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Amyloid- β with isomerized Asp7 cytotoxicity is coupled to protein phosphorylation

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Neuronal dysfunction and loss associated with the accumulation of amyloid- β (A β) in the form of extracellular amyloid plaques and hyperphosphorylated tau in the form of intraneuronal neurofibrillary tangles represent key features of Alzheimer's disease (AD). Amyloid plaques found in the brains of AD patients are predominantly composed of A β 42 and its multiple chemically or structurally modified isoforms. Recently, we demonstrated that A β 42 with isomerised Asp7 (isoA β 42) which is one of the most abundant A β isoform in plaques, exhibited high neurotoxicity in human neuronal cells. Here, we show that, in SH-SY5Y neuroblastoma cells, the administration of synthetic isoA β 42 rather than intact A β 42 resulted in a significantly higher level of protein phosphorylation, especially the phosphorylation of tau, tubulins, and matrin 3. IsoA β 42 induced a drastic reduction of tau protein levels. Our data demonstrate, for the first time, that isoA β 42, being to date the only known synthetic A β species to cause AD-like amyloidogenesis in an animal AD model, induced cell death by disabling structural proteins in a manner characteristic of that observed in the neurons of AD patients. The data emphasize an important role of isoA β 42 in AD progression and provide possible neurotoxicity paths for this particular isoform.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder most common in ageing people and is characterized clinically by memory loss and cognitive decline¹. The neuromorphological hallmarks of AD are cerebral amyloidogenesis, i.e., the accumulation of amyloid- β (A β) in the form of extracellular, insoluble aggregates (so-called amyloid plaques) in specific brain regions, intraneuronal neurofibrillary tangles (the major component of which is hyperphosphorylated tau protein), and neuronal degeneration². According to a widely accepted amyloid hypothesis, the primary process triggering the pathogenesis of AD is the formation of soluble neurotoxic oligomers of A β ³. Human oligomers also induce the hyperphosphorylation of tau at AD-relevant epitopes and cause neuritic dystrophy in cultured neurons⁴. It is suggested that amyloid plaques represent the major source of neurotoxic forms of A β oligomers^{5,6}.

Intracerebral injections of homogenates from the brains of AD patients induce the whole spectrum of AD-specific disruptions in the brains of model animals⁷. Amyloid plaques from an AD brain contain a wide array of other forms of A β peptide (39–43 aa length) and their post-translationally modified isoforms besides the major A β 42 peptide^{8,9}. Furthermore, in various animal models of AD, chemically or structurally modified A β rather than intact A β , drastically accelerates cerebral amyloidogenesis⁷, likely due to the active involvement of physiologically intact, endogenous A β molecules in a chain reaction initiated by a seed-like mechanism¹⁰.

One of the most common component of amyloid plaques is the A β isoform with an isomerized aspartic acid residue at position 7 (isoA β)^{8,11}. We hypothesized that this A β isoform is a major player in AD pathogenesis^{12,13}. We have shown that, in contrast to intact A β 42, a synthetic peptide corresponding to isoA β 42 causes cerebral amyloidogenesis in AD animal models^{14,15}. In addition, the toxic effect of isoA β 42 on neuronal cells is stronger than that of A β 42^{16,17}. Here, using SH-SY5Y neuroblastoma cells as a model for studying the effect of beta-amyloid^{18–21}, we showed that isoA β 42 is more effective than A β 42 in inducing the phosphorylation of a number of proteins, including tau, tubulins and matrin 3.

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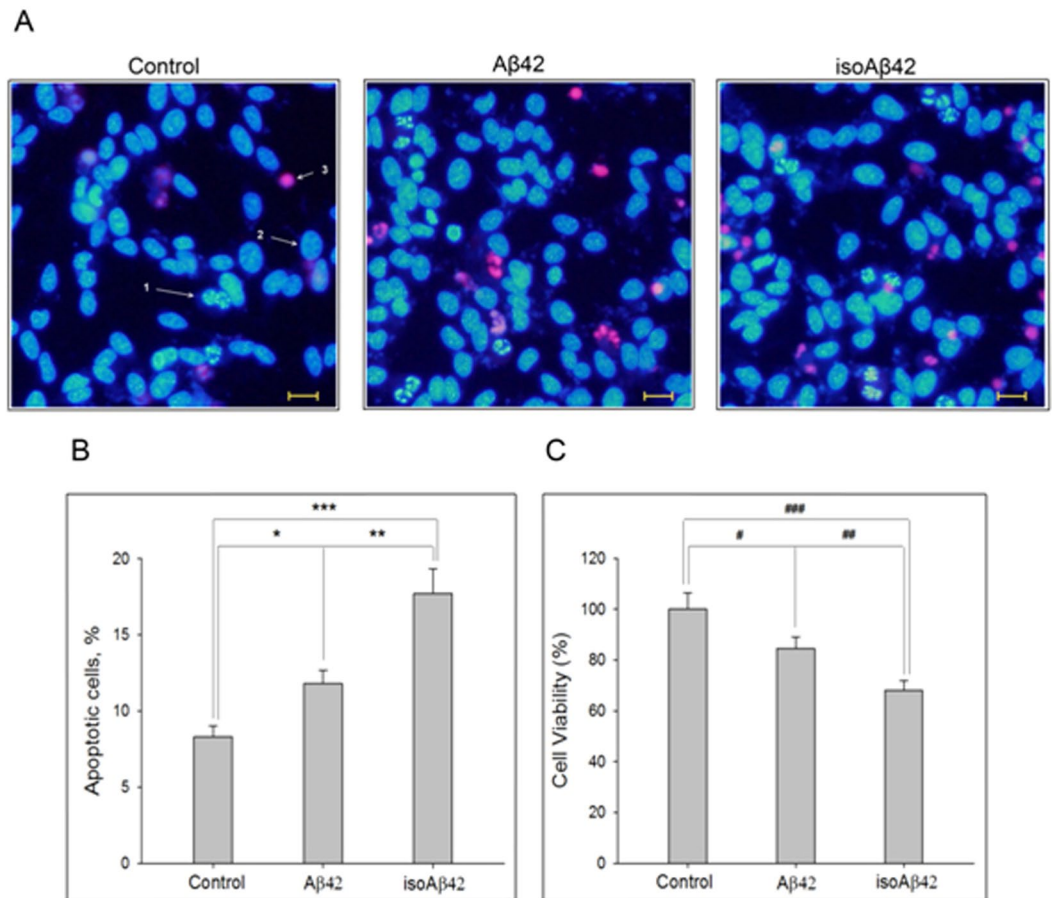


Figure 1. Effects of A β 42 and isoA β 42 (10 μ M) on SH-SY5Y cells. **(A)** Visualization of the cells using fluorescent microscopy. Left, untreated cells. Centre, cells treated with A β 42. Right, cells treated with isoA β 42. Arrows indicate the following: 1 - apoptotic cells stained with Hoechst 33342; 2 - living cells; 3 - necrotic cells stained with propidium iodide. Scale is 20 μ m. **(B)** Number of apoptotic cells in the cell population. Control - untreated cells; isoA β 42 and A β 42 - cells treated with isoA β 42 and A β 42 for 24 h, respectively. **(C)** Viability of the cells in percent, revealed by MTT-test treated with A β 42 or isoA β 42 relative to control without treatment at 48 h. Each value represents mean \pm SD of at least three independent experiments performed in quadruplicate; * p < 0.023, ** p < 0.002, *** p < 0.001, # p < 0.005, ## p < 0.003, ### p < 0.001.

Results

Isomerization of Asp7 increased the apoptogenic properties of A β in SH-SY5Y cells. Both isoA β 42 and A β 42 peptides induced SH-SY5Y cell death after 24 h of treatment; however, isoA β 42 had substantially greater toxic effects (Fig. 1). Relative to the control group of cells, the percentage of apoptotic cells in the group treated with 10 μ M of isoA β 42 increased by 10%, whereas the percentage of apoptotic cells in the group treated with A β 42 increased by only 4% (Fig. 1B). MTT-test confirmed greater toxicity of isoA β 42 in SH-SY5Y cells in comparison with A β 42 (Fig. 1C). Western blot analysis with antibodies to caspase-3 showed that the procaspase-3 level, an apoptosis marker, increased by two- and four-fold in SH-SY5Y cells treated with A β 42 and isoA β 42, respectively (Fig. 2).

Protein phosphorylation levels in SH-SY5Y cells treated with A β 42 and isoA β 42 were different.

We performed 2D analysis of 35 S-labeled protein lysates from control SH-SY5Y cells and cells incubated for 6 hours with A β 42 and isoA β 42 peptides. The treatment of SH-SY5Y cells with A β 42 and isoA β 42 for 6 h changed the isoelectric points (pI) of several proteins. There was a significant shift in the pI values for α - and β -tubulin to the acid zone, which was more drastic in cells treated with isoA β 42 (Fig. 3). Because the hyperphosphorylation of β -tubulin is a hallmark of AD in the brains of patients²², we suggested that the observed shift was at least partially due to A β -induced phosphorylation. Using Western blot analysis with antibodies recognizing phosphoserine, we showed that incubation of SH-SY5Y cells with A β 42 and isoA β 42 led to a significant increase in the phosphorylation levels of a specific set of proteins (Fig. 4); among them were both forms of tubulin (Fig. 5A and B). In contrast to A β 42, isoA β 42 caused the appearance of additional isoforms of phosphorylated β -tubulin (Fig. 5B). IsoA β 42 treatment, but not A β 42 treatment, caused the phosphorylation at serine residues of another vital protein, matrin 3 (Fig. 5A). To confirm the results of Western blot analysis major proteins from this area including β -tubulin, α -tubulin and matrin3 were validated by mass spectrometry followed by a search using Mascot software (Suppl. Table 1 and Suppl. Fig. 1). Additionally, gels after second dimension were stained with pro-Q

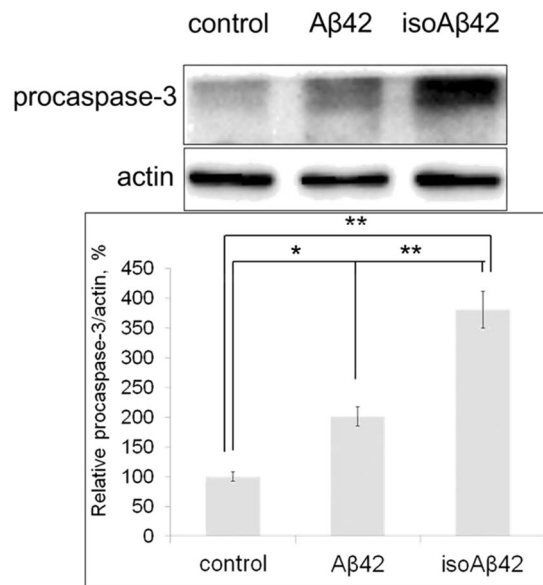


Figure 2. Effects of A β 42 and isoA β 42 (10 μ M, 6 h) treatment on procaspase 3 levels in SH-SY5Y cells. After peptide treatment, the cells were lysed, the isolated proteins were separated by SDS-PAGE, caspase 3 and actin were detected by Western blot using the appropriate antibodies. The bars represent procaspase 3 expression changes. Each value represents the mean \pm SD of at least three independent experiments; * p < 0.02, ** p < 0.005. Here and on the other figures full-length gels and blots are included in a Supplementary Information.

diamond phosphoprotein gel stain. Phosphoprotein staining verified our conclusion that A β 42 and especially isoA β 42 changes phosphorylation level of tubulins and matrin 3 (Fig. 6 and Suppl. Fig. 6).

IsoA β 42 decreased tau protein levels and induced its hyperphosphorylation. The accumulation of hyperphosphorylated tau in damaged brain regions is one of the major hallmarks of AD patients. After 6 h of treatment, neither A β 42 nor isoA β 42 significantly influenced tau phosphorylation levels; isoA β 42 treatment decreased the amount of tau in the cells by 30% (Fig. 7). Treatment with isoA β 42 for 24 h resulted in a drastic drop (53%) of tau levels, while A β 42 treatment did not induce any significant changes in the tau level (Fig. 8A,B). To evaluate the degree of tau phosphorylation, we used antibodies against tau phosphorylated at the S262, S396 and T231 residues. A β 42 treatment increased the phosphorylation of tau at S262 by 23% and at T231 by 34%. IsoA β 42 treatment increased phosphorylation at S262 by 168%, at T231 by 63%, and at S396 by 40% (Fig. 8A,C–E).

Discussion

AD includes a broad spectrum of profound molecular, histological and cognitive disturbances². It is widely accepted that the oligomerization of A β peptides and the occurrence of intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein are responsible for the observed global brain dysfunction²³. In the normal brain, A β peptides are involved in synaptic activity and provide protection against excessive glutamate release^{24,25}. These peptides also participate in the monitoring of cholesterol transport²⁶ necessary for neuronal survival. Importantly, direct injections of synthetic A β 42 into the brains of model animals fail to induce AD-like manifestations⁷. However, the administration of isoA β 42 induces massive amounts of amyloid plaques in the brains of AD-model mice¹⁴ and exerts significantly higher apoptogenic effects on model neurons and neuroblastoma cells (SK-N-SH and SH-SY5Y) than A β 42^{16,17} (Fig. 1). These data suggest that the presence of isoA β 42 in the brain triggers the development of AD pathology. The probability of isoA β 42 formation in the human brain increases significantly with age, when the catalytic activity of protein L-isoaspartyl (D-aspartyl) methyltransferase (PIMT), responsible for isoasparagine repair is diminished²⁷, which may explain the high frequency of sporadic forms of AD observed in ageing people.

A β 42 alters the phosphorylation levels of many proteins²⁸; however, isoA β 42 treatment resulted in significantly higher levels of phosphorylation of many proteins than A β 42 treatment (Figs 4 and 6, Suppl. Fig. 6). Compared with A β 42 treatment, isoA β 42 treatment induced higher numbers of phosphorylated isoforms of β -tubulin (Fig. 5B). In AD brains, β -tubulin is hyperphosphorylated at serine residues²². This event leads to the disturbance of microtubule (MT) assembly. Phosphorylated bovine brain β -tubulin loses its ability to assemble MTs and, when dephosphorylated, tubulin regains this vital ability²⁹. Our experiments demonstrated that the two A β peptides that were used increased α -tubulin phosphorylation (Figs 5 and 6, Suppl. Fig. 6), which increases its solubility and disrupts MT assembly³⁰.

Tau proteins play a major role in regulating neuronal MT assembly and stability^{31,32}. Tau promotes the polymerization of tubulin into MTs³³ and, when bound to MTs, helps stabilize them in their polymerized state³⁴. The deleterious action of A β 42 oligomers is associated with the hyperphosphorylation of tau, which results in neurofibrillar tangles and neuronal cell death²³. We showed here that isoA β 42 administration, in contrast to A β 42,

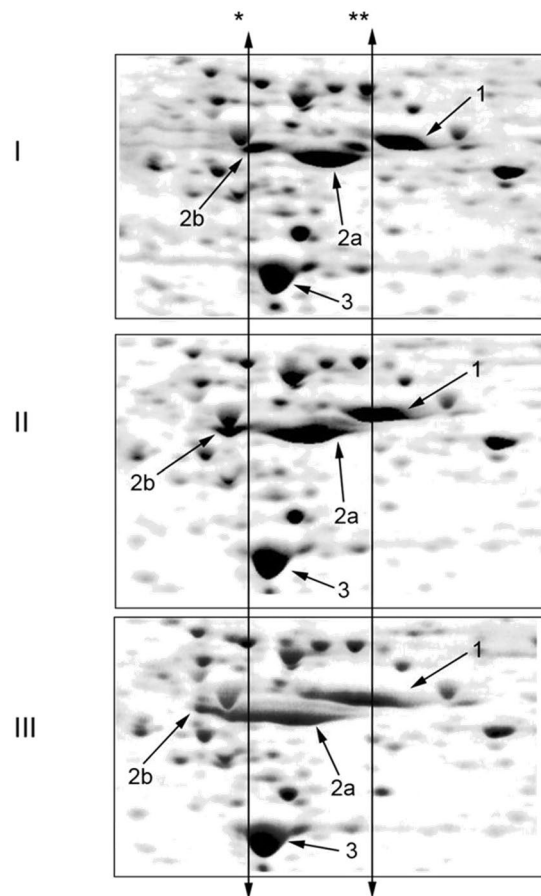


Figure 3. Visualization of changes in the isoelectric points of tubulin proteins after SH-SY5Y cells were treated with A β 42 and isoA β 42 peptides (10 μ M, 6 h). 35 S-labelled proteins from control SH-SY5Y cells (I) and cells treated with A β 42 (II) or isoA β 42 (III) were separated by 2D electrophoresis. The double arrow shows *the position of the acid end of β -tubulin in the control cells and ** the position of α -tubulin in the control cells. Protein identification: 1 - α -tubulin; 2a - β -tubulin (TBB5); 2b - β -tubulin (TBB4); 3 - actin (ACTB).

decreased tau levels in human neuroblastoma cells, which may result in disruption of MT assembly. Normal tau levels are significantly decreased in the supernatants from the brains of AD cases³⁵. However, it has not been demonstrated whether tau aggregation actually lowers the levels of soluble tau *in vivo* in AD patients³². IsoA β 42 treatment induced tau phosphorylation at S262, T231 and S396 residues more efficiently than A β 42 treatment did (Fig. 8). Notably, phosphorylation of all these residues is associated with AD progression. Phosphorylation of S262 inhibits the binding of tau with MTs and concurrently activates a cascade of kinases that phosphorylate tau at other residues³⁶. Furthermore, phosphorylation at T231 regulates the binding of tau with MTs and affects the stability of MT stability^{37,38}, while S396 phosphorylation enhances A β -induced mitochondrial injury, which contributes to neuronal dysfunction and to the pathogenesis of AD³⁹. It is evident that the observed alterations in the phosphorylation of tubulins and tau induced by isoA β 42 should result in severe disturbances in neurotransmission, MT formation and eventually neuronal death. The hyperphosphorylation of matrin 3 at serine residues (Fig. 5), observed after isoA β 42 treatment but not after A β 42 treatment, may be another factor responsible for the higher level of neuronal death detected with isoA β 42 treatment (Fig. 1). Matrin 3 is a highly conserved nuclear matrix phosphoprotein implicated in transcription and interaction with other nuclear matrix protein to form the internal fibrogranular network^{40,41}. Hyperphosphorylation of matrin 3 in neurons leads to their degradation⁴².

In summary, we have shown, for the first time, that the significantly higher cytotoxic effect of isoA β 42 than that of intact A β 42 is associated with the role of isoA β 42 as the inducer of more effective phosphorylation of several structural proteins including tau, the tubulins, and matrin 3. Hyperphosphorylation of these proteins is a hallmark of AD brains and is linked to neuronal death, which suggests a triggering role for isoA β 42 in this pathological cascade.

Materials and Methods

Cells and peptides. The SH-SY5Y neuroblastoma cell line was obtained from the European Collection of Authenticated Cell Culture (ECACC, Public Health England, UK). Cells were cultured in DMEM (Sigma Aldrich) supplemented with 10% heat-inactivated defined foetal calf serum (FCS, HyClone), 2 mM L-glutamine, 100 units/ml of penicillin and 100 μ g/ml streptomycin at 37 $^{\circ}$ C in a humid atmosphere with 5% CO₂. The synthetic peptides, isoA β 42 and A β 42, were purchased from Biopptide and prepared as described previously¹⁶. Cold

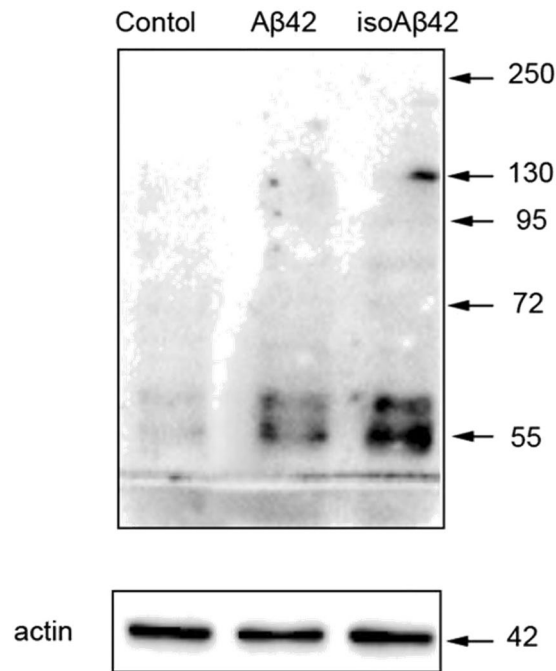


Figure 4. Effects of A β 42 and isoA β 42 (10 μ M, 6 h) treatment on the levels of phosphoserine-containing proteins. After incubation with the amyloid peptides, SH-SY5Y cells were lysed, the proteins were separated using 8% SDS-PAGE, and antibodies against phosphoserine were used to identify phosphoserine-containing protein bands.

hexafluoroisopropanol (Fluka) was added to dry A β 42 or isoA β 42 to a concentration of 1 mM and incubated for 60 min at room temperature. Then this solution was put on ice for 10 min and aliquoted into non-siliconized microcentrifuge tubes (0.56 mg peptide per tube). Peptide in the tubes was dried under vacuum using Eppendorf Concentrator 5301. Dried peptide was stored at -80°C . For addition to cells, 2.5 mM peptide stock solution was prepared by adding 20 μ l of 100% anhydrous DMSO (Sigma-Aldrich) to 0.22 mg peptide and incubating for 1 h at room temperature. For use in the experiments, the peptide was diluted to the required concentration with buffer solution. Equivalent amount of DMSO was added to the control samples in all experiments. Based on our previous data for cell treatment we used peptides in concentration 10 μ M which is optimal for *in vitro* studies of amyloid pathogenic effects on neuroblastoma cells^{16,17,43}.

Cell viability assay. Cell viability was assessed with MTT-test kit (Sigma). Briefly, SH-SY5Y cells were seeded in 96-well plates and cultured for 24 h at 37°C . Then, the cells were treated with isoA β 42 or A β 42 for 48 h followed by incubation with MTT reagent for 4 h at 37°C . The absorbance of samples was measured in a multi-scan FC microplate reader (Thermo Fisher Scientific) at 570 nm. Viability of untreated cells was taken as 100%.

Apoptosis assay by Hoechst 33342 staining. To study apoptosis, cells were collected using trypsin-versene, washed with complete culture medium, counted, and seeded into four-well plates (Nunc, Thermo Fisher Scientific, USA), coated with 0.01% Poly-L-lysine, at 400,000 cells per well in 1 mL of culture medium supplemented with 5% FCS and were cultivated at 37°C in a humid atmosphere with 5% CO_2 for 24 h. After 24 h, the medium with 5% FCS was substituted with FCS-free medium. IsoA β 42 or A β 42 peptides were added to the wells, and the cells were cultured for 24 h at 37°C . Apoptotic cells were detected using fluorescent microscopy and Hoechst 33342 dye⁴⁴, and necrotic cells were detected using propidium iodide. After the culture medium was washed off, the cell layer was stained with 10 μ g/ml Hoechst 33342 dye in phosphate buffer for 30 min at 37°C in the dark. Then, 30 μ M propidium iodide was added, and the cells were visualized using an inverted fluorescent microscope (Keyence BZ8100, Japan). The number of apoptotic cells was calculated as a portion of the number of cells with fragmented DNA (not stained with propidium iodide) out of the total number of cells (100%). Cells that were stained with propidium iodide were considered necrotic. To register apoptosis, at least 20 fields of view were analysed, each containing 250–350 cells. Results of three experiments with four repetitions were pooled and statistically processed.

Probe preparation for 2D analysis. To obtain samples for 2D electrophoresis, SH-SY5Y cells were harvested from culture flasks using a trypsin-EDTA solution (Sigma-Aldrich) and washed twice with culture medium. The pellet was resuspended, and the cells were counted, diluted in serum-free culture medium, and transferred into glass tubes. Then, the cells were treated with either A β 42 (10 μ M) or isoA β 42 (10 μ M) for 6 or 24 h at 37°C and 5% CO_2 . Following the incubation, the cells were placed in an ice bath for 15 min, centrifuged, and washed three times in Hanks' Balanced Salt solution (HBSS, Sigma-Aldrich). The cell pellets were resuspended in HBSS and 2.0×10^6 cells were labelled with 1.85 MBq of L-[^{35}S]methionine (Amersham Biosciences Corp.,

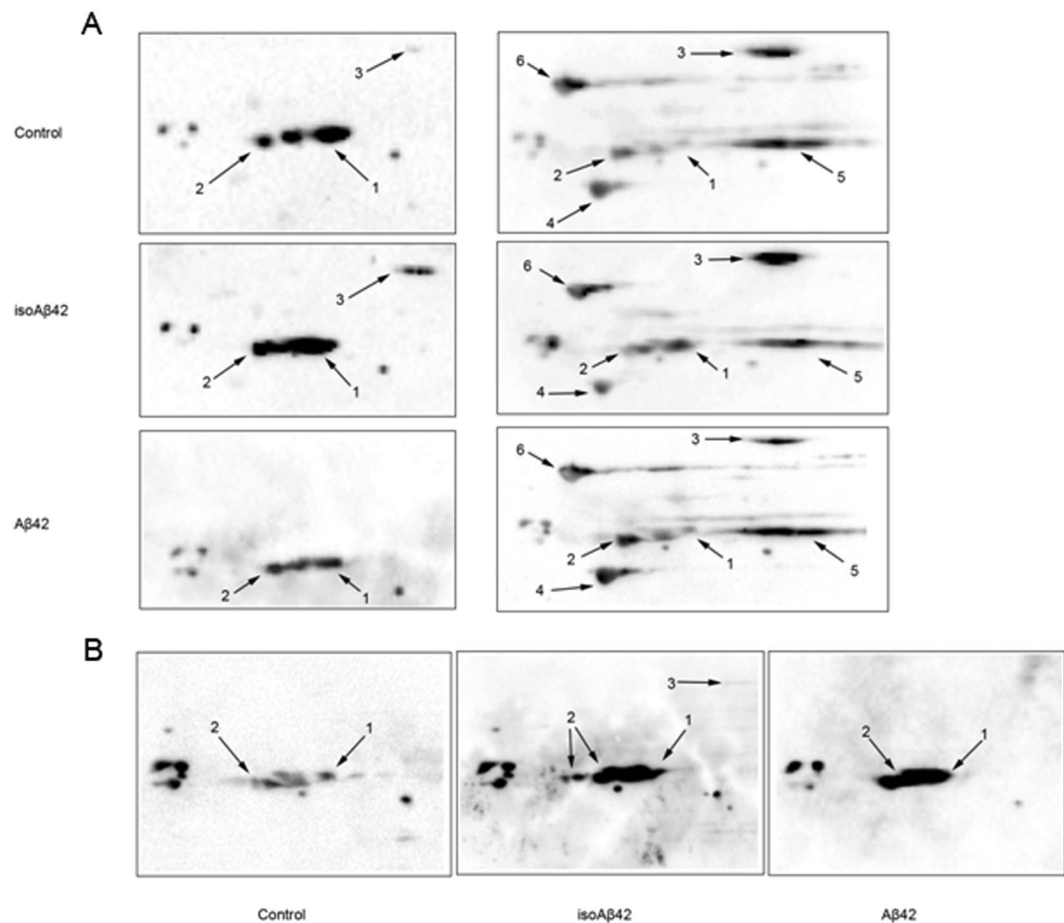


Figure 5. Western blot analysis of 2D-gels from SH-SY5Y control cells and SH-SY5Y cells treated with A β 42 and isoA β 42 (10 μ M, 6 h). (A) A merged image of anti-actin, anti-matrin 3, anti-total tau, anti- β -tubulin, anti- α -tubulin and anti-HSP90 antibodies (1 - α -tubulin, 2 - β -tubulin, 3 - matrin 3, 4 - actin, 5 - total tau, 6 - HSP90). (B) Anti-phosphoserine antibodies (1 - α -tubulin, 2 - β -tubulin, 3 - HNRH1, 4 - matrin 3).

Piscataway, NJ, USA) in HBSS for 1 h at 37 °C. An unlabelled sample was prepared at the same time. The 2×10^6 L-[35 S]methionine-labelled and unlabelled cells were lysed by stirring at 4 °C for 15 min in 70 μ l of O'Farrell lysis buffer with 1% PMSF and 1% protease-inhibitor cocktail (Amresco). Halt Phosphatase Inhibitor Cocktail (Thermo scientific) was added into the samples for staining with pro-Q diamond phosphoprotein gel stain. The probes were then centrifuged at 15,000 g for 10 min, and the supernatant was collected.

2D electrophoresis and data analysis. 2D PAGE was performed by electrofocusing in a polyacrylamide gel at a pH range of approximately 4.5 to 9.5 in the first direction followed by SDS-11% PAGE in the second direction using a modified version of O'Farrell's method^{45,46}. After electrophoresis, the gels were stained with a 0.2% solution of Coomassie Brilliant Blue G250 (CBB), destained, and dried for viewing using the Typhoon FLA 9500 imaging system from GE Healthcare Life Sciences. To reveal phosphorylated proteins, gels after second direction were stained with pro-Q diamond phosphoprotein gel stain (Molecular Probes, Invitrogen, UK) according to manufacturer's instructions and viewed on Bio-Rad ChemiDoc MP gel imaging system. After imaging, gels were stained with a 0.2% solution of Coomassie Brilliant Blue G250. Densitometric analysis was performed using Fiji ImageJ software.

MALDI-TOF mass spectrometry. Mass spectra of the tryptic peptides of from SH-SY5Y cellular proteins were obtained using a matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass (TOF) spectrometer, Ultraflextreme BRUKER (Germany), equipped with a UV laser (Nd) and reflectron at the Human Proteome Shared Facility Centre at the Institute of Biomedical Chemistry (Moscow, Russia). Pieces (2 \times 2 mm) of the proteins of interest from 2D-gels were analysed as described previously⁴⁷. Identification of SH-SY5Y cellular proteins was performed using Mascot software (www.matrixscience.com) and the NCBI database, taking into account the possible oxidation of methionine residues and the modification of cysteine residues by acrylamide.

Analysis of proteins by Western blot. For immunoblot analysis of tau phosphorylation, cells were incubated with 10 μ M A β 42 or isoA β 42 for 24 h and then lysed by stirring at 4 °C for 1 h in RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet-P40, 0.1% SDS, 1% sodium deoxycholate), containing 200 μ M

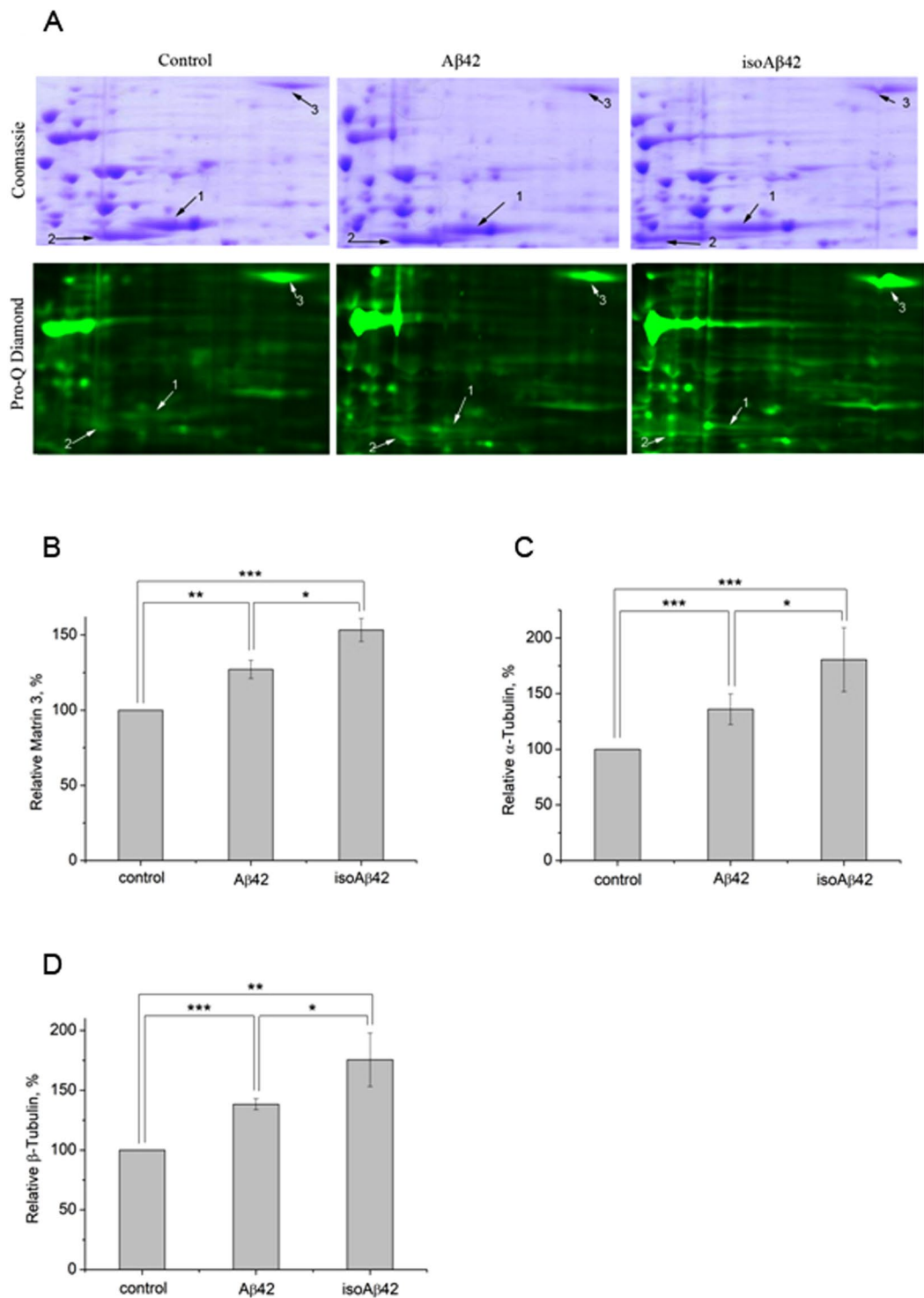


Figure 6. Visualization of the changes in protein phosphorylation levels after SH-SY5Y cells incubation with A β 42 or isoA β 42 (10 μ M, 6 h). Extracted proteins were separated by 2D electrophoresis. In the second dimension, proteins were separated on 11% SDS-PAGE, followed by Pro-Q diamond phosphoprotein staining and after image acquisition were restained with Coomassie R-250 (A). 1 - α -tubulin, 2 - β -tubulin, 3 - matrin 3. Densitometric analysis was performed using Fiji ImageJ software. Phosphorylated matrin 3 (B), α -tubulin (C) and β -tubulin (D) stained with Pro-Q diamond phosphoprotein stain were normalized to its total amount stained with Coomassie R-250. Each value is the mean expressed as a percentage of the protein phosphorylation level in the control group \pm SD of at least three independent experiments; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

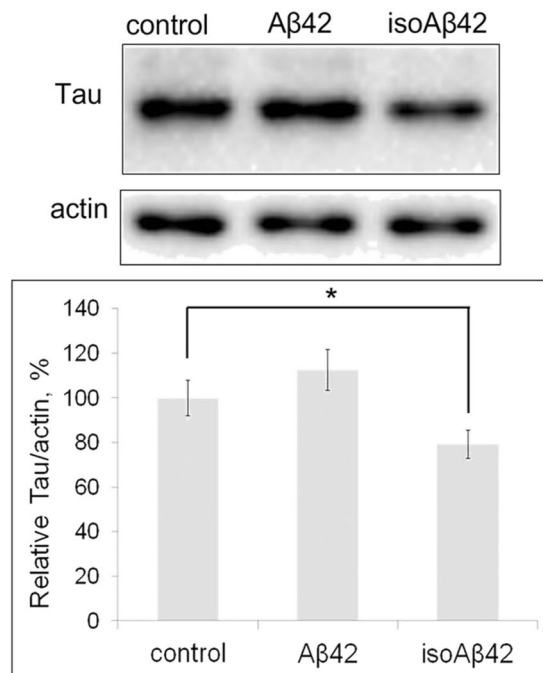


Figure 7. Effect of A β 42 and isoA β 42 (10 μ M, 6 h) treatment on the total tau protein level. After incubation with the amyloid peptides, the SH-SY5Y cells were lysed, the proteins in the cell lysate were separated by SDS-PAGE, and total tau was detected by Western blot using antibodies against total tau. The total tau level is expressed as a percentage of the tau level in the control group, which was not treated with A β s. The bars represent the changes in the total tau level. Each value is the mean \pm SD of at least three independent experiments; * p < 0.02.

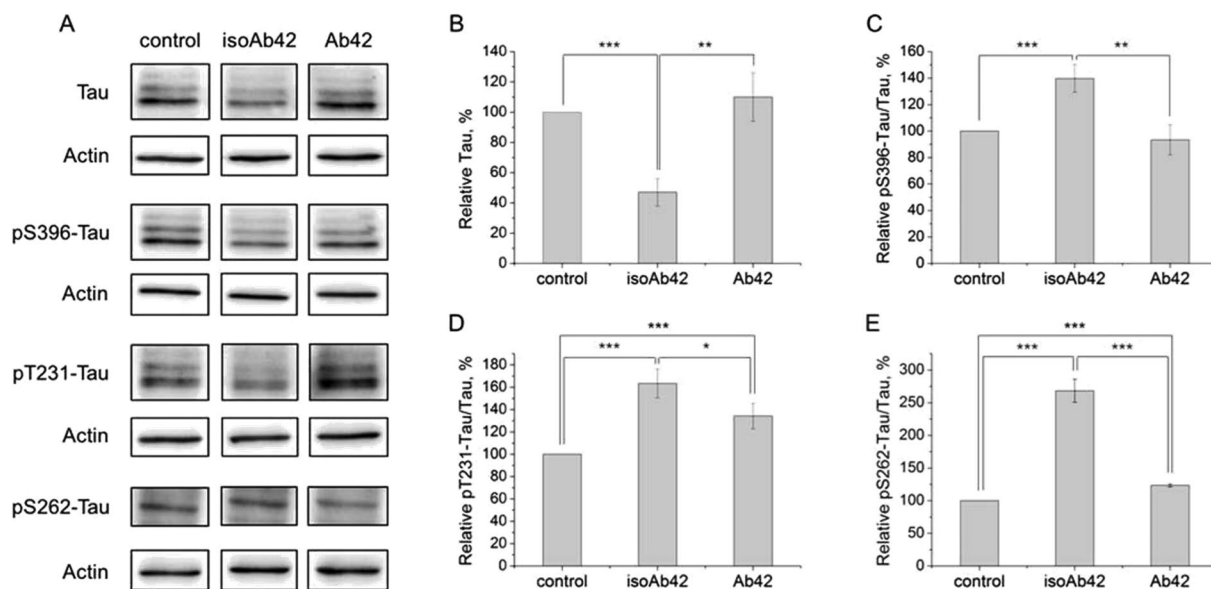


Figure 8. Effect of A β 42 and isoA β 42 treatment (10 μ M, 24 h) on the total tau level and the phosphorylated tau level in SH-SY5Y cells. After incubation with A β 42 or isoA β 42, the cells were lysed, and the proteins were separated by SDS-PAGE and detected by Western blot using antibodies against total tau, phosphorylated S396 tau, phosphorylated T231 tau or phosphorylated S262 tau (A). The bars represent the changes in the total tau level (B), phosphorylated S396 tau (C), phosphorylated T231 tau (D) or phosphorylated S262 tau (E), expressed as a percentage of the total tau level in the control group, which was not treated with A β . Changes to tau phosphorylation levels were normalized to its total amount. Each value is the mean \pm SD of at least three independent experiments; * p < 0.02, ** p < 0.005, *** p < 0.001.

PMSF and a complete protease-inhibitor mixture (Roche). The lysate and probes were then centrifuged at 15,000 g for 10 min, and the supernatant was collected. Proteins from the cell lysates and proteins isolated after the first gel electrophoresis on a 2D gel electrophoresis were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were stained with Ponceau S (Sigma, USA) to monitor transfer efficiency. After the membranes were blocked with 5% nonfat milk or 5% BSA in PBST, primary antibodies (Suppl. Table 2) were used to detect the proteins. Then, the blots were incubated with the appropriate secondary antibodies (Suppl. Table 2). Protein visualization was performed using the appropriate horseradish peroxidase-conjugated secondary antibodies provided by the enhanced chemiluminescence SuperSignal™ West Femto Maximum Sensitivity Substrate kit (Thermo Scientific). Chemiluminescence was detected using a Bio-Rad ChemiDoc MP gel imaging system. Densitometric analysis was performed using Image Lab software (Bio-Rad), and the results are expressed as the ratio of phospho-tau band density to total tau band intensity.

Statistical analyses. The data are presented as the mean \pm standard deviation of at least three independent experiments. The differences between the groups were analysed using One Way ANOVA with Tukey's pairwise comparisons and $p < 0.05$ was considered significant.

Data availability statement. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

O.G.Z., V.A.M. and S.A.K. conceived the project and drafted the paper with contribution from O.I.K., M.B.E. and A.A.M.; O.G.Z., O.I.K., M.M. Yu., M.G.V., M.V.S. and A.P.R. performed the experiments; M.B.E. and A.A.M. coordinated the study and contributed materials and reagents. All authors reviewed the manuscript.

Additional Information

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