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## AMP-activated protein kinase (AMPK) is a tau kinase, activated in response to $\beta$ -amyloid exposure

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Running title: AMPK is a tau kinase

Hyperphosphorylation of tau is a hallmark of Alzheimer's disease and other tauopathies. Although the mechanisms underlying hyperphosphorylation are not fully understood, cellular stresses such as impaired energy metabolism are thought to influence the signalling cascade. The AMPK-related kinases, MARK and BRSK, have been implicated in tau phosphorylation but are insensitive to activation by cellular stress. Here we show that AMPK itself phosphorylates tau on a number of sites including S262 and S396, altering microtubule binding of tau. In primary mouse cortical neurons, CaMKK $\beta$  activation of AMPK in response to  $\beta$ -amyloid ( $A\beta_{1-42}$ ) leads to increased phosphorylation of tau at S262/S356 and S396. Activation of AMPK by  $A\beta_{1-42}$  is inhibited by memantine, a partial antagonist of the *N*-methyl-D-aspartate (NMDA) receptor and currently licensed for the treatment of Alzheimer's disease. These findings identify a pathway in which  $A\beta_{1-42}$  activates CaMKK $\beta$  and AMPK via the NMDA receptor suggesting the possibility that AMPK plays a role in the pathophysiological phosphorylation of tau.

### INTRODUCTION

The physiological role of the microtubule-associated protein tau is to bind to tubulin heterodimers and promote microtubule assembly. Phosphorylation of tau is required for efficient neurite outgrowth and the regulation of axonal transport [1, 2]. However, hyperphosphorylation of tau is believed to underlie the formation of neurofibrillary tangles (NFTs), a neuropathological characteristic of Alzheimer's disease and other "tauopathies" resulting in neurodegeneration [3]. This abnormal phosphorylation allows the release of tau from microtubules, its aggregation into paired helical filaments, a major component of NFTs and promotes microtubule disruption [4]. Events surrounding the initiation of tau hyperphosphorylation *in vivo* are not clearly understood. At least 30 protein kinases have been reported to phosphorylate tau *in vitro*, and of these a number, including glycogen synthase kinase (GSK) 3 $\beta$ , cyclin-dependent kinase 5, casein kinase 1, cAMP-dependent protein kinase A and MAP/microtubule Affinity Regulating Kinases (MARKs) have been suggested to play a role in tau phosphorylation *in vivo* [5]. Despite the complexity of the system, only a limited number of the potential tau kinases have been shown to phosphorylate tau at S262 within the KXGS motif of the microtubule-binding domain. Phosphorylation of S262 is thought to influence the ability of tau to bind to microtubules [6].

MARKs are members of the AMP-activated protein kinase (AMPK)-related kinase family which also includes two recently characterised brain-specific kinases, BRSK1 and BRSK2 [7]. All kinases within the group share amino acid sequence homology within their catalytic

domains and require phosphorylation of a conserved threonine residue within the activation-loop region. LKB1, which was identified as an upstream kinase in the AMPK cascade, phosphorylates and activates all of the AMPK-related kinases, apart from MELK [7]. In addition to LKB1, AMPK is phosphorylated and activated by  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase kinase (CaMKK)  $\beta$ , and this has been shown to mediate activation of AMPK in response to increased intracellular calcium concentrations. [8]. AMPK is also activated in response to an increase in AMP, following a fall in ATP levels within the cell. AMP allosterically activates AMPK as well as protecting the enzyme against dephosphorylation [9]. AMPK is a heterotrimer comprising a catalytic  $\alpha$  subunit and regulatory  $\beta$  and  $\gamma$  subunits. Isoforms of all three subunits exist ( $\alpha 1, \alpha 2, \beta 1, \beta 2, \gamma 1, \gamma 2, \gamma 3$ ) and all twelve heterotrimeric complex combinations have been identified *in vivo*. The physiological significance of the different AMPK isoform complexes is only partly understood, although it seems likely that they allow tissue specific regulation of AMPK as well as tissue specific function (reviewed in [10]).

AMPK is expressed ubiquitously and is a key regulator of metabolic pathways such as fatty acid and cholesterol synthesis [10]. However, delineating the function of neuronal AMPK is still in its infancy. The most detailed studies have taken place in the arcuate nucleus of the hypothalamus where AMPK is involved in co-ordinating the regulation of appetite and satiety [11]. AMPK is active in other brain regions but as yet, it is unclear whether activation of AMPK plays a neuroprotective or neurodegenerative role with data supporting either position [12-15]. More recently, AMPK has been implicated in both beneficial and deleterious modulations of amyloid precursor protein (APP), proteolytic cleavage of which generates  $\text{A}\beta_{1-42}$  peptide, a key component of disease-characteristic amyloid plaques [16]. In response to metformin treatment, activation of AMPK was reported to promote the formation of both intracellular and extracellular amyloid  $\beta$  peptide from APP [17]. Conversely, resveratrol, a plant polyphenol with potential neuroprotective properties, is reported to negatively regulate the production of  $\text{A}\beta_{1-42}$  peptide through an AMPK-mediated pathway [18].

Recent research in both drosophila and mice suggest that the MARKs and to a lesser extent, the related BRSKs, may play a role in early priming phosphorylation of tau. However, to date the physiological regulation of these kinases and the signals that modulate their activity remain unknown. [19]. Currently, there has been little examination of the role of AMPK in the phosphorylation of tau. Here we present data implicating AMPK as a physiological kinase in a pathway phosphorylating tau. We have identified a number of sites in tau phosphorylated by AMPK, many of which are clustered around the microtubule binding domain of tau. Intriguingly, phosphorylation of tau by AMPK regulates its ability to bind to microtubules. In addition, we show that exposure of primary neurons to  $\text{A}\beta_{1-42}$  increases AMPK activity via CaMKK $\beta$ -dependent pathway.  $\text{A}\beta_{1-42}$ -mediated activation of AMPK is blocked by memantine, an NMDA receptor antagonist licensed for treating Alzheimer's disease. These findings place the NMDA receptor upstream of AMPK in a CaMKK $\beta$ -mediated activation pathway resulting in the phosphorylation of tau.

## EXPERIMENTAL

*Preparation of primary neurons* Animal use was in accordance with local rules and with the regulations and guidance issued under the Animals (SP) Act (1986). Primary cortical neurons were prepared from WT and CaMKK $\beta^{-/-}$  E15 animals as previously described [20]. Briefly, cortices from embryos in a single litter were dissected, meninges removed and tissue pooled. Cortices were roughly chopped before incubation in 0.25% trypsin-EDTA followed by trituration. Cells were pelleted by centrifugation, resuspended and plated in Neurobasal media supplemented with B27, 100U/ml penicillin, 100 $\mu$ g/ml streptomycin, 0.25 $\mu$ g/ml amphotericin B, 300  $\mu$ M glutamine and 25  $\mu$ M  $\beta$ -mercaptoethanol. Treatments were carried out on neurons grown for a minimum of 7 days *in vitro* and all media were obtained from Invitrogen.

*Neuronal cell treatment and lysis* Primary cortical neurons were treated with the following compounds for the times indicated: hydrogen peroxide (0.5mM, 15 min, Sigma), ionomycin (2 $\mu$ M, 5 min, Tocris), A $\beta_{1-42}$  (20 $\mu$ M, 30 min, Invitrogen), NMDA (50 $\mu$ M, 15 min, Sigma), STO609 (10 $\mu$ g/ml, 3 hr, Tocris), memantine (10 $\mu$ M, 3 hr, Sigma). In cases in which a 3 hr pretreatment was required (memantine, STO609), activators were subsequently added in the presence of the pretreating compound. Aggregated A $\beta_{1-42}$  peptide was prepared as per manufacturer's instructions (Invitrogen); lyophilised peptide was first resuspended to 6mg/ml in sterile dH<sub>2</sub>O, vortexed thoroughly then diluted to a final concentration of 1mg/ml in PBS. Aggregation was achieved by incubation at 37°C for 24 hr. Treated cells were harvested in HEPES buffer A containing Triton (50 mM HEPES pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 157  $\mu$ g/ml benzamidine, 10% [v/v] glycerol, 1% [v/v] Triton X-100) and protein concentration determined by Bradford assay (Biorad).

*Immunoprecipitation (IP)* 50 $\mu$ g total protein lysate was incubated for 2 hr at 4°C in the presence of protein A- or protein G-sepharose (for rabbit and mouse antibodies respectively) and antibody as follows: AMPK pan  $\beta$  [21], BRSK2 [19], 6His and MARK3 (Abcam), AMPK $\alpha$ 1 and AMPK $\alpha$ 2 (Cell Signalling). Immobilised immune complexes were washed thoroughly in PBS containing 1% triton then used for kinase assay or western blot analysis.

*In vitro phosphorylation* Recombinant bacterially expressed wild-type ( $\alpha$ 1 $\beta$ 1 $\gamma$ 1; 1 $\mu$ g) or kinase inactive ( $\alpha$ 1D157A $\beta$ 1 $\gamma$ 1; 1 $\mu$ g) AMPK was purified as described previously [22]. GSK3 $\beta$  was from New England Biolabs (1 $\mu$ g;), MARK4 was from Cell Signaling (1 $\mu$ g;) and recombinant tau (2N4R, longest human tau isoform, 0.5  $\mu$ g) was from Sigma. Purified protein kinases and recombinant tau (0.5 $\mu$ g) were incubated phosphorylation buffer (50mM Tris-HCl pH7.5, 10mM MgCl<sub>2</sub>, 5mM dithiothreitol, 3mM <sup>32</sup>P-ATP) for 1hr at 37°C. Phosphorylation reactions were resolved by SDS-PAGE, transferred to PVDF membrane and detected by fluorography overnight at -80°C. For western blots of AMPK-phosphorylated recombinant tau, <sup>32</sup>P-ATP was replaced with 3mM unlabelled ATP and 20ng of phosphorylated tau per lane was analysed by phosphotau-specific antibodies..

*Kinase assays* AMPK, BRSK2 and MARK3 activities was measured using the AMARA substrate peptide as described previously [23]. Assays were carried out for 20 min at 37°C.

*Western blot (WB)* 25-50 $\mu$ g of total protein lysate or immobilised immune complexes were boiled in SDS sample buffer and resolved on 10% NuPage BisTris gels (Invitrogen). Proteins were transferred to PVDF and analysed by western blotting with the following antibodies: phospho-tau pS214 (Abcam), 12E8 [24], PHF1 [25] pS199/pT231/pS262/pS356/pS396/pS400/pT404/pS422 (Biosource), Tau5 (Biosource), phosphoAMPK (pT172) and phosphoACC (Cell Signalling), Actin (Sigma). Membranes were washed and incubated with secondary antibodies conjugated to Alexa-Fluor 680 (Invitrogen) or IRDye800 (Li-Cor) and scanned on the Li-Cor Odyssey Infrared Imaging System using Odyssey software 2.0 for band

quantification. Typically, primary antibody incubations took place overnight at 4°C and secondary antibody incubations were for 2 hr at room temperature.

*Tau over-expression* cDNA encoding 2N4R Tau was subcloned into pcDNA3. Mutation of serine 262 to alanine was carried out by site-directed mutagenesis (Invitrogen). pcDNA3-TauWT and -TauS262A were expressed in CCL13 cells by calcium phosphate transfection of 10µg purified plasmid DNA (BD Clontech). Precipitates were removed after overnight incubation and cells harvested 48 hr later. Expressed protein was isolated by immunoprecipitation with anti-His antibodies.

*Mass Spectrometry* Recombinant AMPK-phosphorylated tau (0.5µg) was resolved by SDS-PAGE, excised and sent for analysis to Harvard Medical School, Taplin Facility.

*Microtubule binding* Taxol-stabilised microtubules were generated according to the manufacturer's instructions (Cytoskeleton, Cambridge). Briefly, a stable population of microtubules was generated by polymerising bovine tubulin (100µg) at 37°C for 20 min in the presence of GTP (1mM) and taxol (20µM). AMPK-phosphorylated or non-phosphorylated tau was incubated in the presence of taxol-stabilised microtubules at room temperature for 30 min. The reaction was fractionated by ultracentrifugation through taxol-stabilised cushion buffer (50% glycerol [v/v], 20µM taxol) at 100,000 g for 40 min at room temperature. The resulting fractions were resolved by SDS-PAGE and analysed by western blot using a phosphorylation-independent tau antibody.

*Statistical analyses* Data are expressed as mean ± standard deviation from three or more independent experiments. Statistical analysis was performed using Prism (Graphpad). Statistical significance between two conditions was established with a two-tailed Student's *t*-test whereas significance among multiple datasets was determined by ANOVA followed by *post hoc* Tukey multiple comparison test. Data was considered significant at \**p*<0.05 or \*\**p*<0.01.

## RESULTS

### AMPK is a tau kinase

In transient transfection studies, we previously observed that BRSK1 and BRSK2 were insensitive to activation by a range of pathophysiological stimuli, including those that might occur early in Alzheimer's disease [19]. Here we have extended this study to primary cortical neurons and find that the activities of endogenous MARK3 and BRSK2 are not significantly altered in response to ionomycin (increasing intracellular calcium concentrations) or hydrogen peroxide (to mimic oxidative stress; Figure 1A). Therefore we examined the hypothesis that AMPK itself was a physiological tau kinase as AMPK is activated in response to these and other cellular stresses. We carried out *in vitro* phosphorylation experiments using active and inactive (mutation of D157A in the ATP-binding pocket of AMPK $\alpha$ ) AMPK, GSK3 $\beta$ , MARK4 and recombinant tau (Figure 1B). In addition to GSK3 $\beta$  and MARK4, which have been shown previously to phosphorylate tau, AMPK caused a robust phosphorylation of tau (Figure 1B). An identical experiment analysed by western blot determined that AMPK increased the phosphorylation of S262 (upper panel, Figure 1C), a site previously implicated as a major phosphorylation site for BRSK and MARK kinases [26, 27]. Only weak phosphorylation of S262 was observed for GSK3 $\beta$ , although the migration of tau on SDS-PAGE was retarded suggesting phosphorylation at other sites (lower panel, Figure 1C). To determine whether Ser262 was the predominant phosphorylation site for AMPK on tau, we generated His-tagged wild-type tau, or a construct harbouring mutation of S262 to alanine (S262A) and expressed the proteins in CCL13 cells, a mammalian cell line lacking LKB1 [23]. These cells therefore have the advantage that the basal activity of AMPK is low and overexpression of tau in this line is likely to remain unphosphorylated by AMPK. Tau was isolated from cell lysates by immunoprecipitation and subjected to phosphorylation by AMPK or GSK3 $\beta$ . As expected, mutation of S262A had no detectable effect on the ability of GSK3 $\beta$  to phosphorylate tau. However, tau phosphorylation by AMPK also remained relatively unchanged in the S262A mutant compared to wild-type, indicating the presence of additional AMPK phosphorylation sites within tau (Figure 1D).

### AMPK phosphorylates multiple tau epitopes

In order to study further the phosphorylation of tau by AMPK, we compared phosphorylation of tau by MARK4 or AMPK using a panel of phospho-tau antibodies (Figure 2A). Five phosphorylation sites common to both kinases were identified (T231, S262, S356, S396 and S422). In addition, AMPK, but not MARK4, was capable of phosphorylating S214 (Figure 2A). In blots in which there was no phosphorylated signal detected (S199, S400, S404), a positive control phosphorylation was carried out using GSK3 $\beta$  to confirm antibody recognition of the phospho-specific epitope (Supplementary Figure S1). As western blotting revealed that AMPK phosphorylates a number of sites in tau, we used mass spectrometry to comprehensively identify as many AMPK sites as possible. Briefly, AMPK-phosphorylated tau was subjected to mass spectrometric analysis, achieving 81% coverage of 2N4R tau (Supplementary Figure S2). Using this approach, we were able to assign with confidence over 20 AMPK phosphorylation sites within tau (Figure 2B). Interestingly, 9 were identified within the microtubule binding domain and 7 out of 8 conserved serine residues within the four repeated tubulin binding motifs were identified as AMPK sites (Figure 2B, top panel).

### AMPK phosphorylation inhibits tau binding of microtubules

As the bulk of AMPK phosphorylation sites are clustered around the microtubule binding domain, we investigated the effect of AMPK phosphorylation on binding of tau to microtubules *in vitro*. Tau, before and after phosphorylation by AMPK, was incubated with

microtubules and subjected to ultracentrifugation to pellet microtubule-binding proteins. The majority (75%) of non-phosphorylated tau was detected in the high-speed pellet, indicating efficient binding to the microtubules (Figure 2C). In contrast, only 20% of the AMPK-phosphorylated tau was detected in the microtubule pellet, with 80% remaining in the supernatant fraction.

### AMPK is activated in response to A $\beta$ <sub>1-42</sub> exposure

The deposition of extracellular plaques, of which a short amyloid  $\beta$  peptide (A $\beta$ <sub>1-42</sub>) is a key component, is a pathological characteristic of Alzheimer's disease [16]. Exposure of primary hippocampal neurons to aggregated A $\beta$ <sub>1-42</sub> peptide has been reported to induce tau phosphorylation [28]. To determine whether there is a role for AMPK in the A $\beta$ <sub>1-42</sub> pathway, a mixed cortical/hippocampal culture of primary neurons was prepared and treated with aggregated A $\beta$ <sub>1-42</sub> peptide (20 $\mu$ M) and AMPK activity in immune complexes determined. As shown in Figure 3A there was a significant increase in activity from AMPK isolated from A $\beta$ <sub>1-42</sub>-treated cell lysates compared with control cells. Phosphorylation of AMPK at T172, another direct measure of AMPK activation, was also increased and this correlated with an increase in phosphorylated acetyl Co-A carboxylase (pACC), a well characterised downstream target of AMPK (Figure 3B). As both BRSK and MARK have been implicated in tau phosphorylation [27, 29] the activity of these kinases following A $\beta$ <sub>1-42</sub>-treatment was also determined. In contrast with AMPK, no change in either BRSK2 or MARK3 activity after A $\beta$ <sub>1-42</sub> treatment was detected (activities for control *vs* A $\beta$ <sub>1-42</sub> treatment, BRSK2: 0.0537  $\pm$  0.0103 *vs* 0.0597  $\pm$  0.0083 nmol/min/mg; MARK3: 0.0464  $\pm$  0.0064 *vs* 0.0463  $\pm$  0.0089 nmol/min/mg, N=4). Acute exposure of primary cortical neurons to aggregated A $\beta$ <sub>1-42</sub> peptide induces a rapid increase in intracellular calcium concentrations [30]. Therefore we tested the hypothesis that the regulation of AMPK by A $\beta$ <sub>1-42</sub> was mediated through a CaMKK $\beta$ -signalling pathway. We isolated primary neuronal cultures from CaMKK $\beta$ -knockout mice, and treated these cells with A $\beta$ <sub>1-42</sub>. In marked contrast to wild-type neurons, A $\beta$ <sub>1-42</sub>-treatment had no effect on AMPK activity in the cells derived from the CaMKK $\beta$ -knockout mice (Figure 3C) establishing that CaMKK $\beta$  is required for AMPK activation by A $\beta$ <sub>1-42</sub>. Previous studies by us and others have identified a substrate preference in CaMKK $\beta$  for activation of AMPK $\alpha$ 1- rather than AMPK $\alpha$ 2-containing complexes [31, 32]. To determine which isoform mediates the response to A $\beta$ <sub>1-42</sub>, isoform-specific depletion of AMPK was repeatedly performed on the same lysate until all isoform complexes were removed. AMPK $\alpha$ 1 complexes were significantly activated in response to A $\beta$ <sub>1-42</sub>, whilst there was no significant increase in AMPK $\alpha$ 2 activity (Figure 3D). These results are similar to a previous study, where we only observed significant activation of AMPK $\alpha$ 1 complexes following activation of CaMKK $\beta$  [32].

### Activation of AMPK by A $\beta$ <sub>1-42</sub> increases tau phosphorylation at disease-relevant epitopes

Our initial data suggested that AMPK phosphorylates a number of residues on recombinant tau. As A $\beta$ <sub>1-42</sub> treatment activates AMPK in primary neurons, we determined the downstream effect on the phosphorylation of endogenous tau. We obtained 12E8 and PHF-1 antibodies, commonly used to detect pathologically phosphorylated tau [24, 25], and examined the phosphorylation state of tau in A $\beta$ <sub>1-42</sub>-treated WT and CaMKK $\beta$ <sup>-/-</sup> neurons. We observed a marked increase in signal for both antibodies in the WT neurons indicating that phosphorylation had occurred at S262/S356 (12E8) and S396/S404 (PHF-1; Figure 4A), consistent with our earlier observations identifying S262, S356 and S396 as candidate AMPK phosphorylation sites *in vitro* (Figure 2). Conversely, there was little difference in tau phosphorylation between control and A $\beta$ -treated CaMKK $\beta$ <sup>-/-</sup> neurons after western blot analysis with 12E8. More surprisingly perhaps, was that phosphorylation of S396/S404, as detected by the PHF-1 antibody, was reduced following treatment with A $\beta$ <sub>1-42</sub> in CaMKK $\beta$ <sup>-/-</sup>

cells (Figure 4B). To determine whether this difference was due to reduced total tau or reduced phosphorylation after A $\beta$  treatment, we compared the levels of total tau in WT and CaMKK $\beta^{-/-}$  lysates. Although there was a difference in total tau protein expression in the CaMKK $\beta^{-/-}$  cells compared with WT (32% less tau in CaMKK $\beta^{-/-}$  neurons), there was no change in tau expression between control and A $\beta_{1-42}$  treated neurons (Figure 4C). These data suggest that tau phosphorylation at S396/S404 in response to A $\beta_{1-42}$  is significantly influenced by active CaMKK $\beta$ .

#### **AMPK activation by A $\beta_{1-42}$ is inhibited by memantine**

One mechanism by which A $\beta_{1-42}$  may exert its intracellular effects is through potentiating glutamate-mediated excitotoxicity [33]. Neurodegeneration of the glutamatergic pyramidal neurons of the hippocampus and cortex is an early event in Alzheimer's disease and cultured neurons can be protected from A $\beta_{1-42}$  toxicity by treatment with glutamatergic *N*-methyl-D-aspartate (NMDA) receptor antagonists [34]. Interestingly, we found that AMPK was robustly activated in primary neurons after NMDA treatment, and that this activation was abolished by pre-treatment with STO609, a pharmacological inhibitor of CaMKK $\alpha$  and CaMKK $\beta$  (Figure 5A). Memantine is a non-competitive NMDA receptor antagonist and recently the use of memantine for treatment in moderate to severe Alzheimer's disease has been approved, highlighting the role of this pathway in the pathology of cognitive decline. We found that treatment of primary neurons with memantine prior to exposure to NMDA prevented the activation of AMPK (Figure 5A). Memantine is reported to reduce both the excitotoxic effects of A $\beta_{1-42}$  in rat cortical neurons and the phosphorylation of tau [35]. Therefore we repeated the A $\beta_{1-42}$  treatment on primary neurons with or without memantine pre-treatment. As with NMDA, memantine successfully prevented the activation of AMPK by A $\beta_{1-42}$  (Figure 5B) implying that A $\beta_{1-42}$  acts on AMPK through activation of the NMDA receptor increasing intracellular Ca<sup>2+</sup> and activating CaMKK $\beta$ .

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## DISCUSSION

The hyperphosphorylation of tau is a key event in the progression of Alzheimer's disease yet the mechanisms underlying this event remain unclear. Our data provide the first evidence that AMPK activation may act as a link between exposure of neurons to  $\beta$ -amyloid and subsequent tau phosphorylation.

Mass spectrometry analysis identified a cluster of candidate AMPK phosphorylation sites around the microtubule binding domain of tau including all four conserved serine residues (S262, S293, S324 and S356) that lie within the four KXGS microtubule binding motifs (Fig 2B, expanded panel). This suggested a role for AMPK phosphorylation in the regulation of microtubule binding. In addition, phosphorylation at S214 and T231 was also detected by western blotting and mass spectrometry. In line with previous research suggesting that phosphorylation of these epitopes induces maximal disruption of binding [27, 36], the interaction of tau with microtubules was greatly reduced following phosphorylation by AMPK *in vitro*. Neither mass spectrometry nor western blot analysis identified S404 as a potential AMPK site, suggesting that S396 provides the epitope for recognition by the PHF-1 antibody. Mass spectrometry also highlighted threonine 71 (T71) and threonine 403 as AMPK phosphorylation sites. An online database of tau phospho-epitopes lists both sites as phosphorylated in brain tissue isolated from patients with Alzheimer's disease, but as yet no kinase has been identified (<http://cnr.iop.kcl.ac.uk/hangerlab/tautable>). The N-terminal sequence of tau containing T71 is referred to as the projection domain as it projects away from the microtubule to interact with cytoskeletal proteins linking it to the plasma membrane and may also regulate tau aggregation [37, 38]. Further work is currently underway to determine whether AMPK phosphorylation at T71 regulates these properties of tau.

Exposure of primary neurons to  $A\beta_{1-42}$  rapidly increases AMPK activity but is also reported to alter the activities of other kinases. Exposure of primary rat neuronal cultures to  $A\beta$  for 6-8hr increased cdk5 and p38 activities [39] and in a similar study, Song and colleagues demonstrated increased ERK1/2 activity after a 1 hr  $A\beta$  exposure [35]. In animals, a study describing lentiviral injection of  $A\beta$  into rat motor cortex revealed prolonged activity of both cdk5 and GSK3 $\beta$  [40]. In all cases, tau phosphorylation was reported to increase. In contrast, a very recent study found that  $A\beta$  exposure of primary rat hippocampal neurons for 3hr resulted in a synaptically localised increase of MARK, BRSK, p70S6K and cdk5 activities whereas GSK3 $\beta$  and MAPK activities did not change [41]. An increase in tau phosphorylation at 12E8 sites (pS262, pS356) was also observed, and ascribed to the activities of AMPK-related kinases although the activity of AMPK itself was not determined. These data suggest that further work is required to distinguish the effects of short and long term  $A\beta$  exposure and intracellular localisation on kinase activities.

In our study, we were unable to detect changes in the activities of BRSK2 or MARK3 in response to 30 min  $A\beta$  treatment. The MARK kinase family were originally identified through their ability to regulate the interaction of tau with microtubules [27]. Studies of the MARK orthologue in drosophila, PAR-1, show that overexpression of APP upregulated PAR-1 activation and phosphorylation of tau [42]. In the current study, we show that short term  $A\beta_{1-42}$  exposure exerts its effects through CaMKK $\beta$  activation, consistent with the hypothesis that  $A\beta$  renders the neuron more susceptible to glutamate receptor neurotoxicity by destabilising calcium homeostasis [30]. These findings provide an explanation for the lack of effect of  $A\beta_{1-42}$  treatment on the activity of the AMPK-RKs tested, since a previous study reported that these kinases were not regulated by calcium signalling pathways, including CaMKK $\beta$  [19]. AMPK-

RKs are activated by LKB1 phosphorylation [7] and although the upstream stimulus for this pathway remains unclear, this may be the mechanism by which they play a role in tau phosphorylation.

Antibodies recognising both early (12E8) and late (PHF-1) stage tau phosphorylation were used to determine the effect of A $\beta$ <sub>1-42</sub> treatment on primary neurons. Treatment conditions which activated AMPK also caused a substantial increase in tau phosphorylation at a number of disease-relevant epitopes in wild type neurons. Conversely in CaMKK $\beta$ <sup>-/-</sup> neurons, we did not detect any change in S262/S356 phosphorylation following treatment. We did, however, observe a reduction in phosphorylation of S396/S404 following A $\beta$ <sub>1-42</sub> treatment in CaMKK $\beta$ <sup>-/-</sup> neurons relative to untreated cells. This is not due to reduced levels of tau as the 12E8 epitopes are unaffected and there is no change in the amount of total tau protein expression in CaMKK $\beta$ <sup>-/-</sup> control versus CaMKK $\beta$ <sup>-/-</sup>A $\beta$ <sub>1-42</sub> lysates (Fig 4C; compare lanes 5 and 6 with lanes 7 and 8). As the actions of kinases and phosphatases are tightly balanced, one possible explanation is that removing CaMKK $\beta$  disturbs this balance. Following influx of calcium after A $\beta$ <sub>1-42</sub> treatment, it is possible that calcium-dependent phosphatases are activated, and in the absence of competing activation of CaMKK $\beta$  the equilibrium is pushed in favour of dephosphorylation. Interestingly, S396 and S404 have been identified as substrates for calcium-dependent protein phosphatase (PP) 2B (calcineurin; [43]). Although the activity of a number of major tau phosphatases has been reported to decrease in Alzheimer's disease hence promoting tau hyperphosphorylation [44], it has been suggested that the activity of PP2B may increase [43].

Our report demonstrates robust AMPK activity in response to pharmacological activation of the NMDA receptor, an activation that can be inhibited by the NMDA-R antagonist memantine. A recent study identified another NMDA receptor-mediated pathway for AMPK activation. AMPK is involved in the development of excitotoxic apoptosis through a mechanism requiring BH3-only protein, Bim. Prolonged activation of AMPK leads to increased JNK phosphorylation, increased *bim* mRNA expression and neuronal death [45]. Other groups report both JNK activity and Bim are upregulated on exposure to A $\beta$ <sub>1-42</sub> peptide [46, 47]. Taking these studies together, it is interesting to speculate that these effects may be exerted in part by A $\beta$ <sub>1-42</sub> activation of AMPK.

AMPK activity is reported to be altered in the APP/PS2 animal model of Alzheimer's disease which exhibits amyloidosis, cognitive deficits, impaired synaptic plasticity and perturbed cerebrovascular development [48]. In a study carried out in young APP/PS2 animals (less than 8 months old), where the phenotype is mild, AMPK is abnormally activated compared with control mice [49]. This activation, in association with insulin growth factor (IGF)-1 imbalance contributes to increased vascular endothelial growth factor (VEGF) expression and decreased formation of blood vessels. Over-expression of A $\beta$ <sub>1-42</sub> in these animals is apparent as early as 5 months even though cognitive impairment is not detectable until 8 months. These data fit with our results from primary neurons in which there is a rapid activation of AMPK in response to pathological upregulation of A $\beta$ <sub>1-42</sub>.

Currently there is little information regarding the alteration of AMPK activity in human neurological disease. Indeed, a practical limitation of assessing AMPK activity in neural tissue is the rapidity needed for dissection and homogenisation to avoid spurious increases in AMPK activity caused by anoxia and such samples are rarely available. However, a study published while this manuscript was in review carried out a comprehensive analysis of *post-mortem* human tauopathy samples (including Alzheimer's disease) with phospho-AMPK antibodies.

They observed an increase in phospho-AMPK in both tangle- and pretangle-bearing neurons and confirmed our identification of S396 as a tau epitope phosphorylated by AMPK, using the same PHF-1 antibody [50]. Taken together with our data, it suggests that AMPK may play a key role in governing the early phosphorylation of tau.

In summary, we present data suggesting that AMPK is rapidly activated in primary neurons on exposure to A $\beta$  resulting in an increase in tau phosphorylation at residues critical for microtubule binding. Based on the previously published properties of A $\beta$ , we have identified a key role for the NMDA receptor and the calcium-activated upstream AMPK kinase CaMKK $\beta$  in this pathway. Our work suggests that AMPK deserves close examination as a candidate *in vivo* kinase in the regulation of tau hyperphosphorylation and further studies are required to determine whether AMPK has a clinically relevant role in neurodegeneration.

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## FIGURE LEGENDS

**Figure 1 AMPK is a tau kinase.** A) Primary neurons were treated with vehicle control (DMSO), hydrogen peroxide or ionomycin, lysed and immune complexes of BRSK2, MARK3 or AMPK assayed for kinase activity. Average fold activity compared with control is shown for three independent experiments (\*\* $p < 0.01$ ). B) Recombinant tau was phosphorylated by GSK3 $\beta$ , MARK4 and active or inactive (D157A) AMPK and incorporation of  $^{32}\text{P}$ -ATP into tau determined by fluorography (upper panel). Equivalent amounts of tau was confirmed by comassie blue (CBB) gel staining (lower panel). Additional bands present in lane 1 are from recombinant GSK3 $\beta$ . C) Phosphorylated recombinant tau was analysed by western blot for phospho-tau S262 (pS262 antibody; upper panel) or total tau (lower panel). D) His-tagged cDNA constructs of wild type (WT) or mutated (S262A) tau were transfected into a mammalian cell line. Expressed tau was purified by immunoprecipitation with anti-His antibodies and subjected to phosphorylation by GSK3 $\beta$  (left panels) or AMPK (right panels). Phosphorylation was determined by fluorography as above (upper panels) and equivalent expression of tau confirmed by western blotting (lower panels). Blots and autoradiographs are representative of three independent experiments.

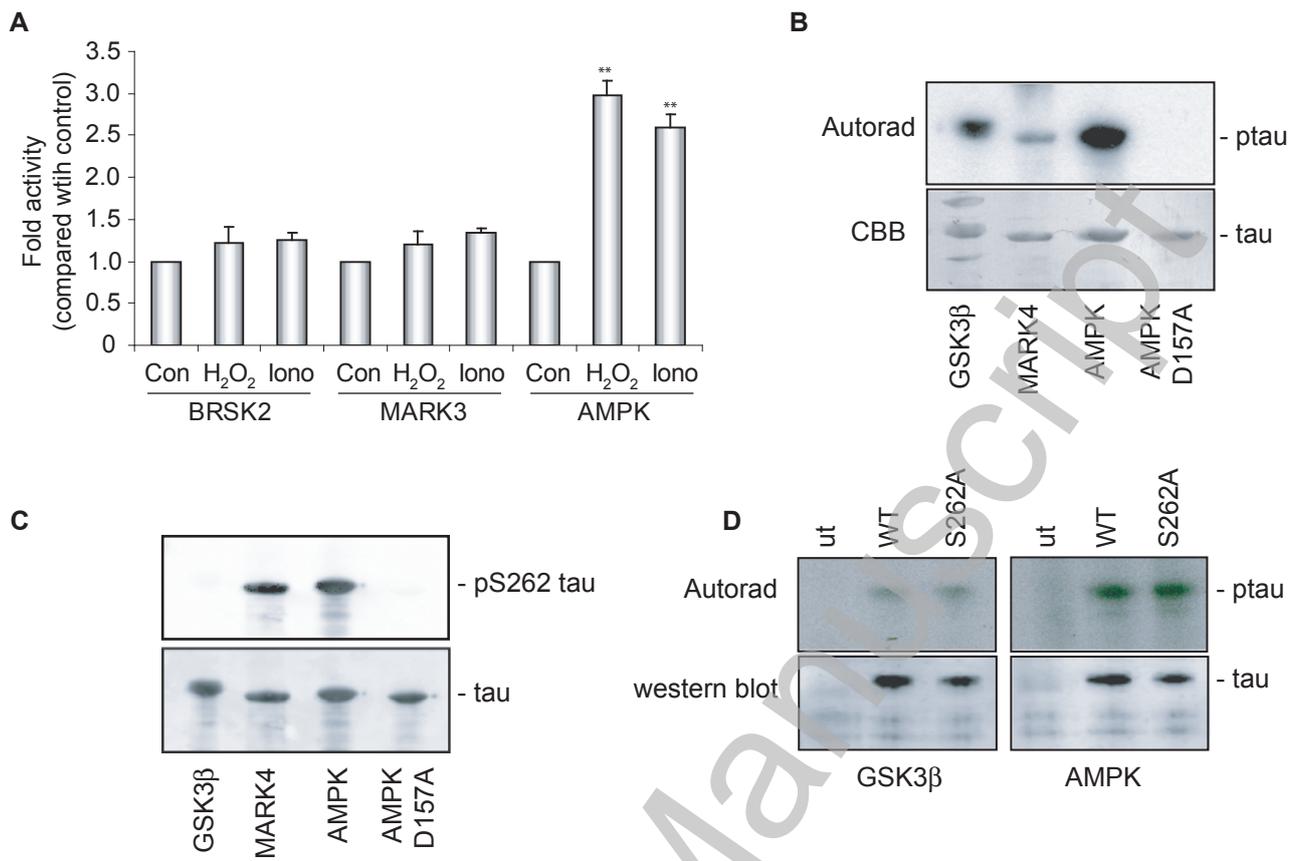
**Figure 2 AMPK phosphorylates multiple tau sites and interferes with microtubule binding.** A) MARK4 (M)- or AMPK (A)-phosphorylated recombinant tau was analysed by western blotting using phospho-specific tau antibodies (epitope indicated above each panel) and total tau (lower panels). B) A schematic of the longest isoform of tau (2N4R; 441 amino acids) showing domains and known phosphorylation sites of serine / threonine kinases or sites that are phosphorylated *in vitro* or in *post-mortem* Alzheimer's disease brain [5, 51]. AMPK sites identified from mass spectrometry analysis are shown in bold. C) Unphosphorylated (tau) or AMPK-phosphorylated (ptau) tau was incubated in the presence or absence of microtubules (MT). Microtubule bound (pellet) or microtubule unbound (supernatant) fractions were generated by ultracentrifugation. Fractions were analysed by western blot for total tau (upper panel). The lower panel shows sedimented microtubules by coomassie staining. The histogram represents the bound versus unbound tau (\*\*p < 0.01) or ptau (<sup>###</sup>p < 0.01) as a percentage of total tau. For A and C, images are representative of four individual experiments.

**Figure 3 AMPK is activated by A $\beta$ <sub>1-42</sub>.** Primary neurons from wild-type (WT) (A,B,D) and CaMKK $\beta$ <sup>-/-</sup> (C) mice were treated with 20 $\mu$ M A $\beta$ <sub>1-42</sub>. Treated and untreated neurons were harvested after 30 min and kinase activity determined in immune complexes of AMPK (A, C). Box plots show the minimum, median and maximum activities obtained for neurons from between 3 and 6 individual experiments (\*\*p < 0.01). B) Lysates from wild-type neurons treated with A $\beta$ <sub>1-42</sub> were analysed by western blot with antibodies recognising phosphorylated (active) AMPK and a known AMPK substrate, acetyl Co-A carboxylase (pACC). Western blot using a pan $\beta$ -AMPK antibody for total AMPK and anti-actin antibodies demonstrate there is no change in the amount of total protein. Representative images shown are neuronal lysates from two of four individual experiments. D) Lysates from treated and untreated neurons were subjected to sequential immunoprecipitation with either AMPK $\alpha$ 1- or AMPK $\alpha$ 2-specific antibodies to isolate all available endogenous isoform-specific AMPK complexes. Results shown represent the total isoform-specific activity from neuronal lysates of three individual experiments (\*p < 0.05).

**Figure 4 Increases in tau phosphorylation in response to A $\beta$ <sub>1-42</sub> are muted in cells lacking CaMKK $\beta$ .** Primary cortical neurons from wild-type (WT) (A, C) and CaMKK $\beta$ <sup>-/-</sup> (B, C) mice were treated with A $\beta$ <sub>1-42</sub> as before and analysed by western blot for phosphorylated tau using 12E8 (pS262/pS356) and PHF-1 (pS396/pS404) antibodies (A,B) or for phosphorylation-independent total tau (C). Quantification of phosphorylation (A,B) and total tau (C) is shown in the histograms normalised relative to actin (\*p < 0.05). Images shown are primary cell lysates from two of four (WT) or two of three (CaMKK $\beta$ <sup>-/-</sup>) individual experiments.

**Figure 5 Activation of AMPK is prevented by the NMDA receptor antagonist memantine.** A) Primary neurons were left untreated, pre-treated with either STO609 or memantine alone, or treated with NMDA. Neurons were harvested and AMPK activity determined from immune complexes. B) Primary neurons either untreated or pre-treated with memantine were treated with A $\beta$ <sub>1-42</sub>. Neurons were harvested and assayed for AMPK activity as above. Box plots show the minimum, median and maximum activities obtained for neurons from four (A, \*\*p < 0.01) or three (B, \*p < 0.05) individual experiments.

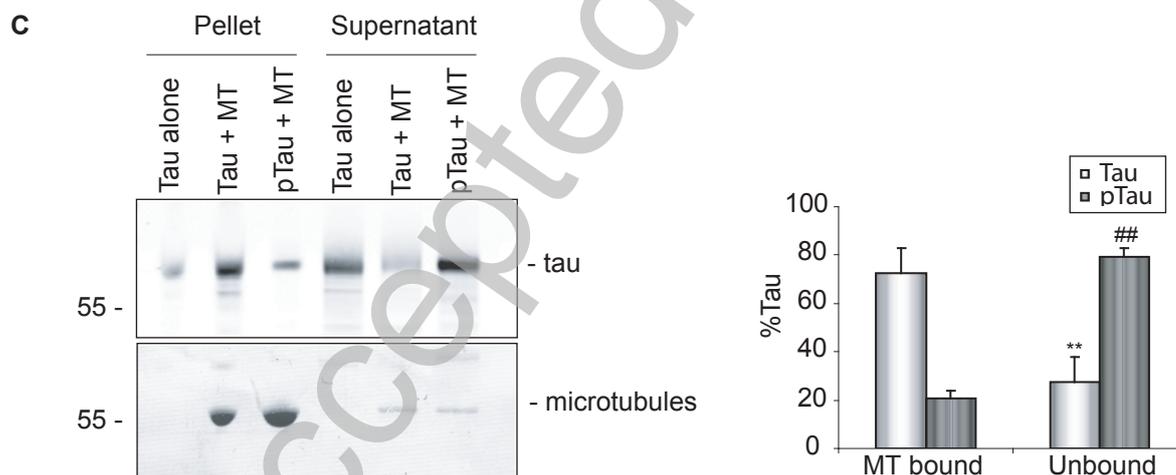
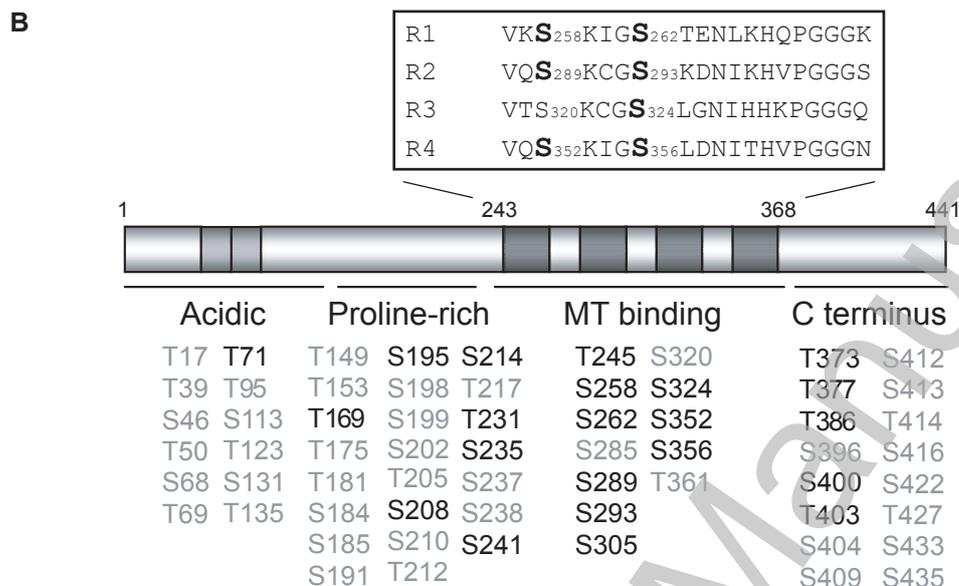
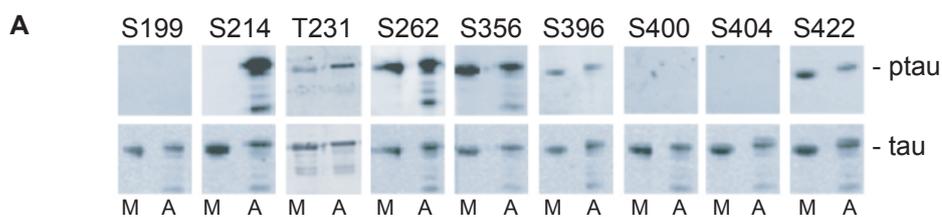
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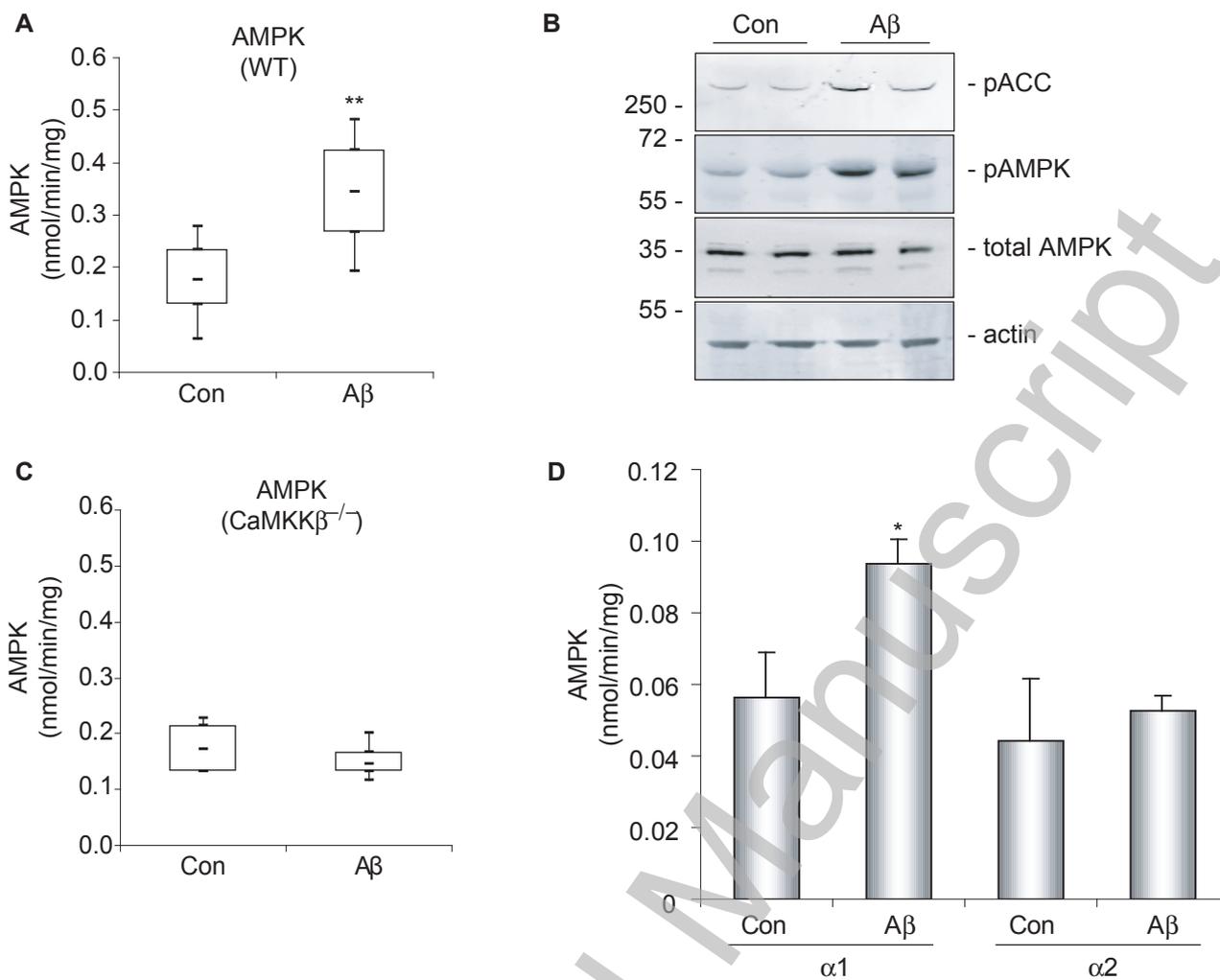
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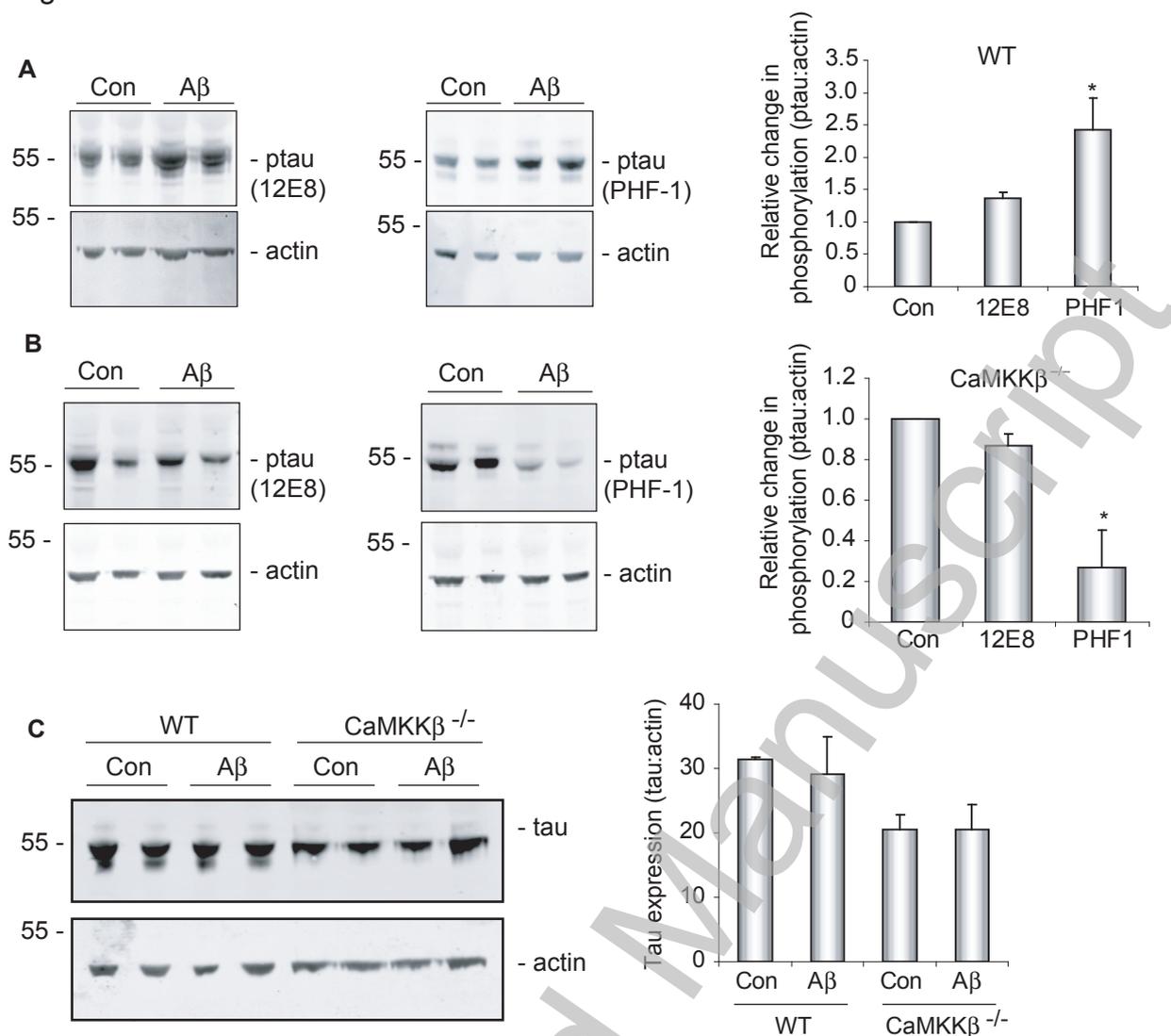
Figure 3 Thornton et al



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