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STOX1A induces phosphorylation of tau proteins at epitopes hyperphosphorylated in Alzheimer's disease

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HIGHLIGHTS

- STOX1A induces tau phosphorylation.
- Stably transfected STOX1A SH-SY5Y cells have enhanced CDK1 activity levels.
- Tau phosphorylation levels are increased during mitosis in SH-SY5Y cells.

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ABSTRACT

Intraneuronal fibrillary tangles are a major hallmark of several neurodegenerative diseases including Alzheimer's disease. The major constituents of these hallmarks are hyper-phosphorylated tau. In this study we used a neuronal cellular model which over-expresses transcription factor STOX1A in combination with the longest human tau isoform to test the effect of STOX1A on tau phosphorylation. Our results show that STOX1A induces phosphorylation of the longest human tau isoform at phospho-epitopes typically found in neurofibrillary tangles in Alzheimer's disease. In conclusion, our results show a STOX1A-dependent effect on tau phosphorylation found in neurodegenerative diseases such as Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease with severe neurodegeneration and cognitive impairment. Classically, AD pathology is characterized by amyloid beta (Aβ) containing senile plaques and intra-neuronal neurofibrillary tangles (NFT). While Aβ is formed by the sequential cleavage of the amyloid precursor protein (APP), intra-neuronal aggregates of NFT are densely packed networks of the hyper-phosphorylated insoluble microtubule associated protein tau (MAPT) [6,11,16]. Normal tau function, which has been shown to be important in stabilizing microtubules, neurite outgrowth and axonal transport, is tightly regulated by a balance between phosphorylation and dephosphorylation [24]. However, hyper-phosphorylation of tau as found in AD and other related neurodegenerative diseases causes failure of tau to bind and stabilize microtubules resulting in neuronal dysfunction [1,4,8,25,33]. Therefore, much effort has been made to identify phosphorylated tau residues to understand the mechanisms that lead to NFT formation as found in AD and other similar neurodegenerative diseases. As a result, currently more that 30 serine/threonine residues have been directly identified in NFT-tau with the use of immunologic studies and mass spectroscopy [2,20,21,28]. Furthermore, several tau protein kinases and the major tau protein phosphatase have been characterized which are capable of regulating the phosphorylation status at many of these tau residues [15,38].

Recently, Storkhead box 1A (STOX1A), a transcription factor structurally and functionally related to the forkhead family of transcription factors has been shown to be expressed abundantly in the brain and to correlate with the severity of Late Onset Alzheimer Disease (LOAD, Braak 3–6) [37]. Transcription factors like STOX1A regulate the expression of hundreds of downstream target genes and we therefore speculated that STOX1A potentially influences transcriptional networks which are associated with phosphorylation and/or de-phosphorylation of tau residues. Furthermore, phosphorylation of tau is known to be driven by kinases which are activated in a cell cycle dependent manner [7,12]. STOX1A has recently been shown to be directly involved in the cell cycle [36]

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which further indicating a role for STOX1A in tau phosphorylation. Together, in this study we tested if there is a potential relationship between the phosphorylation status of tau proteins and STOX1A overexpression. To test this we used a neuronal cellular model in which stably transfected STOX1A cells were stably co-transfected with the longest human tau isoform (hTau40) in the neuroblastoma cell-line SH-SY5Y. With the use of western blot and antibodies recognizing phospho-specifc tau epitopes we found that STOX1A hyperphosphorylates epitopes which are abundantly found in NFT in AD. In conclusion, these results suggest that elevated STOX1A expression in advanced stages of LOAD patients is linked to hyperphosphorylation of tau as found in NFT.

2. Materials and methods

2.1. Human tissues

Human brain specimens of an Alzheimer's disease case (Braak stage 6) and a non-demented control case (Braak stage 1) were obtained as previously described [37] and used as a positive and a negative control for tau phosphorylation in western blot.

2.2. Cell culture and transfection

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All reagents for cell culture were purchased from Invitrogen Life Technologies, Inc. (Burlington, Canada). SH-SY5Y cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum and 100 U/ml penicillin, and 100 g/ml streptomycin. Cells were subcultured in medium every 2–3 days following harvesting by trypsinization (HBSS containing 5% trypsin). The ORF (Open Reading Frame) of the STOX1A gene was sub-cloned into the pF5K-neomycin CMV Flexi vector according to the manufacturers protocol (Promega). The ORF of the longest human tau (hTau40) isoform was obtained from the pNG2 donor vector (Dr. E.M. Mandelkow) by digestion with the restriction enzymes NdeI and BamHI. Both sites were rendered blunt ended using Klenow. The htau40 insert was ligated into the HindIII and BamHI sites of the pcDNA4 vector (Invitrogen) that were blunted with Klenow, to obtain the pcDNA4-htau40 construct.

For transfection the calcium phosphate method was used [34]. Briefly, at the time of transfection, cells were at 70% confluence. By vortexing 2× HEBS (HEPES-buffered saline) with a solution of 2.5 M $CaCl_2$ and 20 μg of plasmid DNA a co-precipitate of DNA and $CaPO_4$ was allowed to form. After incubation for 30 min at room temperature, the precipitate was added to the cells and the medium was changed after 24 h.

Three previously created stably transfected STOX1A SH-SY5Y cell-lines (WT-STOX1A) with STOX1A over-expression of at least 10 fold compared to mock transfected cells (WT-MOCK) [36] were stably co-transfected either with the htau40-pcDNA4 or empty pcDNA4 constructs (STOX1A-htau40 and STOX1A-htau40control). Furthermore, wild type SH-SY5Y cells were stably transfected either with the htau40-pcDNA4 or empty pcDNA4 constructs (WT-htau40 and WT-htau40control). Selection of WT-STOX1A and WT-MOCK cells was possible due to the presence of a Neomycin resistant gene as previously described [36]. Selection of positive clones with either the htau40-pcDNA4 or empty pcDNA4 constructs was possible due to the presence of a Zeocin™ (Invitrogen) resistant gene present in these constructs. Positive clones were maintained in complete IMDM medium supplemented with 300 μg/ml Zeocin™ (Invitrogen) until reaching confluence and sub-cultured every 2–3 days for about 4 weeks. Three stably transfected STOX1A-htau40, STOX1A-htau40control, WT-htau40 and WT-htau40control cell-lines were selected for further analysis. As confirmed with qRT-PCR using TaqMan probes (Applied Biosystems) for Total tau, the STOX1A-htau40 and WT-htau40 had an at least 60 fold htau40 mRNA over-expression compared to STOX1A-htau40control and WT-htau40control, respectively (data not shown).

2.3. Western blot

Protein lysates from stably transfected cells were obtained by directly scraping cells into loading Buffer including β-mercaptoethanol. Lysates were separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto a PVDF-membrane. Rabbit polyclonal antibodies recognizing the STOX1A protein (Sigma), Total tau protein (clone Tau-5, Abcam), Phospho-P44/42 (MAPK, Cell signaling), Phospho-CDK1 (Thr161, Cell signaling) and P35 (Cell signaling) were used in combination with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (DAKO).

Mouse monoclonal antibodies specific for Phospho-Tau S202/T205 (Clone AT8, Pierce Biotechnology), Phospho-Tau S396 (Clone E178, Abcam), P44/42 (Phospho-MAPK, Cell signaling), CDK1 (Clone POH1, Cell signaling) and CDK5 (Clone DC17, Millipore) were used in combination with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO). Endogenous actin used for loading controls was detected with mouse monoclonal anti-actin antibody (Sigma Aldrich) and used in combination with goat anti-mouse horseradish peroxidase-conjugated secondary antibody (DAKO). Protein bands were detected by an enhanced-chemiluminescence assay (GE Healthcare) on a LAS3000.

2.4. Immunofluoresence

For immunofluorescence, WT-htau40, WT-htau40control, STOX1A-htau40 or STOX1A-htau40control cells were grown on glass coverslips. Coverslips were fixed in 4% (PFA) paraformaldehyde for 15 min at room temperature. After fixation, coverslips were rinsed in PBS, 0.1% Triton X-100 and incubated with 1% Triton X-100 in PBS for 15 min at room temperature for permeabilization. Coverslips were washed in wash buffer (PBS, 0.1% Triton X-100, 2% BSA [Bovine serum albumin]) blocked with PBS, 0.1% Triton X-100, 5% BSA for 1 h and incubated with AT8, E178 or Tau-5 antibody (see above) or without a first antibody (serving as a negative control) at 4 °C overnight. After washing with wash buffer coverslips were incubated for 1 h with anti-mouse secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen), washed with washing buffer and mounted with vectashield mounting solution containing DAPI for DNA counterstaining (Vector Laboratories).

2.5. Data analysis

For quantification of westernblots densitometry was used. Westernblot pictures were analyzed with the software ImageJ according to the tutorial described on the website: http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/. Statistical analysis on the obtained density values was carried out with Microsoft Excell and the GraphPad Prism program.

3. Results

3.1. STOX1A induces tau phosphorylation

Several monoclonal antibodies have been created which specifically recognize phosphorylated tau residues as found in NFT of AD