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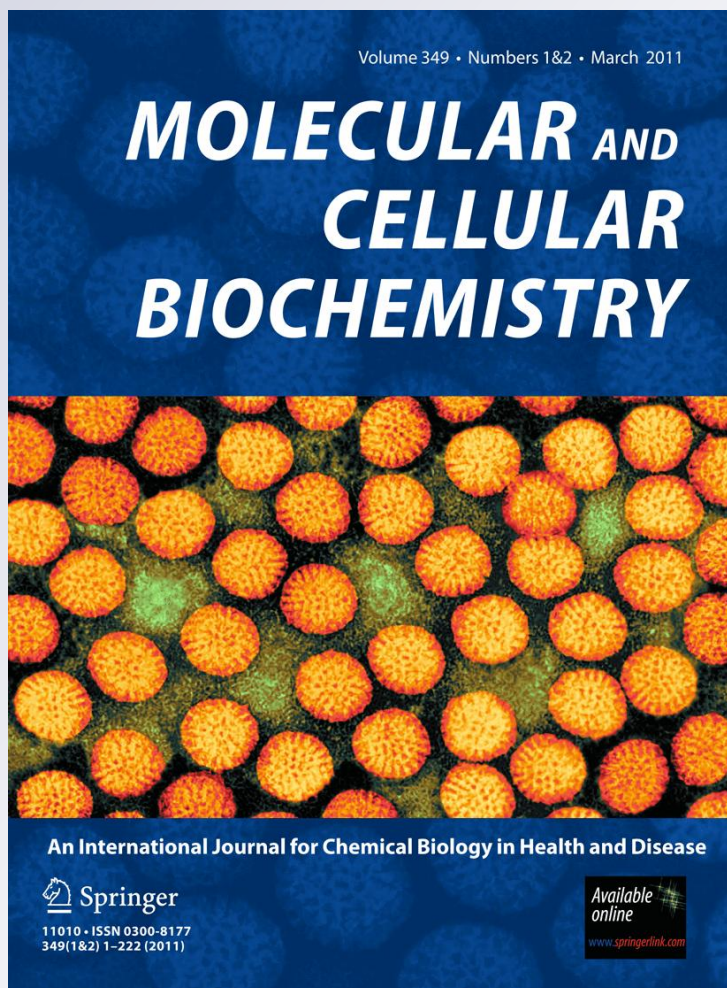
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Pre-aggregated Aβ1–42 peptide increases tau aggregation and hyperphosphorylation after short-term application

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Abstract Neuritic amyloid plaques and neurofibrillary tangles, consisting of hyperphosphorylated tau protein, are the hallmarks of Alzheimer disease. It is not clear so far, how both structures are functionally and physiologically connected. We have investigated the role of Aβ1–42 on hyperphosphorylation and aggregation of tau in SY5Y cells by transfection and overexpression with two tau constructs, a shortened wildtype tau (2N4R) and a point mutation tau (P301L), found in fronto-temporal dementia. It was found that the tau protein becomes hyperphosphorylated and forms large aggregates inside cells, visualized by immunofluorescence, after short incubation of 90 min with preaggregated Aβ1–42. In Addition, Aβ1–42 caused a decrease of tau solubility in both tau constructs in this relatively short time period. Taken together, these experiments suggest that pathological preaggregated Aβ1–42 in physiological concentrations quickly induces hyperphosphorylation and pathological structural changes of tau protein and thereby directly linking the 'amyloid hypothesis' to tau pathology, observed in Alzheimer disease.

Introduction

Alzheimer's disease (AD) is the most common dementia, accounting for 50–70% of cases. In correlation to increasing cognitive deficits senile plaques and microscopic "tangles" (NFTs, neurofibrillary tangles) can be found in post-mortem brain [1, 2], which are considered to be the pathological hallmarks of Alzheimer disease [3, 4]. According to a leading theory, termed as "amyloid hypothesis", extracellular accumulation of Aβ deposits in the brain is the primary cause, driving AD pathogenesis [5, 6]. The other hallmark of AD is an abnormal and excessive phosphorylation of tau proteins. This results in the aggregation of tau into intracellular tangles, consisting of bundles of fibrillar tau proteins (PHFs, paired helical filaments) [7]. Six different isoforms of tau proteins exist in the human adult brain [8]. Tau proteins contain several Ser/Thr-Pro sequences (18%) [9] that are phosphorylation targets for different kinases. Molecular analysis has revealed that an abnormally high phosphorylation might be one of the important events in the process of tau aggregation. Tau protein can be phosphorylated by several kinases. The phosphorylation of Ser-262 within the microtubule-binding region of tau is of particular interest because it is observed only in AD [10]. Its phosphorylation dramatically reduces the affinity for microtubules in vitro [9]. In AD, NFTs show a hierarchical spreading pattern from the allocortex to isocortical association areas with early involvement of the entorhinal region. Comparative studies of neuropathological staging of AD with psychometrically assessed intellectual status (mini-mental state)

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showed a linear correlation [11–15]. Experiments show that injection of Aβ1–42 fibrils into the brains of P301L-mutant tau transgenic mice caused fivefold increase in the numbers of NFTs in cell bodies. These NFTs contained tau phosphorylated at serine 212/threonine 214 and serine 422 [16]. Mutagenesis of the phosphoepitope serine 422 of tau to alanine or glutamate prevented both the Aβ1–42-mediated decrease in solubility and the generation of PHF-like filaments in neuroblastoma cells suggesting a role of serine 422 or its phosphorylation in tau filament formation [17]. Only six phosphorylation sites lie outside the microtubule-binding domain, including Ser199 and Ser202, a binding site, detected by both antibodies AT8 and pSpS 199/205 [18]. Aggregated tau is also hyperphosphorylated at Ser199 in many other tauopathies [16, 19]. The selective phosphorylation of Ser199 residue of tau probably occurs in vitro in mitotic and in apoptotic neuron cells [20, 21]. In tissue culture of human neuroblastoma cells, containing both wild-type and mutant forms of human tau, PHF formation could be induced by incubation with aggregated synthetic β-amyloid [17]. Change of tau hyperphosphorylation and cell viability were detected after 10 min of addition of Aβ1–42 to the cells but still reversible, if Aβ1–42 was removed. After 20 min of treatment changes, were irreversible [22].

The causal connections among Aβ1–42, tau phosphorylation, and tau aggregation were investigated to show that both hypotheses for Alzheimer's pathogenesis are tightly connected. The aim of this was to analyze the effects of short-term exposure of Aβ1–42 peptide on the solubility and the phosphorylation status of tau in tau-overexpressing cells. In this respect, the localization of tau was examined by immunofluorescence and subcellular fractionation.

Materials and methods

Cell culture

SH-SY5Y-cells were cultured in D-MEM Glutamax (Gibco, Eggenstein, Germany), containing 10% FCS (Biochrom, Berlin, Germany) and G418 (200 μg/ml) (Invitrogen, Karlsruhe, Germany). G418 was omitted in the medium of untransfected SY5Y cells. Media were exchanged by 50% every 3–4 days.

Transfection

SH-SY5Y cells were grown for 2–3 days and then transfected with either hTau40 [P301L] or hTau40 [2N4R] in pcDNA3 plasmid (0.8 μg/24-well-plate) using the Lipofectamine 2000-Kit (Invitrogen) according to manufacturer's instructions. Transfected cells were selected by

addition of 400 μg G418/ml medium for 4 weeks to reach a 30-fold tau expression level.

Cell splitting

Growing SY5Y cells were split every 5–6 days to a ratio of 1:4. The medium was removed, and cell culture bottles were rinsed with 37°C PBS. Cells were detached by 5 min incubation with trypsin–EDTA (Biochrom) at room temperature. Floating cells were centrifuged for 5 min at $280 \times g$, resuspended in DMEM and reseeded.

Tau phosphorylation induction by okadaic acid

25 μg of okadaic acid (Calbiochem, Darmstadt, Germany) was dissolved in 30.25 μl H_2O to obtain a 1 mM stock solution. Cells were incubated with 1 μM okadaic acid for 90 min at 37°C.

Preaggregation of synthetic Aβ1–42

Synthetic Aβ1–42 (1 mg) was dissolved in 200 μl sterile PBS by repetitive trituration. The sample was divided in 2×100 μl aliquots and both tubes were incubated for 3 days at 37°C under thorough agitation. The solution was diluted into 11 ml culture medium to obtain a final concentration of 10 μM. This concentration is defined for the monomeric Aβ1–42, since the molarity of the polymerized molecule cannot be calculated but is much lower. This medium was used for incubation of the SY5Y cells in the experiments after removal of the original medium.

Biochemical assays

Lactate dehydrogenase was determined by a commercially available LDH-Assay Kit (Promega, Mannheim, Germany) according to manufacturer's instructions. Total protein concentration of samples was determined by the bicinchonine acid method, using the Micro BCA Protein Assay Reagent Kit (Perbio, Bonn, Germany) according to manufacturer's instructions or a detergent-compatible amido-black B protein stain procedure for proteins, dissolved in SDS sample buffer [23].

Cell fractionation

Cells were collected after trypsinization, washed with PBS and cracked by ultrasonic disintegration (10 × 5 s at 85% max energy level, intermediate cooling in ice water) in RAB (High salt reassembly buffer), containing 2 ml 1 M MES, 100 μl 0.2 M EGTA, 20 μl 0.5 M $MgSO_4$, and 5 ml 3 M NaCl (all Sigma, Steinheim, Germany), and two tablets protease-inhibitor Pep Complete Mini (Roche,

Grenzach-Wyhlen, Germany). Two fractions were obtained by high-speed centrifugation (30 min, 4°C, 16000×*g*): fraction 1 containing the cytosolic and membrane-associated proteins; and fraction 2, the pellet fraction, which contained integral membrane, cytoskeleton: nuclear proteins and larger protein aggregates like tau filaments.

Western blotting

Samples were dissolved in a reducing sample buffer, separated on 10% SDS gels, 0.75 mm thickness, [24], running at a constant current (25 mA/gel) and transferred onto PVDF membrane in a semi-dry blot chamber [25]. PVDF membranes were blocked with 3% nonfat-dry milk in PBS and incubated with respective first and secondary antibodies (anti-rabbit IgG, 1:30000, peroxidase conjugated, Calbiochem). All primary antibodies (pSpS 199/205, pSer262, pSer422, AT8, and HT7) were incubated overnight in PBS—3% milk powder, dissolution 1:4000 at 4°C. PVDF membranes were washed three times with PBS, and secondary antibodies were incubated for 1 h at room temperature. Chemiluminescence detection was conducted using ECL advance kit (GE Healthcare, Munich, Germany) and quantified using a digital camera system (Fluor S-Max, Biorad, Munich).

Immunofluorescence

Coverslips, diameter 12 mm, were autoclaved and placed into 12-well plates, coated with 0.1% collagen overnight at 37°C. Cells were seeded and allowed to grow to 60% confluence after 2–3 days. On the day of the experiment, the cells were incubated at 37°C with 10 μM Aβ1–42 for 90 min. Coverslips were transferred into new plates, washed, and cryofixed with acetone–methanol (1:1) for 10 min. Coverslips were washed twice with PBS, permeabilized with 0.05% triton X-100 (AppliChem, Darmstadt, Germany) for 10 min at room temperature, washed again twice and unspecific binding sites were blocked with Roti-Immunoblock (Roth, Karlsruhe, Germany) for 30 min in a wet chamber. Primary antibodies were applied overnight in a dilution of 1:40 in Roti-Immunoblock at 4°C.

The next day, the coverslips were washed and incubated with secondary antibodies, conjugated to Alexa 488 in a dilution of 1:160. Nuclei were stained with 4′,6-diamidino-2-phenyllindol, 1 μg/ml in PBS-buffer.

Epifluorescence microscopy

Epifluorescence microscopy of FM 1–43 was performed on a Zeiss "Axiovert 135" fluorescence microscope equipped with a "Fluar" objective, 100×, 1.3 NA (both from Zeiss, Jena, Germany), a CCD camera "CoolSnap" (Photometrics, Tucson, USA), and a polychromator Visi-Chrome for single wavelength illumination (Visitron Systems, Puchheim, Germany). Hardware was controlled using Metamorph® 7.5.0.0 imaging software (Universal Imaging Co., Downingtown, USA).

Image processing and analysis

Image analysis was performed using Metamorph® 7.5.0.0. Images were individually background subtracted by subtracting the peak value of the fluorescence distribution histogram before further analysis. The average fluorescence was defined and measured with Metamorph's built-in function "threshold" and "region measurements" functions for each channel where applicable.

Statistical analysis

Standard statistical analysis was performed using Origin 7.5 (OriginLab Corporation, Northampton, USA) and Microsoft Excel 2007 (Microsoft Inc., Redmond, USA). Two-sided Student's *t*-test was used to test significant differences.
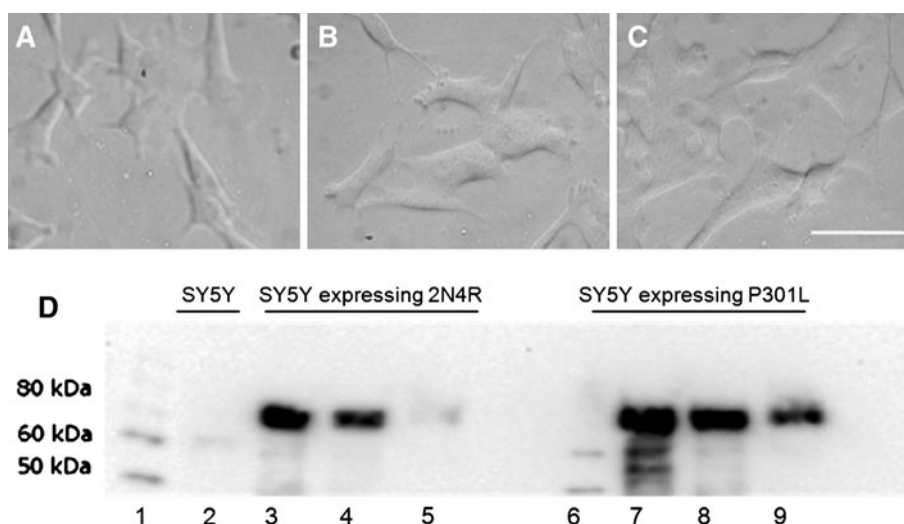
Results

Characterization of transfected cells

Phosphorylation and aggregation of tau constructs were analyzed in a cell culture model. SH-SY5Y neuroblastoma cells were stably transfected with human tau constructs, expressing either a 9 amino acid N-terminal truncated form of the adult tau protein (2N4R) or a tau protein, carrying a point mutation (P301L). Stably transfected cells showed a continuous overproduction of 2N4R and P301L isoforms, respectively.

Figure 1a–c show that all the three SY5Y cell types were morphologically very similar, had short processes and grew to a homogenous layer within a week. All the transfected cells had a clearly higher division rate and grew faster than untransfected SY5Y cells. Western blots with antibody HT7, which recognizes total tau independently of its phosphorylation state, showed that untransfected cells (Fig. 1d, lane 2) expressed embryonic tau only, while 2N4R- and P301L-transfected cells (Fig. 1d, lanes 3–5 and lanes 7–9) overexpressed the higher molecular weight isoform. Different quantities of whole protein extracts from SY5Y cells were blotted and probed with HT7. Untransfected SY5Y-Zellen showed only a weak 59 kD band, when 5 μg of whole protein was loaded onto the gel (Fig. 1d, lane 2). 2N4R-transfected cells overexpressed mainly the larger adult form of tau with a higher molecular

Fig. 1 Overexpression of tau constructs. Overexpressing SY5Y cell show no morphological changes. **a** control cells, **b** 2N4R transfected cells, **c** P301L transfected cells, *scale bar* = 15 μm; and **d** Western blot analysis of overexpressed tau constructs with antibody HT7 against total tau. Lanes 1 + 6: molecular weight marker; lane 2: 5 μg total protein of control cells; lanes 3 + 7: 5 μg; lanes 4 + 8: 0.5 μg; lanes 5 + 9: 50 ng total protein

weight. This band remained still detectable at a tenfold lower protein load (lane 5). P301L-mutant-transfected cells overexpressed this band to an even higher level. Differentiation of the cells with BDNF and retinoic acid did not reveal substantial changes in the amount and the phosphorylation status of tau (data not shown).

Aβ1–42 induces tau phosphorylation and aggregation

The cells were incubated with synthetic Aβ1–42 peptide to investigate site-specific hyperphosphorylation and aggregation of transfected tau protein. Aβ1–42 was preaggregated and administered to the cells culture medium in a final concentration of 10 μM. Figure 2a shows that the cells exhibited no significant toxicity after 90 min and after 24-h incubation with preaggregated Aβ1–42, measured by LDH-cytotoxicity assay. Longer exposure to the peptide caused a slight viability decline after 48 h and a significant $P < 0.05$) reduction of viability after 72 h.

In order to demonstrate hyperphosphorylation of different epitopes on the tau constructs by site-specific anti-phospho tau antibodies, cells were incubated with okadaic acid, a toxin known to induce hyperphosphorylation [23]. Solubilized cell extracts were subsequently probed with HT-7 (antibody against total tau), AT8, pS262, and pS422 on quantitative western blots. Figure 2b presents an overview of the antibody-binding sites on the tau protein. Figure 2c shows the binding of site-specific anti-phospho tau antibodies after induction of hyperphosphorylation by okadaic acid. In this experiment series, the protein loaded onto the gel was normalized for equal tau concentrations, which has been determined by HT-7 antibody before. HT-7 against total tau showed no increase of binding activity in cells, either transfected with 2N4R or with P301L. Significant increase of hyperphosphorylation was detected with all the three site-specific antibodies in P301L-transfected
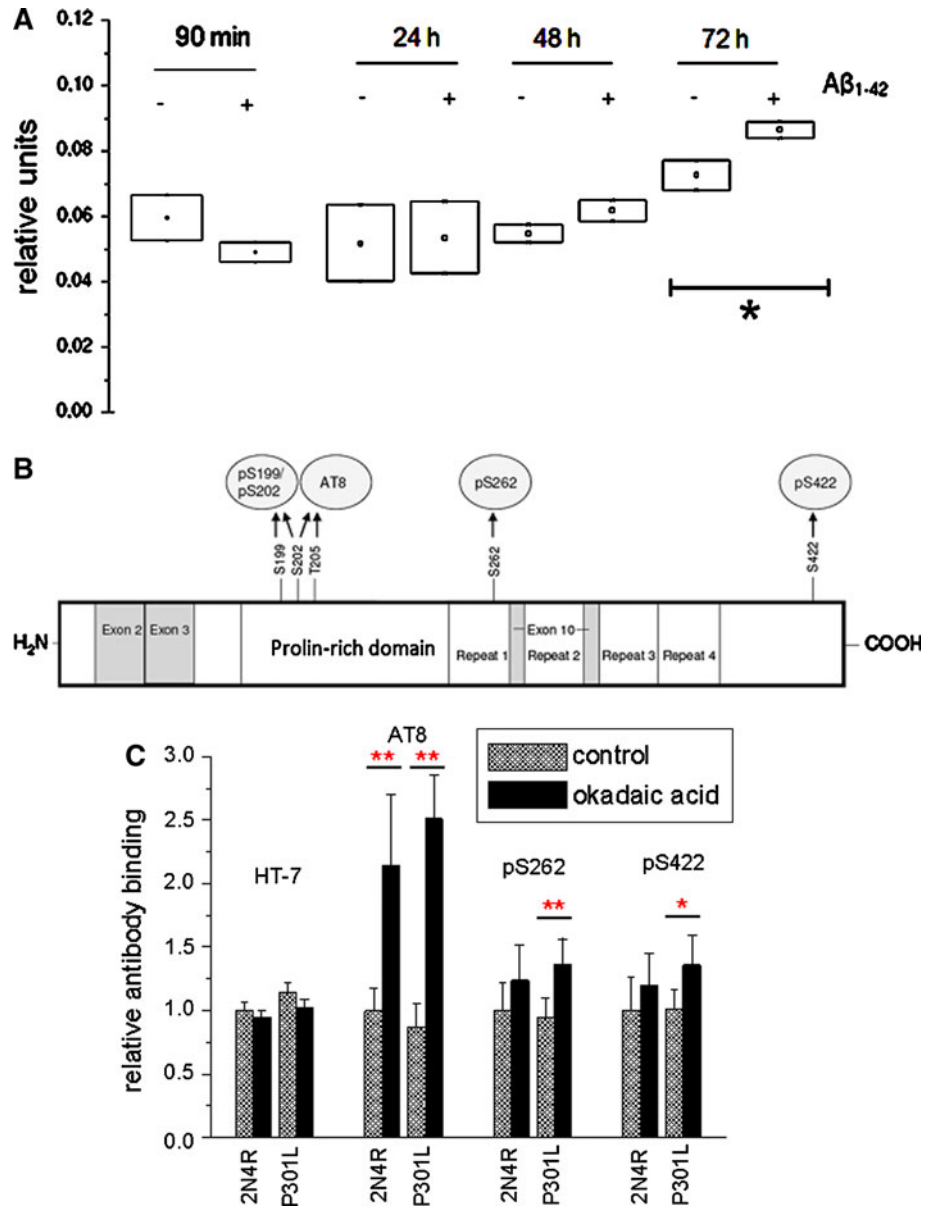
cells and at position Ser199/Ser199/Thr205, which was labeled with AT8 in 2N4R cells. Phosphorylation at sites pS262 and pS422 was increased in 2N4R cells but did not reach the $P < 0.05$ significance level.

SY5Y cells were then incubated with 10-μM preaggregated Aβ1–42 for 90 min and fixed for immunofluorescence. The antibody AT8 was chosen for the experiment because it has shown the biggest difference between okadaic acid-induced tau phosphorylation and unphosphorylated tau (Fig. 2c). Figure 3 shows that phosphorylation increased on epitopes Ser 199/Ser202/Thr205 in untransfected SY5Y control cells (Fig. 3a, d), labeled with AT8 antibody. The endogenous embryonic tau protein became visible only after Aβ1–42 incubation (Fig. 3d) and remained largely homogeneously distributed throughout the cells. Both 2N4R (Fig. 3b, e)- and P301L (Fig. 3c, f)-transfected cell types showed globular aggregates of tau protein after Aβ1–42 treatment that indicated a structural change of tau and an altered subcellular distribution after hyperphosphorylation.

Aβ1–42 decreases the tau solubility

The effect of short-term exposure to Aβ1–42 on tau solubility was investigated by subcellular fractionation. Cells were incubated with 10 μM Aβ1–42 for 90 min. Then they were solubilized by ultrasound disintegration in a high salt buffer and subjected to a high-speed centrifugation. Supernatant (fraction 1) and pellet (fraction 2) were analyzed separately by western blotting, using site-specific anti-phospho tau antibodies. Figure 4 shows the results for 2N4R- and P301L-transfected cells. About 95% of all detectable tau, labeled with antibody HT-7, was found in the soluble high salt fraction 1, independent of the tau construct; short-term exposure to Aβ1–42 did not alter its distribution in between both fractions in control cells (data

Fig. 2 Aβ1–42 toxicity and tau phosphorylation detected by site-specific anti-phospho antibodies. **a** LDH assay shows the relative LDH activity in the culture medium after incubation with Aβ1–42 (+), asterisks indicates a significant ($P < 0.05$, Student's t-test) increase of LDH activity, $n = 3$ per condition. The box plots represent mean (*small dot*) and standard deviation (*box*); **b** binding epitopes of respective site-specific anti-phospho antibodies; and **c** Quantification of western blot analysis with respective antibodies. *Error bars* represent standard deviations All Student's t-test significance level: *$P < 0.05$, **$P < 0.01$
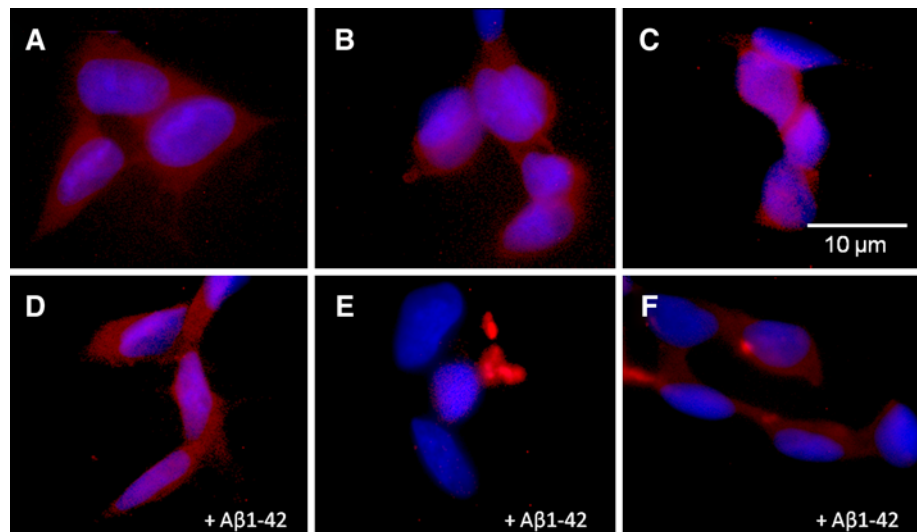

not shown), but it reduced the high salt buffer soluble proportion of phosphorylated tau significantly in 2N4R- and in P301L-transfected cells and doubled its insoluble portion in fraction 2. This redistribution was consistently seen with all the three site-specific anti-phospho tau antibodies. The Aβ1–42 induced shift of phospho tau from fraction 1 to fraction 2 indicated a structural conformation change of the protein that reduced its solubility and increased its phosphorylation in parallel.

Discussion

SY5Y-cells were analyzed to study effects of short-term Aβ1–42 application on tau phosphorylation, subcellular redistribution, and aggregation in vitro. Our cell culture model consisted of SY5Y-cells, which were stably transfected with expression constructs, encoding the longest 4-repeat isoform of human tau, containing the pathogenic FTDP-17 mutation P301L, or a truncated form of the adult tau protein (2N4R). In addition, both transfected cell lines showed also the shorter embryonic tau form in western blots [26]. This shorter tau was also present in the untransfected SY5Y cells, as published earlier [27]. Differentiation of the cells with BDNF and retinoic acid did not reveal substantial changes in the amount and the phosphorylation status of tau, and okadaic acid was used as an inductor for tau hyperphosphorylation [28]. As expected, most analyzed phosphorylation sites became hyperphosphorylated after OA application, and site-specific

Fig. 3 Immunofluorescence with antibody AT8. SY5Y cells, incubated with 10 μM Aβ1–42 (lower panels) and stained with anti phospho-tau antibody AT8 (Alexa-red 568); **a + d**: untransfected cells, **b + e**: 2N4R-tau transfected cells, **c + f**: P301L-tau transfected cells. Nuclei were stained with DAPI and superimposed onto AT8 image. Transfected cells developed phospho-tau aggregates after incubation with Aβ1-42. Experiments were conducted in three independent trials

antibodies showed increased binding activity. Therefore, the three site-specific antibodies were chosen to study the subcellular distribution of hyperphosphorylated tau after incubation with preaggregated Aβ1–42 [9, 16, 29].
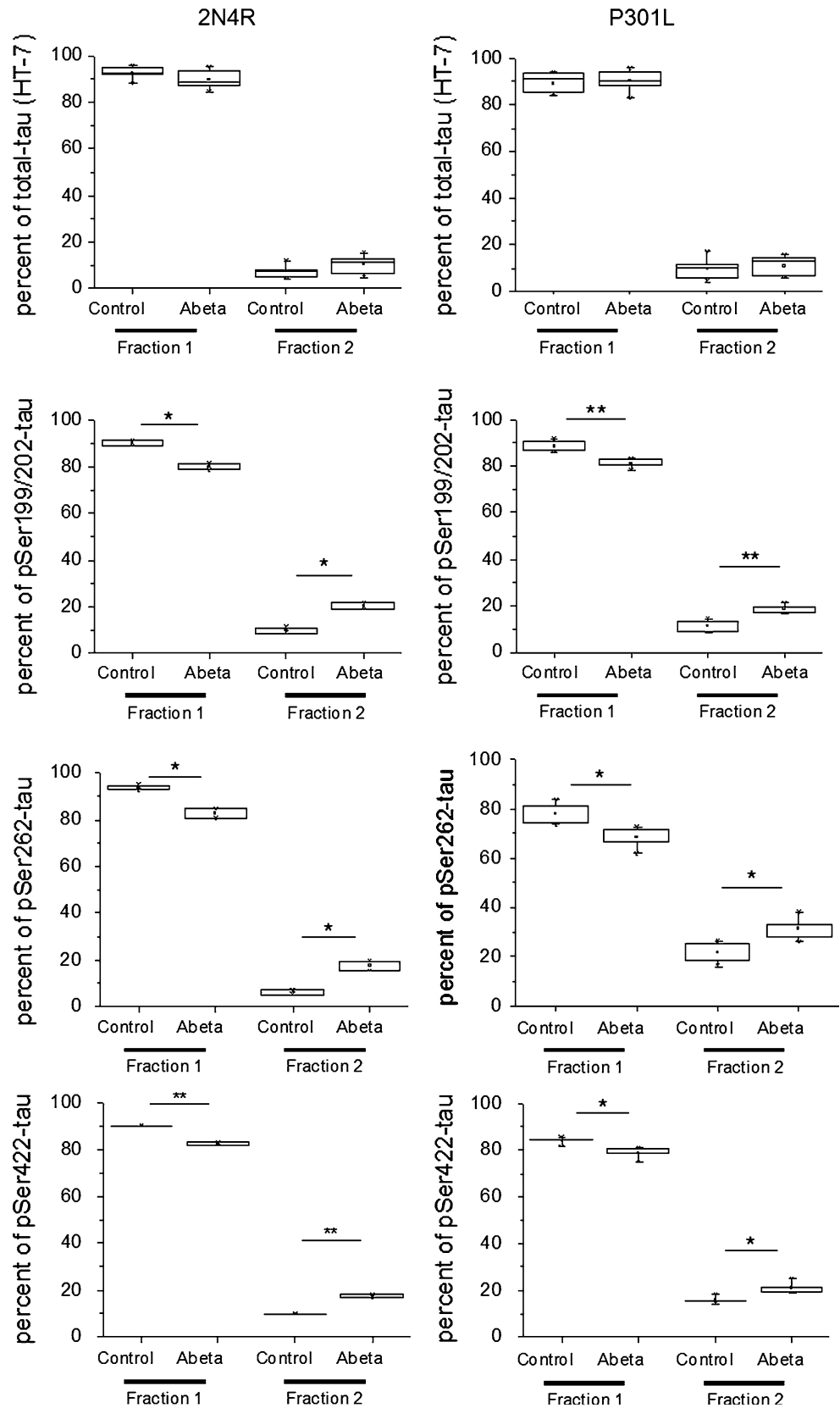
We used a concentration of 10 μM monomeric Aβ1–42, which was subsequently preaggregated and demonstrated that the peptide exerted no toxic effects onto the cells within 90 min. Longer incubations than 24 h showed increasing toxicity at this concentration. However, we chose this relative high concentration to mimic the situation in the brains of Alzheimer patients, where even higher concentrations were found in neuritic plaques and their vicinity [30]. Earlier reports of very fast occurring toxicity after Aβ1–42 application could not be observed in our cells [22].

It has been proposed that neither Aβ1–42 nor phospho-tau alone is the cause of AD, but rather a pathological synergistic combination of both triggers the outbreak of the disease [31]. It has further been shown that Aβ1–42 induces tau aggregation after induction of hyperphosphorylation with monomeric Aβ1–42 which was administered for 5 days [17]. Later studies showed that very short incubation of 30 min with preaggregated Aβ1–42 could induce hyperphosphorylation [22]. We confirm this and add the additional information that the tau solubility decreases upon short-term incubation. We also used additional antibodies and showed the effect by two independent methods (western blot, immunocytochemistry). Furthermore, we investigated, if the short-term hyperphosphorylation, induced by Aβ1–42, is directly responsible for aggregation of tau. Intracellular localization was detected by antibody AT8, because it binds exclusively to tau from Alzheimer patients [9]. AT8 recognized the tau epitope only, when all both two amino acids, Serin202 and Threonin205, were phosphorylated [32], and Serin199 is

optionally phosphorylated. We found large globular aggregates of hyperphosphorylated tau in immunofluorescence images, even after short-term incubation (90 min) of SY5Y cells with preaggregated Aβ1–42. Aβ1–42 had no effect in untransfected cells (Fig. 3a, d). There was only a diffuse staining in the cytosol. This staining was very similar to the untreated 2NR4- and P301L-transfected cells without Aβ1–42 incubation. The major point of this experiment is that the treatment with Aβ1–42 induces the formation of phosphorylated and clustered tau aggregates, visible in Fig. 3e, f only in the transfected mutant cells. This observation supports the hypothesis that Aβ1–42 induced tau aggregation is a fast molecular process, when preaggregated Aβ1–42 is used instead of the monomer as a trigger, and it emphasizes the higher malignancy of the peptide after conformational restructuring. Significant longer incubation (18–96 h) with aggregated Aβ1–40 and artificial Aβ25–35 in concentrations between 0.7 and 60 μM induced also hyperphosphorylation in a cell culture model [33]. Interestingly, embryonic tau did not show the tendency to form aggregates after Aβ1–42 incubation, pointing to a specific vulnerability of adult tau to Aβ-induced tau aggregation and associated neurotoxicity. The potency of preaggregated Aβ1–42 to trigger tau aggregation to form large globular clusters was independently observed in both 2N4R- and P301L-transfected cells. Therefore, these modifications seem not to be crucial for the conformational change of hyperphosphorylated tau. In general, tau phosphorylation in fetal tissue is elevated, because of a higher phosphatase activity; however, in contrast to adult tau, reduced microtubule binding and increased aggregation were not observed [34].

To further quantify tau aggregation, we analyzed the relative proportions of soluble and insoluble tau after Aβ1–42 incubation. The tau distribution in untransfected

Fig. 4 Quantification of aggregated phospho-tau in cytosolic (fraction 1) and particular fraction (fraction 2) after incubation with 10 μM Aβ1–42 for 90 min. Aβ1–42 triggers a shift of phosphorylated tau from the cytosolic fraction 1 to the particular fraction 2. All antibodies were probed $n = 4$ on PVDF membranes, except antibody H7 which was probed $n = 6$. The box plots show the means (*dot*) ± standard deviation (*box*) and 95% range (*whiskers*). Asterisks (*$P < 0.05$, **$P < 0.01$) indicate statistically significant differences in Student's t-test between controls and Aβ1–42 treated cells

cells could not be analyzed due to low endogenous tau concentrations. Most of the anti-phospho tau antibodies did not show sufficient immunoreactivity in either or the both fractions to be analyzed in western blots (for example see Fig. 1). Therefore, it was not possible to quantify the distribution reliably. However, tau overexpressing 2N4R- and P301L-transfected cells were lyzed in a high salt buffer to keep cytosolic and nonintegral membrane-associated proteins in solution. Aggregated, nuclei-, membrane- and organelle-bound proteins were precipitated by high-speed centrifugation. About 90% of total tau was found in the soluble fraction, and incubation with Aβ1–42 did not change this proportion significantly under control conditions. Hyperphosphorylated tau was distributed to a similar proportion between soluble and insoluble conformation structures in both 2N4R- and P301L-transfected cell types, but this ratio was significantly changed after Aβ1–42 incubation. The precipitated proportion doubled to 20–30%, depending on the antibody used, while the soluble fraction was clearly reduced by the respective quota. Obviously both the transfected cell types showed a very similar shift into the insoluble fraction within the same order of magnitude. This is supported by the previous results from immunofluorescence (Fig. 3) that showed the formation of globular phosphor-tau aggregates after Aβ1–42 application in both cell types. The formation of larger insoluble aggregates is, therefore, not limited only to the P301L mutation, but can also occur in the truncated N2R4 tau form. Immunofluorescence data (Fig. 3a, d) support the idea that endogenous wild type tau is not as vulnerable by Aβ1–42 as the truncated form. This observation indicates a universal acting Aβ1–42-triggered mechanism for tau aggregation, which is correlated to hyperphosphorylation of tau on at least three different epitopes. Such a mechanism could be either activated by general activation of kinases or inhibition of phosphatases [35]. Inhibition of phosphatases appear more likely, since there are contradicting data on an activation of kinases by Aβ [36, 37]. Aggregation of tau has also been prevented by specific cleavage by caspase, which is inhibited by tau phosphorylation at position 422 [38]; however, this mechanism appears to be unlikely, since proteases were blocked by an inhibitor cocktail in our experiments.

Taken together, we have demonstrated that the hyperphosphorylation and aggregation of tau can be induced within a short time period of 90 min by preaggregated Aβ1–42 in physiological occurring concentrations. Furthermore, the peptide triggers a general increase of phosphorylation on at least three epitopes and these effects occur in both P301L- and 2N4R-transfected cells. It remains to be determined, whether Aβ1–42 activates a molecular sequence that causes these multiple effects or it just regulates the fine tuning between kinases and phosphatases in the cells.

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