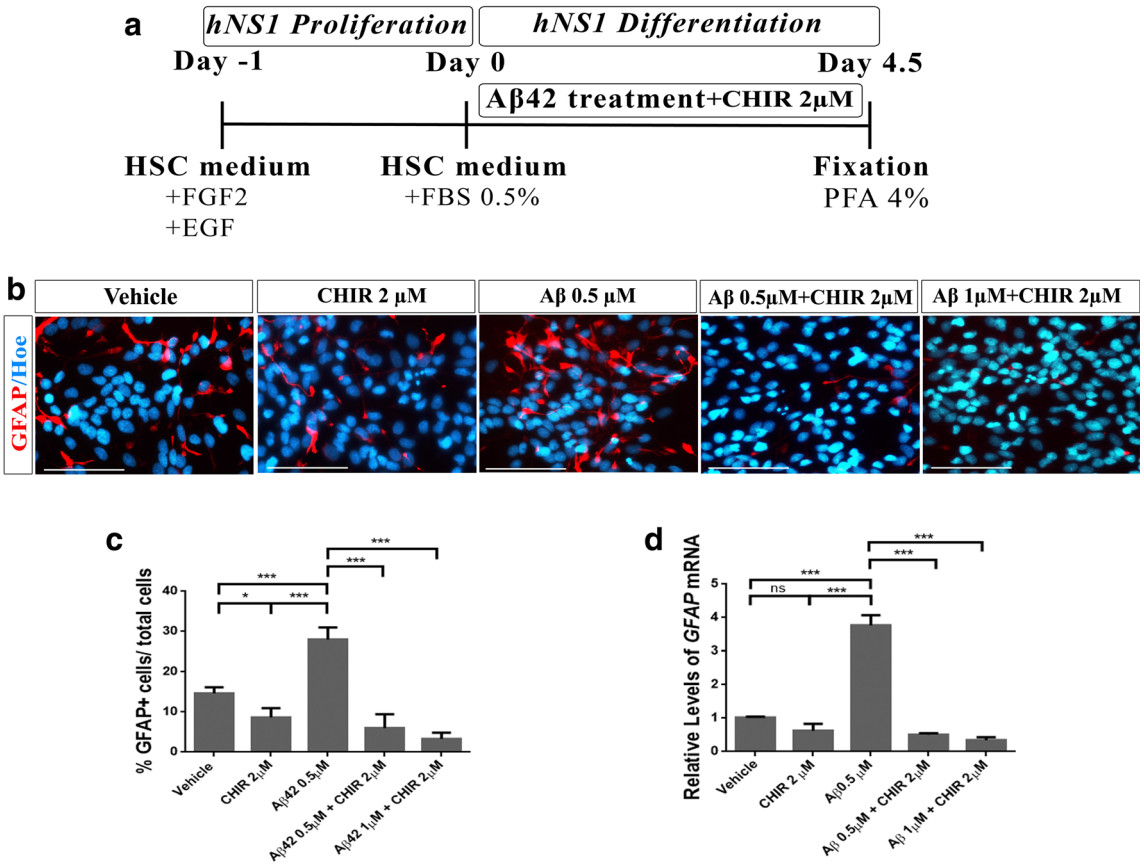

Fig. 7 Effects of Aβ42 + CHIR99021 on cell fate decisions of hNS1 cells. **a** Schematic representation of the experimental protocol. **b** Immunoreactivity for GFAP (red). Scale bar, 50 μm. **c** Analyses of the percentage of GFAP+ cells in the different groups tested after Aβ42 + CHIR 2-μM treatment. **d** Relative expression levels of *GFAP* mRNA obtained by qRT-PCR. Cell nuclei in **b** were counterstained by Höechst (blue). Data are represented as mean ± SD of at least three different experiments ($n = 3$). Statistical significance of one-way ANOVA with the post hoc Tukey test; $*p < 0.05$; $***p < 0.001$; ns not significant

In an attempt to find possible therapies for this disease, much attention has been focused on understanding the pathophysiological causes of AD, including the toxic effects of Aβ peptides in promoting synaptic dysfunction, neuronal death, and the deterioration of the development of neuronal progenitor cells [10, 14, 36].

In this report, we demonstrate that Aβ42 peptide significantly stimulates the proliferation of human NSCs undergoing differentiation (Fig. 3) and commit them to a glial cell fate (Figs. 4 and 5).

This cell fate specification may be mediated by the activation of the GSK3β pathway (Figs. 6 and 7). However, prolonged exposure of these cells to high concentrations of peptide becomes neurotoxic and activates an apoptotic pathway (Fig. 2).

Aβ peptides are involved in the progression of AD, promoting neuronal death and impairing neural progenitor development [10, 14, 36, 37]. It is already known that Aβ42 is one of the predominant forms of peptide that accumulates in the AD brain [44]. In vitro experiments have demonstrated that Aβ42 aggregates into amyloid plaques much more rapidly than Aβ40 [45, 46]. We showed that monomeric Aβ42 induces programmed cell death at the higher concentration tested (1 μM), as confirmed by an increase in caspase 3 activation and in the number of pyknotic nuclei observed. Furthermore, the expression of γH2AFX indicated the presence of DNA damage (Fig. 2). These results are contrary to previous reports [19, 20], which may be due to differences in the cellular systems and experimental conditions tested. In our case, we used differentiating human NSCs that were treated with different concentrations of Aβ42 peptide for 4.5 days. Chen and Dong, however, used undifferentiated NPCs obtained from E18 fetal rat brains and treated cells for 25 h with a single dose of 1.5 μM of Aβ42 peptide. Fonseca et al. used differentiating NSCs obtained from E14.5 fetal mice brains, treated for 24 h, also only testing a single dose, 1.5 μM. The increasing levels of cell death observed in human NSC cells probably reflect the cytotoxicity of Aβ42 peptide at prolonged exposure and higher peptide concentration, since at lower doses, cell death was not significant.

Although the toxic effects of Aβ42 have been described, there is accumulating evidence that Aβ peptides (including Aβ42) may have important functions in a normal physiological context, especially in their monomeric state, before they aggregate and become toxic [47]. Unfortunately, the signaling pathways that regulate Aβ function and the molecular pathways involved are still poorly understood. For this reason, it is important to study the effects of these peptides in their monomeric state, in order to help understand its function in normal physiological contexts, in processes such as neurogenesis and gliogenesis.

Neurogenesis in the adult central nervous system plays an important role in learning, hippocampal memory, and smell [48]. However, in patients with AD, neurogenesis is decreased and there is a progressive loss of neurons in the brain [1] partially due to the extracellular aggregates of Aβ peptide. Furthermore, the formation of plaques activates microglia, leading to exaggerated expression and release of inflammatory cytokines and chemokines with neurodegenerative effects [47, 49]. Gliogenesis consists of the generation of glial populations derived from multipotent NSC and its dysregulation is involved in AD. It has been shown that post mortem tissue of AD brains present increased levels of NCS proliferation [50] and increased gliogenesis [31, 50–52], indicating a disturbance in the normal physiological context of the diseased brain.

A number of studies have shown that Aβ peptide may affect NSC proliferation, depending on the type of cell, peptide used, and the time of treatment. Our results provide evidence that Aβ42 promotes human NSC proliferation in a dose-dependent manner (Fig. 3), as observed by the increase of Ki67 expression and by increased BrdU incorporation. Our findings agree with a previous study showing that monomeric Aβ42 stimulates the proliferation of primary NPCs isolated from the embryonic rat brain [19]. However, Fonseca et al. did not observe alterations in mouse NSCs proliferation after Aβ42 treatment. The reason for these discrepancies has not yet been established.

The function of Aβ peptide is very controversial and differs between already published studies [53–55]. It has been shown that human NSC exposure to oligomeric Aβ peptides decreased proliferative potential of these cells, stimulating their differentiation into a glial cell fate, without affecting neuronal fates [23]. Similar results were obtained by the same authors when analyzing the effects of oligomeric forms of Aβ42 in hNSCs, observing a decrease in proliferation and significant increase in gliogenesis of these cells [23]. This is consistent with a marked effect of Aβ oligomers on hNSCs. Another group found that in NSCs from the rat hippocampus, neurogenesis is induced by Aβ42, and this activity is associated with Aβ oligomers and not with fibrils [24].

However, our results showed that aside from the toxic effects of Aβ42, at lower concentrations, it favors gliogenesis without affecting neurogenesis (Fig. 4). We saw significantly increased expression of $GFAP^+$ in hNS1s treated with Aβ42, indicating an increase in the number of cells obtaining a glial phenotype. This result was concentration-dependent, peaking at lower concentration (0.5 μM). Furthermore, we saw an increase in the number of proliferating precursors ($Ki67^+$) that were also $GFAP^+$, indicating that the increased proliferation observed promoted glial cell fate specification. A similar study showed that there were no significant changes in the number of β-III-$tubulin^+$ cells double-positive for $Ki67^+$, indicating that the increased proliferation observed was specific in increasing gliogenesis without affecting neurogenesis. This is supported by our previous work in hNS1 cells which

showed that in cells overexpressing amyloid precursor protein (APP whose enzymatic processing generates Aβ peptides) increases gliogenesis and inhibits neurogenesis, an effect most likely mediated by *GSK3β* [31].

Accumulating evidence suggest that *GSK3β* interferes with the biology of Aβ [51]. Our results clearly indicate that Aβ42 treatment during hNS1 differentiation promotes the expression of *GSK3β* and we showed that both Aβ42 and *GSK3β* might have an important role in the phenotypic specification of hNS1 cells (Figs. 6 and 7). Although further studies are needed to evaluate the molecular pathways and mechanisms involved in the effects of different Aβ peptides on human NSC biology, our data may help to explain previous findings demonstrating that NSCs from AD individuals or animal models differentiate into astrocytes rather than neurons [31, 50–52]. Earlier studies have begun highlighting the importance of GSK3β signaling for NSCs proliferation and differentiation [43]. In fact, an important focus of AD research is *GSK3β* and its involvement in the pathological symptoms of this disease, Aβ plaque formation, tau hyperphosphorylation, and neurodegeneration. *GSK3β* is starring in a variety of physiological processes such as regulating cell morphology, neuronal outgrowth, and motility and synaptic plasticity [56]. This gene is constitutively active in most tissues and most commonly regulated by inhibitory phosphorylation on SER9. However, the dysregulation of these signal transduction pathways results in failure to adequately repress *GSK3β*, thus allowing it to remain abnormally active, which is believed to contribute to various pathologies [51]. The importance of GSK3β signaling has also been demonstrated in cultured neural progenitor cells where inhibitors of *GSK3β* protect NPCs from apoptosis [57] and facilitate neural progenitor differentiation towards a neuronal phenotype [58]. Furthermore, in vivo overexpression of this gene causes alterations in adult neurogenesis, leading to a depletion of the neurogenic niches and a decrease in the number of mature neurons [59].

Our results indicate that treatment with Aβ42 increases the expression of *GSK3β* in differentiating hNS1 cells. This further indicates that Aβ42, mediated by *GSK3β*, might have an important role in the phenotypic specification of hNS1 cells, since we saw that inhibiting GSK3β activity with CHIR99021 abrogated the effect on gliogenesis.

Conclusively, we have established an effect of Aβ42 peptide on hNSCs, showing that the effects of Aβ42 are concentration-dependent. At higher concentrations, Aβ42 appears to be cytotoxic while at lower concentrations, it promotes the differentiation of hNSCs to glia. Since the cellular and molecular effects involved in AD occur several decades before the first cognitive and clinical symptoms appear, these results could be relevant in discovering new markers for earlier diagnosis and for the development of new therapeutic targets. Taken together, it can help us understand the cellular and molecular processes that occur in the brain of Alzheimer's patients at the beginning of the disease, at which point treatment could be more effective. A major limitation to these types of studies is the lack of models, both in vitro and in vivo, that perfectly mimic an Alzheimer's affected brain [60]. However, our cell system can be a useful tool to study the physiological context of a brain with AD and help clinical progress in stem cell-based therapies to treat this disease. Although more studies are needed, our results provide important information that could help improve the development of future therapies. Understanding the mechanisms that control the differentiation of NSCs in neurodegenerative diseases and understanding the molecular pathways involved can provide valuable information for possible stem cell-based therapies to treat AD.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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