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Alzheimer's disease-type neuronal tau hyperphosphorylation induced by A β oligomers

Fernanda G. De Felice^{1,2}, Diana Wu¹, Mary P. Lambert¹, Sara J. Fernandez¹, Pauline T. Velasco¹, Pascale N. Lacor¹, Eileen H. Bigio³, Jasna Jerecic⁴, Paul J. Acton⁵, Paul J. Shughrue⁵, Elizabeth Chen-Dodson⁵, Gene G. Kinney⁵, and William L. Klein¹

¹Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois, 60208 USA

²Instituto de Bioquímica Médica, Programa de Bioquímica e Biofísica Celular, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21944-590, Brazil.

³Neuropathology Core, Cognitive Neurology and Alzheimer's Disease Center, Northwestern Feinberg School of Medicine, Chicago, IL 60611 USA

⁴Acumen Pharmaceuticals, Inc., South San Francisco, CA 94080 USA

⁵Merck Research Laboratories, Department of Alzheimer's Research, West Point, PA 19486 USA

Abstract

Alzheimer's disease (AD) is characterized by presence of extracellular fibrillar A β in amyloid plaques, intraneuronal neurofibrillary tangles consisting of aggregated hyperphosphorylated tau and elevated brain levels of soluble A β oligomers (ADDLs). A major question is how these disparate facets of AD pathology are mechanistically related. Here we show that, independent of the presence of fibrils, ADDLs stimulate tau phosphorylation in mature cultures of hippocampal neurons and in neuroblastoma cells at epitopes characteristically hyperphosphorylated in AD. A monoclonal antibody that targets ADDLs blocked their attachment to synaptic binding sites and prevented tau hyperphosphorylation. Tau phosphorylation was blocked by the Src family tyrosine kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7(t-butyl)pyrazol(3,4-D)pyrimidine (PP1), and by the phosphatidylinositol-3-kinase inhibitor LY294002. Significantly, tau hyperphosphorylation was also induced by a soluble aqueous extract containing A β oligomers from AD brains, but not by an extract from non-AD brains. A β oligomers have been increasingly implicated as the main neurotoxins in AD, and the current results provide a unifying mechanism in which oligomer activity is directly linked to tau hyperphosphorylation in AD pathology.

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Corresponding author: William L. Klein/ E-mail: wklein@northwestern.edu, Northwestern University, Department of Neurobiology and Physiology, 2205 Tech Drive, Evanston, IL 60208 USA, Telephone 847-491-5510, FAX 847-491-5521, Phone/Fax: (+847) 491-2868.

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Disclosure Statement: WLK is an inventor on ADDLs patents held jointly by Northwestern University, University of Southern California, and Acumen Pharmaceuticals, a biotech company for which he provides consultancy and in which he owns stock. JJ is an employee of Acumen. PJA, PJS, EC-D and GJK are employees of Merck Research Laboratories. Acumen and Merck are involved in the development of ADDLs and related technology for discovery and development of novel Alzheimer's disease diagnostics and therapeutics.

Keywords

Alzheimer's disease; A β oligomers; tau hyperphosphorylation; hippocampal neurons; Src; PI3K

1. Introduction

AD is the most common cause of dementia in aged humans. It is neuropathologically characterized by intraneuronal neurofibrillary tangles consisting of abnormally hyperphosphorylated tau, extracellular accumulation of fibrillar amyloid beta peptide (A β) in senile plaques, and the build-up of soluble A β oligomers (also known as ADDLs) in AD brains [26,53]. There is considerable interest in determining whether these major facets of Alzheimer's pathology may be mechanistically interrelated.

In AD and in the so-called tauopathies, tau undergoes abnormal hyperphosphorylation, which ultimately appears to lead to neurodegeneration [2]. The mechanisms triggering activation of intracellular kinases and tau hyperphosphorylation in AD remain largely unclear, although A β has been implicated. Increased A β levels precede NFTs appearance in AD-affected brain areas [44], while injection of A β fibrils into the brains of non-human primates [13] and of P301L tau transgenic mice [15] induces tau hyperphosphorylation and the formation of neurofibrillary tangles (NFTs). Neurofibrillary degeneration also has been observed in transgenic mice expressing both human amyloid precursor protein (APP) and mutant tau [36] and of mice harboring APP, presenilin and mutant tau transgenes [46]. Additionally, several studies have shown that A β fibrils induce tau hyperphosphorylation *in vitro* [e.g., 8,30,42] and that A β fibrils do not cause degeneration of hippocampal neurons from tau knock-out mice [47], suggesting that tau is one of the major downstream targets of toxic A β .

Although amyloid fibrils found in plaques were originally considered to be responsible for AD pathogenesis, recent evidence indicates that the primary neurotoxic species in AD may actually comprise soluble oligomers of the A β peptide, also known as ADDLs [11,25,29,52]. It has been proposed that these oligomers instigate formation of tangles [20], and increased brain levels of soluble A β correlate with NFT density in AD patients [41]. A β oligomers activate glycogen synthase kinase-3 β [22], one of the kinases that appears to be involved in pathological tau hyperphosphorylation. A recent study has shown that intrahippocampal injection of an anti-oligomer antibody clears both A β pathology and tau pathology in a triple transgenic mouse model harboring mutant human amyloid precursor protein, presenilin 1 and tau [46]. In these mice, extracellular and intracellular A β appear to be in dynamic equilibrium [45]. Additionally, antibodies against A β peptide lead to a decline of soluble A β oligomers, but not insoluble A β , and reduce both glycogen synthase kinase-3 β activation and tau phosphorylation *in vivo* and *in vitro* [39].

We now report direct cell biological evidence that A β oligomers, whether prepared *in vitro* or present in AD brain extracts, stimulate tau hyperphosphorylation at AD-specific epitopes. This hyperphosphorylation is inhibited by antibodies that target pathological but not monomeric forms of A β . The mechanism of oligomer-induced tau phosphorylation depends on binding to specifically-targeted neurons and requires signaling through Src family tyrosine kinases and phosphatidylinositol 3-kinase (PI3K). These findings provide further strong support for the hypothesis [25] that neurologically active A β -derived oligomers, which show a striking elevation in AD-affected brain [14], are the toxins responsible for initiating AD pathogenesis.

2. Materials and Methods

2.1. Materials

A β ₁₋₄₂ was purchased from California Peptide (Napa, CA). Monoclonal antibody 6E10 was from Signet Laboratories (Dedham, MA). Anti-phosphotau antibodies (phosphoepitopes P404, P231 and P181), pre-immune mouse IgG antibody (from serum) and anhydrous DMSO were from Sigma (Sigma Chem. Co., St. Louis, MO). Anti-phosphotau antibody AT8, Coomassie Plus protein assay and SuperSignal West Fento Maximum Sensitivity substrate were from Pierce (Rockford, IL). Cyclophilin B antibody was from Affinity Bioreagents (Golden, CO). PP1 and LY294002 inhibitors were from Biomol International (Plymouth Meeting, PA).

2.2. ADDLs preparation and characterization

A β ₁₋₄₂ was prepared in aliquots as a dried HFIP film and stored at -80 °C as previously described [13,28]. The peptide film was dissolved in neat, sterile DMSO to make a 5 mM solution. The solution was diluted to 100 μ M with phosphate buffered saline (PBS), pH 7.4, and aged overnight at 4 °C. The preparation was centrifuged at 14,000 g for 10 min at 4 °C to remove insoluble aggregates (protofibrils, fibrils), and the supernatants containing soluble A β oligomers were transferred to clean tubes and stored at 4 °C. Protein concentrations were determined using the Coomassie Plus protein assay and BSA as a standard.

Routine characterization of ADDLs preparations was performed by Western immunoblots using NU1, a monoclonal antibody that recognizes trimers, tetramers and high molecular weight oligomers, but not A β monomers [31]. Samples were mixed 1:1 with Tricine sample buffer and resolved on a 10–20% gel with Tris/Tricine/SDS buffer at 120V for 80 min at room temperature. The gel (20 pmoles A β /lane) was electroblotted onto Hybond ECL nitrocellulose using 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.02% SDS, pH 8.3, at 100 V for 1 hr at 4 °C. The blots were blocked with 5% non-fat milk in Tris-buffered saline Tween 20 (TBS-T) (0.1% Tween-20 in 20 mM Tris-HCl, pH 7.5, 0.8% NaCl) for 1 hr at room temperature. Monoclonal antibodies 6E10 or NU1 were diluted (1:5,000) in 5% milk/TBS and incubated with the blots for 90 min at room temperature. Following three 10 min washes with TBS-T, the blots were incubated with HRP-linked anti-mouse IgG (1:40,000 in TBS-T) overnight at 4 °C. The blots were washed 3 times for 10 min with TBS-T, rinsed 3 times with deionized H₂O, developed with SuperSignal West Fento Maximum Sensitivity substrate (1:1 dilution with water) and imaged on a Kodak Image Station.

2.3. A β fibrils preparation

For preparation of fibrils, an aliquot of the dried HFIP film of A β ₁₋₄₂ (see above) was dissolved in neat, sterile DMSO to make a 5 mM solution. This solution was 50-fold diluted in 10 mM HCl in PBS, immediately vortexed for 30 s and incubated for 24 hours at 37 °C.

2.4. Tissue extracts

Samples from frontal cortex and hippocampi from Alzheimer's disease and non-demented control subjects were obtained from the Northwestern Alzheimer's Disease Center Neuropathology Core (Chicago, IL). Soluble extracts from brain tissues were prepared as described previously [14].

2.5. Neuronal cultures

Hippocampal neuronal cultures were prepared and maintained in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA) for 3 weeks as described previously [14]. As indicated in "Results", the cultures were treated for different times with vehicle or

synthetic ADDLs (100 nM or 500 nM), 10 μ M fibrils, or F12-extracted human cortex (1.0 mg protein/ml) from AD or non-demented controls. For studies with AT8, primary hippocampal cultures were prepared from frozen dissociated rat hippocampal cells (Cambrex Corp., East Rutherford, NJ). Cells were thawed and plated according to manufacturer's instructions, maintained in Neurobasal medium supplemented with B27 for 3 weeks and then used for immunocytochemistry studies.

2.6. B103 neuroblastoma cultures

The CNS B103 neuroblastoma cell line was grown in DMEM without phenol red (Gibco), 10% fetal bovine serum (Hyclone), and 1% Pen-Strep (Gibco). Exponentially growing B103 cells were dissociated and plated in 96-well plates at a concentration of 5,000 cells/well. Twenty-four hours after plating, the cells were used to assess tau hyperphosphorylation.

2.7. MTT viability assay

19-day old hippocampal neuronal cultures were used. Cell viability was determined using the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Boehringer Mannheim, Indianapolis, IN). Cells were cultured in 96-well plates and ADDLs or an equivalent amount of vehicle were added directly to the medium. Cells were incubated for 24, 48 or 96 h at 37 °C, and 10 μ l of MTT solution (5 mg/ml of MTT in PBS) was added (to a final concentration of 0.5 mg/ml). The plates were incubated for an additional 4 h and 100 μ l of solubilization solution (10% SDS in 0.01 M HCl) was added and incubated overnight. The amount of formazan dye was quantified at 550 nm, using a microtiter plate ELISA reader (Packard, Meriden, CT). Two independent experiments (each with 6 wells per experimental condition) were carried out with different neuronal cultures. Cell culture solutions were prepared and kept at all times under sterile conditions.

2.8. Western immunoblotting

Primary cultures of E-18 hippocampal neurons were plated on poly-L-lysine coated coverslips and cultured for 20 days (20 DIV). Neurons were maintained for 4 hours at 37 °C in the absence or in the presence of different concentrations of ADDLs or A β fibrils (see "Results"). When present, NU1 antibody was added 30 minutes before addition of ADDLs. The cultures were then rinsed with PBS and lysed in buffer containing 100 mM Tris-HCl, pH 7.5, 1% SDS, 150 mM NaCl, 1 mM EDTA, 20 mM sodium pyrophosphate, 20 mM sodium fluoride, 1 mM sodium orthovanadate and a cocktail of protease inhibitors. Lysates were incubated in a boiling water bath for 5 min. Protein content in the samples was measured by the BCA method using bovine serum albumin as a standard. Samples (10 micrograms of total protein applied per lane) were resolved by SDS-PAGE on 4–20% gels. Cyclophilin B immunostaining was used as an additional loading control to allow direct comparison between the total protein mass applied to different lanes. The gels were transferred to nitrocellulose membranes and the membranes were blocked with 5% non-fat milk in TBS-T overnight, followed by incubation for 24 hours with anti-phosphotau antibodies Ser404 (1: 4,000 dilution), Thr231 (1: 4,000 dilution) or Thr181 (1: 4,000 dilution) and anti-cyclophilin B (1:4,000) at 4 °C. After washing with TBS-T, immunoreactive bands were visualized using peroxidase-conjugated anti-rabbit IgG secondary antibody (1 hour incubation; 1:50,000 dilution) and enhanced chemiluminescence detection (ECL Plus kit, Amersham, Buckinghamshire, England). Densitometric scanning and quantification of the intensities in Western blot bands were carried out using Image J (NIH; Windows version) [1].

2.9. Immunocytochemistry

Primary cultures of E-18 hippocampal neurons were plated on poly-L-lysine coated coverslips and grown for 20 days (20 DIV). Neurons were maintained for 4 hours at 37 °C in the presence of vehicle, ADDLs, A β fibrils, ADDLs + anti-ADDLs monoclonal antibody NU1, ADDLs + PP1, or ADDLs + LY 294002. When present, NU1 antibody, PP1 or LY 294002 were added to the culture media 30 minutes before addition of ADDLs. Alternatively, neurons were maintained for 3 hours at 37 °C in the presence of soluble extracts from AD brains or non-AD controls. Cells were fixed by adding an equal volume of 3.7% formaldehyde (in PBS buffer) to the media for 5 minutes followed by the removal of the entire fix:media solution and replacement with 3.7% formaldehyde for 10 minutes. Cells were rinsed 3 times with PBS, incubated with PBS:10% NGS, 0.1% Triton X-100 for 90 minutes at room temperature and were double immunolabeled by overnight incubation at 4 °C with NU1 (1:1,000) and either pSer404 or pThr231 antibody (1:500) diluted in PBS:10% NGS, 0.1% Triton X-100. Neurons were then rinsed 3 times with PBS and incubated with Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 555 anti-mouse IgG secondary antibodies (1:1000, Molecular Probes, Eugene, OR) diluted in PBS:1% NGS, 1% Triton X-100 for 3 hours at room temperature. Cells were rinsed 5 times with PBS and coverslips were mounted with Prolong Gold mounting media (Molecular Probes). Cells were visualized on a Nikon Eclipse TE 2000-U fluorescence microscope and images were digitally acquired using MetaMorph software (Meta Image Series, Universal Imaging Corporation).

For studies with AT8, B103 neuroblastoma and frozen dissociated rat hippocampal cells were maintained in the presence of 1 μ M biotinylated ADDLs (bADDLs) or vehicle for 1, 6 or 24 hrs at 37°C. After incubation, the cells were washed and fixed with 4% paraformaldehyde (Electron Microscopy Sciences; 16% paraformaldehyde diluted in PBS; 2x 10 min at RT). The cells were permeabilized (4% paraformaldehyde solution with 0.1% Triton-X 100) for 10 min, washed 6 times with PBS, blocked for 1 hr at 37°C with PBS containing 10% bovine serum albumin and then incubated overnight with AT8 antibody (1:500). Cells were washed 3 times with PBS, incubated for 1 hr at RT with Alexa 488-labeled anti-mouse IgG (1:1,000) and Alexa 594-labeled streptavidin (Molecular Probes; 1:500) and washed 5–6 times in PBS. The cell nuclei were then stained with DAPI (1:1,000) according to standard protocols.

Quantitative analysis of the immunofluorescence data was carried out by histogram analysis of the fluorescence intensity at each pixel across the images using Image J (NIH; Windows version) [1]. Briefly, the program analyzes a grayscale image by plots of the intensity histogram and by calculation of average intensity and standard deviation. Appropriate thresholding was employed to eliminate background signal in the images before histogram analysis. The results of the analysis of 20 images acquired under each experimental condition (in three independent experiments with different neuronal cultures) were then combined to allow quantitative estimates of changes in neuronal phosphotau levels.

3. Results

3.1. Biochemical characterization and neurotoxicity of ADDLs

Each ADDL preparation was evaluated for the presence of soluble oligomers by size exclusion chromatography and by SDS-PAGE; gels were processed for silver staining and for western blotting [10,28] using 6E10 (non-selective) or NU1 (oligomer-selective, [31]) monoclonal antibodies. Gels routinely showed A β monomers and SDS-resistant oligomers but no fibrils (Fig. 1A). Ultrafiltration indicated that monomers derived largely from SDS-unstable oligomers [28]; typical ADDL preparations comprised about 40%/60% distribution between molecules >50 kDa and molecules <50 kDa. Absence of fibrillar material was

confirmed (not shown) by non-denaturing gel electrophoresis and atomic force microscopy [10]. For experiments in which A β fibrils were used, Western blots using 6E10 and NU1 revealed that fibrils did not enter the polyacrylamide gel and that such preparations contain monomers that are only recognized by the 6E10 monoclonal antibody (Fig. 1A).

The neurotoxicity of ADDLs was evaluated using the MTT reduction assay in mature hippocampal neuronal cultures (19 DIV) exposed for 24, 48 or 96 hours to different concentrations of ADDLs (Fig. 1B). After 24 hours, no significant toxicity was observed in the presence of 300 nM, 500 nM or 1 μ M ADDLs (based on total A β concentration). At longer incubation times (48 and 96 hours) ADDLs caused a progressive decrease in MTT reduction (Fig. 1B), which can be due to altered trafficking [38] as well as cell death.

3.2. ADDLs induce tau hyperphosphorylation in B103 neuroblastoma cells

Our first experiments examined the effects of A β oligomers on tau phosphorylation in B103 neuroblastoma cells using AT8, an antibody that recognizes tau phosphorylated at residues Ser²⁰² and Thr²⁰⁵ and has been shown to detect abnormal hyperphosphorylated tau in cerebrospinal fluid from AD patients [40]. While vehicle-treated cells (Fig. 2A, B) exhibited low phosphotau immunofluorescence, cells treated with 1 μ M biotinylated ADDLs (bADDLs) for 6 hours (Fig. 2C, D) showed a significant increase in P-tau immunofluorescence (Fig. 2D). A time-course investigation further indicated that the levels of P-tau increased from 1 to 6 hours after bADDLs treatment and were still elevated 24 hours later (Fig. E–H).

We then investigated whether bADDLs also induced tau phosphorylation in rat hippocampal neurons. To this end, we initially used frozen dissociated rat hippocampal cell preparations (Cambrex). Cells were maintained in the presence of 1 μ M bADDLs (Fig. 2I, J) or vehicle (not shown) for 6 hours at 37°C. We observed a marked P-tau immunostaining in a subpopulation of neurons that also had ADDLs bound (Fig. 2I, arrowheads), while cells that did not bind bADDLs had no AT8 staining (2J).

3.3. ADDLs induced neuronal tau hyperphosphorylation at Thr231 and Ser404 in hippocampal neurons

We next carried out more detailed investigations of tau phosphorylation using highly differentiated hippocampal neuronal cultures, prepared in our laboratory from dissociated embryonic rat hippocampus. Following exposure to ADDLs, double-label immunofluorescence microscopy showed high levels of tau phosphorylated at Thr231, which discriminates among AD and non-AD subjects and patients with other forms of dementia [18,19], in neurons with prominent dendritic ADDL binding (detected with NU1, Fig. 2K–M). ADDL binding to synaptic hot-spots in hippocampal neurons is evident in images at higher-magnification (60X objective, Panel L, M). Hippocampal neurons exposed to A β fibrils rather than oligomers also showed elevated P-tau immunofluorescence (Fig. 2N–P). However, although fibrils could be seen attached to neurons, they did not bind in the synaptic pattern observed for ADDLs (Fig. 2O, P).

Oligomer-induced increases in tau phosphorylation were verified by quantitative immunofluorescence microscopy (Fig. 3), with tests extended to an additional p-tau at epitope Ser404, that becomes abnormally phosphorylated in AD brains [18,19]. Vehicle-treated neurons (Fig. 3A and E) exhibited very low phosphotau immunofluorescence, but neurons treated for 4 hours with 500 nM ADDLs showed significantly higher levels in immunofluorescence of P-Ser404 and P-Thr231 tau (Fig. 3B and F, respectively). Neurons treated for 4 hours with 10 μ M A β fibrils also showed an increase in immunofluorescence of P-Ser404 and P-Thr231 tau (Fig. 3C and G, respectively).

3.4. NU1 antibody blocks ADDL-induced tau hyperphosphorylation

Because ADDL preparations may include monomers (Fig.1), we tested whether the stimulation of tau phosphorylation indeed depended on oligomers through selective immunoneutralization experiments [10,31,32]. The antibody used (NU1) discriminates between AD and non-AD cases in immunoblot assays using soluble brain extracts and in immunohistochemistry of brain tissue sections [31]. As shown (Fig. 1A), this antibody has negligible affinity for A β monomers but robustly binds to A β oligomers. NU1 completely blocked the increase in P-Ser404 and P-Thr231 phosphotau levels induced by ADDLs (Fig. 3D–H). Control IgG had no impact on A β oligomer-induced tau hyperphosphorylation (Fig. 3K–M). These results confirm that the tau hyperphosphorylation stimulated by soluble ADDL preparations is indeed oligomer-induced. Tau hyperphosphorylation induced by 10 μ M A β fibrils (Fig. 3N) was partially blocked (Fig. 3O), consistent with shared epitopes between oligomers and fibrils. Quantitative image analysis showed that ADDLs and A β fibrils increased tau hyperphosphorylation by approximately 3-fold at Ser404 and 2.8-fold at Thr231 (Fig.3I and J, respectively); ADDL-stimulated levels of phosphotau were completely blocked (Fig. 3I and J) and fibril-stimulated phosphotau partially blocked (Fig. 3P). Control IgG treatment gave levels of phosphotau indistinguishable from those found with ADDLs alone (Fig. 3P).

We note that these observed increases in tau phosphorylation occurred well before neuronal death (Fig. 1B), consistent with this pathology representing an early stage in neurodegeneration.

Verification of the findings from immunofluorescence microscopy was provided by Western blot analysis of hippocampal neuronal lysates with P404, P231 and P181 anti-phosphotau antibodies. A 4 hour exposure to 500 nM ADDLs resulted in a significant increase in tau phosphorylated at the three epitopes, to levels similar to those observed after exposure to 10 μ M A β fibrils (Fig. 4A–D). Addition of 100 nM ADDLs to the medium had no effect on tau phosphorylation levels in hippocampal neuronal cultures (Fig. 4). It could not be determined whether the steep concentration dependence reflects cooperative effects localized at the cellular level or the consequence of structural rearrangements of oligomers in the culture medium; no structural changes were observed in Western blots (not shown), but newly-formed large oligomer species typically are SDS unstable [7]. Additionally, Western blots of ADDLs incubated for 4 hr at 37°C in conditioned medium provided no evidence for the formation of fibrils (data not shown). The difference between the values representing the increase in P-tau obtained by biochemical analysis (Fig. 4) and by immunocytochemistry (Fig. 3) can be explained by a partial loss in previously phosphorylated sites that may occur during the processes of cell lysis and boiling of the cells lysates. Furthermore, the blotting analysis takes in consideration the overall population of cells, while the immunocytochemistry analysis takes in consideration a representative randomly chosen population of cells. Western blot analysis also confirmed that NU1 completely blocked ADDL-induced tau hyperphosphorylation (Fig. 4A–D).

3.5. Involvement of Src family protein kinases and PI3 kinase on ADDL-induced tau hyperphosphorylation

In order to identify possible cellular signaling pathways involved in tau hyperphosphorylation in response to ADDLs, we next investigated the involvement of Src family protein kinases and phosphatidylinositol 3-kinase (PI3K) activation, both of which have been implicated in the action of fibrillar A β [55]. Interestingly, we found that tau hyperphosphorylation at Thr231 was completely blocked by the Src family tyrosine kinase inhibitor, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazol(3,4-D)pyrimidine (PP1), and by the phosphatidylinositol 3-kinase inhibitor, LY 294002 (Fig. 5). Inhibition occurred even though

ADDLs were still bound to cell surfaces, indicating that those kinases are involved in signal transduction coupling between ADDL binding and tau hyperphosphorylation. Fig. 5E shows a typical NU1 labeling of ADDL-treated neurons. In line with our previous report [14], numerous small puncta corresponding to ADDL-attachment sites are seen in the neurites. NU1 completely prevented ADDL binding to synaptic hot-spots (Fig. 5N), as shown by the absence of dendritic puncta. Moreover, the presence of large extracellular aggregates in NU1-treated cultures (Fig. 5N) suggests that the antibody effectively sequesters ADDLs and prevents their interactions with neurons (Fig. 5O). No inhibition of ADDL binding was associated with PP1 and LY 294002 (Fig. 5H, I and K, L respectively), but both kinase inhibitors effectively blocked ADDL-induced tau hyperphosphorylation (Fig. 5G and J).

3.6. Soluble extract from AD brain induces tau hyperphosphorylation in neurons in culture

Direct cell biological evidence linking the presence of A β oligomers to neuropathology in AD is lacking. ADDLs previously were shown to occur in soluble extracts of AD brain [14] and found to exhibit the same patterns of attachment to cultured hippocampal neurons as synthetic ADDLs, binding to synapses in small clusters with ligand-like specificity [14]. We therefore next investigated whether extracts from AD brains could induce AD-type tau hyperphosphorylation. Consistent with the results obtained with synthetic ADDLs, we found that treatment of mature hippocampal neuronal cultures with a soluble AD brain extract led to a significant increase in P231 tau phosphorylation (Fig. 6D) compared to cultures treated with a non-AD brain extract (Fig. 6A). Importantly, pre-incubation of AD brain extracts with NU1 significantly blocked the increase in Thr231 phosphotau immunofluorescence (Fig. 6G), establishing the tau hyperphosphorylation was induced by A β oligomers in the AD brain extracts. NU1 also prevented the binding of brain-derived ADDLs to synaptic hot-spots (Fig. 6H, I). In NU1-treated cultures, the presence of large extracellular aggregates indicates that the antibody sequesters ADDLs and prevents their interactions with neurons (Fig. 6I). Attempts to recover AD brain-derived ADDLs following immunoprecipitation for toxicology experiments were not successful.

4. Discussion

There is increasing interest in the possibility that soluble A β oligomers (ADDLs) could be the proximal neurotoxins in AD [49]. It therefore is important to determine directly whether neurons exposed to oligomers undergo pathological changes characteristic of AD brain. In the current study, we have verified that a significant pathological consequence of ADDL binding to CNS neurons is tau hyperphosphorylation. ADDL-induced tau hyperphosphorylation was found at epitopes characteristically phosphorylated in AD, namely Ser404, Thr231, Thr 181, Ser 202 and Thr 205 [18,19,40]. Importantly, we show that tau hyperphosphorylation is induced not only by synthetic ADDLs, but also by soluble extracts containing ADDLs obtained from AD brains. These findings provide strong evidence for the role of human A β oligomers in the induction of tau hyperphosphorylation in AD.

We have previously shown that not all neurons in culture are targeted by ADDLs [28]. As shown here, this binding to specific neurons is required for ADDL-induced tau hyperphosphorylation. Image analysis revealed a marked increase in P-tau levels in a subpopulation of neurons that exhibited ADDL binding (Fig. 2 I–M), but not in neurons that were ADDL-free. This cell-to-cell specificity in binding and evoked pathology would presumably provide a basis for selective neuronal vulnerability in AD. The molecular basis for this specificity depends on ADDL attachment to trypsin-sensitive sites on neuronal membranes [29]. The identity of these sites is currently under investigation.

Consistent with the requirement for ADDL binding to neuronal surfaces, tau hyperphosphorylation induced by synthetic ADDLs (Fig. 3, 4) or soluble AD brain extracts (Fig. 6) was blocked by NU1. This monoclonal antibody targets pathological assemblies of A β and blocks their binding to cell surfaces [31]. Use of monoclonal antibodies that target and neutralize A β oligomers remains a promising alternative to active vaccination as a strategy for AD therapeutics. Although A β immunization approaches originally aimed to clear amyloid plaques [4,24,43,50], the mounting evidence suggests that immunotherapy should be directed at blocking the early toxic action of A β oligomers, maximizing the chances of slowing or reversing the cognitive and memory deficits in AD. Transgenic mouse models of AD responds to anti-A β monoclonal antibodies with improved memory performance prior to any impact on insoluble A β deposits [12,27]. Recent studies have extended these investigations to pathology. A single intrahippocampal injection of an anti-oligomer antibody is sufficient to clear both A β pathology and tau pathology in a triple transgenic mouse model harboring mutant human amyloid precursor protein, presenilin 1 and tau [46]; related studies showed extracellular A β was cleared before intracellular A β [45]. In addition, it has been shown that other antibodies can produce a decline of soluble A β oligomers, but not insoluble A β , and reduce both glycogen synthase kinase-3 β activation and tau phosphorylation both *in vivo* and *in vitro* [39]. Along with the present results, these observations indicate that anti-oligomer antibodies may protect against tau pathology and neuritic dysfunction in AD.

We note that the current studies maximized ADDL concentrations in order to investigate effects that might be cumulative and potentially take longer times *in vivo*. Most likely, the active species are 12mers, which have been shown to accumulate in AD affected human brain [14] and more recently in tg-mice models [34]. The 12mers, which appear to represent only a fraction of the ADDL prep, can be readily detected in Western blots of synthetic preparations because of the sensitivity of the NU1 antibody (Fig. 1), but detection in silver stain requires further concentration [14]. Recent procedures have been described that enhance formation of SDS-stable 12mers from synthetic A β [7]. The ability of crude AD brain extracts to stimulate tau hyperphosphorylation in an antibody-sensitive manner suggests it would be possible to immunoisolate the neurologically active species, but efforts to recover active toxins has so far been unsuccessful.

With respect to the cellular mechanism, ADDL-induced tau phosphorylation was found to be blocked by the Src family tyrosine kinase inhibitor, PP1, and by the PI3K inhibitor, LY294002 (Fig. 5). In accord with these results, phosphorylation of neuronal proteins including tau [30] and focal adhesion kinase (FAK) [56] has been shown to be induced by fibrillar A β and blocked by PP1 and LY294002 [55]. Phosphorylation of FAK is believed to be central in a number of important signaling pathways involving Src family kinases, PI3K and Akt. FAK serves as a regulated adaptor protein, recruiting other proteins by autophosphorylation of Tyr397, a high-affinity binding site for SH2 domains of Src proteins including Fyn [57] and PI3K [9,48]. Binding of these kinases to FAK in turn enables them to phosphorylate tyrosine residues in the C-terminal region of FAK and also other cytoskeletal proteins associated with FAK. Phosphorylation is followed by recruitment of other proteins, resulting in the formation of multiprotein complexes that trigger a number of different signaling pathways. For example, activation of Fyn/FAK/PI3K/MAPK/extracellular signal-regulated kinase (ERK) pathways is associated with protective (e.g., anti-apoptotic, neuritogenic) responses under normal physiological conditions [e.g., 23,33,51,54]. Although previous studies suggest that activation of PI3K/Akt may protect against neuronal death induced by A β [e.g. 3,37], those same signaling pathways also have been reported to be activated by A β [e.g., 5,16,21,57]. Therefore, it is possible that ADDL binding to neuronal target receptors leads to aberrant activation of trophic signaling and to an incomplete set of downstream events that lead to tau hyperphosphorylation and neuronal

dysfunction. In this regard, recent studies suggest a potential role of aberrant control of Akt and PI3K signaling in AD. For example, increased Akt activation and hyperphosphorylation of critical Akt substrates has been demonstrated in AD brain [17]. Moreover, it has been suggested that excessive activation of the PI3K/Akt pathway contributes to neuronal degeneration in a mouse model of Niemann-Pick type C [6], a neurodegenerative disease also characterized by the presence of neurofibrillary tangles. Furthermore, transient PI3K inhibition has been shown to protect neurons from oxidative stress via suppression of ERK activation, suggesting that the PI3K pathway may serve opposing roles, acting at distinct kinetic phases to either promote or limit a slowly developing program of cell death [35].

ADDLs have previously been shown to trigger rapid synaptic dysfunction, blocking LTP and the reversal of LTD while inducing aberrant expression of the memory-linked protein Arc [13,27,28]. Recently, ADDLs have also been shown to induce excessive generation of reactive oxygen species [11]. These effects of ADDLs seem likely to be related to early AD memory failure. Here we show that selective binding of ADDLs to neurons also stimulates tau hyperphosphorylation, a major facet of AD pathology. The properties of ADDLs thus appear capable of accounting for key features of AD pathology and cognitive failure, consistent with the unifying hypothesis that soluble oligomers of A β act as proximal neurotoxins in AD.

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Fig. 1. Biochemical characterization and neurotoxicity of ADDLs. A, SDS-PAGE separation followed by Western blot with 6E10 and NU1 shows that ADDLs preparations contain a mixture of monomers, trimers, tetramers and higher molecular weight oligomers. Western blot with 6E10 and NU1 shows that A β fibril preparations contain A β oligomers, that can only be detected by 6E10 antibody. B, Time-dependent toxicity of ADDLs to hippocampal neurons. Mature hippocampal E-18 cultures (19 days *in vitro*) were incubated with ADDLs or vehicle for 24, 48 or 96 hours. Values represent means $\pm$ SD of the relative absorbance measured using 6 wells for each experimental condition for three independent experiments.

Fig. 2.

ADDLs induce tau hyperphosphorylation in B103 neuroblastoma cells B103 neuroblastoma cells (Panels A–D) were maintained for 6 hours at 37 °C in the presence of vehicle (Panels A, B) or 1 μM biotinylated ADDLs (Panels C, D). Panels A and C shows DAPI staining of vehicle (A) or biotinylated ADDLs treated (C) cells. Panels B and D show cells treated with vehicle and biotinylated ADDLs, respectively, for 6 hours and immunostained with AT8 P-tau antibody. A time-course investigation on ADDL-induced tau phosphorylation is shown in Panels E–H. Panels I and J: Primary hippocampal cultures prepared from frozen dissociated rat hippocampal cells (21 days *in vitro*) were maintained for 6 hours at 37 °C in the presence of vehicle (Panel I) or 1 μM biotinylated ADDLs (Panel J). ADDLs-induced tau phosphorylation (AT8 labeling, panel J arrowheads) is detected in cells that are also labeled with ADDLs (Panel I, arrowheads). Panel K shows a typical image of Thr231 tau immunofluorescence in mature hippocampal neurons treated with 500 nM ADDLs. Double labeling using an ADDL specific antibody (Panel L) reveals that only the neuron that has ADDLs bound shows high P-tau fluorescence (Panel K, M). A cell that does not have ADDL labeling (circled in Panel L and indicated by an arrow in Panel M) also does not have P-tau fluorescence. Panel N shows a typical image of Thr231 tau immunofluorescence in mature hippocampal neurons treated with 10 μM Aβ fibrils. Double labeling using NU1 (Panel O, P) reveals the presence of large extracellular aggregates that are not specifically bound synapses.

Fig. 3.

ADDL-induced neuronal tau hyperphosphorylation at Ser404, Thr231 and Thr181 is blocked by NU1 antibody. Primary cultures of E-18 hippocampal neurons (20 days *in vitro*) were maintained for 4 hours at 37 °C in the presence of vehicle, 500 nM ADDLs, 10 μM Aβ fibrils or 500 nM NU1 + 500 nM ADDLs, as indicated. When present, NU1 antibody was added 30 minutes before addition of ADDLs. Panels A–D, P-Ser404 tau immunofluorescence in mature hippocampal neurons treated with vehicle (Panel A), 500 nM ADDLs (Panel B), 10 μM Aβ fibrils (Panel C) or 500 nM NU1 + 500 nM ADDLs. Panels E–H and K–O, P-Thr231 tau immunofluorescence in mature hippocampal neurons treated with vehicle (Panel E), 500 nM ADDLs (Panel F), 10 μM Aβ fibrils (Panel G or N), 500 nM NU1 + 500 nM ADDLs (Panel H), 500 nM IgG + 500 nM ADDLs (Panel M) or 10 μM Aβ fibrils + 500 nM NU1 (Panel O). Cells were immunostained for tau as described in “Methods”. In order to allow direct comparison of tau immunofluorescence, identical conditions and exposure times for image acquisition were employed for the different experimental conditions. This explains the faint appearance of the images of control cultures. Scale bar corresponds to 20 μm. Panels I and J show, respectively, quantitative analysis of P-Ser404 and P-Thr231 tau immunofluorescence obtained in at least three separate experiments (60 images analyzed per experimental condition) using Image J software (NIH Windows version). * indicates a statistically significant ($p < 0.005$) increase compared to control cultures.

Fig. 4.

NU1 antibody prevents ADDL-induced tau hyperphosphorylation. Primary cultures of E-18 hippocampal neurons (20 days *in vitro*) were maintained for 4 hours at 37 °C in the presence of vehicle, 100 nM ADDLs, 500 nM ADDLs, 10 μM Aβ fibrils or 500 nM NU1 + 500 nM ADDLs as indicated. Panel A, Western blots showing the immunoreactivity for 2 different phosphorylated tau epitopes in hippocampal culture lysates. Identical protein amounts (10 μg) were applied to each lane. As a loading control, cyclophilin B was used to allow direct comparison between the total protein mass applied to different lanes. Panels B–D shows the quantification of the ratio between phosphorylated tau at Ser404/cyclophilin B (Panel B), Thr231/cyclophilin B (Panel C), Thr181/cyclophilin B (Panel D). Bars represent means ± SD values from densitometric analysis of the bands obtained in four separate experiments using Image J software (NIH Windows version). ** indicates a statistically significant ($p < 0.007$) and * indicates a statistically significant ($p < 0.05$) increase compared to control cultures.

Fig. 5.

PP1 and LY 294002 block ADDLs-induced tau hyperphosphorylation. Primary cultures of E-18 hippocampal neurons (20 days *in vitro*) were maintained for 4 hours at 37 °C in the presence of vehicle, 500 nM ADDLs, 500 nM NU1 + 500 nM ADDLs, 10 μ M PP1 + 500 nM ADDLs or 5 μ M LY 294002 + 500 nM ADDLs. When present, NU1 antibody, PP1 and LY 294002 were added 30 minutes before addition of ADDLs. PP1 and LY 294002 were added again immediately before ADDLs addition. Double labeling using P-Thr231 (green) and NU1 (red) antibodies in neurons treated with vehicle (Panels A and B), 500 nM ADDLs (Panels D and E), 10 μ M PP1 + 500 nM ADDLs (Panels G and H), 5 μ M LY 294002 + 500 nM ADDLs (Panels J and K) or 500 nM NU1 + 500 nM ADDLs (Panels M and N). Scale

bar corresponds to 20 μm . Panel P shows quantitative analysis of P-Thr231 tau immunofluorescence for neurons treated with vehicle, 500 nM ADDLs, 10 μM PP1 + 500 nM ADDLs, 5 μM LY 294002 + 500 nM ADDLs, 500 nM NU1 + 500 nM ADDLs obtained in three separate experiments (60 images analyzed per experimental condition) using Image J software (NIH Windows version). * indicates a statistically significant ($p < 0.005$) increase compared to control cultures.

Fig. 6.

Soluble extract from AD brain containing ADDLs induces neuronal tau hyperphosphorylation *in vitro*. Primary cultures of E-18 hippocampal neurons (20 days *in vitro*) were maintained for 3 hours at 37 °C in the presence of aqueous soluble extracts from AD- or non-AD brains. When present, NU1 antibody was added 30 minutes before addition of soluble brain extracts. Double labeling using anti-P-Thr231 (green; Panels A, D and G) and NU1 (red; Panels B, E and H) antibodies are shown for neurons treated with 1 mg/ml non-AD soluble extract (Panels A–C), 1 mg/ml AD soluble extract (Panels D–F), or 1 mg/ml AD soluble extract + 500 nM NU1 (Panels G–I). Merge images of phosphotau and NU1 labeling for neurons treated with non-AD soluble extract and soluble AD extract are shown

in Panels C, F and I, respectively. Scale bar corresponds to 20 μ m. Panel J shows quantitative analysis of P-Thr231 tau immunofluorescence in neurons treated with 1 mg/ml non-AD soluble extract, 1 mg/ml AD soluble extract or 1 mg/ml AD soluble extract + 500 nM NU1 obtained from three separate experiments (60 images analyzed per experimental condition) using Image J software (NIH Windows version). * indicates a statistically significant ($p < 0.02$) increase compared to cultures exposed to Non-AD extract. # indicates a statistically significant ($p < 0.03$) decrease compared to cultures exposed to AD soluble extract.