

Triple Validation of a Neuronal Cell Model and Standardized Amyloid- β Aggregation Uncovers Key Sources of Variability in A β 1-42 Toxicity Assessment

Anne Cécile Van Baelen

CEA Paris-Saclay

Clément Poteaux

CEA Paris-Saclay

Philippe Robin

CEA Paris-Saclay

Xavier Iturrioz

CEA Paris-Saclay

Steven Panek

Bielefeld University

Norbert Sewald

Bielefeld University

Denis Servent

CEA Paris-Saclay

Nicolo Tonalì

`nicolo.tonali@cea.fr`

CEA Paris-Saclay

Article

Keywords: SH-SY5Y cells, neuronal differentiation, amyloid β 1–42, cytotoxicity

Posted Date: October 27th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10392606/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Reliable *in vitro* evaluation of amyloid- β (A β) toxicity remains a major challenge for the development of anti-amyloid therapeutics, largely due to the lack of standardized experimental workflows. In our previous work, we established a robust and reproducible protocol for the synthesis, physicochemical characterization and controlled aggregation of highly pure A β 1–42 species. Here, we provide a comprehensive validation of the cellular component by combining, for the first time, a triple characterization strategy of the neuronal model, a standardized amyloid aggregation process, and a direct comparison of toxicity readouts under identical experimental conditions. SH-SY5Y cells were differentiated using retinoic acid and brain-derived neurotrophic factor, and the neuron-like phenotype was validated by immunofluorescence, gene expression profiling and increased resistance to H₂O₂-induced oxidative stress. Using this fully characterized platform, we compared the effects of non-aggregated and pre-aggregated A β 1–42 on differentiated and non-differentiated cells through two complementary viability assays (MTT and LDH). Our results demonstrate that A β -induced toxicity strongly depends on peptide aggregation state, neuronal maturation and the analytical method employed. This integrated approach highlights critical sources of variability in amyloid research and provides a standardized framework to improve reproducibility and translational relevance of *in vitro* anti-amyloid screening strategies.

1. INTRODUCTION

Alzheimer's disease (AD) is a major global health issue, affecting almost 60 million people worldwide, and remains an incurable progressive neurodegenerative disorder ¹. AD is defined by intracellular neurofibrillary tangles of hyperphosphorylated tau and extracellular beta-amyloid (A β) peptides plaques, ultimately leading to neuronal loss and brain atrophy ². Among A β peptides, A β 1–42 is the most aggregation-prone, forming soluble oligomers, protofibrils and fibrils ³⁴. Increasing evidence indicate that soluble species are most closely associated with early neuronal dysfunction, but their precise structural nature remains debated ^{5 6 7 8 9}. However, understanding their cytotoxic mechanisms remains challenging mainly due to difficulties in reproducibly controlling A β 1–42 aggregation, and in limitations of conventional neuronal cell models.

Indeed, the self-assembly of A β 1–42 is a complex mechanism in which unfolded monomers undergo a transition through transient oligomeric intermediates leading to fibril formation by on-pathway or off-pathway routes ^{10 11 12}. Although fibrils were traditionally considered the main pathological hallmark, increasing attention is given to oligomers, which reduce functional monomer availability, and are thought to contribute to synaptic toxicity and neurodegeneration ⁴⁵. Mastering this complex process is essential, and it is important to ensure the use of a well-established *in vitro* protocol for controlled aggregation ^{13 14}.

The development of novel therapeutic agents targeting amyloid- β aggregation relies heavily on in vitro screening platforms to evaluate the toxicity of A β assemblies¹⁵. (However, the vast majority of these studies are still performed using undifferentiated neuroblastoma cell lines, such as SH-SY5Y, which only partially recapitulate the phenotype and physiology of mature human neurons¹⁶. Consequently, the biological responses observed in these simplified models may not accurately reflect neuronal susceptibility to A β aggregates, raising concerns about the reliability and reproducibility of current in vitro screening strategies for anti-amyloid compounds.

Reliable neuronal cell culture models remain a central challenge. While primary rodent neurons provide high physiological relevance, with authentic morphology and synaptic activity, they are limited by non-human origin and ethical constraints¹⁷. Also, human pluripotent stem cells (ESCs and iPSCs) offer a comprehensive model of neurogenesis and are expandable, but require complex, costly protocols that limit widespread and high-throughput use^{18 19 20}. Consequently, there is sustained interest in alternative *in cellulo* models that are cost-effective, reproducible and scalable. The immortalized human neuroblastoma cell lines SH-SY5Y is a widely used neuronal cellular model^{21 22}. SH-SY5Y cells are relatively easy to culture, exhibit unlimited proliferative capacity and grow robustly under standard conditions, but in their undifferentiated state they display only limited neuronal characteristics²³. However, these cells retain the capacity for differentiation to induce a more neuronal phenotype, using serum restriction combined with retinoic acid (RA), frequently supplemented with neurotrophic factors such as brain-derived neurotrophic factor (BDNF) or nerve growth factor, promoting neurite outgrowth and the expression of markers associated with mature neurones^{24 25 26 27 28}. Differentiated SH-SY5Y cells thus provide a valuable intermediate model for screening studies, offering enhanced neuronal features, while maintaining lower technical complexity, cost-effectiveness and scalability compared to primary neurons or iPSC-derived systems, but with slightly reduced physiological relevance²¹.

However, despite this interest, there is still a lack of studies investigating the effects of well-controlled A β 1–42 aggregates in differentiated SH-SY5Y cells. Most A β 1–42 toxicity studies are performed on non-differentiated SH-SY5Y cells, especially in the context of amyloid aggregation inhibitors, and their use remains controversial^{29 30 31}. A few studies have nevertheless highlighted a distinct behaviour of A β 1–42 in differentiated vs non-differentiated cells but have not explored the full relationship between toxicity and aggregation state^{32 33 34}. In addition, the differentiation state of SH-SY5Y cells is rarely systematically validated through morphological analysis, gene expression profiling, and functional assays, leaving aspects of their functional maturation incompletely understood. Such validation is essential for establishing robust and relevant *in cellulo* systems, in particular in comparison with iPSCs, to better understand how distinct A β 1–42 aggregate species influence neurodegenerative mechanisms.

In this context, we propose three independent levels of analysis to validate differentiation methodology that offers a reliable framework for investigating neuronal outcomes in the presence of well controlled A β 1–42 species. We evaluated the effects of these A β 1–42 aggregates on (i) cytotoxicity, assessing both membrane integrity and metabolic activity, and (ii) cellular differentiation, including the expression

of neuronal markers. This approach enhances the scientific relevance of differentiated SH-SY5Y cultures and broadens their use as a reproducible, accessible, and high-throughput-compatible model for studying neuronal differentiation and the cellular effects of A β 1–42 aggregates. Rather than simply comparing differentiated and undifferentiated neuronal cells, this work provides a systematic evaluation of the principal experimental variables that influence the interpretation of A β toxicity assays. We demonstrated that controlled aggregation protocol, together with a triple-level validated neuronal model is an essential prerequisite for generating reliable and biologically meaningful data during the preclinical evaluation of anti-amyloid therapeutic candidates.

2. MATERIALS & METHODS

2.1 Cell culture and differentiation – SH-SY5Y cells (CRL-2266 purchased from ATCC) were cultured in a maintenance medium composed of DMEM/F12 (GIBCO 61965059) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/mL), streptomycin (50 μ g/mL) (P/S) and glutaMAX-CTS (0.5 mM), and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Culture medium was changed every 2–3 days, and cells were subcultured upon reaching approximately 70–80% confluence using trypsin-EDTA.

For differentiation experiments, cells were seeded at an appropriate density (2×10^4 cells/cm²) to avoid over confluence during the treatment period. After cell attachment (typically 24 h post-seeding), differentiation was initiated by replacing the maintenance medium with low-serum (1% FBS) differentiation medium containing retinoic acid (RA, 10 μ M, Thermo Scientific 302-79-4) and/or brain-derived neurotrophic factor (BDNF, 50 ng/mL, Sigma Aldrich B3795). Differentiation medium was changed every 48 h. Control conditions included treatment with RA alone, BDNF alone, or vehicle.

- 2.2 Immunofluorescence** – For immunofluorescence experiments, SH-SY5Y cells were seeded in a 24-wells plate (2×10^4 cells/cm²) on a coverslip before differentiation process typically 24 h after seeding. Then, cells were fixed with 4% paraformaldehyde for 10 min at room temperature (rt), permeabilized in PBS-Triton X-100 0.1% (PBST) for 10 min at rt and blocked prior to antibody incubation with 5% bovine serum albumin (BSA) in PBS for 45 min at rt. Cells were incubated with primary antibody (rabbit anti-calnexin, Cell Signalling Technology (C5C9) at 1/200 for 1h30 at rt; rabbit anti-RCAS1, Cell Signalling Technology (D2B6N) at 1/200 for 1h30 at rt; rabbit anti-TH, Proteintech (284171AP) at 1/500 for 1h30 at rt; rabbit anti-synaptophysin, Cell Signalling Technology (D8F6H) at 1/200 for 1h30 at rt); and/or direct staining with the anti- β -III-tubulin primary antibody (Thermo Fisher 2G10-TB3 at 1/1000 for 30 min at rt) or with ReadyProbes™ Reagent F-Actin phalloidin red/orange (Invitrogen R37112 at 1/100 for 5 min at rt). The Alexa Fluor 555–conjugated goat anti-rabbit IgG secondary antibody (Life Technologies) was applied at a dilution of 1/10,000. Nuclei were counterstained with DAPI, and samples were mounted using Vectashield (Sigma Aldrich F6057). Fluorescence imaging was performed using a Nikon Eclipse Ti microscope.
- 2.3 RNA extraction** – RNA extraction from SH-SY5Y cells (1 well seeded at 2×10^4 cells/cm²) was performed using the RNeasy Mini Kit (Qiagen 74014) according to the manufacturer's instructions.

RNA was solubilized in 20 µL of RNase-free water.

3. **2.4 RT-qPCR** – First-strand cDNA was generated by reverse transcription (RT) using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher, K1672) according to the manufacturer's instructions. The cycling conditions consisted of an initial denaturation at 95°C for 1 min. Subsequently, 40 amplification cycles were carried out, with denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min, using the C1000 Touch thermocycler (Biorad, CFX96 Real-Time system). Data are expressed as the mean of ratio of $\frac{\frac{2^{-\Delta C_q \text{ test condition gene}}}{2^{-\Delta C_q \text{ reference gene}}}}{\frac{2^{-\Delta C_q \text{ control condition gene}}}{2^{-\Delta C_q \text{ reference gene}}}}$. Primers were bought from ThermoFischer: RPLP0 Hs00420895_gH, SYP Hs00300531_m1, TUBB3 Hs00801390_s1, MAP2 Hs00258900_m1, DLG4 Hs01555373_m1, MAPT Hs00902194_m1, RBFOX3 Hs01370654_m1, BCL2 Hs04986394_s1, TFAM Hs00273372_s1, MFN2 Hs00208382_m1, DCX Hs00167057_m1.
4. **2.5 MTS and LDH test** – Cells were seeded into 96-well plates (2×10⁴ cells/cm²) before differentiation process typically 24 h after seeding. Following treatment with the indicated compounds (H₂O₂ from Sigma Aldrich H1009 and Aβ1–42 from Bachem 4014447) for the specified duration, 20 µL of MTS solution (Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay, G3580) was added to each well, and plates were incubated for 1h30. For LDH cytotoxicity test, 50 µL of cell culture supernatants were collected and transferred to a fresh 96-well plate. Then, 50 µL of CytoTox 96® reagent (Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay, G1780) was added according to the manufacturer's instructions, and the plate was incubated for 30 min at rt protected from light before adding 50 µL of the Stop solution. Plates were stored protected from the light at rt for 30 min before reading. In each case, absorbance was measured at 490 nm using a CLARIOstar Plus plate reader (BMG LABTECH). Cell viability was expressed as a percentage relative to control cells.
5. **2.6 Aβ1–42 aggregates preparation** – The Aβ1–42 peptide was obtained commercially (Bachem 4014447) and handled according to previously published procedures¹³. Initially, the peptide was dissolved in 0.16% ammonia in H₂O (prepared from a 25% ammonia stock solution) at a concentration of 2 mg/mL for aliquoting and was subsequently lyophilized. To disaggregate preformed aggregates, the Aβ1–42 sample was resolubilized in 60 mM NaOH to a final concentration of 1 mg/mL, followed by sonication for 5 min and centrifugation at 5000 rpm for 5 min. The sample was then immediately lyophilized and aliquots were stored at -20°C until use. Briefly, 20 µg of the monomeric lyophilized peptide was first solubilized in 60 mM HCl prepared in phosphate buffer at 1 µg/µL, then diluted at 50 µM into phosphate buffer (10 mM at pH 7.4) to initiate controlled aggregation. Fibril formation was induced by incubation under conditions favouring self-assembly (37°C, shaking at 250 rpm, 24 h), while parallel preparations were applied directly to cell cultures in OptiMEM medium (Thermo Fischer 31985062) with 1% FBS and 1% P/S, to assess biological effects at a final 10 µM concentration of Aβ1–42.

3. RESULTS

3.1 Differentiation protocol and kinetic of differentiation

To induce and characterize the differentiation of SH-SY5Y cells, we established and compared several differentiation protocols. This step was critical to obtain a more mature neuron-like phenotype suitable for downstream functional and molecular analyses. Cells were treated with retinoic acid (RA) and/or brain-derived neurotrophic factor (BDNF) using different treatment durations and combinations (Table 1). RA is known to promote neuronal commitment and initiate differentiation, whereas BDNF supports neuronal maturation, neurite outgrowth and synaptic functionality^{24 27}. By varying both the combination of these factors and the duration of exposure, we aimed to determine the conditions that most effectively drive the transition toward a stable and functionally mature neuronal phenotype. This comparative approach allowed us to identify differentiation conditions to obtain this neuron-like phenotype and that maximize thereafter reproducibility for subsequent experiments. Structural and transcriptional changes associated with neuronal maturation were assessed throughout the differentiation process.

Table 1

Comparison of the protocols tested for SH-SY5Y differentiation. SH-SY5Y cells were exposed to different differentiation conditions using retinoic acid (RA, 10 μ M) and/or brain-derived neurotrophic factor (BDNF, 50 ng/mL). The table summarizes the presence or absence of each treatment, the duration of exposure, the starting day of treatment, and the total length of the differentiation protocol for each condition (a–f).

Conditions	RA (10 μ M)	Duration of RA (days)	Starting day	BDNF (50 ng/mL)	Duration of BDNF (days)	Starting day	Total days of treatment
a	yes	7	1	no			7
b	no			yes	7	1	7
c	yes	7	1	yes	7	1	7
d	yes	15	1	no			15
e	no			yes	15	1	15
f	yes	15	1	yes	10	5	15

Immunofluorescence (IF) analyses (Fig. 1a) showed that non-differentiated SH-SY5Y cells after 15 days of culture displayed a typical neuroblast-like morphology, characterized by rounded cell bodies and very limited neurite extension. Cells were visualized using F-actin staining, which highlights the cytoskeletal organization and allows clear observation of cell shape and neurite-like outgrowth. Neurite-like processes began to appear as early as 7 days following differentiation with RA alone but this produced relatively modest morphological changes, with only limited neurite-like outgrowth (condition a). At this time point, treatment with BDNF alone induced no visible modification of the shape, with cells even less abundant (condition b). The effect was slightly more pronounced when RA was combined with BDNF for

7 days (condition **c**), suggesting an enhanced response to the dual treatment; however, these early changes remained partial.

At later time points, clearer morphological alterations were observed. Treatment with RA alone for 15 days (condition **d**) led to the appearance of increased neurite-like extensions, indicating partial differentiation of the cells. In contrast, cells were scarcer and showed minimal neurite-like extension when treated with BDNF alone for 15 days (condition **e**) suggesting that this condition was not favourable for cell health or differentiation. The most pronounced neuron-like morphology was observed when RA and then BDNF were combined, particularly after 15 days of treatment (condition **f**), with increased neurite-like outgrowth and reduced cell body size compared with the 7 days condition, consistent with more advanced differentiation. It should be noted that the nucleus of the differentiated cells is relatively smaller compared to non-differentiated cells.

These structural observations were supported by qPCR analyses using markers specific of neuron-like cells (Fig. 1b). Microtubule-associated protein 2 (MAP2) is a cytoskeletal protein primarily localized in dendrites and is widely used as a marker of neuronal structural maturation and dendritic development ³⁵. Microtubule-associated protein Tau (MAPT) is involved in the stabilization of microtubules within axons and plays an important role in neurite extension and neuronal polarity ³⁶. Synaptophysin (SYP) is an integral membrane protein of synaptic vesicles and is commonly used as a marker of synaptic formation and neuronal connectivity ³⁷. Together, the increased expression of these markers reflects both structural and functional aspects of neuronal differentiation.

Conditions **a**, **b**, **c**, **d** and **e** induced no significant variation in the expression of these markers in comparison to non-differentiated cells (Fig. 1b). Condition **d** showed a tendency to increase MAPT expression levels but not for SYP and MAP2. Condition **e**, as mentioned in the IF analysis, shows a tendency to a weaker effect on genes expression. In contrast, an increase in the expression of the neuronal differentiation markers SYP, MAPT, and MAP2 was observed in condition **f** compared with non-differentiated cells. Overall, these results were consistent with the morphological observations and further indicated that the differentiation protocol used in condition **f** promotes the most pronounced neuron-like phenotype.

Based on the morphological and transcriptional analyses, the differentiation protocol **f** was selected as the final validated strategy to obtain neuron-like SH-SY5Y cells. In this protocol **f** (Fig. 2a), SH-SY5Y cells were first maintained under proliferation conditions and then exposed to RA (10 μ M) to initiate neuronal differentiation. RA treatment was applied for 5 days to promote early neurite formation and commitment toward a neuron-like phenotype. Subsequently, cells were co-treated with RA and BDNF (50 ng/mL) until day 15 to support further differentiation. This sequential treatment resulted in pronounced neurite outgrowth, increased neuronal network complexity, and reduced cell body size, features consistent with neuron-like morphology. Fluorescence staining for β -III-tubulin (a neuronal cytoskeleton protein associated with neurite formation) confirmed the neuron-like identity of differentiated cells revealing extensive neuritic processes and interconnected networks (Fig. 2Figure 2b). Together, these

observations validated the 15-day protocol combining RA priming followed by RA and BDNF co-treatment as the most effective condition to induce robust neuronal differentiation of SH-SY5Y cells.

3.2 Triple-level validation of SH-SY5Y differentiation for neuron-like phenotype acquisition

To ensure the reliability of the differentiation strategy (condition **f**), it was important to validate the neuronal phenotype using complementary approaches that assess different aspects of cellular identity and function. Structural changes alone are not sufficient to confirm neuronal differentiation, as neurite-like structures can appear under spontaneous differentiation or stress-related conditions. Therefore, the differentiation protocol was validated using three independent levels of analysis: immunofluorescence to highlight neuronal shape and organelles dynamic, RT-qPCR to quantify the transcriptional upregulation of established neuronal differentiation markers, and functional assays to assess cellular responses relevant to neuronal physiology. This multiparametric validation provided a robust framework to confirm that SH-SY5Y cells acquired a stable neuron-like phenotype under the optimized differentiation conditions.

Cell structure validation: cellular shape changed in differentiated SH-SY5Y cells.

Immunofluorescence assay was performed to compare differentiated (protocol **f**) and non-differentiated cells by visualizing specific cellular organelles and morphological markers (Fig. 3a). As evoked Fig. 2b, staining with β -III-tubulin confirmed the extension of neurites in differentiated cells, with a shape drastically different from non-differentiated. Cells were also stained for RCAS1 (Receptor-binding cancer antigene), a protein associated with the Golgi apparatus and involved in protein processing and secretion; for calnexin, an endoplasmic reticulum (ER) membrane chaperone involved in protein folding and quality control; and for synaptophysin. Based on qualitative assessment of fluorescence intensity, a qualitatively moderately stronger RCAS1 labelling was observed in differentiated cells, which may suggest an increased secretory and protein trafficking activity associated with neuronal maturation. Similarly, calnexin labelling seemed moderately increased in differentiated cells, which could be indicative of enhanced ER activity and protein processing during differentiation. In contrast, synaptophysin labelling showed slight difference between differentiated and non-differentiated cells, this protein is already expressed in non-differentiated cells and the differentiation induces slight increase in this expression as we saw in the previous RT-qPCR results (Fig. 1b).

Transcriptional validation: upregulation of selected markers in differentiated SH-SY5Y cells.

In addition to the immunofluorescence analyses described above, gene expression profiling was performed as another approach to validate the differentiation protocol (Fig. 3b). During the differentiation process, increased expression of several neuronal and cellular markers was observed, including β -III-tubulin, DCX (doublecortin), a microtubule-associated protein involved in neuronal migration and early neuronal differentiation, and DLG4 (disc large homolog 4), a postsynaptic scaffolding

protein important for synaptic organization. In addition, the anti-apoptotic gene BCL2, mitochondrial-related genes mitofusin-2 (MFN2), which regulates mitochondrial fusion; and TFAM, a key factor in mitochondrial DNA maintenance and transcription, were also upregulated. Together, these changes supported the acquisition of a neuron-like phenotype and suggest adaptations in mitochondrial function and cellular survival pathways during differentiation.

Functional validation: differentiated SH-SY5Y cells were resistant to H_2O_2 mediated toxicity.

The H_2O_2 toxicity test is typically performed as a positive control and model validation step. Hydrogen peroxide is a well-established inducer of oxidative stress, a mechanism strongly implicated in neuronal damage and neurodegenerative processes. By exposing cells to H_2O_2 , it is possible to compare the stress sensitivity of differentiated vs non-differentiated cells by two distinct viability assays (Fig. 3c). The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was used to assess metabolic activity thus cell viability, and lactate dehydrogenase (LDH) assays measured the LDH release as an indicator of cytotoxicity and cell lysis. The observed increase in LDH release in non-differentiated SH-SY5Y cells indicated that oxidative stress can disrupt plasma membrane in these cells, while the absence of a reduction in MTS signal suggested that mitochondrial metabolic activity was not immediately affected. Conversely, the lack of significant toxicity in differentiated SH-SY5Y cells supported the idea that neuronal differentiation conferred greater resistance to oxidative stress, possibly through improved antioxidant defences or more developed cellular stress-response pathways. This validation step therefore strengthened the relevance of the model before investigating the effects of A β 1–42 toxicity.

3.3 Assessment of A β 1–42 toxicity in differentiated cells under controlled aggregation kinetic

Assessing A β 1–42 toxicity in differentiated cells provides a more relevant neuron-like context for investigating its cellular effects. In this study, we aimed at evaluating the effects of both non-aggregated A β 1–42 (the soluble form) and pre-aggregated A β 1–42 (the fibrillar form) species. In our previous work, we reported the synthesis of highly pure A β 1–42 together with a standardized preparation protocol enabling reproducible and controlled aggregation kinetics. This work addressed one of the major, yet often overlooked, sources of variability in amyloid research: the quality and physicochemical state of the peptide itself.¹³ The aggregation kinetics of A β 1–42 species were characterized using complementary techniques, including fluorescence spectroscopy, circular dichroism, transmission electron microscopy, dot blot, single-molecule fluorescence imaging and NMR spectroscopy. Together, these methods provided convergent evidence of the progressive structural transitions of A β 1–42 throughout the aggregation pathway¹³. Cellular viability and membrane integrity were assessed using MTS and LDH assays. Integrating these measurements in both cell models allowed a comprehensive evaluation of A β 1–42 toxicity and facilitates the assessment of their respective relevance for studying its mechanism of action.

Divergent LDH and MTS responses revealed state-specific A β 1–42 toxicity in differentiated SH-SY5Y cells.

Interestingly, differential responses to A β 1–42 species were observed between non-differentiated and differentiated SH-SY5Y cells and according to distinct toxicity tests. In this case, A β 1–42 species were incubated at the end of differentiation. In the LDH assay, non-differentiated cells were predominantly sensitive to pre-aggregated A β 1–42, both at 48 h and 72 h of incubation, but less sensitive to non-aggregated A β 1–42 (Fig. 4a). Conversely, differentiated cells showed increased cell death following exposure to non-aggregated A β 1–42, while aggregated A β 1–42 did not induce significant membrane damage (Fig. 4a'), suggesting that soluble A β 1–42 species had preferential toxicity toward interference with membrane. In the MTS assay, non-differentiated cells displayed minimal reductions in MTS signal, with only a slight decrease observed following soluble A β 1–42 treatment after 72 h of incubation (Fig. 4b). In contrast, differentiated SH-SY5Y cells exhibited reduced metabolic activity following exposure to A β 1–42 fibrils, indicating mitochondrial dysfunction in response to these species (Fig. 4b'). When toxicity was observed, it occurred at least after 48 h of incubation into the cells in both cytotoxicity assays.

Selective gene expression changes in differentiated SH-SY5Y cells exposed to A β 1–42.

Following the functional toxicity assays, RT-qPCR analysis was performed to assess the molecular responses of differentiated SH-SY5Y cells to A β 1–42 exposure. Differentiated SH-SY5Y cells were treated with pre-aggregated or non-aggregated A β 1–42 at the end of differentiation and analysis revealed selective alterations in gene expression with no clear differences between the type of aggregation state (Fig. 5Figure 5). Regarding cytoskeletal and synaptic markers, we observed a significant decrease of MAP2 expression for both forms, and β -III-tubulin, MAPT and SYP expression showed a tendency to decrease, suggesting that A β 1–42 can induce early perturbations in neuronal structural and synaptic components. In contrast, genes related to mitochondrial function and cell survival (TFAM, MFN2, and BCL2) as well as the postsynaptic scaffolding gene DLG4, remained stable, indicating that A β 1–42 exposure at this stage did not significantly compromise mitochondrial integrity, apoptosis regulation, or postsynaptic organization. Interestingly, the expression of RBFOX3 (NeuN) and DCX highlighted a significant increase after incubation with the both forms and the soluble one respectively, which may reflect compensatory mechanisms or a relative enrichment of cells expressing early neuronal markers in response to A β 1–42 stress. The observed changes in gene expression primarily reflects early structural or differentiation-related responses rather than overt cytotoxicity. Notably, these findings also suggested that soluble A β 1–42 induced more pronounced early damage to the cells compared with what was observed in the previous LDH assays.

4. DISCUSSION

For more than two decades, amyloid- β aggregation inhibitors have been evaluated using experimental workflows that differ considerably among laboratories. Variations in peptide preparation, aggregation

protocols, cellular models and toxicity assays have generated a literature that is often difficult to compare and, in many cases, difficult to reproduce.

Although tremendous efforts have been devoted to discovering compounds capable of inhibiting A β aggregation, much less attention has been paid to the robustness and standardization of the experimental workflows used to evaluate these molecules.

As a consequence, compounds reported as promising in one laboratory frequently fail to reproduce similar biological effects in another, raising questions not only about the compounds themselves but also about the experimental conditions under which they were evaluated.

In this context, we reported a triply validated neuron-like cell model for the mechanistic and toxicity study of A β 1–42. Differentiation was confirmed using complementary morphological, transcriptional and functional assays. In parallel, a controlled workflow for the aggregation of A β 1–42 was developed, providing robust and reproducible framework for studying A β 1–42 toxicity in a more relevant model ¹³.

At the morphological level, after 7 days of RA treatment, early neurite-like extensions appear, indicating the start of neuronal commitment but with limited growth, and after 7 days of BDNF treatment, almost no transition was observed. A slight improvement with combined RA and BDNF suggests a synergistic effect, although short-term exposure remains insufficient for full differentiation. After 15 days, RA alone induced partial differentiation, whereas BDNF alone failed to promote differentiation. The most robust neuronal phenotype was achieved with the sequential RA and BDNF protocol. This sequential effect reflects distinct but complementary signalling pathways, and reinforce previous reports that RA preconditioning is required for efficient BDNF responsiveness, while further supporting the importance of treatment duration and sequence for achieving robust neuronal phenotypes ^{38 39}. qPCR data support these structural observations with increase in MAP2, MAPT and SYP indicating concurrent structural and functional maturation ^{40 41 42}. The absence of significant changes in intermediate conditions supports that partial treatments are insufficient to activate the full transcriptional program required for neuronal maturation ^{25 43}. Furthermore, as supported by others, non-differentiated SH-SY5Y cells already have a relatively high basal expression of some neuronal markers, so the dynamic range for further induction is limited. Because of this, differentiation tends to produce modest but biologically meaningful changes, rather than large fold increases seen in less specialized systems ^{21 24}. However, these moderate changes can still reflect coordinated maturation, especially when multiple markers increase together. The validated 15-days differentiation strategy therefore appears to recapitulate key aspects of neuronal development *in cellulo*. The confirmation of extensive β -III-tubulin-positive neuritic networks further supports the acquisition of a neuron-like identity. Despite these features, SH-SY5Y-derived neurons remain a simplified model that does not fully recapitulate the complexity of primary or iPSC-derived neurons, although they provide a robust and controlled system ^{20 44 28}.

Differentiation into a neuron-like phenotype was validated through complementary morphological, transcriptional, and functional analyses, indicating a coordinated maturation process. Morphologically,

moderate but consistent changes in organelle-associated markers were observed. However, these observations are based on qualitative assessment of immunofluorescence intensity and were not quantitatively measured. Slight qualitative increased RCAS1 labelling suggests enhanced Golgi activity, consisting with elevated trafficking demands during neuronal maturation⁴⁵. Similarly, slight qualitative increased of calnexin expression indicates enhanced endoplasmic reticulum activity⁴⁶. Synaptophysin levels remained relatively stable in agreement with previous reports indicating that SH-SY5Y cells often exhibit incomplete synaptic maturation and its basal expression in undifferentiated cells²¹. This observation aligns with prior qPCR results showing only modest transcriptional upregulation, suggesting that while synaptic components begin to emerge, full synaptic maturation may not yet be achieved under the applied differentiation conditions. Transcriptional profiling further supports the acquisition of neuron-like characteristics. The upregulation of β -III-tubulin and DCX confirms cytoskeletal remodelling and early neuronal differentiation^{47 48}, while upregulation of DLG4, coding for PSD95 a key postsynaptic scaffolding protein, indicates the initiation of synaptic organization, consistent with studies showing early synaptic gene induction preceding full synaptogenesis⁴⁹. Differentiation was also associated with enhanced cellular resilience. Elevated expression of BCL2 is consistent with its role in survival during neuronal maturation and increased expression of MFN2 and TFAM reflects mitochondrial adaptations critical for neuronal bioenergetics^{50 51}. These changes are particularly relevant given the high demands of neurons and the central role of mitochondria in supporting neuronal activity and survival⁵². Functionally, differentiated cells exhibited increased resistance to H₂O₂-induced stress. While non-differentiated SH-SY5Y cells showed increased LDH release following oxidative challenge, differentiated cells maintained both membrane integrity and metabolic activity, indicating a more robust stress response. This increased resilience is consistent with enhanced antioxidant defences, stress-response pathways, and upregulation of BCL2, along with mitochondrial adaptations involving MFN2 and TFAM^{24 25 52}. Additionally, mitochondrial adaptations reflected by increased MFN2 and TFAM expression may contribute to maintaining cellular homeostasis under stress conditions. Overall, these findings demonstrate that SH-SY5Y differentiation induces coordinated changes in organelle function, gene expression, and stress response, supporting the acquisition of a more neuron-like phenotype. This highlights the fact that not all cell models are equivalent, and that a toxic response can vary depending on the cells stage of differentiation.

In this context, this validated system therefore provides a robust platform for studying neuronal function and vulnerability, particularly in the context of neurodegenerative processes such as A β 1–42 toxicity. The present study demonstrates that the toxicity of A β 1–42 in SH-SY5Y cells is strongly dependent on (i) the aggregation state, (ii) the differentiation state of the cells and (iii) the assay used to assess cell viability. The combined use of LDH and MTS assays provides important insight into the temporal and mechanistic aspects of A β 1–42 aggregates toxicity. In this study, LDH assays revealed that differentiated cells (but not non-differentiated cells) are more sensitive to non-aggregated A β 1–42, highlighting the membrane-disruptive properties of these species, when fibrillar A β 1–42 did not induce significant membrane damage. In contrast, MTS measurements showed higher impact of fibrillar

species on these differentiated cells (but not on non-differentiated cells). This divergence underscores the importance of assay selection when interpreting cytotoxic effects, as different readouts capture distinct stages of cellular stress. The apparent discrepancies between LDH and MTS results reflect the distinct cellular processes measured by these assays, suggesting that A β 1–42 toxicity in SH-SY5Y cells manifests through distinct cellular mechanisms depending on both differentiation status and peptide aggregation state.

At the same time, the modest alterations observed in structural and synaptic markers suggest that A β 1–42 primarily induces subtle, early-stage toxicity rather than overt neurodegeneration in this model. Interestingly, exposure to A β 1–42 resulted in a marked increase in RBFOX3 expression, accompanied by a modest upregulation of DCX, despite the presence of functional toxicity. Interestingly, it suggests that A β 1–42 does not immediately suppress neuronal identity but instead triggers adaptive cellular responses. RBFOX3 upregulation may reflect a stress-induced adaptation in RNA processing, while increased DCX expression suggests activation of cytoskeletal remodelling and plasticity-related pathways. Importantly, the absence of significant changes in mitochondrial (TFAM, MFN2) and anti-apoptotic (BCL2) gene expression indicates that these responses occur independently of major transcriptional reprogramming of survival or metabolic pathways. Together, these findings suggest that A β 1–42 does not markedly alter the transcription of key mitochondrial and survival genes under the tested conditions, and that its exposure induces an early adaptive phase characterized by reinforcement of neuronal identity and structural plasticity, preceding the activation of irreversible degenerative processes. A β 1–42 toxicity is not purely degenerative, it also triggers adaptive neuronal responses and this depends on peptide aggregation and cells differentiation states. Basically, in this system, there is a first phase highlighted by adaptive neuronal stress response, followed by functional impairment leading to assumingly a last apoptotic phase that we have not reached yet.

Differentiated SH-SY5Y cells represent an intermediate neuron-like state characterized by partial maturation, intrinsic neuronal features, and increased resistance to apoptosis. This profile makes them particularly suitable for modelling early stages of neurodegenerative processes, where subtle functional and structural alterations precede overt cell death. In this context, exposure to A β 1–42 induces mild but detectable changes in neuronal integrity, especially in response to soluble species, while largely preserving mitochondrial function and viability under the tested conditions. These findings reinforce the relevance of this model for investigating early pathogenic events associated with A β 1–42 toxicity. More broadly, they highlight that cellular responses to A β 1–42 are highly context-dependent, varying with differentiation status and methodological readouts, which may contribute to the variability observed across studies. Altogether, this work emphasizes the importance of combining well-characterized models with complementary assays to accurately capture the complexity of A β 1–42-induced neurotoxicity.

RESOURCE AVAILABILITY:

Lead contact: Further information and requests for resources and reagents should be directed to the lead contact, Nicolo Tonali (nicolo.tonali@cea.fr).

Abbreviations

BCL2

B-cell lymphoma 2

BDNF

brain-derived neurotrophic factor

DCX

doublecortin

DLG4

disc large homolog 4

ER

endoplasmic reticulum

ESC

embryonic stem cell

FBS

fetal bovine serum

HCl

hydrochloric acid

iPSC

induced pluripotent stem cell

LDH

lactate deshydrogenase

MAP2

microtubule-associated protein 2

MAPT

microtubule-associated protein tau

MFN2

mitofusin-2

NMR

nuclear magnetic resonance

MTS

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NaOH

sodium hydroxide

P/S

penicillin/streptomycin

PBS

phosphate buffer saline
RA
retinoic acid
RCAS1
receptor-binding cancer antigene
RT-qPCR
reverse transcription-quantitative polymerase chain reaction
SYP
synaptophysin
TFAM
mitochondrial transcription factor A

Declarations

CONFLICT OF INTEREST STATEMENT:

The authors declare no conflict of interest.

CONSENT STATEMENT

Consent was not necessary.

FUNDING:

This work was supported by the Agence Nationale de la Recherche and the Deutsche Forschungsgemeinschaft (DFG) through the joint ANR–DFG program (ANR-22-CE92-0076-01, DFG SE 609/20 – 1), as well as by PASREL (Université Paris-Saclay), France 2030 programme (ANR-11-IDEX-003) and by BIOPROBE (Université Paris-Saclay), France 2030 programme (ANR-11-IDEX-003).

Author Contribution

A.V. and N.T. conceived the project. A.V. and C.P. performed the experiments. A.V. and C.P. analyzed the data. A.V. wrote the manuscript. X.I., P.R., S.P., N.S., D.S., N.T. edited and revised the manuscript.

Acknowledgement

We are grateful to many participants, researchers and staff who contributed significantly to this study (Marion Chaigneau, Peggy Barbe). This work was supported by the Agence Nationale de la Recherche

and the Deutsche Forschungsgemeinschaft (DFG) through the joint ANR–DFG program (ANR-22-CE92-0076-01, DFG SE 609/20-1), as well as by PASREL (Université Paris-Saclay), France 2030 programme (ANR-11-IDEX-003) and by BIOPROBE (Université Paris-Saclay), France 2030 programme (ANR-11-IDEX-003).

Data Availability

Lead contact: Further information and requests for resources and reagents should be directed to the lead contact, Nicolo Tonalì (nicolo.tonali@cea.fr). Materials availability: All unique/stable reagents generated in this study are available from the lead contact without restriction. Data and code availability • All data reported in this paper will be shared by the lead contact upon request. • This paper does not report original code. • Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Materials availability:

All unique/stable reagents generated in this study are available from the lead contact without restriction.

Data and code availability • All data reported in this paper will be shared by the lead contact upon request. • This paper does not report original code. • Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

References

1. GBD 2019 Dementia Forecasting Collaborators. Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019. *Lancet Public. Health.* **7** (2), e105–e125. 10.1016/S2468-2667(21)00249-8 (2022).
2. DeTure, M. A. & Dickson, D. W. The neuropathological diagnosis of Alzheimer’s disease. *Mol. Neurodegener.* **14** (1), 32. 10.1186/s13024-019-0333-5 (2019).
3. Silvers, R. et al. Aggregation and Fibril Structure of A β M01-42 and A β 1–42. *Biochemistry* **56** (36), 4850–4859. 10.1021/acs.biochem.7b00729 (2017).
4. Tay, W. M., Huang, D., Rosenberry, T. L. & Paravastu, A. K. The Alzheimer’s amyloid- β (1–42) peptide forms off-pathway oligomers and fibrils that are distinguished structurally by intermolecular organization. *J. Mol. Biol.* **425** (14), 2494–2508. 10.1016/j.jmb.2013.04.003 (2013).
5. Benilova, I., Karran, E. & De Strooper, B. The toxic A β oligomer and Alzheimer’s disease: an emperor in need of clothes. *Nat. Neurosci.* **15** (3), 349–357. 10.1038/nn.3028 (2012).
6. Haass, C. & Selkoe, D. J. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer’s amyloid beta-peptide. *Nat. Rev. Mol. Cell. Biol.* **8** (2), 101–112. 10.1038/nrm2101 (2007).

7. Olajide, O. J., La Rue, C., Bergdahl, A. & Chapman, C. A. Inhibiting amyloid beta (1–42) peptide-induced mitochondrial dysfunction prevents the degradation of synaptic proteins in the entorhinal cortex. *Front. Aging Neurosci.* **14**, 960314. 10.3389/fnagi.2022.960314 (2022).
8. Han, X. J. et al. Amyloid β -42 induces neuronal apoptosis by targeting mitochondria. *Mol. Med. Rep.* **16** (4), 4521–4528. 10.3892/mmr.2017.7203 (2017).
9. Cha, M. Y. et al. Mitochondria-specific accumulation of amyloid β induces mitochondrial dysfunction leading to apoptotic cell death. *PloS One.* **7** (4), e34929. 10.1371/journal.pone.0034929 (2012).
10. Knowles, T. P. J., Vendruscolo, M. & Dobson, C. M. The amyloid state and its association with protein misfolding diseases. *Nat. Rev. Mol. Cell. Biol.* **15** (6), 384–396. 10.1038/nrm3810 (2014).
11. Muschol, M. & Hoyer, W. Amyloid oligomers as on-pathway precursors or off-pathway competitors of fibrils. *Front. Mol. Biosci.* **10**, 1120416. 10.3389/fmolb.2023.1120416 (2023).
12. Herrera, M. G., Ciccone, L., Moleiro, L. H., Tonali, N. & Dodero, V. I. Endogenous A β and Exogenous Wheat Gluten Nanostructures: Understanding Peptide Self-Assembly in Disease. *ACS Nano.* **19** (34), 30688–30719. 10.1021/acsnano.5c01662 (2025).
13. Panek, S. et al. Cyclopeptide-Based Fluorescent Conjugates for Monitoring Prefibrillar A β Nanostructures. *J Med. Chem Published online March.* **11** 10.1021/acs.jmedchem.6c00059 (2026).
14. Faller, P. & Hureau, C. Reproducibility Problems of Amyloid- β Self-Assembly and How to Deal With Them. *Front. Chem.* **8**, 611227. 10.3389/fchem.2020.611227 (2021).
15. Pinheiro, N. R. et al. The human neuroblastoma SH-SY5Y cell line as a model to assess β -amyloid neurotoxicity: A systematic review and meta-analysis. *bioRxiv*. Preprint posted online May 23, 2026:2025.10.20.683497. doi:10.1101/2025.10.20.683497
16. Lopez-Suarez, L., Awabdh, S. A., Coumoul, X. & Chauvet, C. The SH-SY5Y human neuroblastoma cell line, a relevant in vitro cell model for investigating neurotoxicology in human: Focus on organic pollutants. *Neurotoxicology* **92**, 131–155. 10.1016/j.neuro.2022.07.008 (2022).
17. Tonazzini, I., Cecchini, A., Elgersma, Y. & Cecchini, M. Interaction of SH-SY5Y cells with nanogratings during neuronal differentiation: comparison with primary neurons. *Adv. Healthc. Mater.* **3** (4), 581–587. 10.1002/adhm.201300216 (2014).
18. Stathakos, P. et al. A monolayer hiPSC culture system for autophagy/mitophagy studies in human dopaminergic neurons. *Autophagy* **17** (4), 855–871. 10.1080/15548627.2020.1739441 (2021).
19. Bukhteeva, I. et al. Effects of Natural Lithium and Lithium Isotopes on Voltage Gated Sodium Channel Activity in SH-SY5Y and iPSC Derived Cortical Neurons. *BioRxiv Prepr Serv Biol.* Published online June 1, 2025:2025.05.28.656602. 10.1101/2025.05.28.656602
20. Vassal, M., Cruz, A. C., Rebelo, S. & Martins, F. Neurons in a Dish: A Review of In Vitro Cell Models for Studying Neurogenesis. *J. Neurochem.* **170** (1), e70344. 10.1111/jnc.70344 (2026).
21. Xicoy, H., Wieringa, B. & Martens, G. J. M. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Mol. Neurodegener.* **12** (1), 10. 10.1186/s13024-017-0149-0 (2017).

22. Bell, M. & Zempel, H. SH-SY5Y-derived neurons: a human neuronal model system for investigating TAU sorting and neuronal subtype-specific TAU vulnerability. *Rev. Neurosci.* **33** (1), 1–15. 10.1515/revneuro-2020-0152 (2022).
23. Biedler, J. L., Roffler-Tarlov, S., Schachner, M. & Freedman, L. S. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res.* **38** (11 Pt 1), 3751–3757 (1978).
24. Cheung, Y. T. et al. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology* **30** (1), 127–135. 10.1016/j.neuro.2008.11.001 (2009).
25. Encinas, M. et al. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. *J. Neurochem.* **75** (3), 991–1003. 10.1046/j.1471-4159.2000.0750991.x (2000).
26. Jensen, L. M., Zhang, Y. & Shooter, E. M. Steady-state polypeptide modulations associated with nerve growth factor (NGF)-induced terminal differentiation and NGF deprivation-induced apoptosis in human neuroblastoma cells. *J. Biol. Chem.* **267** (27), 19325–19333 (1992).
27. Hromadkova, L. et al. Brain-derived neurotrophic factor (BDNF) promotes molecular polarization and differentiation of immature neuroblastoma cells into definitive neurons. *Biochim. Biophys. Acta Mol. Cell. Res.* **1867** (9), 118737. 10.1016/j.bbamcr.2020.118737 (2020).
28. Targett, I. L., Crompton, L. A., Conway, M. E. & Craig, T. J. Differentiation of SH-SY5Y neuroblastoma cells using retinoic acid and BDNF: a model for neuronal and synaptic differentiation in neurodegeneration. *Vitro Cell. Dev. Biol. - Anim.* **60** (9), 1058–1067. 10.1007/s11626-024-00948-6 (2024).
29. Riegerová, P. et al. Expression and Localization of A β PP in SH-SY5Y Cells Depends on Differentiation State. *J. Alzheimers Dis. JAD.* **82** (2), 485–491. 10.3233/JAD-201409 (2021).
30. Datki, Z. et al. Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells. *Brain Res. Bull.* **62** (3), 223–229. 10.1016/j.brainresbull.2003.09.011 (2003).
31. Milton, N. G. N., Chillumuri, A., Rocha-Ferreira, E., Nercessian, A. N. & Ashioti, M. Kisspeptin prevention of amyloid- β peptide neurotoxicity in vitro. *ACS Chem. Neurosci.* **3** (9), 706–719. 10.1021/cn300045d (2012).
32. Krishtal, J., Bragina, O., Metsla, K., Palumaa, P. & Tõugu, V. In situ fibrillizing amyloid-beta 1–42 induces neurite degeneration and apoptosis of differentiated SH-SY5Y cells. *PLoS One.* **12** (10), e0186636. 10.1371/journal.pone.0186636 (2017).
33. Krishtal, J., Metsla, K., Bragina, O., Tõugu, V. & Palumaa, P. Toxicity of Amyloid- β Peptides Varies Depending on Differentiation Route of SH-SY5Y Cells. *J. Alzheimers Dis. JAD.* **71** (3), 879–887. 10.3233/JAD-190705 (2019).
34. Özdemir, A. Y., Hofbauerová, K., Kopecký, V., Novotný, J. & Rudajev, V. Different amyloid β 42 preparations induce different cell death pathways in the model of SH-SY5Y neuroblastoma cells. *Cell. Mol. Biol. Lett.* **29**, 143. 10.1186/s11658-024-00657-8 (2024).

35. Lyu, J., DeMarco, A. G., Sweet, R. A. & Grubisha, M. J. MAP2 phosphorylation: mechanisms, functional consequences, and emerging insights. *Front. Cell. Neurosci.* **19**, 1610371. 10.3389/fncel.2025.1610371 (2025).
36. Caceres, A. & Kosik, K. S. Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. *Nature* **343** (6257), 461–463. 10.1038/343461a0 (1990).
37. Valtorta, F., Pennuto, M., Bonanomi, D. & Benfenati, F. Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis? *BioEssays News Rev. Mol. Cell. Dev. Biol.* **26** (4), 445–453. 10.1002/bies.20012 (2004).
38. Maden, M. Retinoic acid in the development, regeneration and maintenance of the nervous system. *Nat. Rev. Neurosci.* **8** (10), 755–765. 10.1038/nrn2212 (2007).
39. Park, H. & Poo, M. ming. Neurotrophin regulation of neural circuit development and function. *Nat Rev Neurosci.* ;14(1):7–23. (2013). 10.1038/nrn3379
40. Dehmelt, L. & Halpain, S. The MAP2/Tau family of microtubule-associated proteins. *Genome Biol.* **6** (1), 204. 10.1186/gb-2004-6-1-204 (2005).
41. Weingarten, M. D., Lockwood, A. H., Hwo, S. Y. & Kirschner, M. W. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. U S A.* **72** (5), 1858–1862. 10.1073/pnas.72.5.1858 (1975).
42. Tarsa, L. & Goda, Y. Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. *Proc. Natl. Acad. Sci. U S A.* **99** (2), 1012–1016. 10.1073/pnas.022575999 (2002).
43. Shipley, M. M., Mangold, C. A. & Szpara, M. L. Differentiation of the SH-SY5Y Human Neuroblastoma Cell Line. *J. Vis. Exp. JoVE.* (108), 53193. 10.3791/53193 (2016).
44. Şahin, M., Öncü, G., Yılmaz, M. A., Özkan, D. & Saybaşılı, H. Transformation of SH-SY5Y cell line into neuron-like cells: Investigation of electrophysiological and biomechanical changes. *Neurosci. Lett.* **745**, 135628. 10.1016/j.neulet.2021.135628 (2021).
45. Subkhangulova, A. & Mikhaylova, M. The Golgi apparatus: adaptations to neuronal shape and functions. *EMBO J.* **45** (2), 358–373. 10.1038/s44318-025-00658-z (2025).
46. Vásquez, G. E., Medinas, D. B., Urra, H. & Hetz, C. Emerging roles of endoplasmic reticulum proteostasis in brain development. *Cells Dev.* **170**, 203781. 10.1016/j.cdev.2022.203781 (2022).
47. Ayanlaja, A. A. et al. Distinct Features of Doublecortin as a Marker of Neuronal Migration and Its Implications in Cancer Cell Mobility. *Front. Mol. Neurosci.* **10**, 199. 10.3389/fnmol.2017.00199 (2017).
48. Radwitz, J. et al. Tubb3 expression levels are sensitive to neuronal activity changes and determine microtubule growth and kinesin-mediated transport. *Cell. Mol. Life Sci. CMLS.* **79** (11), 575. 10.1007/s00018-022-04607-5 (2022).
49. Bustos, F. J. et al. Epigenetic editing of the Dlg4/PSD95 gene improves cognition in aged and Alzheimer’s disease mice. *Brain* **140** (12), 3252–3268. 10.1093/brain/awx272 (2017).

50. Callens, M. et al. The role of Bcl-2 proteins in modulating neuronal Ca²⁺ signaling in health and in Alzheimer's disease. *Biochim. Biophys. Acta Mol. Cell. Res.* **1868** (6), 118997. 10.1016/j.bbamcr.2021.118997 (2021).
51. Markham, A., Bains, R., Franklin, P. & Spedding, M. Changes in mitochondrial function are pivotal in neurodegenerative and psychiatric disorders: How important is BDNF? *Br. J. Pharmacol.* **171** (8), 2206–2229. 10.1111/bph.12531 (2014).
52. Schneider, L. et al. Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress. *Free Radic Biol. Med.* **51** (11), 2007–2017. 10.1016/j.freeradbiomed.2011.08.030 (2011).

Figures

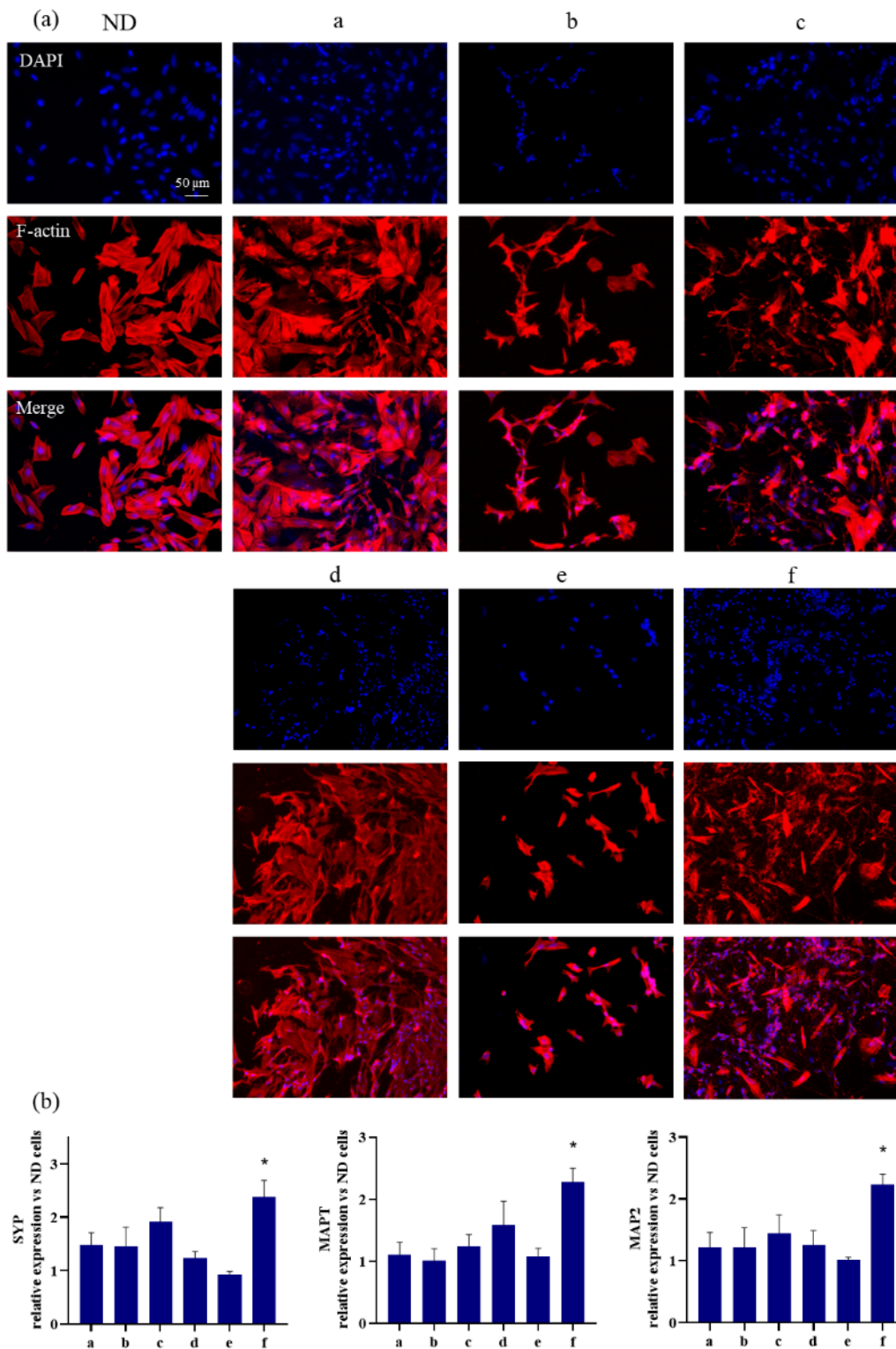


Figure 1

Morphological assessment of SH-SY5Y differentiation under different RA and BDNF treatment protocols. (a) Representative immunofluorescence images of SH-SY5Y cells subjected to the differentiation protocols described before (treatments a-f). Cell nuclei are stained with DAPI (blue) and the actin cytoskeleton is stained with phalloidin red/orange fluorescent conjugate (red). Merge panels show the combined signals. Imaging was performed with a $\times 10$ objective and analysed in the same conditions

with ImageJ. (b) Quantification of relative mRNA expression levels measured by RT-qPCR for neuronal markers SYP, MAPT and MAP2 across the different differentiation conditions, compared to the control non-differentiated cells. Data are presented as mean $\pm$ SEM. $n = 3$ independent experiments, each performed in experimental triplicates. Statistical analysis was analyzed using one-sample t-tests against the theoretical value of 1. * $p < 0.05$. ND for non-differentiated cells.

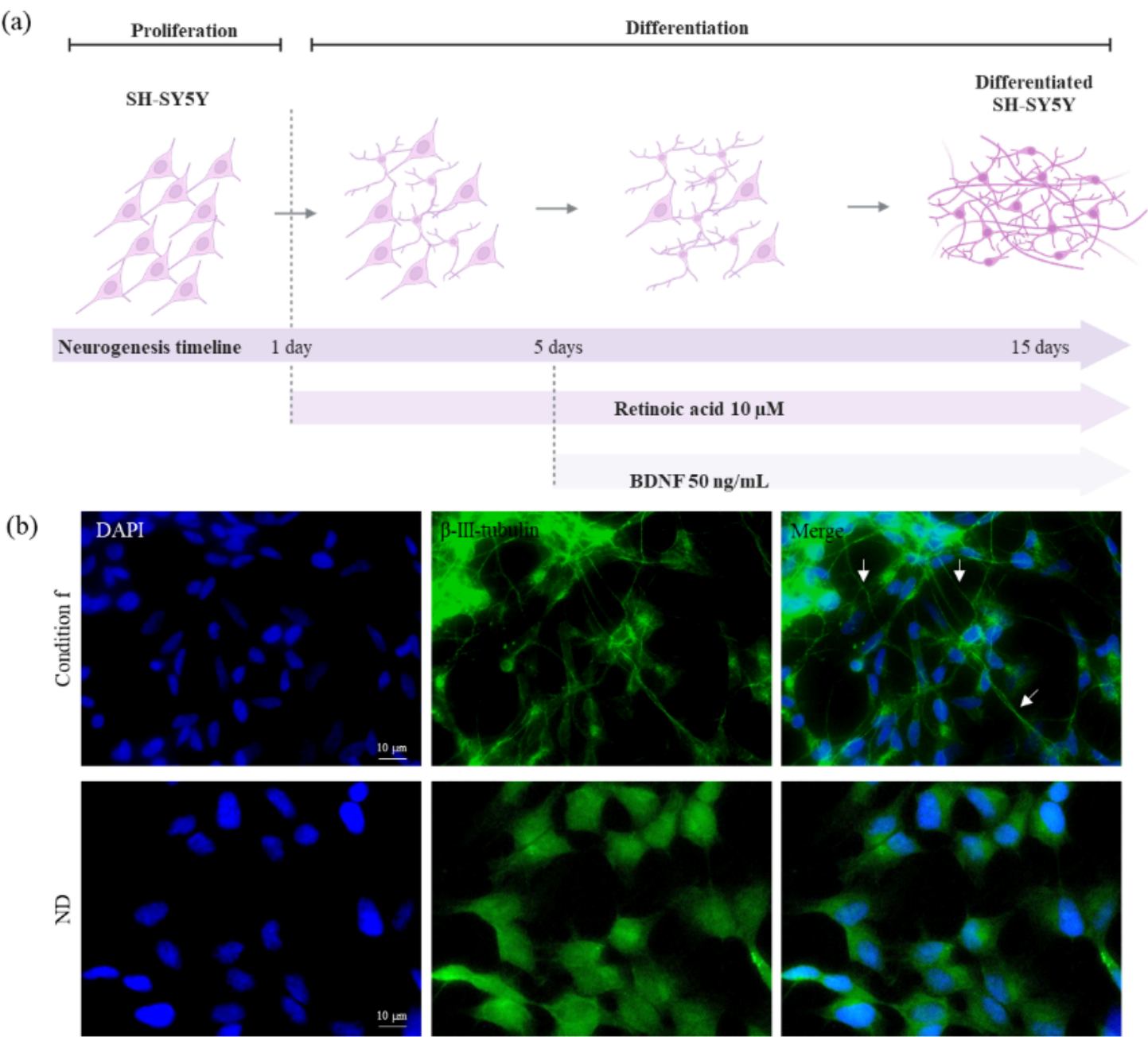


Figure 2

Final protocol of differentiation (protocol f). (a) Scheme representing chosen differentiation protocol. Created with BioRender.com (b) Fluorescence images of SH-SY5Y cells subjected to the differentiation protocol f. Cell nuclei are stained with DAPI (blue) and the β -III-tubulin is detected using specific antibody (green). Merge panels show the combined signals. White arrowheads indicate representative neurite-like

extensions. ND for non-differentiated cells. Imaging was performed with a ×60 objective with water immersion.

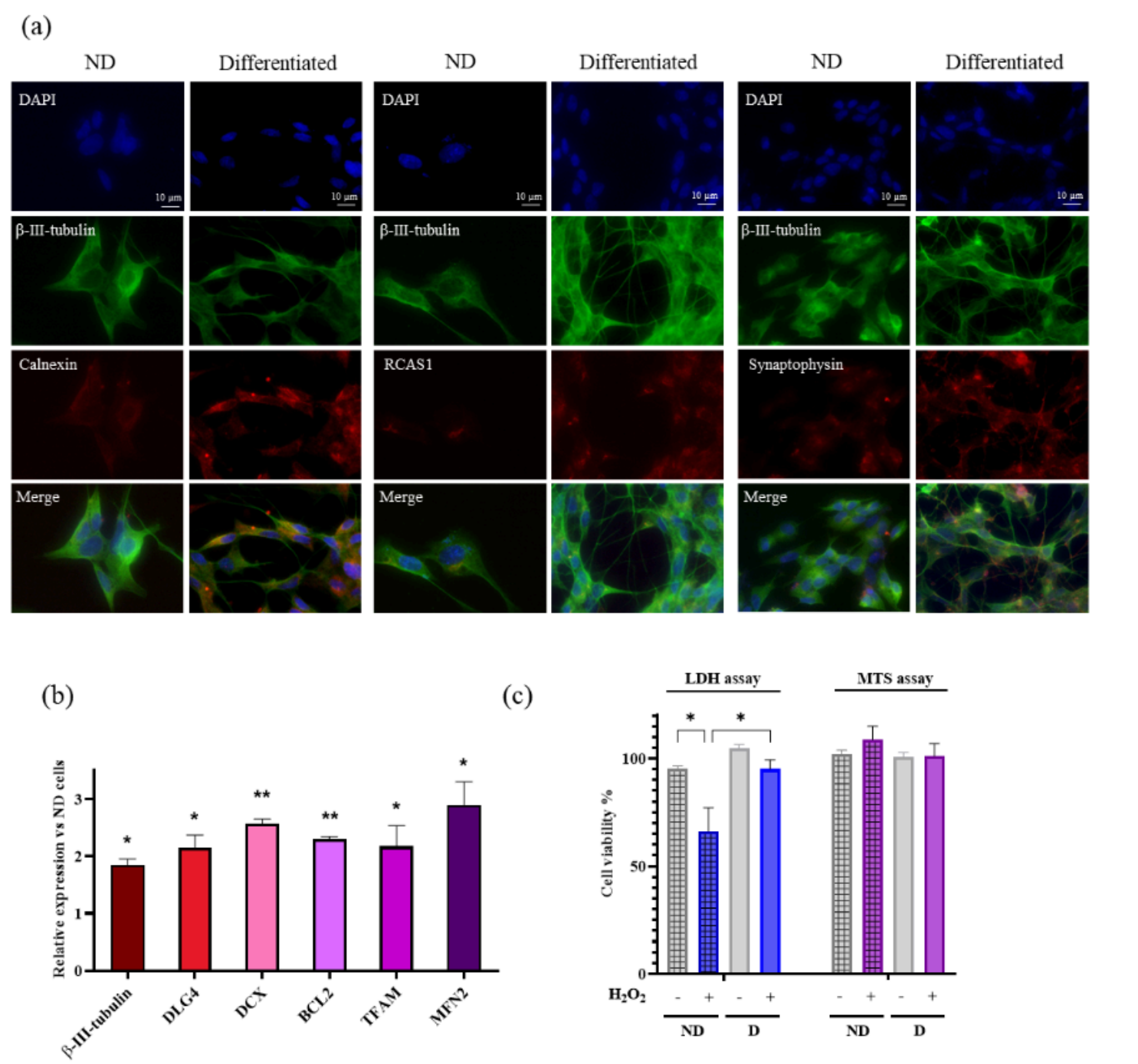


Figure 3

Validation of SH-SY5Y differentiation under protocol f. (a) Representative immunofluorescence images of SH-SY5Y cells subjected to the differentiation protocol f. Cell nuclei are stained with DAPI (blue) and the β-III-tubulin is detected using specific antibody (green), Golgi apparatus (RCAS1), ER (calnexin) and synaptophysin are detected using specific antibody and labelled with secondary antibody (red). Merge panels show the combined signals. Imaging was performed with a ×60 objective with water immersion. (b)

Quantification of relative mRNA expression levels measured by RT-qPCR for neuronal markers β -III-tubulin, DLG4, DCX, BCL2, TFAM and MFN2 compared to the control non-differentiated cells (value = 1). (c) Functional test for cell viability assay in presence of H_2O_2 . Data are presented as mean $\pm$ SEM. n = 3 independent experiments, each performed in experimental quadruplicates. Statistical analysis was performed using for (b) one-sample t-tests against the theoretical value of 1 and for (c) two-way ANOVA followed by multiple comparison tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ND for non-differentiated cells. D for differentiated cells.

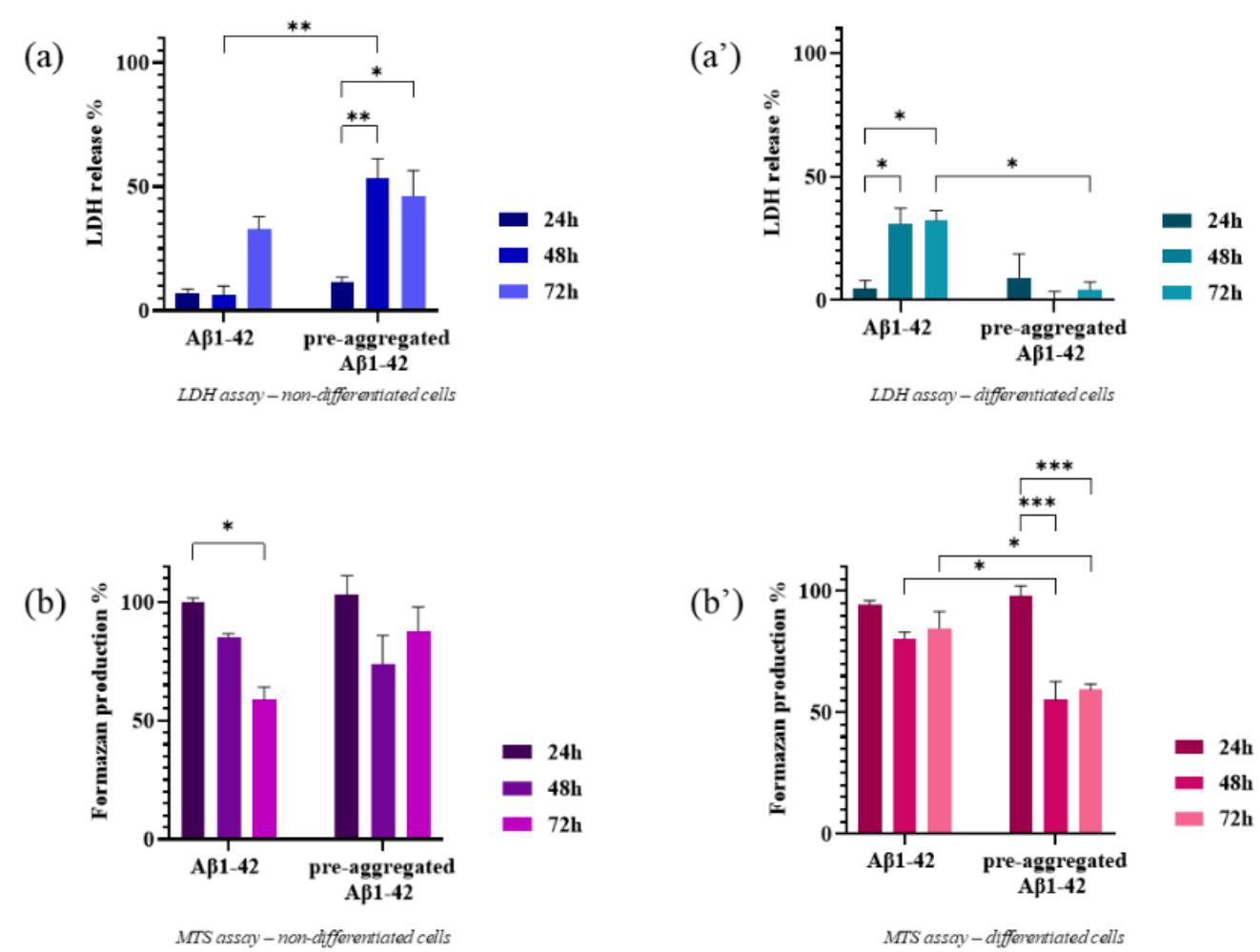


Figure 4

Kinetic of cytotoxic effects of Aβ1-42 pre-aggregated or not on differentiated and non-differentiated SH-SY5Y cells measured by LDH and MTS assays. (a, a') Cytotoxicity evaluated with LDH test. (b, b') Cytotoxicity evaluated with MTS test. Aβ1-42 was pre-aggregated or non-aggregated and incubated into the cells for 24 h, 48 h or 72 h. Data are presented as mean $\pm$ SEM. n = 3 independent experiments, each performed in experimental quadruplicates. Statistical analysis was performed using two-way ANOVA followed by multiple comparison tests.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

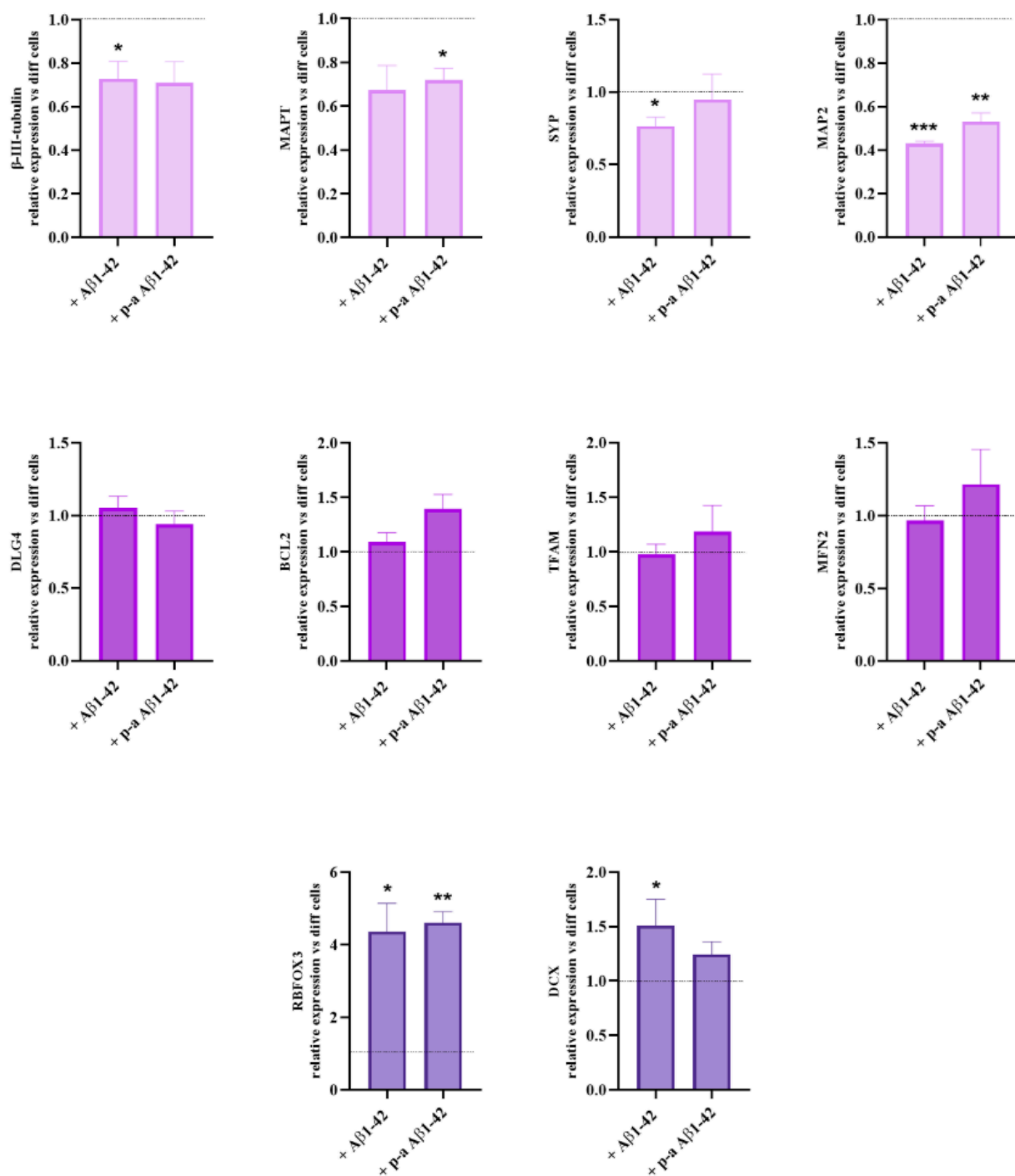


Figure 5

Effect of Aβ1-42 on specific markers. Analysis compared to the control differentiated cells without any treatment (value = 1). p-a for pre-aggregated. Data are presented as mean ± SEM. n = 3 independent experiments, each performed in experimental triplicates. Statistical analysis was performed using one-sample t-tests against the theoretical value of 1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.