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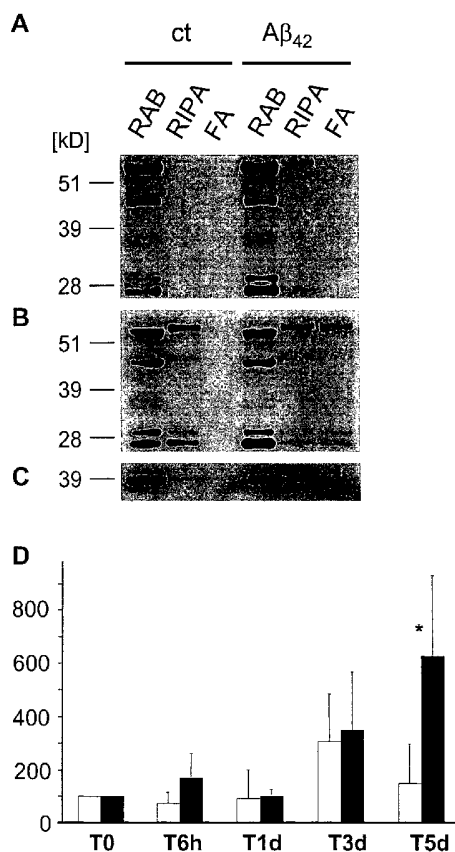
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(54) Title: A CELLULAR ASSAY SYSTEM FOR NEUROFIBRILLARY TANGLE FORMATION



(57) Abstract: The present invention discloses a cell culture assay system useful for the screening and testing of modulating agents of neurodegenerative diseases associated with the formation of neurofibrillary tangles, in particular Alzheimer's disease. Based on the surprising finding that contacting of cells growing in culture and expressing human recombinant tau with b-amyloid causes the formation of Alzheimer-like paired helical filaments in said cells, the present invention further provides methods and applications useful for the identification and testing of compounds and therapeutic agents for the treatment of neurodegenerative diseases.



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A CELLULAR ASSAY SYSTEM FOR NEUROFIBRILLARY TANGLE FORMATION

The present invention relates to a method of inducing the formation of neurofibrillary tangles in cells and said cells as such. An assay is provided whereby agents, modifiers and compounds are tested to determine their potential efficacy as therapeutics for the treatment of disorders associated with neurofibrillary tangles, in particular Alzheimer's disease and tauopathies.

Neurodegenerative diseases, in particular Alzheimer's disease, have a severely debilitating impact on a patient's life. Furthermore, these diseases constitute an enormous health, social, and economic burden. Alzheimer's disease is the most common age-related neurodegenerative condition affecting about 10 % of the population over 65 years of age and up to 45 % over age 85 (for a recent review see Vickers et al., *Progress in Neurobiology* 2000, 60:139-165). Presently, this amounts to an estimated 12 million cases in the US, Europe, and Japan. This situation will inevitably worsen with the demographic increase in the number of old people ("aging of the baby boomers") in developed countries. The neuropathological hallmarks that occur in the brain of individuals suffering from Alzheimer's disease are senile plaques, composed of amyloid- β protein, and profound cytoskeletal changes coinciding with the appearance of abnormal filamentous structures and the formation of neurofibrillary tangles. AD is a progressive disease that is associated with early deficits in memory formation and ultimately leads to the complete erosion of higher cognitive function. Currently, there is no cure for AD, nor is there an effective treatment to halt the progression of AD or even a method to diagnose AD ante-mortem with high probability. The late onset and complex pathogenesis of neurodegenerative disorders pose a formidable challenge to the development of therapeutic and diagnostic agents. Therefore, it is very important to develop suitable cellular models and assay systems of neurodegenerative disease which may be useful in the development of such therapeutic and diagnostic agents.

Paired helical filaments (PHF) in neurofibrillary tangles (NFT) are filamentous aggregates of hyperphosphorylated forms of the microtubule-associated protein tau (Lee et al., 2001, *Annu Rev Neurosci*, 24:1121-1159; Götz, 2001, *Brain Res Brain Res Rev*, 35:266-286). In addition to Alzheimer's disease (AD), NFT are abundant in several other neurodegenerative diseases, including frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17), caused by mutations in the tau gene (Hutton et al., 1998, *Nature*, 393:702-705; Poorkaj et al., 1998, *Ann Neurol*, 43:815-825; Spillantini et al., 1998, *Proc Natl Acad Sci USA*, 95:7737-7741). Moreover, NFT can be generated in transgenic mice by expressing pathogenic tau mutations (Lewis et al., 2000, *Nat Genet*, 25:402-405; Götz et al., 2001, *J Biol Chem*, 276:529-534). Besides NFT, β -amyloid is the principal histopathological hallmark of AD. β -amyloid consists of hydrophobic A β peptides which have a strong tendency to aggregate into various oligomeric and filamentous states, with SDS-stable oligomers and protofibrils being most toxic to neuronal function (Hartley et al., 1999, *J Neurosci*, 19:8876-8884; Walsh et al., 2002, *Nature*, 416:535-539). The presence of tau may be essential to β -amyloid-induced neurotoxicity (Rapoport et al., 2002, *Proc Natl Acad Sci USA*, 99:6364-6369), and injection of β -amyloid into brains of P301L mutant tau transgenic mice, as well as co-expression of APP and tau mutants in mice, established a causal relationship between β -amyloid and NFT *in vivo* (Lewis et al., 2001, *Science*, 293:1487-1491; Götz et al., 2001, *Science*, 293:1491-1495).

In comparison to a recently demonstrated *in-vivo* system of A β_{42} -induced filament formation in transgenic mice (Götz et al., 2001, *Science*, 293:1491-1495), previous attempts in generating a cell culture system for the formation of fibrillar aggregates of tau and neurofibrillary tangles were only partially successful in respect to the formation of aggregates and tangles with characteristic features observed in human disease. Shorter and smaller fibrillar aggregates of tau were previously observed in Chinese hamster ovary cells that had been transfected with triple mutant tau expression constructs (Vogelsberg-Ragaglia et al., 2000, *Mol Biol Cell*, 11:4093-4104). In a related study, combined treatment with okadaic acid and 4-hydroxynonenal of SH-SY5Y cells induced 2 to 3 nm wide fibrillar tau polymers (Perez et al., 2002,

Eur J Biochem, 269:1484-1489). However, a cell culture system for the formation of genuine NFT has not been reported.

It is an object of the present invention to provide cells and a cellular assay system useful for the screening and testing of modulating agents of neurodegenerative diseases associated with the formation of neurofibrillary tangles, in particular Alzheimer's disease. Based on the surprising finding that contacting of cells growing in culture and expressing human recombinant tau with β -amyloid causes the formation of Alzheimer-like paired helical filaments in said cells, the present invention further provides methods and applications useful for the identification and testing of compounds and therapeutic agents for the treatment of neurodegenerative diseases.

The singular forms "a", "an", and "the" as used herein and in the claims include plural reference unless the context dictates otherwise. For example, "a cell" means as well a plurality of cells, and so forth. The term "fragment" as used herein is meant to comprise e.g. an alternatively spliced, or truncated, or otherwise cleaved transcription product or translation product. The term "derivative" as used herein refers to a mutant, or an RNA-edited, or a chemically modified, or otherwise altered transcription product, or to a mutant, or chemically modified, or otherwise altered translation product. For instance, a "derivative" may be generated by processes such as altered phosphorylation, or glycosylation, or, acetylation, or lipidation, or by altered signal peptide cleavage or other types of maturation cleavage. These processes may occur post-translationally. The term "modulator" as used in the present invention and in the claims refers to a molecule capable of changing or altering the level and/or the activity of a gene, or a transcription product of a gene, or a translation product of a gene. Preferably, a "modulator" is capable of changing or altering the biological activity of a transcription product or a translation product of a gene. Said modulation, for instance, may be an increase or a decrease in enzyme activity, a change in binding characteristics, or any other change or alteration in the biological, functional, or immunological properties of said translation product of a gene. The terms "agent", "reagent", or "compound" refer to any substance, chemical,

composition or extract that have a positive or negative biological effect on a cell, tissue, body fluid, or within the context of any biological system, or any assay system examined. They can be agonists, antagonists, partial agonists or inverse agonists of a target. Such agents, reagents, or compounds may be nucleic acids, natural or synthetic peptides or protein complexes, or fusion proteins. They may also be antibodies, organic or inorganic molecules or compositions, small molecules, drugs and any combinations of any of said agents above. They may be used for testing, for diagnostic or for therapeutic purposes. The term "variant" as used herein refers to any polypeptide or protein, in reference to polypeptides and proteins disclosed in the present invention, in which one or more amino acids are added and/or substituted and/or deleted and/or inserted at the N-terminus, and/or the C-terminus, and/or within the native amino acid sequences of the native polypeptides or proteins of the present invention. Furthermore, the term "variant" shall include any shorter or longer version of a polypeptide or protein. Variants comprise proteins and polypeptides which can be isolated from nature or be produced by recombinant and/or synthetic means. Native proteins or polypeptides refer to naturally-occurring truncated or secreted forms, naturally occurring variant forms (e.g. splice-variants) and naturally occurring allelic variants.

Neurodegenerative diseases or disorders according to the present invention comprise Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Pick's disease, fronto-temporal dementia, progressive nuclear palsy, corticobasal degeneration, cerebro-vascular dementia, multiple system atrophy, and mild-cognitive impairment. Further conditions involving neurodegenerative processes are, for instance, ischemic stroke, age-related macular degeneration, narcolepsy, motor neuron diseases, nerve injury and repair, and multiple sclerosis.

In one aspect the present invention features a method of producing neurofibrillary tangles (NFT) in a cell comprising providing a cell, or a plurality of cells in a sample, expressing in said cell, or plurality of cells, a recombinant gene coding for tau protein, contacting said cell, or plurality of cells, with amyloid precursor protein (APP) or a fragment, or derivative, or variant

thereof, in particular β -amyloid, incubating said cell, or plurality of cells, for a period of time, and allowing said cell, or plurality of cells, to produce neurofibrillary tangles. A recombinant gene coding for tau protein can be expressed in said cell, or plurality of cells, in a stable or transient manner. In a preferred embodiment, the transient expression of such a recombinant gene coding for tau protein can be accomplished by transient transfection of an expression vector comprising a gene coding for tau protein. Alternatively, such an expression vector can be stably integrated into a cell, generating a cell line expressing recombinant tau. Methods for transient or stable expression of transgenes are well known to a person skilled in the art. It is preferred that said cell, or plurality of cells in a sample, are growing in cell culture.

In another aspect, the present invention features a method of generating a cell with neurofibrillary tangles comprising providing a cell, or a plurality of cells in a sample, expressing in said cell, or plurality of cells, a recombinant gene coding for tau protein, contacting said cell, or plurality of cells, with APP or a fragment, or derivative, or variant thereof, in particular β -amyloid, incubating said cell, or plurality of cells, for a period of time, and allowing said cell, or plurality of cells, to form neurofibrillary tangles.

In a preferred embodiment, said β -amyloid is $A\beta_{42}$. It is further preferred that said cell, or plurality of cells, are contacted with a pre-aggregated or aggregated form of $A\beta_{42}$.

In one further embodiment of the present invention, said cell, or plurality of cells, express a recombinant human tau gene, in particular the human 4-repeat isoform of tau. Preferably, said human 4-repeat isoform of tau is bearing the P301L mutation.

In a preferred embodiment of the invention, said contacting of said cell, or plurality of cells in a sample, is accomplished by addition of amyloid precursor protein, or a fragment, or derivative, or variant thereof, in particular β -amyloid, to the cell culture medium. In one example, said cell, or plurality of cells, are

incubated for a period of at least 2 days after contacting with APP, or a fragment, or derivative, or variant thereof, in particular β -amyloid. In a preferred example, said cell, or plurality of cells, are incubated for a period of 3 to 7 days after said contacting. Most preferably, said cell, or plurality of cells, are incubated for a period of five days after said contacting.

In one embodiment of the invention, the cell used in the claimed method is a neural cell, in particular of human origin. Preferably, said neural cell is a neuroblastoma cell. In a most preferred embodiment, said neuroblastoma cell is an SH-SY5Y cell.

It is one feature of the instant inventive method that the generated neurofibrillary tangles comprise fibrillar polymers of 10 to 30 nm in width, preferably 20 nm in width.

In a preferred embodiment of the present invention, the formation of NFT in a cell which expresses a recombinant gene coding for tau protein and which has been contacted with APP, or a fragment, or derivative, or variant thereof, in particular β -amyloid, is determined by using electron microscopy or other appropriate methods. One such other appropriate method comprises the utilization of conformation-dependent antibodies that are capable of recognizing and discriminating the tau molecule in the context of neurofibrillary tangles from tau molecules existing in other states of aggregation and that can be detected, for instance, by fluorescence resonance energy transfer (FRET) technology. The formation of neurofibrillary tangles could also be detected by the use of other optical methodologies such as fluorescence polarisation spectroscopy, fluorescence correlation spectroscopy, fluorescence cross-correlation spectroscopy, fluorescence intensity distribution analysis, fluorescence lifetime measurements, fluorescence anisotropy measurements, or combinations thereof. For instance, an assay to monitor and quantify the formation and aggregation of paired helical filaments in solution has been described by Friedhoff et al. (1998, *Biochemistry*, 37:10223-30). In a preferred embodiment, said conformation-dependent antibodies are optically labeled, preferably

fluorescently labeled. Alternatively, in another embodiment, antibodies that are capable of recognizing phosphorylated epitopes of tau, in particular epitopes whose degree of phosphorylation correlates with the state of tau aggregation and neurofibrillary tangle formation as observed in Alzheimer's disease, in particular the tau epitope S422, can be used.

The present invention features a cell, preferably a neural cell, preferably a neuroblastoma cell, in particular an SH-SY5Y cell, which expresses a recombinant gene coding for tau, wherein said cell is capable of producing NFT after contacting with APP or a fragment thereof, in particular β -amyloid. In a preferred embodiment, said cell is of human origin.

In one further aspect, the present invention features a method of screening for an agent or agents capable of modulating NFT formation in a cell, comprising i) providing a cell, or a plurality of cells in a sample, expressing in said cell, or plurality of cells, a recombinant gene coding for tau protein, contacting said cell, or plurality of cells, with amyloid precursor protein (APP) or a fragment, or derivative, or variant thereof, in particular β -amyloid, incubating said cell, or plurality of cells, for a period of time, and allowing said cell, or plurality of cells, to produce neurofibrillary tangles, ii) contacting said cell, or plurality of cells, with a putative modulatory agent, or multitude of putative modulatory agents, iii) incubating said cell, or plurality of cells, with said agent or agents for a period of time, and iv) assessing the ability of said agent, or multitude of agents to modulate a biological function selected from the group consisting of NFT formation or phosphorylation of the tau epitopes, particularly tau epitope S422. This type of screening method preferentially allows for the identification of an agent that possesses a modulatory effect on already formed or pre-existing neurofibrillary tangles. In an alternative screening method, a cell, or a plurality of cells in a sample, subject to screening are incubated with a putative modulatory agent, or multitude of putative modulatory agents, prior to the contacting of said cell, or plurality of cells, with APP or a fragment, or derivative, or variant thereof, in particular β -amyloid. This type of screening method preferentially allows for the identification of an agent that is capable of modulating the *de novo* production

of neurofibrillary tangles right from the outset of the formation process. In a preferred instance, this type of screening assay allows for the identification of an agent that prevents or slows down the process of neurofibrillary tangle formation.

Other features and advantages of the invention will be apparent from the following description of figures and examples which are illustrative only and not intended to limit the remainder of the disclosure in any way. In this respect, it is conceivable that the underlying idea of the instant invention, namely the development of a cell culture assay system characterized by the formation of intracellular aggregates of molecules that are implicated in neurodegenerative diseases through contacting of cells, expressing a gene encoding such molecules, with APP or a fragment, or derivative, or variant thereof, in particular β -amyloid, can be extended to other prominent neurodegenerative diseases. An example for such a cell culture assay system, particularly relevant to Parkinson's disease, could comprise a cell expressing α -synuclein, wherein the formation of α -synuclein aggregates, for instance in the form of Lewy bodies, is effected through contacting said cell with APP or a fragment, or derivative, or variant thereof, in particular β -amyloid. Likewise, a cell culture assay system, particularly relevant to polyglutamine diseases such as Huntington's disease, could comprise a cell expressing a polyglutamine-repeat containing protein such as huntingtin, wherein the formation of polyglutamine protein aggregates is effected through contacting said cell with APP or a fragment, or derivative, or variant thereof, in particular β -amyloid.

Figure Legends

Figure 1: $A\beta_{42}$ aggregates are deposited on tau-expressing cells. A, Western blot analysis of neuronally differentiated cells using a human tau-specific antibody reveals comparable levels of transfected 4-repeat tau in wild-type, P301L, P301L/S422E, and P301L/S422A cells (arrow). Undifferentiated cells express comparable levels of 4-repeat tau (data not shown). Levels of 4-repeat tau are more than tenfold increased when compared to endogenous 3-

repeat tau in the mock-transfected control cells (arrows). β -actin staining is included to confirm equal loading. *B*, Confocal analysis of cells shows that $A\beta_{42}$ (4G8, in green) forms large clumps on the cell bodies and processes of neuronally differentiated tau-expressing cells (HT7, in red). Intracellular accumulation of $A\beta_{42}$ is not detectable. *C*, Electron-microscopy of an $A\beta_{42}$ deposit in contact with a cell (low magnification) illustrates the filamentous structure of $A\beta_{42}$ in the two boxed areas shown at higher magnification in (*D*) and (*E*). The inset in *E* shows sarkosyl-extracted $A\beta_{42}$ fibrils that are 7 to 10 nm wide and lack regular periodicities. Scale bar: 20 μ m (*B*), 3 μ m (*C*), and 40 nm (inset in *E*).

Figure 2: Solubility of tau in transfected SH-SY5Y cells. *A*, Sequential extraction of both untreated and $A\beta_{42}$ -treated undifferentiated wild-type tau expressing cells in high-salt RAB buffer, RIPA buffer and FA (formic acid) reveals, already after a one day incubation with $A\beta_{42}$, a small fraction of tau in the RIPA fraction, in contrast to untreated cells. *B*, After 5 days of incubation with $A\beta_{42}$, the solubility of tau is significantly decreased as a substantial fraction of tau is present both in the RIPA and FA fraction, whereas in untreated cells, after a five day culture period, tau is present in the RIPA fraction, but absent in the FA fraction. *C*, As loading control, GAPDH (39 kDa) has been included. *D*, To monitor the kinetics of tau insolubility, tau in the FA fraction is shown after incubation with PBS (0) or $A\beta_{42}$ (+) for 0 hr, 6 hr, 1 d, 3 d and 5 d. For each experiment, the ratio of the intensities for each time point was determined relative to the value at time-point T0. Intensity values are given in percentages. The data represent the mean of three independent experiments for time points T6h, T1d, and T5d, and two for T3d. A two-tailed students t-test reveals statistically significant differences (* $p=0.03$) after 5 days of incubation with $A\beta_{42}$.

Figure 3: $A\beta_{42}$ does not cause increased transcription of tau mRNA. After incubation with PBS for 5 days (shown in grey), slight increases of tau mRNA levels (as shown by Δ Ct values for tau compared with the reference gene GAPDH) are found for both mock- and P301L-transfected cells, compared with time-point T0 (shown in white). These are statistically not significant

(two-tailed students t-test; $p > 0.38$). After incubation with $A\beta_{42}$ for 5 days (shown in black), levels are slightly decreased in both cell-types. These differences are again not statistically significant (two-tailed students t-test; $p > 0.14$) demonstrating that $A\beta_{42}$ does not cause increased tau transcription. In addition, this analysis reveals approximately 60-fold higher tau mRNA levels in P301L- compared with mock-transfected cells (as shown by the lower ΔC_t values).

Figure 4: Generation of PHF-like tau filaments in cultured human neuroblastoma cells. Tau-expressing SH-SY5Y cells were incubated for 5 days with aggregated $A\beta_{42}$ preparations, extracted with sarkosyl and protein extracts analyzed by immuno-electron microscopy. *A*, A typical PHF extracted from an AD brain has a width of 20 nm and a periodicity of 80 nm. *B-C*, Tau filaments extracted from wild-type tau-expressing cells have a width of 20 nm and a periodicity of 150 to 160 nm. To reveal the periodicity, part of the filament in *C* is shown at a higher magnification in (*C'*). *D-E*, Filaments extracted from undifferentiated P301L tau-expressing cells have a width of 20 nm and a periodicity of 130 to 140 nm, resembling the narrow twisted ribbons identified in human P301L carriers. Both types of filaments are labeled with antibody Tau-5A6. Scale bar: 40 nm (*A-C, D-E*), 20 nm (*C'*).

Figure 5: Cryo-electron microscopy of ultrathin sections. *A-B*, Undifferentiated wild-type tau expressing cells were treated with $A\beta_{42}$ for five days, and cryosections were obtained and incubated with the human tau-specific antibody, Tau-5A6, followed by a gold-labeled secondary antibody. Two sections obtained from $A\beta_{42}$ -treated cells are shown. As controls, untreated cells were included and the primary antibody was omitted. Immuno-electron microscopy reveals many gold-decorated aggregates in the cytoplasm of neurons that were treated with $A\beta_{42}$. *C*, For a semiquantitative analysis, a cut-off of 15 gold particles was defined and groups of gold particles were counted per cell in $A\beta_{42}$ -treated (+) compared with untreated cells (0) revealing an increase in the number of filamentous tau aggregates in $A\beta_{42}$ -treated cells. Scale bar: 300 nm (*A*), 200 nm (*B*).

Figure 6: Role of S422 epitope of tau. A-C, Immunocytochemistry of differentiated wild-type tau expressing cells incubated for five days with A β ₄₂ using the human tau-specific antibody HT7 (A, in red) and the pS422 antiserum specific for tau phosphorylated at S422 (B, in green) shows the distribution of phosphorylated tau relative to total tau (C, merge). D, Wild-type tau expressing cells require a treatment for 5 days with A β ₄₂ (+) to increase the ratio of pS422-positive cells relative to total tau expressing, HT7-positive cells, compared with vehicle-treated cells (0). In contrast, incubation of P301L expressing cells with A β ₄₂ already for 1 day causes a more than twofold increase in the number of pS422-positive cells. 200 cells were counted for each experimental condition. Mann-Whitney U-test: (*) $P < 0.05$ comparing A β ₄₂-treated wild-type cells with vehicle-treated wild-type cells, and (**) $P < 0.002$ comparing A β ₄₂-treated P301L cells with vehicle-treated P301L cells. The AT8 antibody directed against phosphorylated S202/T205 stains all HT7-positive cells, irrespective of A β ₄₂-treatments (data not shown). E, In contrast to P301L mutant tau expressing cells, tau is not present in the FA fraction of extracts obtained from A β ₄₂-treated P301L/S422A and P301L/S422E cells. Scale bar: 20 μ m (A-C).

Example

A β ₄₂ decreases the solubility of tau: To assess the role of A β ₄₂ in tau aggregation and filament formation in the tissue culture system of the instant invention, we added preparations of aggregated synthetic A β ₄₂ to the media of SH-SY5Y cells that had been stably transfected with expression constructs encoding the longest 4-repeat isoform of human tau, together with or without the pathogenic FTDP-17 mutation P301L (Fig. 1A). Confocal analysis confirmed the presence of A β ₄₂ aggregates deposited on both cell bodies and neurites (Fig. 1B). Neither the size nor the number of these aggregates varied within 1 and 5 days of incubation. Electron-microscopic analyses demonstrated that A β ₄₂ aggregates contained the expected amorphous fibrous material that was similar to that extracted from brain β -amyloid

plaques (Fig. 1C-E). In this assay system the initial 5 days of treatment with A β ₄₂ were not accompanied by any obvious increases in cell death, and lactate dehydrogenase (LDH) assays did not reveal increased LDH release, as compared to vehicle controls.

Western blots confirmed that cellular levels of human tau were similar in all transfected conditions, both in undifferentiated cells and cells that were sequentially differentiated with retinoic acid and BDNF (Fig. 1A).

To determine the role of A β ₄₂ in the aggregation of tau, we used undifferentiated cells that expressed wild-type human tau. We extracted cellular proteins in high-salt RAB buffer to generate the RAB fraction. We then homogenized the RAB-insoluble pellet in RIPA buffer to obtain RIPA-soluble proteins, and extracted the RIPA-insoluble pellet with formic acid (FA) to obtain the FA-soluble protein fraction (Fig. 2). Equal amounts of total protein of the treated and untreated fractions were loaded, but the FA fractions contained less protein than the RAB and RIPA fractions. The signals in the three fractions can not be added up to determine the total amount of tau, as tau has been sequentially extracted. After one day of A β ₄₂ treatment, most tau was present in the RAB fraction, only a small fraction was RAB-insoluble and found in the RIPA fraction, as compared to none in the RIPA fraction of the vehicle-treated cells (Fig. 2A). During 5 days of incubation with A β ₄₂, the solubility of tau decreased significantly because a substantial amount of it appeared in the RIPA and FA fractions. In contrast, in untreated control cells, tau was absent from the FA fraction after 5 days of incubation with the vehicle (Fig. 2B). In addition to full-length tau, we observed, at variable degrees, truncated tau with a molecular weight of 28 kDa. Probing of the blots (Fig. 2B) with a GAPDH-specific monoclonal antibody confirmed equal amounts of protein in the RAB fraction. Moreover, we could exclude a carryover of soluble proteins into the FA-fraction (Fig. 2C). An A β ₄₂-specific ELISA (Innogenetics, Gent, Belgium) was included for a subset of the samples. This assay showed that substantial amounts of A β ₄₂ were in the RIPA and FA fraction. Therefore, by loading equal amounts of protein of the A β ₄₂- and PBS-treated FA fractions, the amount of FA-soluble tau is likely to be underestimated in the A β ₄₂-treated compared with the PBS-treated FA fractions. Next, to monitor the kinetics of tau assembly, combined levels of FA-soluble full-length and

truncated tau were quantified after incubation with A β ₄₂ for 0 h, 6 h, 1 d, 3 d and 5 d, and compared with PBS-treated controls. Three independent experiments revealed an increase of tau in the FA fraction with longer exposure to A β ₄₂ that was statistically significant at 5d (* two-tailed students t-test; P=0.03) (Fig. 2D).

To determine, whether A β ₄₂ affects tau transcription, we performed a quantitative RT-PCR analysis. When undifferentiated P301L cells were compared with mock-transfected cells at time-point 0, we found that incubation with A β ₄₂ for 5 days did not alter tau mRNA levels, neither in mock- nor P301L-transfected cells (Fig. 3).

A β ₄₂ induces PHF-like tau filaments: To determine whether the decreased solubility of tau in response to exposure to A β ₄₂ was associated with the formation of PHF, we analyzed sarkosyl protein extracts by negative contrast electron microscopy (Fig. 4). As a control, we included sarkosyl extracts from a human brain with a confirmed NFT pathology. It contained many PHF with expected widths of 20 nm and periodicities of 75 to 80 nm (Fig. 4A) (Crowther, 1991, *Proc Natl Acad Sci USA*, 88:2288-92).

Immuno-electron microscopy of sarkosyl protein extracts of undifferentiated SH-SY5Y cells expressing wild-type human tau with the monoclonal antibody Tau-5A6, identified many twisted filaments with widths of up to 20 nm, periodicities of 150 to 160 nm, and lengths of up to 1200 nm (Fig. 4B and C, Table 1). They resembled the PHF extracted from NFT in human neurodegenerative diseases and are best described as narrow twisted ribbons (Spillantini et al., 1998, *Am J Pathol*, 153:1359-63).

As the ultrastructural characteristics of abnormal filaments in human neurodegenerative diseases vary with pathogenic mutations, we analyzed the abnormal filaments generated by P301L mutant tau. Again, A β ₄₂ caused the generation of PHF-like filaments both in differentiated and undifferentiated cells stably transfected with P301L mutant tau (Fig. 4D and E, Table 1). These PHF had shorter periodicities of 130 to 140 nm with similar widths, as compared to the wild-type tau filaments, consistent with the fact that mutations can affect the phenotype of the tau filaments.

To exclude that the PHF-like filaments contained β -amyloid fibrils derived from our $A\beta_{42}$ preparation, we showed that the anti- $A\beta_{42}$ antibody 4G8 failed to decorate the PHF, whereas it clearly decorated β -amyloid fibrils extracted with the identical sarkosyl protocol. Moreover, we found with electron microscopy that our β -amyloid fibrils were 7 to 10 nm wide and lacked regular periodicities (Fig. 1E, inset) (Hartley et al., 1999, *J Neurosci*, 19:8876-84). Control experiments showed that neither vehicle controls, nor identical preparations of the reverse peptide $A\beta_{42-1}$, caused the formation of PHF.

Neuronally differentiated SH-SY5Y cells transfected with either wild-type or P301L tau also formed many PHF-like filaments after five days exposure to the $A\beta_{42}$ preparation (Table 1). This may be due to similar cellular levels of human tau in both differentiated and undifferentiated cells, in that cellular levels of tau in the transfected cells exceeded those of endogenous 3-repeat tau (Uberty et al., 1997, *Neurosci Lett*, 235:149-53) by more than ten times (Fig. 2A).

A quantitative analysis of filament formation in sarkosyl extracts by negative staining electron microscopy is impossible, as the adherence of the sample to the grid surface is irregular, due to the irreproducibility of the surface charge of the carbon-coated grids. Thus, filaments may cluster in one field of a grid, whereas another field may be completely empty.

A semi-quantitative analysis can be achieved by immuno-electron microscopy on ultrathin cryosections of cells. We treated undifferentiated wild-type tau expressing cells either with $A\beta_{42}$ or PBS, and obtained cryosections that were incubated with the human tau specific antibody Tau-5A6 followed by a gold-labeled secondary antibody. As a control, the primary antibody was omitted. Cryo-electron microscopy has the disadvantage that cellular structures are not resolved, but antibodies can penetrate the sections more easily. Many gold-decorated aggregates were revealed in the cytoplasm of neurons that were treated with $A\beta_{42}$ (Fig. 5A and B). For a quantification, an arbitrary cut-off of 15 gold particles was defined, to increase the stringency and to reduce the background. Groups of gold particles were counted per cell in $A\beta_{42}$ -treated compared with untreated cells. This revealed a strong increase in the number of tau aggregates in $A\beta_{42}$ -treated cells (Fig. 5C).

The data disclosed in the instant invention are in line with previous results of A β ₄₂-induced filament formation in transgenic mice (Götz et al., 2001, *Science*, 293:1491-1495), but in addition to the *in vivo* model, A β ₄₂-induced PHF formation also occurred with wild-type human tau in tissue culture. This may be related to the species difference and points to the possibility that human cells in culture may be more susceptible to the formation of abnormal tau filaments as compared to murine cells *in vivo*. Fibrillar aggregates of tau were previously observed in Chinese hamster ovary cells that had been transfected with triple mutant tau expression constructs (Vogelsberg-Ragaglia et al., 2000, *Mol Biol Cell*, 11:4093-4104). In a related study, combined treatment with okadaic acid and 4-hydroxynonenal of SH-SY5Y cells induced 2 to 3 nm wide fibrillar tau polymers (Perez et al., 2002, *Eur J Biochem*, 269:1484-1489). The PHF in the tissue culture system of the instant invention clearly differ from both of the above in that they are much longer, about 10 times wider, and had readily identifiable twisted structures. This difference could well be related to the prolonged exposure to A β ₄₂ in our experiments, for we could not observe any filamentous structures for up to two days of exposure to A β ₄₂ (Table 1).

Role of epitope S422 of tau in aggregation and filament formation: In transgenic mice expressing P301L tau, S422 was among the epitopes that were selectively phosphorylated in response to A β ₄₂ injections. Therefore, we stained both neuronally differentiated P301L and wild-type tau expressing cells with the phospho-S422 specific antiserum pS422 and included the phospho-S202/T205 specific antibody AT8 as a control. 200 cells were counted for each experimental condition. A β ₄₂-treatment did not cause increases in the numbers of AT8-positive cells, as all HT7-positive tau-expressing cells were also AT8-positive, even in the absence of A β ₄₂. In contrast, incubation of P301L expressing cells with A β ₄₂ for 1 day, caused a more than twofold increase in the number of pS422-positive cells compared to HT7-positive cells, whereas wild-type tau expressing cells required a treatment for 5 days with A β ₄₂ to increase the ratio of pS422-positive cells (19) (Fig.6A-D). This difference was statistically significant (* wild-type cells treated with A β ₄₂ for 5 days: 29.81% $\pm$ 7.04% pS422 positive cells/HT7

positive cells; wild-type cells treated with vehicle for 5 days: $18.75\% \pm 9.49\%$; $P < 0.05$; ** P301L cells treated with $A\beta_{42}$ for 1 day: $64.66\% \pm 12.93\%$ pS422/HT7 positive cells; P301L cells treated with vehicle for 1 day: $27.93\% \pm 5.60\%$; $P < 0.002$; Mann-Whitney U-test).

To better characterize the role of S422 in tau filament formation in the tissue culture system of the instant invention, we mutated S422 to alanine or glutamic acid and expressed either P301L/S422A or P301L/S422E double mutants both, in undifferentiated and in neuronally differentiated cells at similar levels as compared to the wild-type and P301L single mutant cells (Fig. 1A). The S422 mutations were introduced into the P301L mutant and not into the wild-type tau expression constructs as the P301L cells showed the strongest phospho-S422 staining. Next, we incubated the undifferentiated cells for five days with $A\beta_{42}$ and fractionated tau into the RAB fraction, the RIPA-soluble fraction, and the FA-soluble fraction. In contrast to $A\beta_{42}$ -treated P301L tau expressing cells, tau was not present in the FA fraction of P301L/S422A and P301L/S422E extracts indicating that mutagenesis of S422 blocked the $A\beta_{42}$ -mediated decrease in the solubility of tau (Fig. 6E).

Finally, we determined whether treatments with $A\beta_{42}$ would induce PHF-like tau filaments in either double mutant cell line. We did not find any evidence for PHF-like tau filament formation both in the presence of the S422A, as well as the S422E mutation (Table 1). Together, these data suggest an important role of S422 in $A\beta_{42}$ -induced tau filament formation.

In summary, the present invention discloses a tissue culture system for the generation of *bona fide* PHF which closely resembled those extracted from brains of AD patients. The results provided by this system are compatible with an important role of $A\beta_{42}$ in the generation of NFT in AD, and with a pivotal role of phosphorylation of tau at S422. PHF formation was achieved within five days, much faster than in current transgenic mouse models (Lewis et al., 2001, *Science*, 293:1487-1491; Götz et al., 2001, *Science*, 293:1491-1495). Therefore, this cell culture system will be useful to screen for, and validate, compounds designed to treat AD and related disorders, to map phospho-epitopes of tau involved in tau filament formation, and to characterize modifiers of tau-related pathology.

METHODS:

Site-directed mutagenesis: The longest human 4-repeat tau isoform, htau40, was cloned into the pRc/RSV expression vector (Invitrogen, Switzerland), and site-directed mutagenesis (Quick Change Kit, Stratagene, Switzerland) was performed to generate the tau mutant P301L, and the double mutants P301L/S422E, and P301L/S422A. The sequences of the mutagenized oligonucleotides were as follows:

P301L:

5'-GGTTTGTAGACTATTTGCACACTGCCGCCTCCC7GGACGTGTTTG-3';

S422E: 5'-GCATCGACATGGTAGACGAGCCCCAGCTCGCCACGC-3';

S422A: 5'-GCATCGACATGGTAGACGCGCCCCAGCTCGCCACGC-3'.

All constructs were sequenced to confirm the absence of randomly introduced mutations.

Cell culture: Human SH-SY5Y neuroblastoma cells were transfected with either wild-type or mutant htau40 cDNA constructs using LipofectAmine 2000 (Invitrogen, Switzerland) and selected with 125 µg/ml G418 for 30 days. Pools of selected clones were seeded on collagen type I coated dishes at a density of $0.3\text{--}2.0 \times 10^4$ cells/cm², depending on the type of experiment (low density culture for immunocytochemistry, and high density culture for EM and Western blot analysis). To normalize for cell numbers, undifferentiated cells were seeded at a density which, after Aβ₄₂ incubation for five days, was below 50% and thus comparable to numbers of differentiated cells at the time of Aβ₄₂ treatment. To induce neuronal differentiation, cells were treated with 20 µM all-trans retinoic acid (Sigma, Buchs, Switzerland) for five days in standard culture medium, and cultivated for additional five days in serum-free medium complemented with 50 ng/ml brain-derived neurotrophic factor (Peprotech, Lucerne, Switzerland) according to Encinas et al. (2000, *J Neurochem*, 75:991-1003).

Neuronal properties were confirmed by the presence of neuronal markers and the absence of glial markers. Moreover, the cells expressed synaptophysin

when cultured on murine organotypic hippocampal slices. Two independent lots of A β ₄₂ (Nos. 524548 and 535120) and A β ₄₂₋₁ (Lot No. 536763) were synthesized by Bachem (Switzerland), reconstituted in PBS at calculated concentrations of 220 μ M, shaken at 1000 rpm for 24 hours at 37°C and analyzed by EM. This preparation contained β -amyloid fibrils along with protofibrils and SDS-stable oligomers (Hartley et al., 1999, *J Neurosci*, 19:8876-8884). Suspensions of A β ₄₂ preparations corresponding to 10 μ M soluble peptide were added to each culture for one to two days for immunohistochemistry, or for two to five days for sarkosyl extractions and EM. Confocal analysis revealed that after adding A β ₄₂ to the cells, it rapidly formed clumps of aggregated material that was deposited on the surface of tau-expressing cells (Fig. 2b, c). A β ₄₂ treatment was accompanied by the phosphorylation of S422 of tau (Fig. 2d, e) (Haque et al., 1999, *Brain Res*, 838:69-77). A β ₄₂ treatment was not accompanied by obvious increases in cell death, and lactate dehydrogenase (LDH) assays (Sigma) did not reveal increased LDH release as compared to vehicle control.

Immunofluorescence analysis and antibodies: For immunocytochemistry, cells were grown on collagen-coated coverslips and fixed with 4% PFA in microtubule-stabilizing buffer at RT for 30 min, followed by permeabilization with 0.3% Triton-X-100. The following antibodies were used for immunocytochemistry (IC), western blotting (WB), and electron microscopy (EM): HT7 (Innogenetics Inc, Belgium, amino acids 159-163, IC: 1:200, WB: 1:50) and Tau-5A6 (Developmental Studies Hybridoma Bank, EM: 1:500) were used to specifically detect human tau (Johnson et al., 1997, *J Neurochem*, 68:430-433). Ser422P #988 (Dr. Andre Delacourte, IC: 1:1000) and pS⁴²² (Biosource Inc., IC: 1:1000) were used to detect tau phosphorylated at S422 (Bussiere et al., 1999, *Acta Neuropathol (Berlin)*, 97:221-230). Antibodies for neuronal markers included NF200 (Sigma, IH: 1:400), NSE (Dako, IC: 1:200), and MAP2 (Chemicon, IC: 1:400), and as a glial marker GFAP (Sigma, IC: 1:100) was used. Monoclonal antibody 4G8 (Signet Laboratories, MA, IC: 1:500, EM: 1:50) was used to detect A β amyloid peptide. Secondary antibodies for immunofluorescence were obtained from Jackson Laboratories

(Westgrove, PA, USA). For a quantification, two independent experiments were performed and 200 cells were counted for each experimental condition.

Western blot analysis, A β -ELISA and quantitative RT-PCR: Tau expressing cells were grown on collagen coated 10 cm dishes, and extracted in RIPA buffer as previously described (Probst et al., 2000, *Acta Neuropathol (Berlin)*, 99:469-481). Proteins were separated on a 10% NuPAGE gel (Invitrogen, Switzerland), blotted onto a nitrocellulose membrane and probed with HT7 and a β -actin-specific antibody (Abcam, 1:5000). The levels and solubility of tau protein were determined by extracting A β -treated and control cells using buffers with increasing ionic strengths (Probst et al., 2000, *ibid*). The cells were homogenized in RAB high-salt buffer to generate the RAB-insoluble fractions. Following centrifugation, the pellets were homogenized in RIPA buffer and centrifuged. The RIPA-insoluble pellets were extracted with 70% formic acid (FA) to give the FA fractions. Centrifugation was done at 50,000 g for 20 min. Protein concentrations of the samples from each fraction were adjusted to equal levels and the same amounts of protein run on a 10% NUPAGE gel and blotted as described above. As loading control and to exclude contamination of the FA fraction with soluble proteins, the blots were probed with a GAPDH-specific monoclonal antibody (BioDesign, Saco, Maine, 1:500). Furthermore, an A β_{42} -specific ELISA (Innogenetics, Gent, Belgium) was included for a subset of the samples.

For quantification, the western blots were probed with the tau-specific antibody HT7 and analyzed by densitometry. The same exposure time was chosen for all blots. The X-ray films were scanned at 400 ppi and the images analyzed with the AlphaEase software (Alpha Innotech Corp., San Leandro, USA). For the purpose of graphic representation, for each blot, the intensity values of all time points were normalized using the value at time-point T0. Statistical analysis of the intensity differences between PBS- and A β_{42} -treated samples was done with the original intensity values, as only the relative and not the absolute differences are relevant for the two-tailed students t-test. The real-time quantitative PCR for tau was performed with HPLC-purified primers and an HPLC-purified probe:

[forward primer: 5'-CAAGACCAAGAGGGTGACACG-3', reverse primer: 5'-TCAGAGCCCGGTTCCTCAG-3' (Metabion GmbH, Martinsried, Germany), TaqMan probe: 5'-6-FAM-CTGGCCTGAAAGAATCTCCCCTGCA-BHQ-1-3' (Biosearch Technologies Inc., Novato, USA)]. As reference gene, the TaqMan rodent GAPDH control n°4308313 (Applied Biosystems, Perkin Elmer, Rotkreuz, Switzerland) was used and ΔC_t values (cycle threshold values) were determined.

Sarkosyl extraction and immuno-electron microscopy: Sarkosyl extractions of tau were done as previously described (Goedert et al., 1992, *Neuron*, 8:159-168). The extracts were placed on formvar/carbon-coated 300-mesh grids and incubated with the primary antibody in PBS/0.1% gelatin for 90 min at room temperature. Antibody 5A6 was used at a 1:500 dilution. As a positive control, AD brain extracts were included; the secondary antibody used for AD extracts was either conjugated to gold or not. In addition, extracts were incubated with the A β 42-specific antibody 4G8 at 1:50 dilutions. Again, controls were included, in which the primary antibody had been omitted. Grids were incubated with 6 nm Au-conjugated or unconjugated secondary antibodies (Sigma) and stained with 2% phosphotungstic acid. Micrographs were recorded on a Philips model CM12 electron microscope.

Thin section immuno-electron microscopy: To visualize β -amyloid on the cells, samples were fixed in 4% paraformaldehyde and 0.1 % glutaraldehyde. Following fixation, tissue blocks were epoxy-embedded, sectioned with a Reichert Ultracut E microtome (Leica, Germany), placed on 200-mesh copper grids, and stained with uranyl acetate and lead citrate. To visualize tau filaments, cells were fixed in 3% PFA and 0.1% glutaraldehyde for 30 min at RT, washed with NH₄Cl 50 mM in PBS, embedded in 3% agar (Noble, Difco, Michigan, USA), and submerged in sucrose-PVP 2M (Sigma) for 3 days. Samples were mounted and cut on a Reichert Ultracut S cryostat (Leica, Germany) to obtain ultra-thin sections. Grids were processed for immuno-labelling as follows: After a blocking step in 5% goat serum in PBS for 20 min, the primary anti-tau antibody Tau-5A6 was added in 1% goat serum in PBS at a 1:50 dilution for 2 h, followed by a 6 nm gold-coupled goat anti-mouse

antiserum (Aurion) in 1% BSA in PBS at a 1:5 dilution for 1 h. Controls were included, in which the primary antibody had been omitted. Washes were done with 1% BSA in PBS for 10 min. Finally, the grids were stained with 3% uranyl acetate and 2% methylcellulose. Micrographs were recorded on a Philips model CM12 electron microscope. To determine the number of gold-decorated tau filamentous aggregates per cell, a cut-off of 15 gold particles per aggregate was defined and groups of gold particles were counted per cell in 30 A β ₄₂-treated compared with 30 untreated cells.

Table 1: Tau filament formation after 5 days of treatment with A β ₄₂ preparations.

	undifferentiated		differentiated	
	PBS	A β ₄₂	PBS	A β ₄₂
mock	ND	ND	no	no
wild-type tau	no	yes	no	yes
P301L tau	no	yes	no	yes
P301L/S422E tau	no	no	no	no
P301L/S422A tau	no	no	no	no

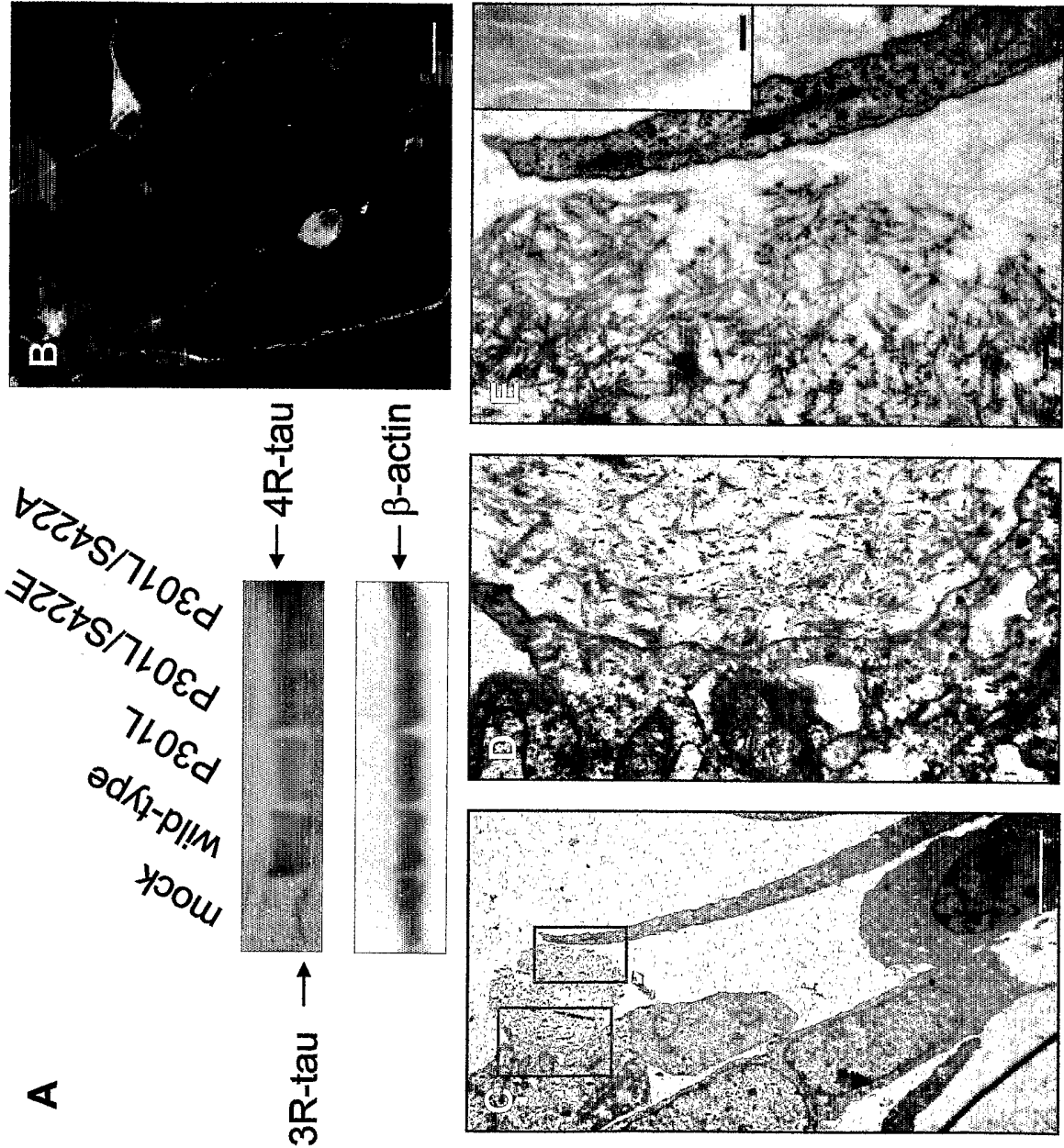
Claims

1. A method of producing neurofibrillary tangles in a cell comprising
 - a) providing a cell, or a plurality of cells in a sample,
 - b) expressing in said cell, or plurality of cells, a recombinant gene coding for tau protein,
 - c) contacting said cell, or plurality of cells, with amyloid precursor protein or a fragment, or derivative, or variant thereof, in particular β -amyloid,
 - d) incubating said cell, or plurality of cells, for a period of time, and
 - e) allowing said cell, or plurality of cells, to produce neurofibrillary tangles.
2. A method of generating a cell with neurofibrillary tangles comprising
 - a) providing a cell, or a plurality of cells in a sample,
 - b) expressing in said cell, or plurality of cells, a recombinant gene coding for tau protein,
 - c) contacting said cell, or plurality of cells, with amyloid precursor protein or a fragment, or derivative, or variant thereof, in particular β -amyloid,
 - d) incubating said cell, or plurality of cells, for a period of time, and
 - e) allowing said cell, or plurality of cells, to produce neurofibrillary tangles.
3. The method according to claims 1 or 2 wherein said β -amyloid is $A\beta_{42}$.
4. The method according to claims 1 to 3 wherein said cell, or plurality of cells, are contacted with a pre-aggregated or aggregated form of $A\beta_{42}$.
5. The method according to claims 1 or 2 wherein contacting said cell, or plurality of cells, is accomplished by addition of amyloid precursor protein, or a fragment, or derivative, or variant thereof, in particular β -amyloid, to the cell culture medium.

6. The method according to claims 1 or 2 wherein said recombinant tau gene is a recombinant human tau gene.
7. The method according to claim 6 wherein said recombinant human tau gene is the 4-repeat isoform bearing the P301L mutation.
8. The method according to claims 1 or 2 wherein said cell, or plurality of cells, are incubated for a period of at least 2 days after contacting with amyloid precursor protein, or fragment, or derivative, or variant thereof, in particular β -amyloid.
9. The method according to claims 1 or 2 wherein said cell, or plurality of cells, are incubated for 3 to 7 days after said contacting.
10. The method according to claims 1 or 2 wherein said cell, or plurality of cells, are incubated for a period of five days after said contacting.
11. The method according to claims 1 or 2 wherein said cell is a human cell.
12. The method according to claims 11 wherein said human cell is a neural cell.
13. The method according to claim 12 wherein said human neural cell is a neuroblastoma cell.
14. The method according to claim 13 wherein said neuroblastoma cell is an SH-SY5Y cell.
15. The method according to claims 1 or 2 wherein said neurofibrillary tangles comprise fibrillar polymers of 10 to 30 nm in width, preferably 20 nm in width.

16. A cell, preferably a neural cell, preferably a neuroblastoma cell, in particular an SH-SY5Y cell, which expresses a recombinant gene coding for tau, wherein said cell is capable of producing neurofibrillary tangles after contacting with amyloid precursor protein, or fragment thereof, in particular β -amyloid.
17. A method of screening for an agent or agents capable of modulating the formation of neurofibrillary tangles in a cell, comprising
 - i) providing a cell, or a plurality of cells in a sample, according to one of claims 1 or 2,
 - ii) contacting said cell, or plurality of cells, with a putative modulatory agent, or multitude of putative modulatory agents,
 - iii) incubating said cell, or plurality of cells with said agent or agents for a period of time, and
 - iv) assessing the ability of said agent, or multitude of agents to modulate a biological function selected from the group consisting of neurofibrillary tangle formation or phosphorylation of the tau epitope S422.
18. A medicament comprising an agent according to claim 17.
19. Use of an agent according to claim 17 for preparation of a medicament for treating a neurodegenerative disease associated with neurofibrillary tangle formation.

Fig. 1



-2/6-

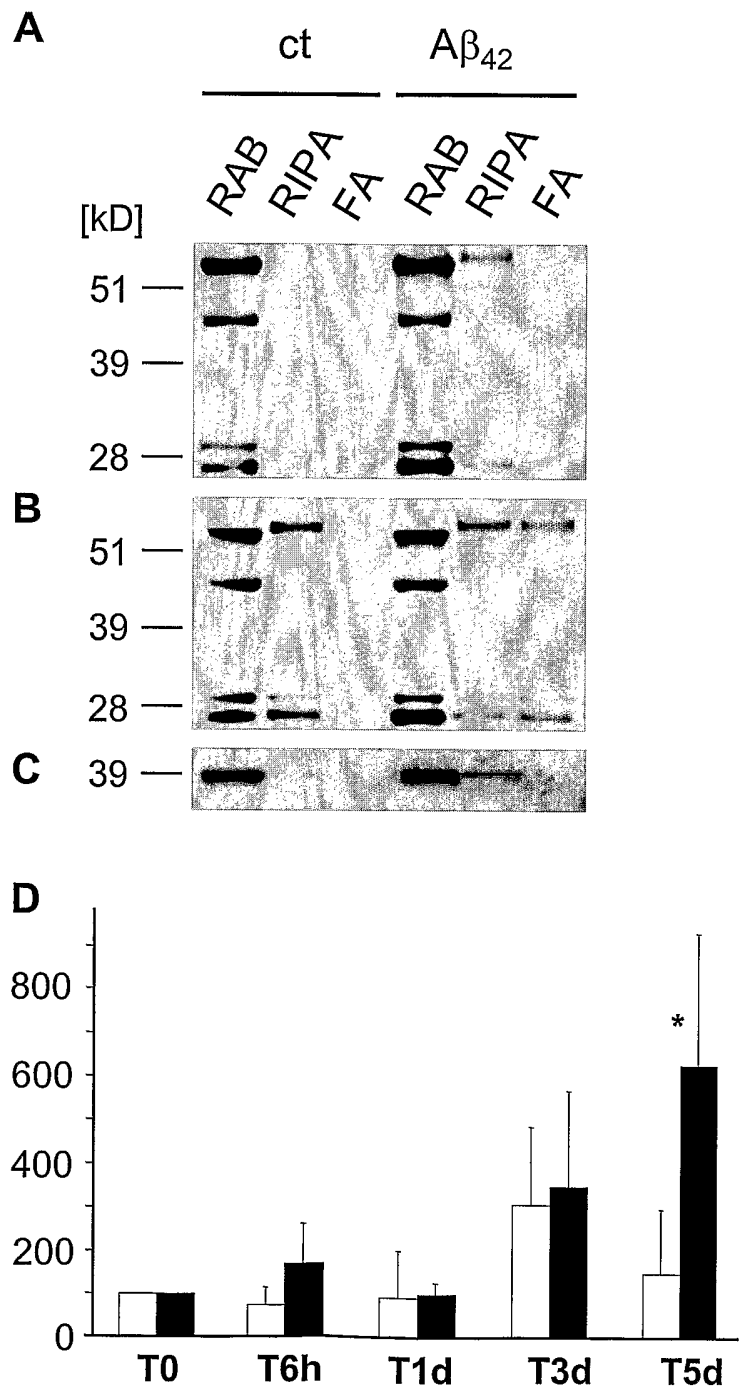
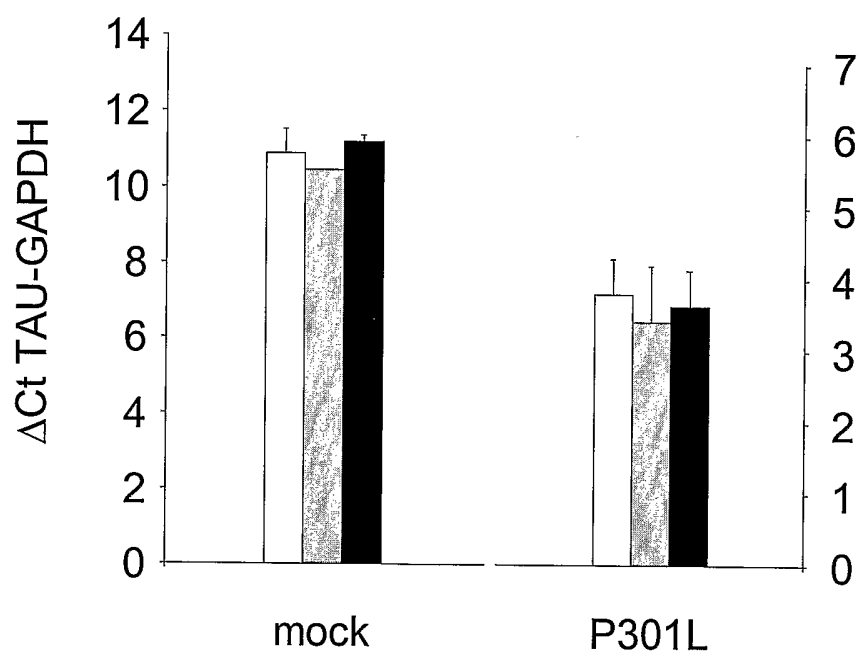


Fig. 2

-3/6-**Fig. 3**

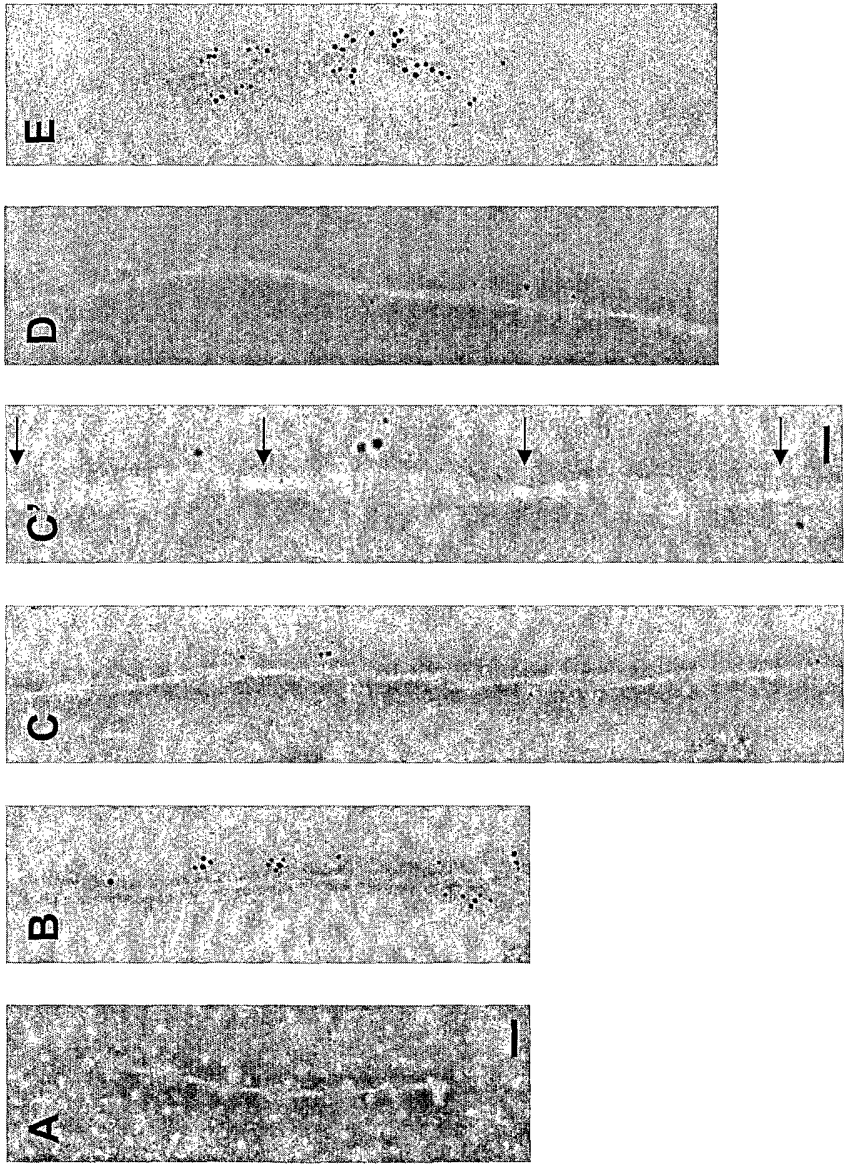
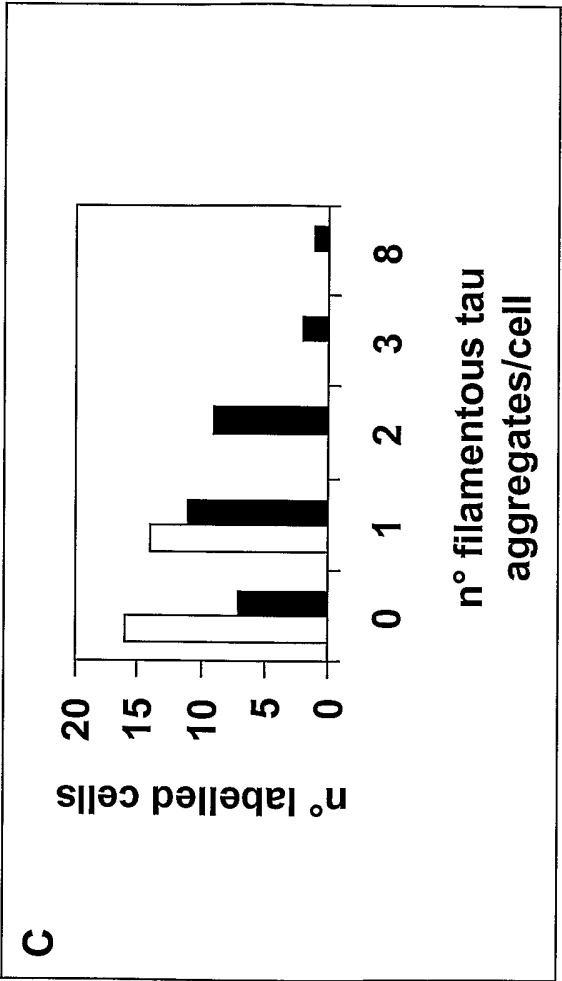
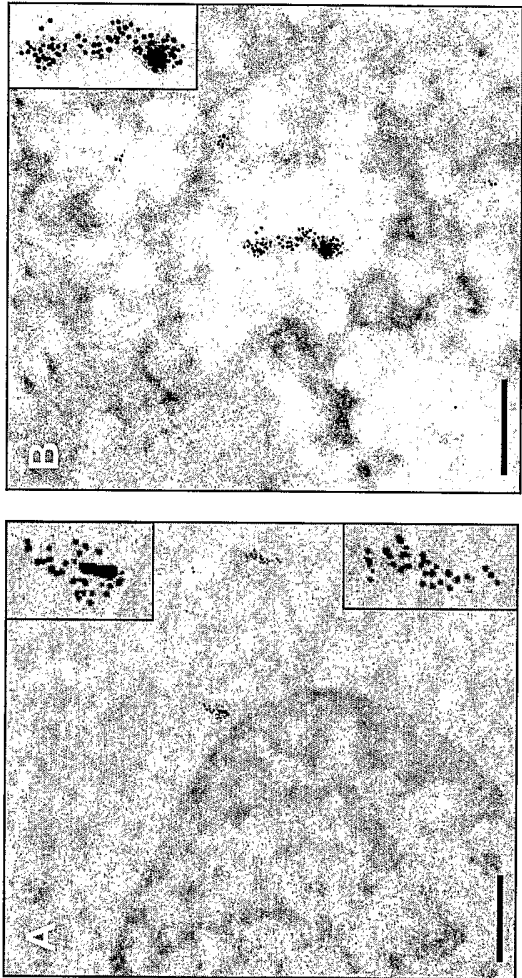
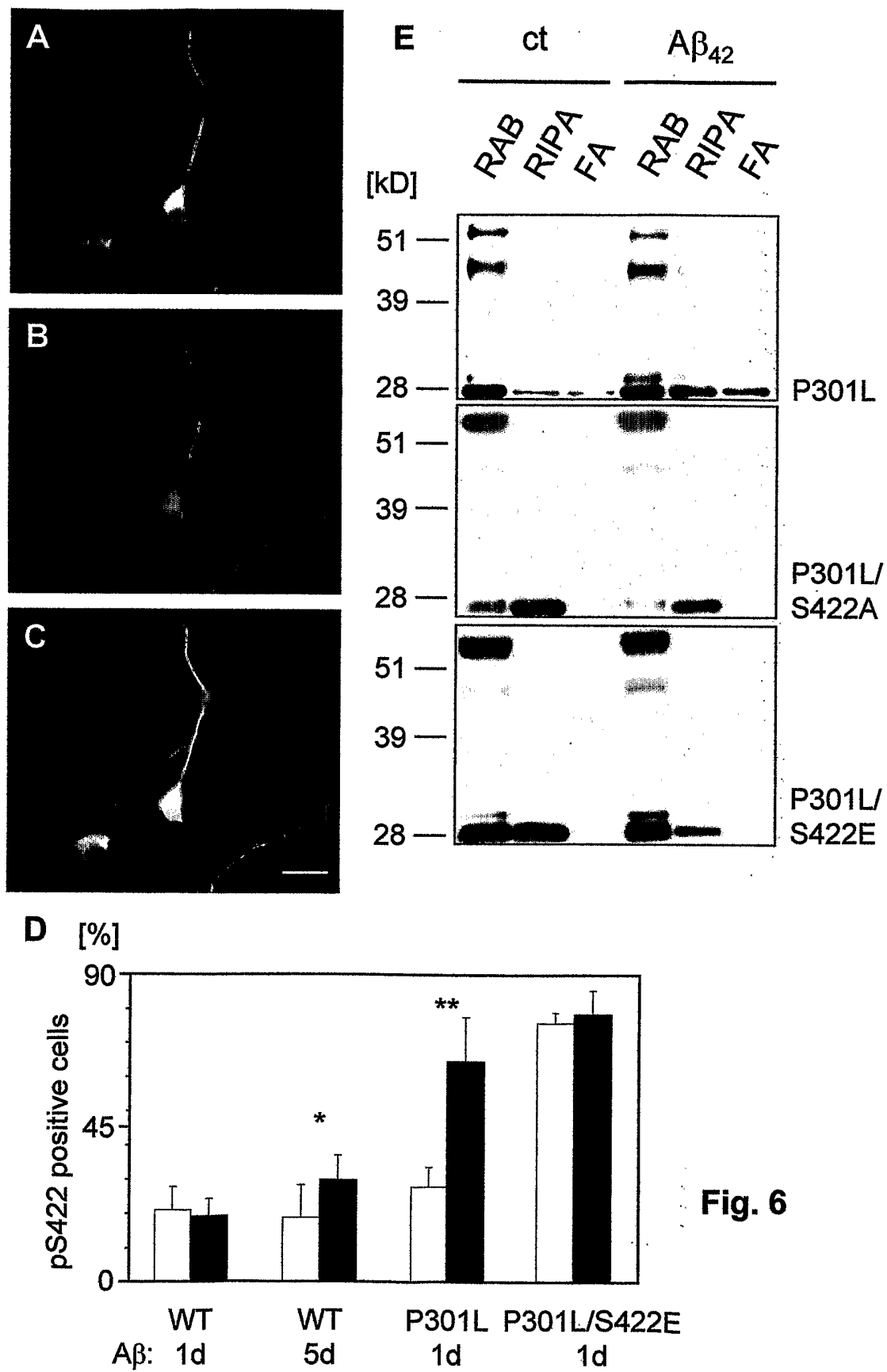


Fig. 4

Fig. 5



-6/6-

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/08983

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 G01N33/68 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, EMBASE, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VOGELSBERG-RAGAGLIA V ET AL: "Distinct FTDP-17 missense mutations in tau produce tau aggregates and other pathological phenotypes in transfected CHO cells." MOLECULAR BIOLOGY OF THE CELL. UNITED STATES DEC 2000, vol. 11, no. 12, December 2000 (2000-12), pages 4093-4104, XP002224857 ISSN: 1059-1524 cited in the application page 4094, left-hand column, last paragraph -right-hand column, line 22 page 4098, left-hand column, paragraph 2 -page 4100, left-hand column, line 3 figures 6,8-10 page 4102, left-hand column, last paragraph -right-hand column, paragraph 1 --- -/--	1-17

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

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Date of the actual completion of the international search

6 January 2004

Date of mailing of the international search report

28/01/2004

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/08983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RANK K B ET AL: "Direct interaction of soluble human recombinant tau protein with Abeta 1-42 results in tau aggregation and hyperphosphorylation by tau protein kinase II" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 514, no. 2-3, 13 March 2002 (2002-03-13), pages 263-268, XP004347709 ISSN: 0014-5793 Sections 2.3 and 3.4 abstract; figure 3 ----	1-17
Y	GOTZ J ET AL: "Formation of neurofibrillary tangles in P301L tau transgenic mice induced by Abeta42 fibrils." SCIENCE (WASHINGTON D C), vol. 293, no. 5534, 2001, pages 1491-1495, XP002224858 ISSN: 0036-8075 cited in the application the whole document ----	1-17
Y	LEWIS JADA ET AL: "Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP." SCIENCE (WASHINGTON D C), vol. 293, no. 5534, 2001, pages 1487-1491, XP002224859 ISSN: 0036-8075 cited in the application the whole document ----	1-17
Y	PÉREZ MAR ET AL: "Formation of aberrant phosphotau fibrillar polymers in neural cultured cells." EUROPEAN JOURNAL OF BIOCHEMISTRY / FEBS. GERMANY MAR 2002, vol. 269, no. 5, March 2002 (2002-03), pages 1484-1489, XP002224860 ISSN: 0014-2956 cited in the application page 1486, right-hand column, paragraph 1 -page 1481, left-hand column, paragraph 1; figures 3-5 ---- -/--	1-17

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/08983

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAQUE N ET AL: "Regulation of expression, phosphorylation and biological activity of tau during differentiation in SY5Y cells." BRAIN RESEARCH. NETHERLANDS 14 AUG 1999, vol. 838, no. 1-2, 14 August 1999 (1999-08-14), pages 69-77, XP002224861 ISSN: 0006-8993 cited in the application the whole document ---	
A	SAMBAMURTI KUMAR ET AL: "Advances in the cellular and molecular biology of the beta-amyloid protein in Alzheimer's disease." NEUROMOLECULAR MEDICINE. UNITED STATES 2002, vol. 1, no. 1, February 2002 (2002-02), pages 1-31, XP002224862 ISSN: 1535-1084 the whole document ---	
A	TAKASHIMA A ET AL: "Amyloid beta peptide (25-35) induces tau phosphorylation and decreases microtubule-forming ability in rat hippocampal culture." SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 21, no. 1-3, 1995, page 1719 XP002224863 25th Annual Meeting of the Society for Neuroscience; San Diego, California, USA; November 11-16, 1995 ISSN: 0190-5295 abstract ---	
A	IRVING N G ET AL: "Tau phosphorylation in cells transfected with wild-type or an Alzheimer's disease mutant Presenilin 1." NEUROSCIENCE LETTERS. IRELAND 31 JAN 1997, vol. 222, no. 2, 31 January 1997 (1997-01-31), pages 71-74, XP002224864 ISSN: 0304-3940 page 73, right-hand column, paragraph 1 ---	
T	FERRARI ALESSANDRA ET AL: "beta-Amyloid induces paired helical filament-like tau filaments in tissue culture." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 278, no. 41, 10 October 2003 (2003-10-10), pages 40162-40168, XP002266170 ISSN: 0021-9258 the whole document -----	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 18, 19

Present claims 18, 19 relate to an agent defined by reference to a desirable characteristic or property, namely capable of modulating the formation of neurofibrillary tangles.

The claims cover all agents having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such agents. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the agent by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search impossible. Consequently, no search has been carried out for said claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 03/08983

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 18, 19
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.