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Activated c-Jun is present in neurofibrillary tangles in Alzheimer's disease brains

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Abstract

Alzheimer's disease (AD) pathology is characterized by the presence of insoluble β-amyoid deposits and neurofibrillary tangles containing hyperphosphorylated tau. Increased expression of the immediate early gene product c-Jun has also been reported in post-mortem AD brains, and the presence of upstream regulators of c-Jun has been described in tangle formations. Here, we report the presence of c-Jun specifically phosphorylated on ser-63, but not ser-73, in tangle-bearing neurons and in 'late-stage' extracellular tangles in AD brains. Western blot analysis confirmed the presence of c-Jun phosphorylated on ser-63 but not on ser-73 in AD brain tissue. The expression of differentially phosphorylated c-Jun in the AD brain may reflect the contradictory roles of these phosphorylation sites in neurons. Furthermore, the inappropriate sequestration of phosphorylated c-Jun in tangles in AD brains may contribute to AD pathology and neurodegeneration.

Keywords: Neurofibrillary tangle; Phosphorylation; Transcription factor

The neurological hallmarks of Alzheimer's disease (AD), the most common cause of dementia, are neurofibrillary tangles containing hyperphosphorylated tau protein, and senile plaques consisting mostly of the amyloidogenic 42 amino acid peptide β-amyloid 1-42. As well as the appearance of tangles and plaques, AD is characterized by neuronal cell loss that originates in the medial temporal lobe which then extends into the hippocampus, temporal cortex and into the frontal and parietal regions and subcortices [8].

Previous studies have identified an increase in expression of the activator protein-1 (AP-1) transcription factor c-Jun in AD brains [1,2,18]. c-Jun is an inducible transcription factor known to play a key role in both nerve cell death and survival, depending on contextual expression [28] and is phosphorylated most efficiently by c-Jun N-terminal kinases (JNK) on ser-63 and ser-73 [27,30]. Phosphorylation of c-Jun has also been ascribed to extracellular related kinase (ERK) 1/2 activity in some cell types as well as to p38 [6,26,27,30,37]. Phosphorylation of c-Jun is required to enhance its ability to promote transcription from target genes, including *c-jun* itself [3]. An increase in the presence of the following upstream activators of c-Jun in AD brains has previously been noted: JNK, ERK, p38, JNK kinase 1 (JKK), ERK kinase 1 (MEK1) and mitogen activated protein kinase (MAPK) kinase 6 (MKK6) [13,23,24,33,41–43,45,46]. In addition, JNK, ERK and p38 activity is implicated in tau hyperphosphorylation and the resulting generation of insoluble tau aggregates in AD and associated tauopathies [4,25,44]. We have extended previous analyses to investigate the expression of two species of phosphorylated c-Jun, c-Jun-ser^{63} and c-Jun-ser^{73} (c-Jun phosphorylated on ser-63 and ser-73, respectively), the down-stream targets of the JKK/JNK and MKK/MEK/ERK kinase cascade, in post-mortem AD brain tissue, compared with neurologically normal cases.

For this study human brain tissue was obtained from the Neurological Foundation of New Zealand Human Brain Bank (Department of Anatomy with Radiology, The University of Auckland, Auckland, New Zealand). All protocols used were approved by The University of Auckland Human Subjects Ethics Committee and all cases used were diagnosed by Dr. B. Synek (Neuropathologist at Auckland Hospital) to exclude other brain

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Table 1
Human cases used in this study

Case	Age (years)	Sex	Post-mortem delay (h)	Cause of death
Neurologically normal cases (controls)				
3070	84	F	7	Ischaemic heart disease
4229	76	F	21	Ischemic heart disease
4236	76	F	18	Ischaemic cardiomyopathy
H103	70	M	5	Myocardial infarction
H104	69	M	14	Ischemic heart disease
H109	81	M	8	Coronary atherosclerosis
H112	79	M	8	Bleeding stomach ulcer
H123	78	M	7.5	Ruptured aortic aneurism
H127	59	F	21	Pulmonary emobolism
H132	63	F	12	Ruptured aorta
Alzheimer's disease cases				
AZ23	71	F	10	Broncho-pneumonia
AZ25	73	M	47	Broncho-pneumonia
AZ28	75	F	8	Pneumonia
AZ29	76	F	20	Pneumonia
AZ37	83	M	4	Broncho-pneumonia
AZ39	74	M	12	Pseudomonas Bacteraemia
AZ42	60	M	7	Alzheimer's disease
AZ52	68	F	36	Broncho-pneumonia
AZ53	85	F	2	Broncho-pneumonia
AZ54	84	M	3	Broncho-pneumonia
AZ55	51	M	4	Broncho-pneumonia
AZ57	82	F	14.5	Broncho-pneumonia

pathology and confirm control cases and AD (diagnosed according to the CERAD neuropathology protocol [19]). For analysis of c-Jun-ser^{63} and c-Jun-ser^{73} immuno-reactivity in AD post-mortem tissue two methods were employed, immunohistochemistry and Western blot analysis. Tissue from AD cases was obtained from 12 subjects (average age 73.5 years; range 51–85 years; average post-mortem interval 14 h); for control cases, tissue was obtained from 10 subjects (average age 74 years, range 59–84 years; average post-mortem interval 12 h). Details of each case are listed in Table 1.

Where possible, the forebrain of each brain was divided in half in order to obtain fixed tissue and unfixed tissue from the same case. Blocks of tissue, including the middle temporal gyrus (MTG) and hippocampus, were cut from the unfixed half of the forebrain and quickly frozen in crushed dry ice before storing at −80 °C. For preparation of fixed tissue for immunohistochemical procedures, the other hemisphere was perfused with formalin (15% in 0.1 M PB) through the cerebral arteries before blocks were cut from the same areas as the fresh frozen side. Blocks were further fixed in 15% formalin for 24 h, before immersion in sucrose solution (20% in 0.1 M phosphate buffer; PB) for 2 days, and then transferred to 30% sucrose for a minimum of 2–3 days. For long-term storage, blocks were snap frozen with crushed dry ice and kept at −80 °C.

Free-floating sections (50 μm) were cut from frozen fixed blocks using a sliding microtome (Leica) and soaked overnight in phosphate-buffered saline containing 0.2% Triton X-100 (PBS-T; Sigma, St. Louis, MO) at 4 °C. The following day, sections were again washed in PBS-T then incubated in 50% methanol containing 1% H_2O_2 for 20 min. The tissue was then washed twice in PBS-T and left in a third wash for 6–8 h at room temperature. The sections were incubated with primary antibodies (primary antibody to c-Jun-ser^{63} was a mouse monoclonal antibody obtained from Santa Cruz Biotechnology, Santa Cruz, CA, 1:500; the primary antibody to c-Jun-ser^{73} was a rabbit polyclonal antibody obtained from Cell Signalling Technology, Beverly, MA, 1:250; and the primary antibody to tau was a mouse monoclonal from DAKO, Denmark, 1:10000) for 48–72 h at 4 °C with gentle shaking. The primary antibodies were then washed off with PBS-T and the sections then incubated with the appropriate species-specific biotinylated secondary antibodies (Sigma; both at 1:500) for 24 h at room temperature. Following this, sections were again washed and then incubated with ExtraAvidin™ (Chemicon, Temecula, CA; 1:1000) for 4 h at room temperature. Finally, sections were washed before being immersed in DAB solution (0.5 mg/mL, in 0.1 M PB, with 0.01% hydrogen peroxide; BDH, Poole, Dorset, UK; added just before use) for 10–20 min until a brown reaction-product was visualised. Sections were washed three times in PBS before mounting onto chrome-alum coated slides, air dried overnight then immersed briefly in water and dehydrated through a graded alcohol series. Sections were coverslipped using Hystomount (BDH).

For Western blot analysis, tissue (0.1–0.2 g) was cut from unfixed frozen sections using a cryostat and 1 mL of homogenisation buffer (150 mM sucrose, 15 mM Hepes buffer pH 7.9, 60 mM KCl, 5 mM EDTA, 1 mM EGTA, with 1 Complete mini tab; Roche, per 10 mL buffer) was added. Tissue was homogenised briefly and Triton X-100 was added to a final concentration of 1% before incubation on ice for 1 h. Homogenates were then centrifuged for 10 min at 14,000 rpm (4 °C) and the supernatant retained and stored at −80 °C until required. The protein concentration of homogenates was determined using the Bio-Rad D_C Protein assay and 50 μg of each sample was electrophoresed. Laemlli buffer (Sigma) was added to samples before being heated to 95 °C for 10 min prior to loading onto 4–12% Bis-Tris acrylamide gels (NuPAGE® Novex, Invitrogen, Carlsbad, CA). Blots were electrophoresed, transferred and processed for visualisation of protein bands using standard techniques.

Using immunohistochemistry, sections from the middle temporal gyrus of AD and control brains were examined. The structures immuno-stained by the primary antibody to c-Jun-ser^{63} in the AD tissue resembled tau-positive neurofibrillary tangles and neurites observed in post-mortem AD brains [11]. c-Jun-ser^{63}-positive tangles were found throughout the gray matter of the middle temporal gyrus samples (Fig. 1). The intensity of the immuno-labelling varied between cases and structures. Most tangles were densely stained fibrillar structures with no nuclei (Fig. 1A); however, several cases also contained peri-nuclear threads within cells containing intact nuclei as shown in Fig. 1B. Some neurons with fainter c-Jun-ser^{63}-immuno-staining containing intact nuclei were also observed. The primary antibody to c-Jun-ser^{63} also detected a proportion of neuritic threads and dystrophic neurites. No staining was detected in either AD or control cases with the primary antibody to c-Jun-ser^{73}. Controls were performed in which the primary antibody was omitted. No