Increased level of active GSK-3ß in Alzheimer's disease and accumulation in argyrophilic grains and in neurons at different stages of neurofibrillary degeneration.

Karelle Leroy, Zehra Yilmaz, Jean-Pierre Brion

Laboratory of Histology, Neuroanatomy and Neuropathology, Université Libre de Bruxelles, Campus Erasme, 808 route de Lennik, B-1070 Brussels, Belgium.

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Send correspondence to:
Dr Jean-Pierre Brion
Laboratory of Histology and Neuropathology
Université Libre de Bruxelles, School of Medicine. 808, route de Lennik, Bldg GE
1070 – Brussels, BELGIUM
Tel: 32 - 2 – 5556505. Fax: 32 - 2 – 5556285.
E-mail: jpbrion@ulb.ac.be

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Abstract

The somatodendritic accumulation of hyperphosphorylated tau proteins is an early event preceding the appearance of neurofibrillary tangles (NFT) in Alzheimer's disease (AD) and might be necessary for their formation. Glycogen synthase kinase-3ß (GSK-3ß) is a physiological kinase for tau that generates many tau phosphorylation sites identified in NFT and in other tau-positive inclusions. We have studied the cellular distribution and the expression of the active form of GSK-3ß (GSK-3 pTyr216) in AD patients, in argyrophilic grain disease and in diffuse Lewy body disease. By western blotting analysis, a significant increase in the level of GSK-3 (pTyr216) was observed in the frontal cortex of AD patients. A population of neurons showed a somatodendritic accumulation of GSK-3 (pTyr216) but not of the inactive form of GSK-3ß (GSK-3 pSer9). Most of these GSK-3 (pTyr216) positive cells were positive for 6 different phosphotau epitopes known to be generated by GSK-3ß. By using a quadruple labelling method using GSK-3 (pTyr216) and phosphotau immunolabelling combined with Gallyas and DAPI staining, we examined neurons containing a somatodendritic GSK-3 (pTyr216) immunoreactivity at different stages of neurodegeneration. A majority of neurons at the pretangle stage without Gallays positive inclusions were GSK-3 (pTyr216) positive and this GSK-3 (pTyr216) immunoreactivity remained in most cells containing Gallyas and phosphotau positive inclusions excepted in extracellular NFT. A GSK-3 (pTyr216) immunoreactivity was present in argyrophilic grains but not in cortical Lewy bodies. These results directly suggest that the activity of GSK-3ß is increased in AD and that somatodendritic accumulation and activation of GSK-3ß is an early event preceding and accompanying the formation of NFT and of other tau-positive inclusions.

Key words: glycogen synthase kinase-3ß – tau – phosphorylation – neurofibrillary tangles – argyrophilic grains – Lewy body - Alzheimer's disease

Introduction

Neurofibrillary tangles and senile plaques are the two neuropathological hallmarks of Alzheimer's disease. Neurofibrillary tangles are constituted of bundles of abnormal filaments called « paired helical filaments » (PHF) that accumulate in neurons in selected brain areas. The PHF are composed of hyperphosphorylated forms of the microtubule-associated protein tau (PHF-tau proteins). Hyperphosphorylation of tau is thought to be necessary to promote fibrillar aggregation of tau in vitro [1] and the accumulation of this non-aggregated tau constitute an early, pretangle stage in the evolution of the neurofibrillary pathology [2, 3]. The hyperphosphorylation of tau decreases its ability to bind to and to stabilize microtubules and is consequently thought to affect the neuronal microtubule network, leading to disturbances of axoplasmic transports. Hyperphosphorylated tau proteins, eventually in the forms of small oligomers, might also be neurotoxic per se. Phosphorylation sites on tau can be generated by many protein kinases including glycogen synthase kinase-3ß (GSK-3ß), cyclin-dependent protein kinase 5, extracellular signal-regulated protein kinases (ERK), Jun protein kinases, protein kinases A and C, calcium/calmodulin dependent kinase II, casein kinases, p38 kinases and microtubule affinity-regulating kinase [4]. GSK-3ß appears to be a key physiological protein kinase involved in the hyperphosphorylation of tau proteins [5]. GSK-3ß has a neuronal localization [6, 7] and has been observed to generate many phosphorylation sites on tau in vitro [6, 8], in cultured cells [9], and in transgenic animals [10-12]. GSK-3ß has also been observed to co-localize with some neuropathological lesions. GSK-3ß is associated to granulovacuolar degeneration [13] and to neurofibrillary tangles in AD [13-16] and in other tauopathies [17]. The association of GSK-3ß with neurofibrillary tangles in these studies was however reported to be variable, not involving all NFT, or even absent [18]. These observations possibly indicated that this association changed during NFT evolution. In this study, we have quantitatively assessed the subpopulations of neurons with a GSK-3ß somatodendritic immunoreactivity in relationship with markers of neuronal degeneration, using a methodology allowing simultaneous assessment in the same neurons of GSK-3ß immunoreactivity, tau phosphorylation, tau aggregation and nuclear staining. To further document the potential involvement of GSK-3ß in other neurodegenerative conditions with and without tau-positive inclusions, we have also investigated GSK-3ß immunoreactivity in argyrophilic grain disease and in Lewy body disease.

Material and methods

Tissue samples

Brains were obtained post-mortem in 6 non-demented control subjects, in 7 demented subjects with probable AD, in 2 patients with argyrophilic grain disease (AGD) and in 3 patients with diffuse Lewy body disease (DLBD), and all studies performed with the approval of the ethical committee of the medical School of the Free University of Brussels. The age, sex and post-mortem delays for each case are described in table 1. No significant differences were observed for age ($p= 0.10$) and post-mortem delays ($p= 0.37$) with Student t test. One hemisphere was frozen in liquid N2 and kept at -80°C for biochemical analysis. The other hemisphere was fixed by immersion in 10 % formalin (v/v) for 3 weeks to 3 months. Tissue blocks of the hippocampus and the parahippocampal gyrus were embedded in paraffin and cut in tissue sections with a thickness of 10 µm. For neuropathological examination, histological sections were stained with the Nissl method, the Gallyas silver technique and immunolabelled with antibodies to tau protein, to Aß, to ubiquitin and to α-synuclein (see below). The neuropathological examination indicated the presence of numerous plaques and a severe neurofibrillary pathology (stages V-VI according to the staging of [19] in the AD cases. In 2 control subjects, moderate neurofibrillary pathology was present (stages I and II). The two cases of AGD were diagnosed on the basis of the presence of numerous argyrophilic grains in the CA1 sector and in the entorhinal cortex [20, 21]. The DLBD cases showed brainstem and cortical Lewy bodies (transentorhinal, cingulate and temporal cortex) positive with ubiquitin and synuclein antibodies in presence of moderate AD changes, consistent with the pathological classification of limbic subtype [22].

Antibodies

The AT8, AT180 and AT100 mouse monoclonal antibodies (purchased from Innogenetics) are specific for tau phosphorylated at Ser202 and Thr205 (AT8) [23] at Thr231 (AT180) [24] and a specific PHF-tau epitope involving phosphorylation at Thr212 and Ser214 (AT100) [25]. The tau HT9 antibody is a mouse monoclonal antibody whose epitope maps at the N-terminus of tau and was obtained from Innogenetics. The mouse monoclonal antibody PHF-1 (kindly provided by Drs. P. Davies and S. Greenberg, New York) is specific for tau phosphorylated at Ser396/404 [26]. The tau-1 monoclonal antibody (Boehringer) recognises an epitope that needs unphosphorylation of Ser199/202 [27]. TauS404 and TauS413 are rabbit

polyclonal antibodies that recognize tau phosphorylated on Ser404 and Ser413, respectively (purchased from Biosource). The mouse monoclonal antibody to ubiquitin was purchased from Chemicon. The anti-α-synuclein rabbit antibody was obtained from Affiniti. The anti-Aß42 rabbit antibody was from Bio-Source.

Three GSK3 antibodies have been used in this study: the TPK1 monoclonal antibody was generated to the N-terminal domain of GSK-3ß and was purchased from Transduction Laboratories. The rabbit polyclonal phospho-GSK-3α/ß (Tyr216/279) antibody (purchased from Biosource) reacts specifically with the active form of GSK-3 phosphorylated on tyrosine 216 (GSK-3ß) and on Tyr 279 (GSK-3α). The phospho-GSK-3ß (Ser9) rabbit polyclonal antibody (Cell Signaling) detects GSK-3ß only when phosphorylated at serine 9, a phosphorylation inhibiting its activity. The anti-ß tubulin isotype III antibody, specific for neuronal tubulin, is a mouse monoclonal antibody purchased from Sigma (clone SDL.3D10).

Immunocytochemistry

The chromogenic single immunocytochemical labelling was performed as previously reported [13]. Briefly, after blocking of endogenous peroxidase, tissue slides were sequentially incubated with normal goat serum 10 % (v/v) in TBS (Tris 0.01 M, NaCl 0.15 M pH 7.4) for 1 h and overnight with the primary antibodies at the following dilutions in TBS containing 1 % (v/v) normal goat serum: anti-GSK-3 (pTyr216): 1/500: anti-GSK-3 (pSer9): 1/ 100, TPK1: 1/100, AT8: 1/ 100; PHF-1: 1/500 ; AT100: 1/100; AT180: 1/100; tau-1: 1/100; TauS404: 1/100; TauS413: 1/100; HT9: 1/100; anti-ubiquitin: 1/500; anti-α-synuclein: 1/2000; anti-Aß; 1/2000. They were then incubated with a goat anti-rabbit antibody conjugated to biotin (Vector) for 30 minutes, with the ABC complex (Vector) for 30 min and finally with 3,3'diaminobenzidine as chromogen (Dako). For double fluorescent immunolabelling (GSK-3ß and phosphotau), the primary antibodies were incubated together and the GSK-3 antibody was detected by a goat anti-rabbit antibody conjugated to biotin (Vector) followed by the ABC complex and incubation with Alexa488-tyramide (Molecular Probes); the phosphotau antibody was detected by a goat anti-mouse antibody conjugated to Alexa594. Immunocytochemical controls were performed by omitting the primary antibody. Tissue sections were examined under UV illumination with a Zeiss Axioplan microscope and digital images acquired using an Axiocam HRc digital camera.

Gallyas and DAPI staining

After fluorescent immunolabelling and image acquisition, the Gallyas silver staining method [28] was used to assess for the presence of argyrophilic and aggregated material on the same tissue sections. Briefly, tissue slides were sequentially incubated in lanthanum nitrate for 1 h, in alkaline Ag iodide for 1 min., in acetic acid 0.5 % for 3 min. and in a mixed solution of A (NH_4NO_3 25 mM, $AgNO_3$ 12 mM, tungstosilicic acid 3 mM and formaldehyde 0.4 %) and B (Na_2 CO_3 0.5 M). Slides were then incubated in acetic acid 0.5 % for 3 min., 1% and in Na thiosulfate 1 % for 5 min. For DAPI nuclear staining, the sections were stained with DAPI (1 µg/ml) for 10 min.

Relationship between GSK-3 (pTyr216) immunoreactivity, tau phosphorylation and tau aggregation

A quadruple labelling procedure was used to study in the same neurons the relationship between GSK-3 (pTyr216) immunoreactivity, phosphotau immunoreactivity, presence of Gallays positive material inclusions, and presence or absence of a nucleus. A double fluorescent labelling was first performed using the AT8 antibody and the GSK-3 (pTyr216) antibody, followed by DAPI staining, on coronal tissue sections of the hippocampus and adjacent parahippocampal gyrus from AD patients. Ten fields of 204 x 163 μm randomly disposed in the pyramidal layer of the Ammon's horn and in adjacent subicular areas were selected for acquisition (in each field) of successive digital images of AT8 labelling, GSK-3 (pTyr216) labelling and DAPI staining. The same tissue slides were then stained with the Gallyas silver staining method and new digital images of the same fields acquired. This method was chosen rather than direct combination of immunofluorescence and Gallyas staining, because initial assays showed that strong Gallyas staining tended to mask weak fluorescent labelling. In each field, all AT8 and all Gallyas positive neuronal profiles were identified (approximately 50 neuronal profiles for each AD case) and characterized for their GSK-3 (pTyr216) immunoreactivity and DAPI staining by looking at the four digital images.

Using this methodology, GSK-3 (pTyr216) immunoreactivity was assessed in four populations of degenerating neurons, according to their pattern of AT8 labelling, DAPI staining, and Gallyas staining, a classification resembling the one proposed by [3]. Type 1 neurons showed a diffuse, sometimes granular, somatodendritic AT8 immunoreactivity and absence of Gallyas positive material; these neurons correspond to pretangle neurons. Type 2 neurons contain a faintly argyrophilic material, sometimes in the form of a small rod and were AT8 positive. Type 3 neurons contain a well-developed classic argyrophilic NFT and were

AT8 positive. Type 4 neurons (ghost tangles) contain less tightly packed argyrophilic material and no AT8 immunoreactivity. All of these neurons contain a DAPI positive nucleus, except the type 4.

Immunoblotting

Samples of frontal cortex were homogenized in a buffer containing proteases and phosphatase inhibitors (50 mM Tris, EDTA 10 mM, NaCl 100 mM, pH 7.4, PMSF 1 mM, leupeptin 25 µg/ml, pepstatin 25 µg/ml, Na_3VO_4 1 mM, $Na_4P_2O_7$. 10 H_2O 10 mM, NaF 20 mM). Homogenates (100 µg protein/lane) were run on 5 % (w/v) polyacrylamide gels (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and proteins were electrophoretically transferred to a nitrocellulose membrane. Nitrocellulose membranes were blocked in non-fat milk (10 % (w/v) in TBS) and incubated with the primary antibodies overnight followed by biotin-conjugated anti-rabbit or anti-mouse immunoglobulins followed by streptavidin-conjugated alkaline phosphatase. Densitometry analysis was performed by using the NIH Image J program. Statistical comparisons were made between control and AD cases with the Student t test.

Results

Levels of active GSK-3 (pTyr 216) are increased in AD frontal cortex.

We have investigated by immunoblotting the expression of total levels of GSK-3ß with the TPK1 antibody (fig 1A), of the active form of GSK-3ß with the GSK-3 (pTyr 216) antibody (Fig 1B) and of the inactive form of GSK-3ß with the GSK-3 (pSer9) antibody (Fig 1C). In brain homogenates of control and AD subjects these antibodies identified an immunoreactive band of 47 kDa, the expected molecular weight of GSK-3ß. The levels of total GSK-3ß, of GSK-3 (pTyr216) and of GSK-3 (pSer9) were quantified by densitometry analysis, after normalisation with the levels of tubulin ßIII (a neuronal protein) (fig 1D). Despite a tendency to increase in AD cases, the levels of total GSK-3ß were not significantly different between control (107.6 +/- 14.8, mean +/- SEM) and AD cases (142.4 +/- 38.3) (Fig 1E). The levels of GSK-3 (pSer9) were not significantly different between control and AD cases (Fig 1G). However, we observed a significant increase ($p < 0.01$) of expression of GSK-3 (pTyr 216) in AD cases (180.3 +/- 24.4) by comparison with controls (99.6 +/- 9.1) (Fig 1F).

Active GSK-3 (pTyr216) co-localizes with several phosphotau epitopes in the somatodendritic compartment

Most neurons in the hippocampus and the adjacent temporal cortex showed a weak diffuse pericaryal labelling both in control and AD subjects with the GSK-3ß antibodies. As previously reported, a strong labelling with GSK-3ß antibodies of the granules of the granulovacuolar degeneration of Simchowicz was observed in neurons in pyramidal neurons in the hippocampus both in control and AD subjects (Fig 2). In addition, we also observed an intense labelling of the somatodendritic compartment of a subset of neurons with the GSK-3 (pTyr 216) antibody; some neurons contained classical neurofibrillary tangles that were intensely labelled with the GSK-3 (pTyr216) antibody (Fig 2) confirming previous results obtained with the TPKI antibody, a pan-GSK-3ß antibody [13]. This intense somatodendritic GSK-3 (pTyr216) immunoreactivity co localized with immunoreactivity for several phosphotau epitopes (Fig 3), as shown by double immunolabelling with the GSK-3 (pTyr216) antibody in combination with phosphotau antibodies AT180 (Fig 3A and B), PHF-1 (Fig 3C and D), AT100 (Fig 3E and F), AT8 (Fig 4B and C), S404 and S413 (not shown). Cells showing increased somatodendritic phosphotau immunoreactivity did not show an increased labelling with the GSK-3 (pSer9) antibody (Fig 3G and H).

The active GSK-3 (pTyr216) accumulates in neurons from the pretangle stage to the late "classical " stage

The relationship between GSK-3 (pTyr216) immunoreactivity and the evolution of neurofibrillary changes was investigated by using a method of quadruple identification (phosphotau and GSK-3 (pTyr216) double immunolabeling combined with Gallyas and DAPI staining) on the same tissue section (Fig 4).

At the pretangle stage (type 1 neurons, arrow in Fig 4 A to E), defined by absence of Gallyas positive material (Fig 4A) in presence of a somatodendritic phosphotau immunoreactivity (Fig 4B) a strong GSK-3 (pTyr216) immunoreactivity was observed in the somatodendritic compartment (Fig 4C). This co-localisation was observed in 82.1% +/- 14.2% (mean+/- SEM) of neuronal profiles showing AT8 immunoreactivity in absence of Gallyas positive material. A similar frequency of neuronal profiles with a co-localization of GSK-3 (pTyr216) and somatodendritic phosphotau in absence of Gallyas material was observed when using the PHF-1 (94.1% +/- 4.4%), AT180 (100%) or AT100 (80.6% +/- 13.7%) phosphotau antibodies. A high frequency of co-localization of AT8 and GSK-3 (pTyr216) immunoreactivities was also observed in many neuronal profiles containing faintly Gallyas positive material (type 2 neurons, arrow in Fig 4F to J) (62.0 % +/- 8.9%, mean+/- SEM) or containing a well-developed Gallyas positive NFT (type 3 neurons, arrowheads in Fig 4F to J) (65.4% +/- 18.4%). NFT with less tightly packed Gallyas positive material, no AT8 immunoreactivity and absence of nucleus, corresponding to ghosts tangles (type 4, arrow in Fig 4K to O), did not show a GSK-3 (pTyr216) immunoreactivity. The percentages of GSK-3 (pTyr216) positive neuronal profiles in each group (except for ghosts tangles) were not statistically different (Fig 5).

A reverse situation was observed when combining immunolabelling with tau-1 (recognizing tau unphosphorylated on Ser199) and GSK-3 (pTyr216) antibodies with Gallyas staining (not shown). Neuronal profiles with a strong somatodendritic GSK-3 (pTyr216) labelling and no Gallyas positive material were not tau-1 positive and an increasing proportion of tau-1 positive neuronal profiles with faint and classical Gallyas positive NFT was found. Ghost tangles were almost systematically tau-1 positive. This result suggests that loss of AT8 labelling in ghosts NFT was most probably due to tau dephosphorylation with disappearance of the AT8 phosphotau epitope depending on ser 199 phosphorylation.

Argyrophilic grains but not Lewy bodies are GSK-3 (pTyr216) immunoreactive

Argyrophilic grains share some immunoreactivities and staining properties with NFT and were tested for their GSK-3 (pTyr216) immunoreactivity by combining tau and GSK-3 (pTyr 216) immunolabelling in three cases of argyrophilic grain disease. The double immunolabelling showed the co-localization of tau and GSK-3 (pTyr 216) immunoreactivities in grains (Fig 6 A and B).

Lewy bodies show significant antigenic differences with NFT and were thus also tested for their GSK-3 (pTyr216) immunoreactivity by combining α–synuclein or ubiquitin immunolabelling (two markers of Lewy bodies) with GSK-3 (pTyr216) immunolabelling. Lewy bodies positive for α-synuclein (not shown) or ubiquitin were not GSK-3ß positive (arrow in Fig 6 C and D).

Discussion

In the present study, we have studied the cellular distribution and the expression of GSK-3ß and of its active form (GSK-3 pTyr216) in AD patients, in argyrophilic grain disease and in diffuse Lewy body disease. In AD, we investigated the detailed relationship between neuronal accumulation of active GSK-3ß and presence of other markers of neuronal degeneration in AD, i.e. accumulation of phosphorylated tau protein, accumulation of argyrophilic, aggregated material and disappearance of the nucleus. A strong somatodendritic GSK-3 (pTyr216) immunoreactivity was observed in a subset of neurons in the hippocampus and the temporal cortex of AD patients. This somatodendritic GSK-3 (pTyr216) immunoreactivity co-localized with somatodendritic immunoreactivity for 6 different phosphotau epitopes (PHF-1, AT8, AT180, AT100, S404, and S413) needing phosphorylation of tau on Ser202/Thr205 (AT8), Thr212/Ser214 (AT100), Thr231 (AT180), Ser396/404 (PHF-1) and Ser413 (S413). In vitro, GSK-3ß phosphorylates tau on these amino acids [4, 29, 30] and generates the corresponding phosphotau epitopes detected by these antibodies [6, 8, 24, 29, 31]. Increased activity of GSK-3ß also generates several of these phosphotau epitopes in cellular models [9, 32] and in transgenic models [10-12]. GSK-3 (pTyr216) somatodendritic accumulation in pretangles was not associated to a tau-1 immunoreactivity, in agreement with the observation that the tau-1 epitope disappears upon phosphorylation by GSK-3ß [6, 8]. Only the active form of the kinase, phosphorylated on tyrosine 216 (GSK-3ß) and not the inactive form, phosphorylated on Ser9 (GSK-3ß), was observed to accumulate. This cellular accumulation of GSK-3 (pTyr216) was paralleled by the western blot densitometry analysis of brain homogenates showing an increase in the relative level of GSK-3 (pTyr216) in AD patients, but not of GSK-3 (pSer9). Altogether, these results strongly suggest that GSK-3ß is responsible for the generation of increased tau phosphorylation in these cells. This accumulation does not preclude a role for other protein kinases, e.g. the AT100 phosphotau epitope is generated by the consecutive action of GSK-3ß and protein kinase A [31]. In addition, many substrates of GSK-3ß are more efficiently phosphorylated after priming phosphorylation by other kinases.

The relationship of GSK-3ß immunoreactivity with NFT has been studied in some previous studies. The GSK-3ß immunoreactivity was demonstrated in neurons containing NFT identified with yellow-green birefringence after staining with Congo red [6, 14], with the tau-1 antibody (after dephosphorylation of tissue sections) [15], tau antibodies to phosphoserine

413 [16], AT8 antibody [13, 33] and a pan tau antibody [17]. These studies however did not quantitatively investigate the association of active GSK-3ß with several phosphotau epitopes and other markers to unequivocally identify populations at different stages of neurofibrillary degeneration. Our staining methodology combining Gallyas and DAPI staining with GSK-3 (pTyr216) and phosphotau immunolabelling allowed assessing the proportion of GSK-3 (pTyr216) positive cells containing or not argyrophilic aggregates, phosphotau epitopes and a nucleus. We observed that the majority of phosphotau positive cells without Gallyas positive inclusions, corresponding to the pretangle stage, were GSK-3 (pTyr216) positive indicating that the accumulation of GSK-3ß occurs at an early stage in the progression of the neurofibrillary pathology, preceding tau aggregation. The presence of GSK-3ß in pretangles neurons was suggested before on the basis of co-localization of phosphotau and GSK-3ß immunoreactivity [16, 33]. The study of [33] showed a co-localization of active GSK-3ß and phosphotau (AT8) immunoreactivity in neurons in the form of three patterns of immunoreactivity without (pretangle stage) or with increasing content in AT8 positive inclusions. The additional use of Gallyas and DAPI staining methods afford to prove that tau accumulation is associated to tau aggregation and allow easy identification of cells with or without a nucleus. In addition, we also observed a GSK-3 (pTyr216) immunoreactivity in neurons containing limited or abundant Gallyas material, indicating that the kinase remained active and accumulated in the cell bodies during more advanced stages of neurofibrillary degeneration. Cell-death associated to end-stage neurofibrillary degeneration, i.e. ghosts tangles, represented by extracellular Gallyas positive material, absence of AT8 labelling and absence of nucleus, did not show any GSK-3 (pTyr216) labelling.

In addition to increasing tau phosphorylation, accumulation of an active GSK-3ß might induce additional cell perturbations by acting on other substrates, independently of filament formation. Induction of cell death cascades in cultured neural cells is associated to activation of GSK-3ß and increase in levels of GSK-3 (pTyr216) [34]. Transgenic mice conditionally overexpressing GSK-3ß showed somatodendritic accumulation of phosphorylated tau and neuronal death, in absence of tau filament formation [11].

We also observed that GSK-3 (pTyr216) is also associated to phosphotau positive grains observed in argyrophilic grain disease. AGD differs also biochemically from AD by showing a enrichment in 4 repeats tau isoforms relative to 3 repeats tau isoforms in insoluble tau [35, 36] This association of GSK-3 pTyr216 with argyrophilic grains was not reported before and complements previous observations showing that a GSK-3ß immunoreactivity is associated to other neuropathological lesions found in different tauopathies, i.e. Pick’s bodies, tau-

containing astrocytes and coiled bodies in oligodendrocytes in corticobasal degeneration and in progressive supranuclear palsy [17], although the latter study found a co-localization with GSK-3ß pSer9, i.e. the inactive form of the kinase and not GSK-3ß pTyr216 as we observed in the present study. In addition, we observed that GSK-3ß was not associated to another inclusion body, i.e. cortical Lewy bodies in diffuse Lewy body disease. In contrast, cyclin-dependent kinase-5 was previously found to be associated to Lewy bodies [37]. This suggest that association to GSK-3ß is specific for tau-positive inclusions, whatever their tau isoforms composition.

The total levels of GSK-3ß were however not increased in AD patients by comparison with controls, indicating that increased levels of GSK-3 pTyr216 resulted more probably from either activation of signalling pathways leading to Tyr 216 phosphorylation in GSK-3ß or to inhibition of tyrosine phosphatase. This tyrosine phosphorylation could be mediated by several kinases: e.g. the mammalian orthologue of the tyrosine kinase Zak1, a kinase important for activation of GSK-3 during Dictyostelium development [38], or the calcium-sensitive proline-rich tyrosine kinase 2 [39]. This activation of GSK-3ß could also be mediated by the Fyn tyrosine kinase [40]. The Fyn tyrosine kinase has been reported to be upregulated in AD brain [41]. Interestingly, the Fyn tyrosine kinase [42] and the c-Abl tyrosine kinase [43] can also increase tyrosine phosphorylation of tau proteins and tyrosine phosphorylation of PHF-tau proteins in AD brain has been observed [43-45].

In conclusion, this study indicates that somatodendritic accumulation of active GSK-3ß in association with phosphotau epitopes typically generated by this kinase is an early event, but that also accompany the evolution of neurofibrillary degeneration in AD. The association of active GSK-3ß with argyrophilic grains is an additional indication that GSK-3ß is characteristically associated to tau-positive inclusions found in different tauopathies and might play an important role in the pathogenesis and dysfunction associated to these cellular lesions.

Legends of figures

Figure 1

A – D: Immunoblotting analysis of homogenates of the frontal cortex of two representative control subjects (lanes 1 and 2) and two representative AD cases (lanes 3 and 4) with the GSK-3ß (TPK1) antibody (A), the GSK-3 (pTyr 216) antibody (B), the GSK-3 (pSer9) antibody (C), and with the anti-tubulin ßIII antibody (D). Control cases did not show neurofibrillary changes (stage 0) and AD cases showed severe neurofibrillary changes (stages V and VI). All lanes have similar loading of proteins. Bars on the left indicate the position of molecular weight marker: 58.1 kDa (catalase), 39.8 kDa (alcohol dehydrogenase).

E – G: Densitometry analysis of GSK-3ß (E), GSK-3 (pTyr 216) (F), and GSK-3 (pSer9) (G) expression, expressed as the ratio of GSK-3 to tubulin ßIII immunoreactivities, in control subjects (n = 6) and AD cases (n = 7). For normalization purposes, ratios were transformed in percentages by comparison with a control case. * *: $p < 0.01$ compared with control cases, by Student's t-test.

Figure 2

Tissue section of the hippocampus in an AD case. Immunolabelling with the GSK-3 (pTyr216) antibody. Several NFT are strongly labelled. Granules of the granulovacuolar degeneration (arrow) in a neuron are also labelled.

Scale bar: 15 µm.

Figure 3

Tissue section of the hippocampus in an AD case.

A –F: Double immunolabelling with the GSK-3 (pTyr216) antibody (A, C, E) and different phosphotau antibodies (B: AT180; D: PHF-1; F: AT100). These neurons show a somatodendritic accumulation of GSK-3 (pTyr216) (active form of the kinase) immunoreactivity co-localized with phosphotau immunoreactivity.

G and H: Double immunolabelling with the GSK-3 (pSer9 antibody) (G) and the AT8 antibody (H). Two neurons show somatodendritic phosphotau immunoreactivity but no immunoreactivity with the GSK-3 (pSer9) antibody (inactive form of the kinase).

Scale bar: 10 µm.

Figure 4

Tissue section of the hippocampus in an AD case. Patterns of association of somatodendritic GSK-3 (pTyr216) immunoreactivity with phosphotau immunoreactivity and tau aggregation. Tissue sections were stained with the Gallyas silver staining method (A, F, K) and with DAPI (D, I, N) after immunolabelling with the AT8 phosphotau antibody (B, G, L) and the GSK-3 (pTyr216) antibody (C, H, M). Type 1 neurons (arrow in A to E) show a co-localization of somatodendritic GSK-3 (pTyr216) and AT8 immunoreactivities in absence of Gallyas positive material and correspond to pretangles. Type 2 neurons (arrow in F to J) show a somatodendritic GSK-3 (pTyr216) and AT8 immunoreactivities and presence of discrete clumps of Gallyas positive material. Type 3 neurons (arrowheads in F to J) show GSK-3 (pTyr216) and AT8 immunoreactivities and strongly Gallyas positive NFT. Type 4 neurons (arrow in K to O) do not show GSK-3 (pTyr216) nor AT8 immunoreactivities, no nucleus (DAPI staining) but persistence of Gallyas positive material and correspond to ghosts tangles. The superposition of GSK-3 (pTyr216), AT8 and DAPI staining is shown in E (B, C, D), J (G, H, I) and O (M, N, O).

Scale bar: 25 μm.

Figure 5

Percentage of GSK-3 (pTyr 216) immunoreactive neurons (mean +/- SEM) according to their patterns of phosphotau (AT8) immunoreactivity and Gallyas staining (types 1 to 4 neurons). +/- 50 neurons were counted for each type (n=5).

Figure 6

Tissue section of the temporal cortex in a case of argyrophilic grains disease (A and B) and in a case of diffuse Lewy body disease (C and D).

A and B: Double immunolabelling with the HT9 antibody (A) and the GSK-3 pTyr216 antibody. A GSK-3 pTyr216 immunoreactivity is co-localized with phosphotau immunoreactivity in grains (arrowheads) and in an adjacent NFT (arrow).

C and D: Double immunolabelling with the anti-ubiquitin antibody (C) and the GSK-3 pTyr216 antibody (D). A cortical Lewy body (arrow) is labelled by the anti-ubiquitin antibody but not by the GSK-3 pTyr216 antibody.

Scale bar: 15 μm.

Table 1: Characteristics of human subjects used in this study. AD: Alzheimer's disease; DLBD: Diffuse Lewy body disease; AGD: Argyrophilic grain disease.

Cases	N°	Age	Sex	Post-mortem delay	NFT staging
Control	1	61	M	8	0
	2	65	M	4,5	0
	3	72	M	24	0
	4	77	F	48	0
	5	71	F	7	I-II
	6	88	F	6	I-II
	Mean+/- SEM	72,3 +/-3,9		16,2 +/- 7,0	
AD	7	72	F	24	V-VI
	8	70	F	21	V-VI
	9	73	F	22	V-VI
	10	91	F	5,5	V-VI
	11	84	M	7	V-VI
	12	89	F	7	V-VI
	13	75	M	10	V-VI
	Mean+/- SEM	79,1 +/- 3,3		13,8 +/- 3,1	
DLBD	14	70	M	25	
	15	60	F	24	
	16	88	F	24	
	Mean+/- SEM	72,7 +/- 8,2		24,3 +/- 0,3	
AGD	17	85	F	17	
	18	80	M	7	
	Mean+/- SEM	82,5 +/- 2,5		12 +/- 5,0	

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Figure 1

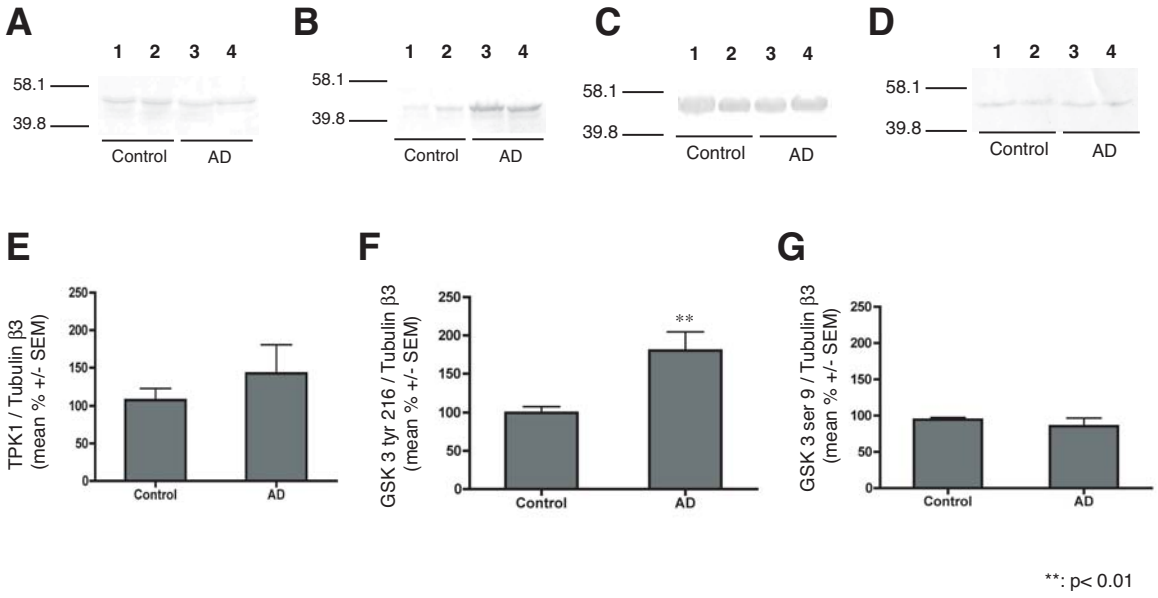
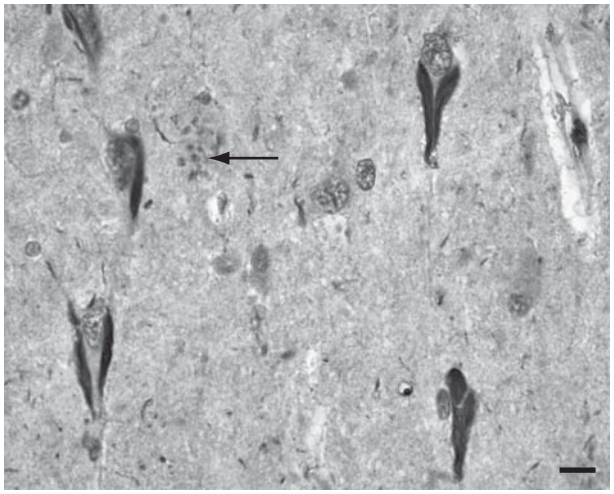


Figure 2



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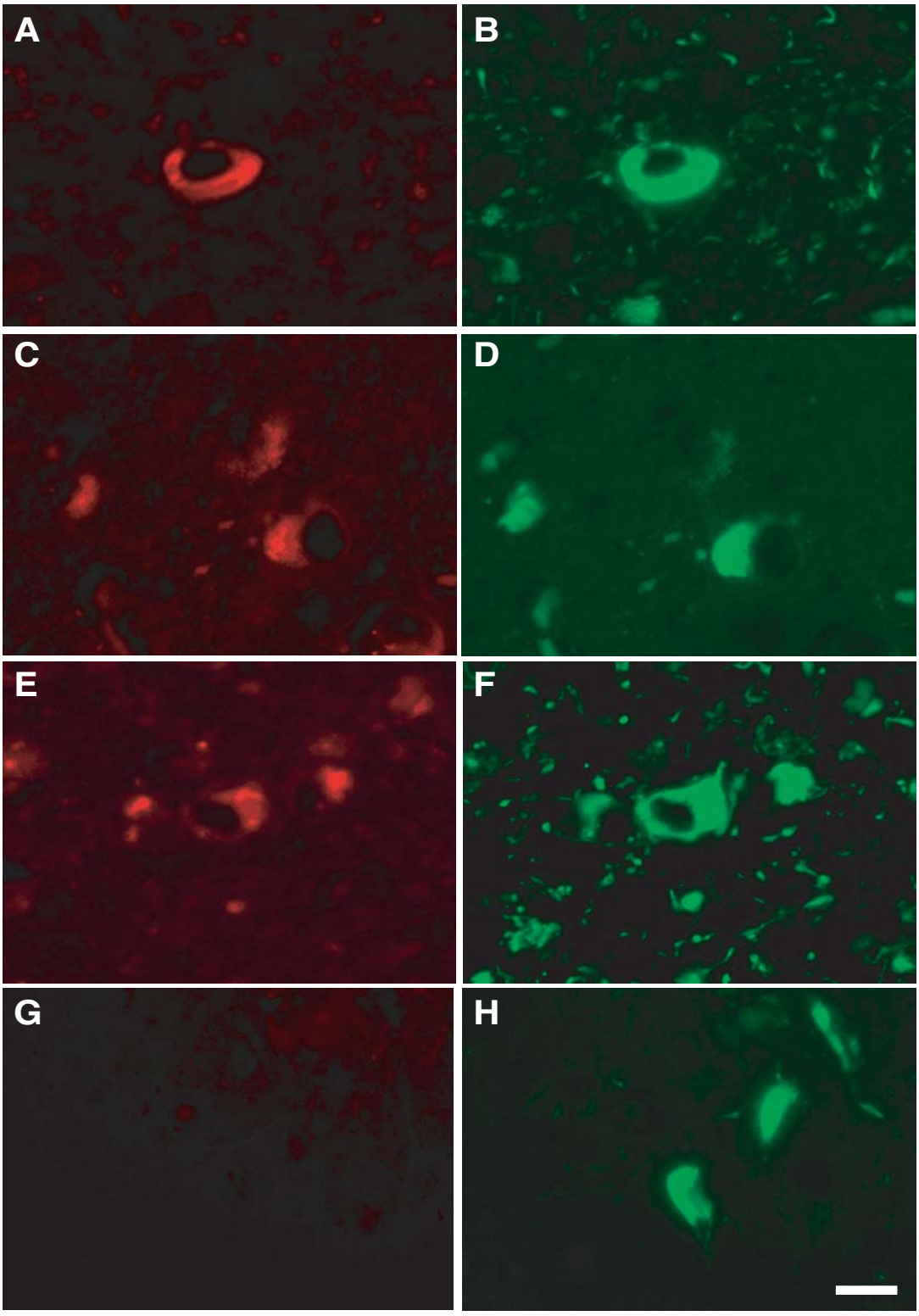

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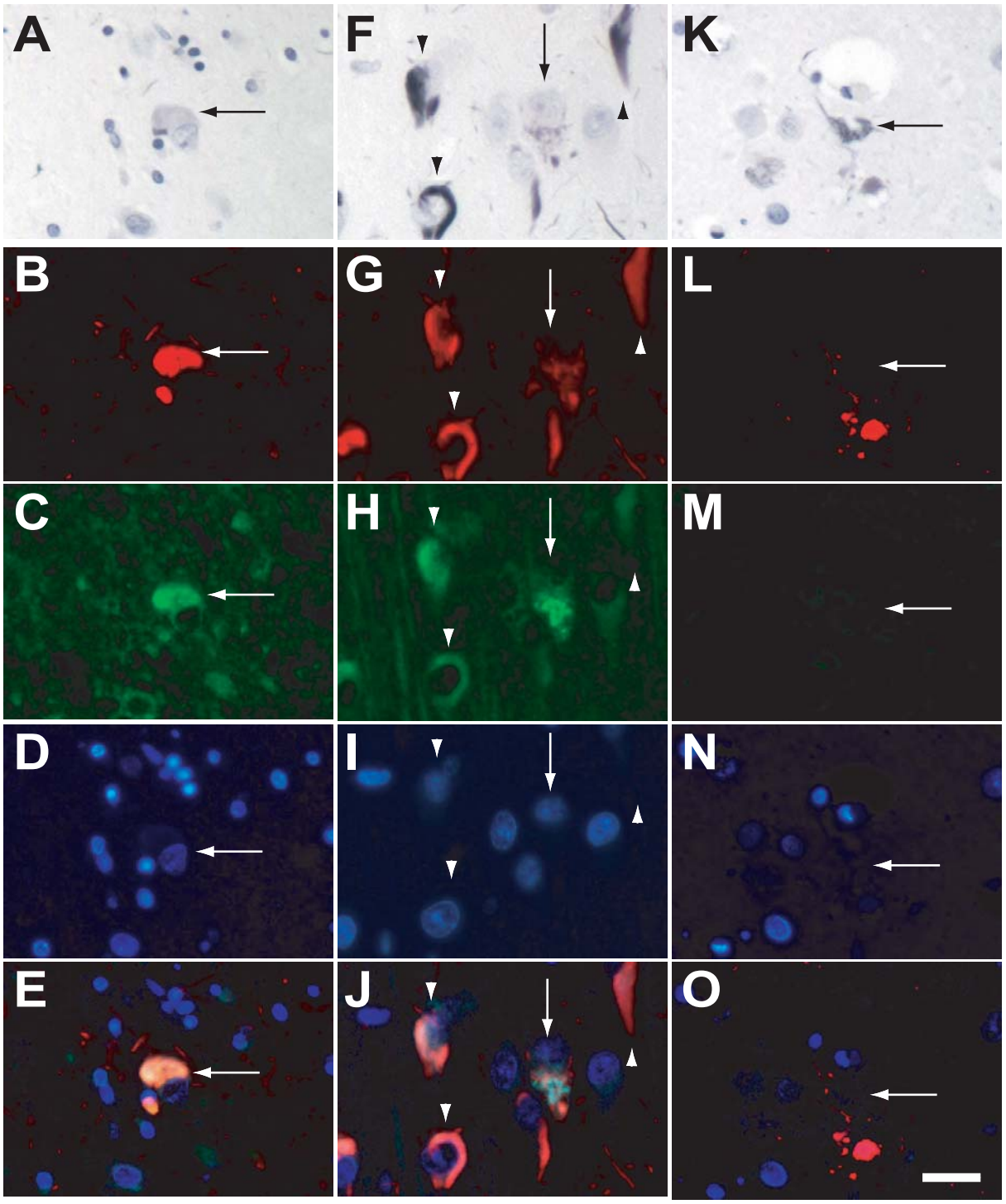

Figure 5

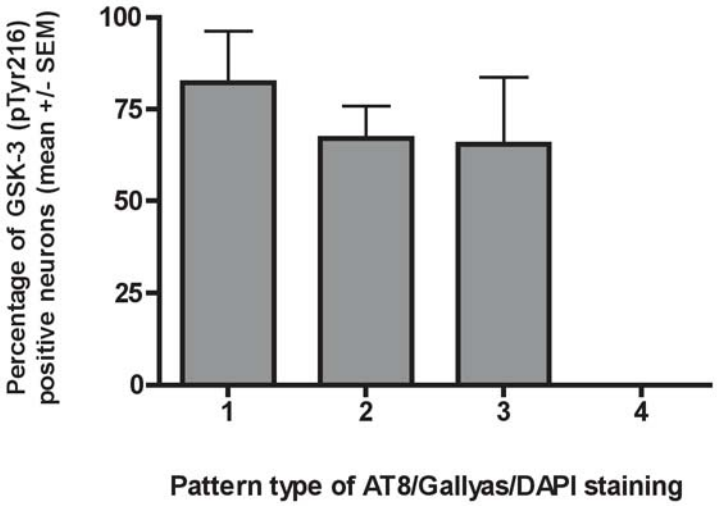

Figure 6