EJN European Journal of Neuroscience

FENS Federation of European Neuroscience Societies

European Journal of Neuroscience, Vol. 44, pp. 1987–1997, 2016 doi:10.1111/ejn.13277

MOLECULAR AND SYNAPTIC MECHANISMS

Early glycogen synthase kinase-3β and protein phosphatase 2A independent tau dephosphorylation during global brain ischaemia and reperfusion following cardiac arrest and the role of the adenosine monophosphate kinase pathway

Shohreh Majd,[1] John H. T. Power,[2] Simon A. Koblar[3] and Hugh J. M. Grantham[1]
[1]Centre for Neuroscience, Neuronal Injury and Repair Laboratory, School of Medicine, Flinders University of South Australia, Adelaide, SA 5042, Australia
[2]Department of Human Physiology, School of Medicine, Flinders University of South Australia, Adelaide, SA, Australia
[3]School of Medicine, The Queen Elizabeth Hospital (TQEH) Campus, University of Adelaide, Adelaide, SA, Australia

Keywords: adenosine monophosphate kinase protein, glycogen synthase kinase-3β, microtubule-associated protein, neuronal energy stress, phosphorylation, protein phosphatase 2A

Edited by Paul Bolam
Received 6 December 2015, revised 27 April 2016, accepted 28 April 2016

Abstract

Abnormal tau phosphorylation (p-tau) has been shown after hypoxic damage to the brain associated with traumatic brain injury and stroke. As the level of p-tau is controlled by Glycogen Synthase Kinase (GSK)-3β, Protein Phosphatase 2A (PP2A) and Adenosine Monophosphate Kinase (AMPK), different activity levels of these enzymes could be involved in tau phosphorylation following ischaemia. This study assessed the effects of global brain ischaemia/reperfusion on the immediate status of p-tau in a rat model of cardiac arrest (CA) followed by cardiopulmonary resuscitation (CPR). We reported an early dephosphorylation of tau at its AMPK sensitive residues, Ser^{396} and Ser^{262}after 2 min of ischaemia, which did not recover during the first two hours of reperfusion, while the tau phosphorylation at GSK-3β sensitive but AMPK insensitive residues, Ser^{202}/Thr^{205} (AT8), as well as the total amount of tau remained unchanged. Our data showed no alteration in the activities of GSK-3β and PP2A during similar episodes of ischaemia of up to 8 min and reperfusion of up to 2 h, and 4 weeks recovery. Dephosphorylation of AMPK followed the same pattern as tau dephosphorylation during ischaemia/reperfusion. Catalase, another AMPK downstream substrate also showed a similar pattern of decline to p-AMPK, in ischaemic/reperfusion groups. This suggests the involvement of AMPK in changing the p-tau levels, indicating that tau dephosphorylation following ischaemia is not dependent on GSK-3β or PP2A activity, but is associated with AMPK dephosphorylation. We propose that a reduction in AMPK activity is a possible early mechanism responsible for tau dephosphorylation.

Introduction

Cognitive and memory impairment following stroke and cardiac arrest (CA) have been reported (Tatemichi *et al.*, 1992; Pohjasvaara *et al.*, 1998; Madureira *et al.*, 2001; Mateen *et al.*, 2011). Reperfusion after ischaemia is thought to precipitate neurodegenerative events such as beta amyloid (Aβ) accumulation and the over-expression of phosphorylated tau (p-tau) in the hippocampus (Geddes *et al.*, 1994) and cortex (Dewar & Dawson, 1995).

Hyperphosphorylated tau has been associated with ischaemic insults (Mailliot *et al.*, 2000; Castro-Alvarez *et al.*, 2011), and is responsible for its loss of biological activity, and neurofibrillary degeneration in Alzheimer's disease (AD) (Iqbal *et al.*, 1998), where paired helical filaments and neurofibrillary tangles (NFTs), are hallmarks of AD brain lesions (Ballatore *et al.*, 2007; Noble *et al.*, 2011).

Glycogen Synthase Kinase-3 (GSK-3β) and Protein Phosphatase 2A (PP2A) are thought to control the levels of tau phosphorylation (Spittaels *et al.*, 2000; Eldar-Finkelman, 2002; Liu *et al.*, 2005a). GSK-3β is linked to tangle-bearing neurons (Pei *et al.*, 1999; Lovell *et al.*, 2004), and phosphorylates tau in about 40 residues; however there are some residues insensitive to its action (Guerra-Araiza *et al.*, 2007; Hanger *et al.*, 2007; Hanger & Noble, 2011). GSK-3β could be influenced by ischaemia, 5 min of brain ischaemia increased GSK-3β phosphorylation at Ser^{9} and decreased its activity (Brywe *et al.*, 2005; Endo *et al.*, 2006). Increased phosphorylation

Correspondence: Shohreh Majd, as above.
E-mail: shohreh.majd@flinders.edu.au

of Ser^{9} in GSK-3β has also been reported following traumatic brain injury (Brywe *et al.*, 2005).

Protein Phosphatase 2A reduces p-tau and works in balance with GSK-3β (Liu *et al.*, 2005a). Phosphorylation at Tyr^{307} or down-regulation of PP2A increases p-tau (Chen *et al.*, 1992; Qian *et al.*, 2010; Martin *et al.*, 2013). Reduced PP2A activity has been reported post-mortem in patients with AD (Gong *et al.*, 1995; Sontag *et al.*, 2004), where phosphorylated PP2A is found in tangle-bearing neurons (Liu *et al.*, 2008). PP2A is sensitive to hypoxia and its reduction is suggested to be a responsible factor for phosphorylation of tau leading to neuronal death (Koh, 2011).

Tau dephosphorylation was reported following ischaemia. Tau can rephosphorylate at Ser^{396} during reperfusion while Ser^{262}/Ser^{356} remains dephosphorylated (Shackelford & Yeh, 1998; Mailliot *et al.*, 2000). Ser^{262} is an insensitive residue to GSK-3β (Guerra-Araiza *et al.*, 2007), which implies the involvement of other kinases in this scenario.

AMPK, the master controller of cellular metabolism and energy status affects tau phosphorylation at many residues such as $Ser^{369, 262}$ (Trinczek *et al.*, 2004), however the extent of its role in changing p-tau status in response to ischaemia has not been examined (Bright *et al.*, 2008). In this study, we used a cardiac arrest/reperfusion model to evaluate the role of AMPK, GSK-3β and PP2A in time-dependent phosphorylation of tau at $Ser^{396, 262}$, (AMPK-dependent), and Ser^{202}/Thr^{205} (AMPK-independent, GSK-3β-dependent). We tested the involvement of AMPK, GSK-3β and PP2A in regulating p-tau by assessing the phosphorylation of GSK-3β at (Ser^{9}), phosphorylation of PP2A (Tyr^{307}) and phosphorylation of AMPK (Thr^{172}). Catalase, a downstream substrate of AMPK was also assessed during the same periods of ischaemia/reperfusion to provide further signs of AMPK activity.

Materials and methods

Animal experiments

The animal experiments in this study were approved by the Animal Ethic Committee of Flinders University of South Australia and were completed in accordance with the South Australian Prevention of Cruelty to Animals Act 1985 and followed the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 2004.

Animal preparation

Sprague–Dawley rats were supplied by Laboratory Animal Services of the University of Adelaide. The rats were kept in the Flinders University Animal Facility until they reached the minimum age of 3 months. Female adult rats with the body weight of 250–350 g and 3 months of age were used in this study. The animals had free access to food and water. Twelve hours before the experiment the rats were fasted with free access to water. The experimental time point has been shown in Fig. 1. On the day of the experiment, anesthesia was initiated by intraperitoneal injection of Ketamine (Sigma, 343099) and Xylazine (Sigma, X1251), 100 mg/kg and 10 mg/kg body weight, respectively. The tail vein was cannulated using a 22G 0.90 mm intravenous catheter for drug delivery. The chest was shaved to provide a clear area for defibrillator electrodes' attachment and the electrocardiogram was recorded constantly via chest leads using a defibrillator/monitor (Philips HeartStart MRX; Philips Healthcare Inc., USA). Oxygen saturation and pulse rate were monitored constantly and recorded every 5 min via a Pulse-oximeter attached to the animal's paw. Ventilation was performed via endotracheal intubation, using a 16-gauge cannula inserted in the trachea and connected to a specific volume-controlled small animal ventilator with supplemental oxygen at 70 bpm and tidal volume adjusted to 6 mL/kg. Cardiac arrest (CA) was achieved, using two phases of transoesophageal alternating current (AC) (with modifications to the previous method used by Chen, *et al.* (Chen *et al.*, 2007). A pacing catheter (5F) with two end ring electrodes and a 0.5 cm gap was inserted into the oesophagus to a depth of 6–6.5 cm and connected to a current generator ensuring that the current was applied close to the heart without generating irreversible respiratory muscle paralysis. Two phase electrical stimulation using AC current consisting of 50 Hz AC 24V (phase 1), followed by 50 Hz AC 18V (phase 2) in order to generate the least oesophageal thermal injury. Ventilation was stopped during the period of CA. CA was confirmed through the electrocardiogram monitor, showing high voltage AC current and a loss of pulse detected by the oximeter within a few seconds after applying the current. Different durations of CA (2, 4 and 8 min) were studied followed by CPR (60, 70, 90, 120 min: short-term recovery; 4w: long-term recovery). At the end of the CA, if the heart rhythm was ventricular fibrillation (VF), the rats were defibrillated via external electrodes connected to a defibrillator (Philips HeartStart MRx), using 8 Joules of current. Standard CPR was performed supported by intravenous injection of adrenaline (0.01–0.03 mg/kg) via the tail vein with manual cardiac compressions at a rate of approximately 200–250/min. Compression depth was about 30% the anterio-posterior chest diameter. Effective circulation was confirmed by a pulse oximeter reading that relies on a pulse through a capillary bed to read oxygen saturation. Ventilation was started immediately and performed using a volume-controlled small animal ventilator with supplemental oxygen at 70 breaths per minute and tidal volume adjusted to 6 mL/kg. Restoration of circulation under resuscitation was defined as a CPR pulse or natural pulse with a mean oxygen saturation of 85–95% or greater during the time of resuscitation after CA. The animals were monitored for the time of resuscitation of 60, 70, 90, 120 min (short-term recovery) and 4 weeks (long-term recovery). For short-term recovery groups, the pulse rate and oxygen saturation were

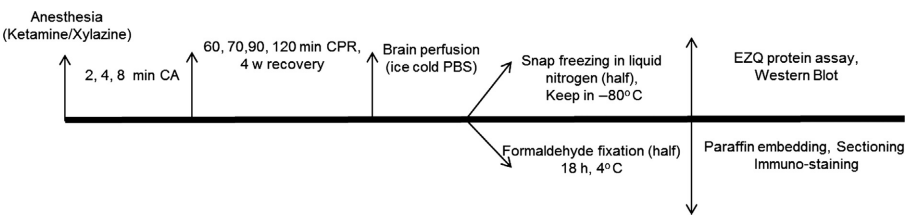

FIG. 1. Experimental time point of the study including the cardiac arrest (CA), reperfusion and recovery periods, brain perfusion and separation, protein assessment and Western blot, and immuno-staining.

monitored constantly and were recorded every 5 min. The animals in the long-term recovery group were also constantly monitored every 5 min until the achievement of a satisfactory cardiac output (confirmed via monitoring the heart rate and oxygen saturation) and a normal conscious state. The animals were then returned to their cages (one rat in each cage) and were returned to the animal house. Post-resuscitation, the rats were supervised continuously until physiological stability is demonstrated. The room temperature was set at 22 °C. Corneal lubricant application was administered during the recovery period until spontaneous blinking was resumed. In the post-resuscitation phase observation of the physiological parameters such as capillary refill time, body weight, physical appearance and movement were assessed twice a day for 4 weeks. To obtain brain samples at the end of recovery period (short and long-term), under general anesthesia and the brains were perfused with cold Phosphate Buffered Saline (PBS) the animals were killed painlessly and the brains collected for the further analysis. The specimens were divided into two with half being frozen in liquid nitrogen immediately after isolation and kept in −80 °C freezer. The remaining half was fixed in 10% paraformaldehyde for 48 h and subsequently prepared in paraffin blocks, which were sectioned using a microtome at 5 μm thickness. The sections were placed on Poly-d-Lysine coated slides, deparaffinized in xylene, and hydrated in a graded series of Ethanol.

Antibodies

Phosphorylated tau rabbit polyclonal antibody (Ser^{396}; sc-101815, Ser^{262} sc-101813), p-GSK-3β goat polyclonal antibody (Ser^{9}; sc-11757), p-PP2A-Cα/β mouse monoclonal antibody (F-8; sc-271903), tau mouse monoclonal antibody (A-10; sc-390476), GSK-3β rabbit polyclonal antibody (H-76; sc-9166) and PP2A-Aα goat polyclonal antibody (C-20; sc-6112) were purchased from Santa Cruz. Mouse monoclonal GFAP and Phospho-PHF-tau $pSer^{202}/Thr^{205}$ Antibody (AT8) were obtained from Associate Professor John Power from the Alzheimer's and Parkinson's laboratory of Flinders University. The p-AMPK rabbit polyclonal antibody (Thr^{172}, #2531) and AMPK rabbit polyclonal antibody (#2532) were purchased from Cell Signalling Australia. Catalase sheep affinity purified antibody was purchased from the Proteomics Facility of Flinders University. Mouse NeuN antibody (MAB377) was purchased from Merck Millipore Australia. Secondary antibodies were purchase from Jackson Immuno Research, USA (HRP donkey anti-mouse, anti-rabbit, anti-goat, Biotinylated donkey anti-rabbit, Alexa Fluor® 488 Donkey anti-rabbit, Alexa Fluor® CY3 Donkey anti-mouse and Alexa Fluor® CY3 Donkey anti-Sheep).

Brain homogenate

The middle 1/3 (0.3–0.35 g) of the frozen brain containing parietal cortex and hippocampus was homogenized in homogenizing extraction buffer containing protease inhibitors of Pepstatin A (Sigma, P5318, 1 μg/mL) and Leupeptin (Sigma, L2884, 1 μg/mL). The homogenate was centrifuged at 1000 ***g*** for 5 min at 4 °C and the supernatants were stored at −80 °C until analysed.

Protein quantification

The amount of total protein in each sample was calculated, using an EZQ assay following the approved protocol (BioRad, Hercules, CA, USA). Briefly, 10 μL of smaple, 25 μL of four times sample buffer (100% glycerol, 1M Tris/HCl pH 6.8, SDS, beta-mercaptoethanol, H_2O) and 65 25 μL H_2O were mixed. A quantity of 10 μL of this solution was added to 90 μL of H_2O, thereafter 1 μL of each sample and the standard solution (serial dilutions of ovalbumin) were loaded on to the assay paper in triplicate each in 96-well plates and absorbance was measured, using an Image Master VDS-CL (Amersham Biosciences) and quatified by CareStream molecular imaging software.

Western blot analysis

To analyse electrophoretic mobility of p-tau, tau, p-GSK-3β, GSK-3β, p-PP2A, PP2A, p-AMPK and AMPK, 30 μg of each sample in the sample buffer was loaded to each well of Any kD™ TGX Stain-free gel (Bio-Rad; 569033), along with 1 well of 5 μL Precision Plus Protein™ Dual Color Standards (Biorad). The current (100V, 300 mA) was applied to the gel for 20 min, to separate the proteins based on their molecular weights. After standard SDS-PAGE separation, the proteins were transferred onto Polyvinylidene Difluoride (PVDF) membrane at 100 V for 30 min. After electroblotting, the membranes were blocked for 1 hour at room temperature in a solution of 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (pH 7.6). The separate membranes were incubated overnight at 4 °C with primary antibodies of rabbit p-tau polyclonal antibody at Ser^{396} (1:250), rabbit anti-p-tau (Ser^{262}, 1:250), mouse Phospho-PHF-tau (Ser^{202}/Thr^{205}, 1:1000), p-GSK-3β polyclonal antibody at Ser^{9} (1:500), p-PP2A-Cα/β monoclonal antibody (1:500), tau monoclonal antibody (1:250), GSK-3β polyclonal antibody (1:500), PP2A-Aα polyclonal antibody (1:500), p-AMPK polyclonal antibody (1:1000), AMPK polyclonal antibody (1:1000) and sheep affinity purified anti-catalase (1:300). On the following day, the membranes were incubated for 1 hour at room temperature with the HRP secondary antibodies (donkey anti- mouse, 1:3000; donkey anti-rabbit, 1:3000, donkey anti-goat, 1:10 000). The blots were then developed using an ECL and the chemiluminescence signal detection was performed using Fuji LAS4000 imager and quatified by CareStream molecular imaging software, and were corrected by actin levels.

Immunohistochemistry

DAB-metal-enhanced immunohistochemistry and Immunofluorescence staining were undertaken by incubating 5μm brain sections of parietal cortex and hippocampus with rabbit anti-phosphorylated tau (Ser^{396}, 1:250), or rabbit anti-phosphorylated (AMPK (Thr^{172}, 1:250) for 18 h at 4° C to detect the phosphorylated tau and active form of AMPK through DAB immunohistochemistry and immunofluorescence staining, respectively. The sections were subsequently incubated for 1 h at room temperature with the secondary antibodies of Biotinylated donkey anti-rabbit for p-tau (1:1000, Jackson) and Goat anti-rabbit (Alexa Flour 488, 1:500). The fluorescent staining was visualized using a Leica SP5 5-channel laser scanning confocal microscope from Flinders University Microscopy Facility.

Statistical analysis

All of the data in this study were analysed using IBM SPSS Statistics version of SPSS Software and are expressed as the mean ± SD. One-way ANOVAS was used to assess the differences between the means of the groups followed by *post hoc* Tukey's. Significance was defined as $P < 0.05$.

Results

ECG pattern at the termination of current

Cardiac arrest produced VF in 3 rats. Eight min CA was associated with an asystolic rhythm when the AC current was terminated. In the rest of the animals, AC current generated normal sinus rhythm or sinus bradycardia (Fig. 2). Resuscitation involved defibrillation, CPR, mechanical ventilation, manual compression and IV adrenaline injections, attaining an average SPO_2 of 85 ± 5% and an average generated or natural pulse of 200 ± 40/min (Table 1).

Effect of global ischaemia and different periods of reperfusion on p-tau [($Ser^{396,\ 262}$, Ser^{202}/Thr^{205} (AT8))]

Western blot analyses were carried out using parietal cortical and subcortical hippocampus homogenates to investigate the levels of p-tau and total tau protein in ischaemic groups vs. control group (anaesthesia only) and in ischaemic/reperfusion groups relative to control group. The level of tau phosphorylation at Ser^{396} was reduced (One Way ANOVA, followed by TUKEY HSD, $F_{4,20} = 14.53$, $^*P < 0.001$ in 2 min ischaemia, 2 and 8 min ischaemia followed by 120 min reperfusion and 4 min ischaemia followed by 60 min reperfusion vs. control). It was also reduced significantly in 2 min ischaemia followed by 60, 70 and 90 min reperfusion (One Way ANOVA, followed by TUKEY HSD, $F_{3,16} = 26.11$, $^*P < 0.001$), whilst β-actin levels reflected equal loading across all lanes (Fig. 3). Tau showed some rephosphorylation after 4 weeks recovery. The difference between this group and control group as well as the short-term reperfusion groups was not statistically significant, however, p-tau distribution after 4 weeks recovery was mainly within the soma which in control groups showed the soma as well as neurites' distribution (Fig. 4B). The level of tau phosphorylation at Ser^{262}, the other residue which is phosphorylated by p-AMPK, was also reduced after short periods of ischaemia (one-way ANOVA, followed by TUKEY HSD, $F_{5,24} = 26.54$, $^*P < 0.001$ in 2 min ischaemia, and 2 min ischaemia followed by 60, 70, 90, and 120 min reperfusion vs. control). The phosphorylation of Ser^{202}/Thr^{205}, the sites that are not phosphorylated by p-AMPK did not show any significant changes (one-way ANOVA, $F_{8,36} = 0.42$, $P = 0.90132$, non-significant).

To examine any underlying changes in tau protein, we evaluated the level of total tau in the brain samples of the ischaemic group and the groups with different periods of ischaemia and reperfusion. The global ischaemia and reperfusion did not affect the tau protein

TABLE 1. Two main physiological parameters of heart rate and oxygen saturation that were recorded by Pulse-oximeter and chest leads using a defibrillator/monitor in control rats (anaesthesia only, no CA) and CA-treated rats

Animals	HR/min (Baseline)	Oxygen saturation% (Baseline)	HR/min (During Procedure)	Oxygen saturation% (During Procedure)
Control	320 ± 30	100–5	320 ± 20	100–2
CA	320 ± 30	100–5	200 ± 40	85 ± 10

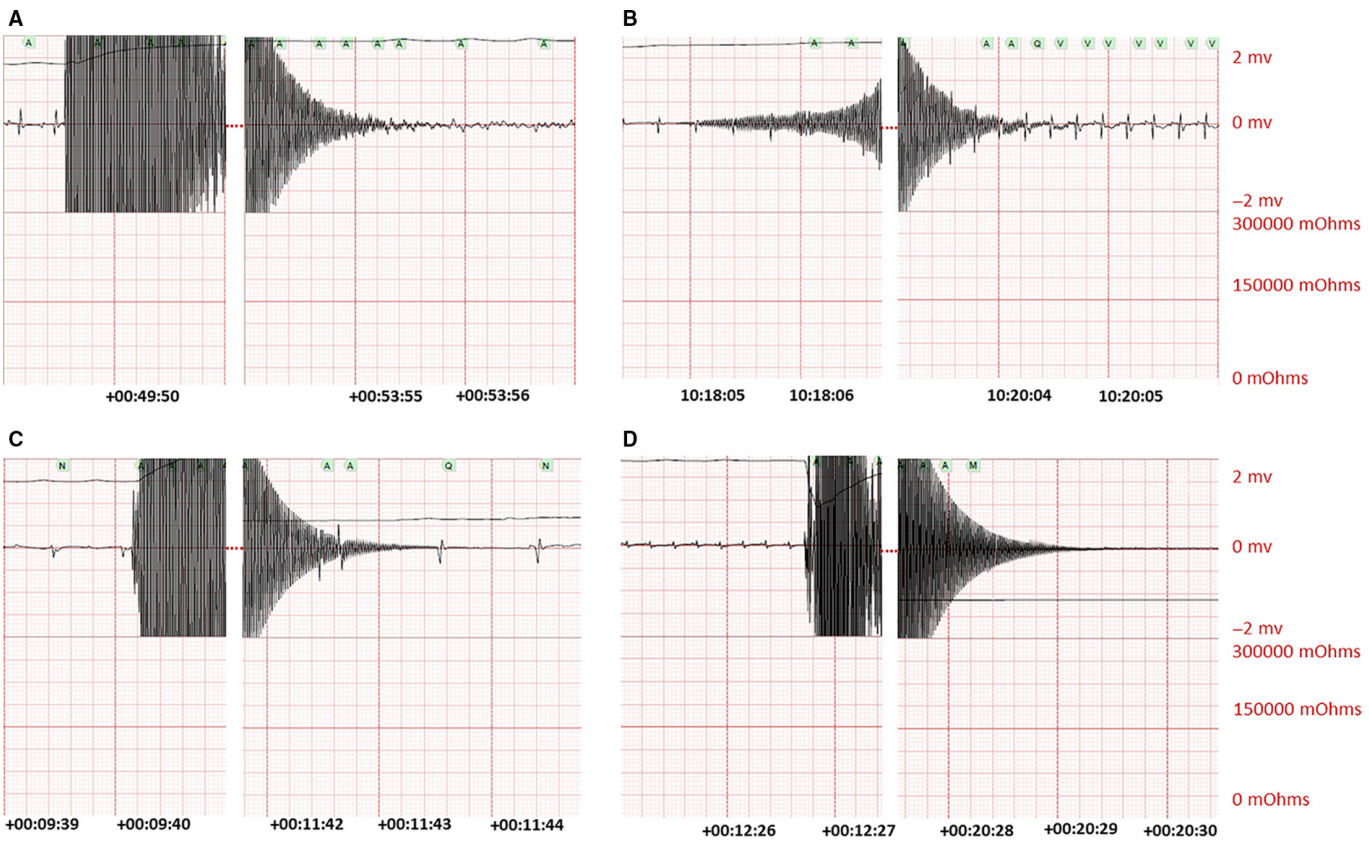

FIG. 2. (A) 4 min of high frequency AC generated the ventricular fibrillation. (B) Normal sinus rhythm after 2 min of CA, while the rat stayed alive for 2 h. (C) Sinus bradycardia after 2 min CA, and (D) Asystole after 8 min CA of 2 phases of high frequency AC.

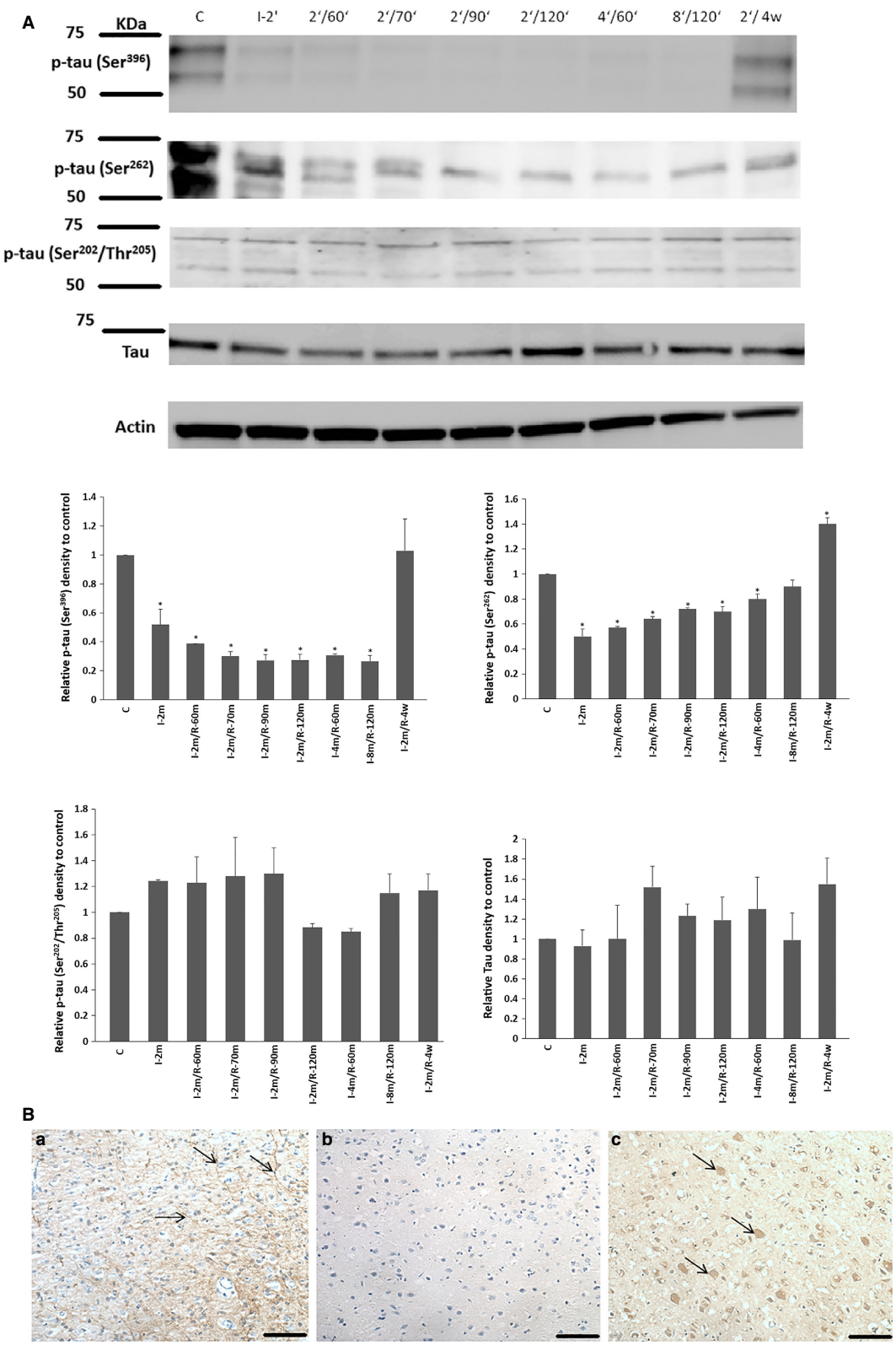

FIG. 3. (A) Tau phosphorylation at Ser^{396} and Ser^{262} and Ser^{202} /Thr^{205} after 2, 4 and 8 min ischaemia followed by 60, 70, 90 and 120 min reperfusion and 4 weeks recovery. Total amount of Tau is shown in all experimental groups. I-2': 2 min ischaemia, 2'/60': 2 min ischaemia followed by 60 min reperfusion, 2'/70': 2 min ischaemia followed by 70 min reperfusion, 2'/90': 2 min ischaemia followed by 90 min reperfusion, 2'/120': 2 min ischaemia followed by 120 min reperfusion, 4'/60': 4 min ischaemia followed by 60 min reperfusion, 8'/120': 8 min ischaemia followed by 120 min reperfusion, 2'/4 w: 2 min ischaemia followed by 4 weeks recovery period. *$P < 0.001$ all groups vs. control (One Way ANOVA). Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the Actin level. (B) Immunohistochemistry staining of tau from cortex and hippocampus of control group (a, b) 2 min ischaemia followed by 120 min of reperfusion and (c) long-term survivals of CA after 4 weeks of recovery following 2 min of CA. Scale bars, 100 μm.

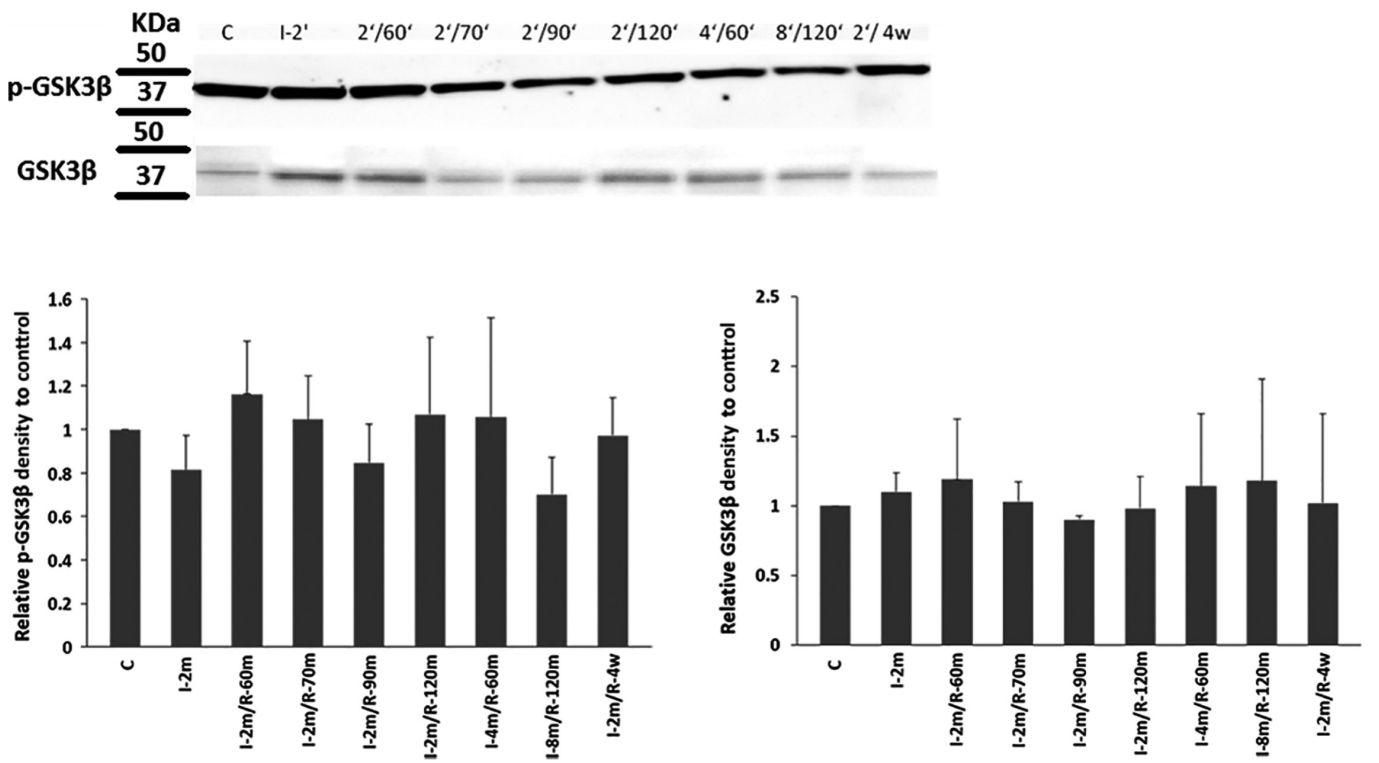

FIG. 4. Western blot analysis of the brain samples have been performed for p-GSK-3β at Ser^{9} and total protein of GSK-3β assessment from control group and during early ischaemia/reperfusion and after 4 weeks of recovery. All values are expressed as percent change relative to control group and were corrected by the Actin level. I-2': 2 min ischaemia, 2'/60': 2 min ischaemia followed by 60 min reperfusion, 2'/70': 2 min ischaemia followed by 70 min reperfusion, 2'/90': 2 min ischaemia followed by 90 min reperfusion, 2'/120': 2 min ischaemia followed by 120 min reperfusion, 4'/60': 4 min ischaemia followed by 60 min reperfusion, 8'/120': 8 min ischaemia followed by 120 min reperfusion, 2'/4 w: 2 min ischaemia followed by 4 weeks recovery period. Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the Actin level.

expression as no significant change in total tau between different ischaemia/reperfusion groups and between the ischaemia/reperfusion groups and the control group was observed (one-way ANOVA, $F_{8,36} = 1.43$, $P = 0.21776$, non-significant) (Fig. 3).

GSK-3β activity during global ischaemia/reperfusion

We examined the level of phosphorylated GSK-3β (p-GSK-3β) at Ser^{9} (inactive form) and the un-phosphorylated GSK-3β (active form) to study the role of this enzyme in the early state of tau phosphorylation. There was no significant change in p-GSK-3β between the control and ischaemic groups (one-way ANOVA, $F_{1,8} = 0.69$, $P = 0.43025$, nonsignificant). During reperfusion of 60, 70, 90, 120 min and 4 weeks, following 2, 4 and 8 min ischaemia, p-GSK-3β remained unchanged (one-way ANOVA, $F_{7,33} = 0.57$, $P = 0.77478$, nonsignificant). The total GSK-3β was also unchanged between the groups (one-way ANOVA, $F_{8,36} = 0.25$, $P = 0.97764$, non-significant) (Fig. 4), indicating that the activity of GSK-3β was constant, suggesting another mechanism rather than GSK-3β was involved in tau dephosphorylation during ischaemia and reperfusion.

PP2A activity during global ischaemia/reperfusion

PP2A levels were assessed to investigate the role of this main tau phosphatase in tau phosphorylation patterns after early ischaemia and during reperfusion. Phosphorylated PP2A (p-PP2A) at Tyr^{307} (inactive form) did not show any significant change (one-way ANOVA, $F_{8,36} = 2.11$, $P = 0.06043$, no significant difference between all groups vs. control). PP2A (active form) remained at the same levels with no significant change between different times of ischaemia (2, 4 and 8 min) followed by 60, 70, 90, 120 min and 4 weeks of reperfusion (One Way ANOVA, $F_{8,36} = 1.98$, $P = 0.07757$, non-significant) (Fig. 5). This suggests that tau dephosphorylation occurred immediately after ischaemia and in the early reperfusion times was independent of PP2A activity.

AMPK phosphorylation in ischaemia and reperfusion

The level of p-AMPK (active form) phosphorylated at Thr^{172} significantly decreased during ischaemia (2 and 4 min) followed by 60, 70, 90 and 120 min reperfusion (one-way ANOVA, followed by TUKEY HSD, $F_{4,20} = 6.8$, $P = 0.00126$, $^{*}P < 0.005$, 2 min ischaemia and 2 min ischaemia followed by 60, 70 and 90 min reperfusion vs. control; One Way ANOVA, followed by TUKEY HSD, $F_{2,12} = 121.41$, $^{*}P < 0.001$, 2 min ischaemia followed by 120 min reperfusion and 4 min ischaemia followed by 60 min reperfusion vs. control) with almost the same pattern as p-tau. AMPK phosphorylation was increased in the 8 min CA group and after 4 weeks recovery compare with the control group (one-way ANOVA, followed by TUKEY HSD, $F = 13.86$, $^{*}P < 0.001$), whilst β-actin levels reflected equal loading across all lanes (Fig. 6A). The ischaemia and reperfusion did not affect the AMPK protein expression as the total AMPK remained unchanged during ischaemia/reperfusion (One Way ANOVA, $F_{8,36} = 0.12$, $P = 0.99808$, no significant difference between all groups vs. each other and vs. control) (Fig. 6A). Immunofluorescent results showed AMPK activation in control groups as well as positive staining for p-AMPK after 4 weeks of recovery, which was mostly detected in neurons rather than glial

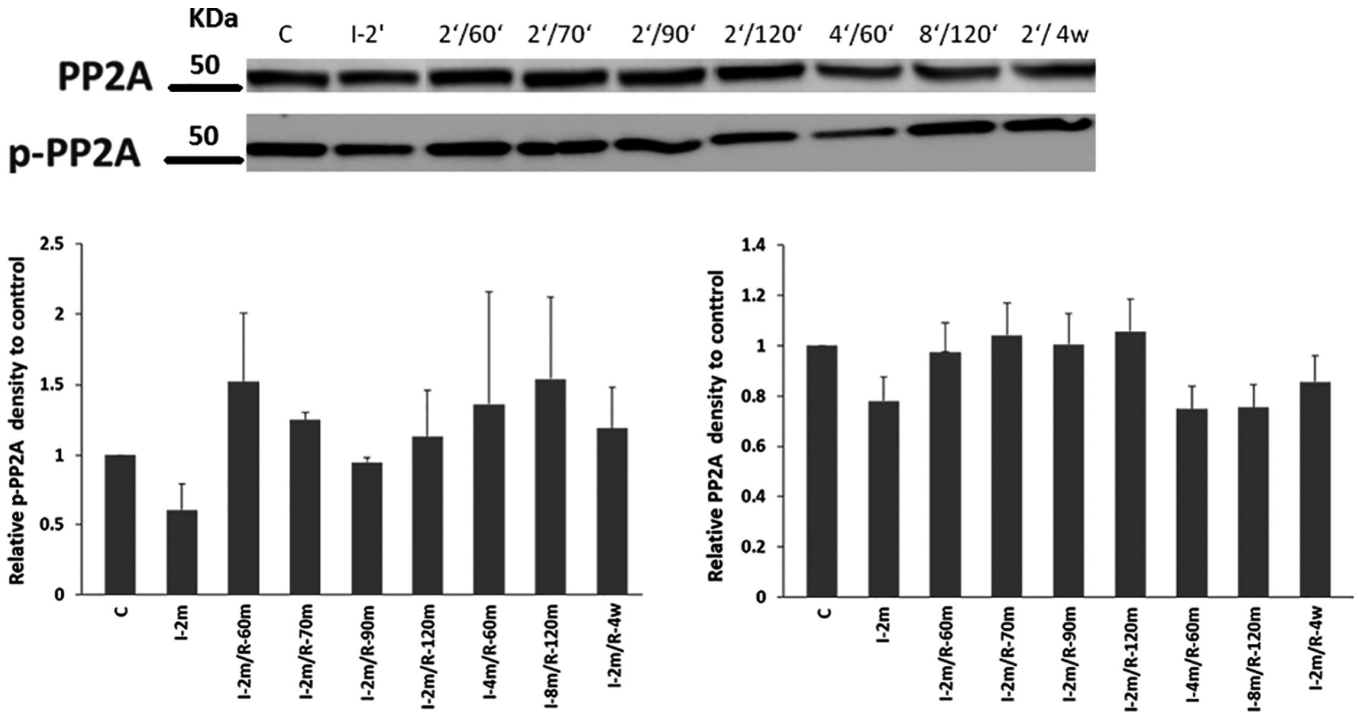

FIG. 5. Western blot analysis of the brain samples have been performed for PP2A and p-PP2A assessment from control group and during ischaemia and reperfusion and after 4 weeks of recovery. I-2': 2 min ischaemia, 2'/60': 2 min ischaemia followed by 60 min reperfusion, 2'/70': 2 min ischaemia followed by 70 min reperfusion, 2'/90': 2 min ischaemia followed by 90 min reperfusion, 2'/120': 2 min ischaemia followed by 120 min reperfusion, 4'/60': 4 min ischaemia followed by 60 min reperfusion, 8'/120': 8 min ischaemia followed by 120 min reperfusion, 2'/4 w: 2 min ischaemia followed by 4 weeks recovery period. Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the Actin level.

cells, however a positive AMPK activation was missing in the early response to ischaemia/reperfusion (Fig. 6B). Changes in p-tau in response to ischaemia compared with control groups follows the same pattern of p-AMPK alteration within the same periods of ischaemia/reperfusion, which suggests another evidence for AMPK kinase activity on tau following ischaemia, when the level and activity of GSK-3β and PP2A have not been change.

Effect of ischaemia and reperfusion on catalase, the downstream substrate of AMPK

One of the known upstream regulators of catalase is AMPK. We examined the effect of ischaemia on catalase levels in the different groups by Western blot. Catalase reduced following 2 min ischaemia (one-way ANOVA, followed by TUKEY HSD, $F_{1,8} = 98.75$, $^*P < 0.001$) and also in ischaemia and reperfusion groups versus control group (one-way ANOVA, followed by TUKEY HSD, $F_{7,32} = 546.21$, $^*P < 0.001$) (Fig. 6A). AMPK activation in hypoxic situations is known to increase the cellular level of catalase (Sengupta *et al.*, 2011; Awad *et al.*, 2014). A lower level of catalase after short periods of ischaemia is consistent with the lower levels of AMPK activation that we have observed.

Discussion

This study, revealed an almost instant dephosphorylation of tau protein as one of the first events following a global ischaemia in the brain, in two residues of Ser^{396} and Ser^{262}, the latest one is specifically affected by kinase activity of AMPK. Our CA model of global cerebral ischaemia in the rat provided us with a similar situation to CA in human beings, which could be followed by reperfusion due to cardiopulmonary resuscitation. Our results are inconsistent with a previous study, showing the tau dephosphorylation in a transient ischaemic model of stroke (Shackelford & Yeh, 1998). Song *et al.* (Song *et al.*, 2013) reported that dephosphorylated tau after 15 min of ischaemia had been rephosphorylated during reperfusion, however in our study, shorter periods of ischaemia (up to 8 min) caused tau dephosphorylation at Ser^{396} which had not shown rephosphorylation within a reperfusion time of 60, 70, 90 and 120 min. This suggests that shorter ischaemic periods possibly cause dephosphorylation through a different mechanism to that involved in phosphorylation control after longer ischaemic times.

The leading cause of changes in the neuronal status of tau between phosphorylated, hyperphosphorylated and dephosphorylated forms has been known as a deregulated activity of tau protein kinases and phosphatases, with a documented role in AD pathology (Liu *et al.*, 2005a; Qian *et al.*, 2010). The main tau kinase is GSK; its GSK-3β isoform can phosphorylate tau at multiple sites, including Ser^{235}, Ser^{396}, Ser^{202}/Thr^{205}, Thr^{175} and Thr^{181} (Takashima, 2006; Hanger *et al.*, 2009; Hernandez *et al.*, 2013), but not at Ser^{262} (Guerra-Araiza *et al.*, 2007) Animal *in vivo* studies have also shown that activation or over-expression of GSK-3β affects microtubule assembly leading to their disruption due to tau hyperphosphorylation (Lucas *et al.*, 2001; Hernandez *et al.*, 2013). Human post-mortem results from AD brains have reported the co-existence of activated GSK-3β with NFTs in dystrophic neurites and astrocytes (Ferrer *et al.*, 2002). Tau phosphorylation at Ser^{262} is one of the principal causes for loss of tau's physiological ability to attach to microtubules, which is mediated by AMPK rather than GSK-3β (Iijima *et al.*, 2010). Among the many different phosphatases, PP2A is the dominant phosphatase responsible for 70% of tau dephosphorylation, its inhibited activity has been suggested as

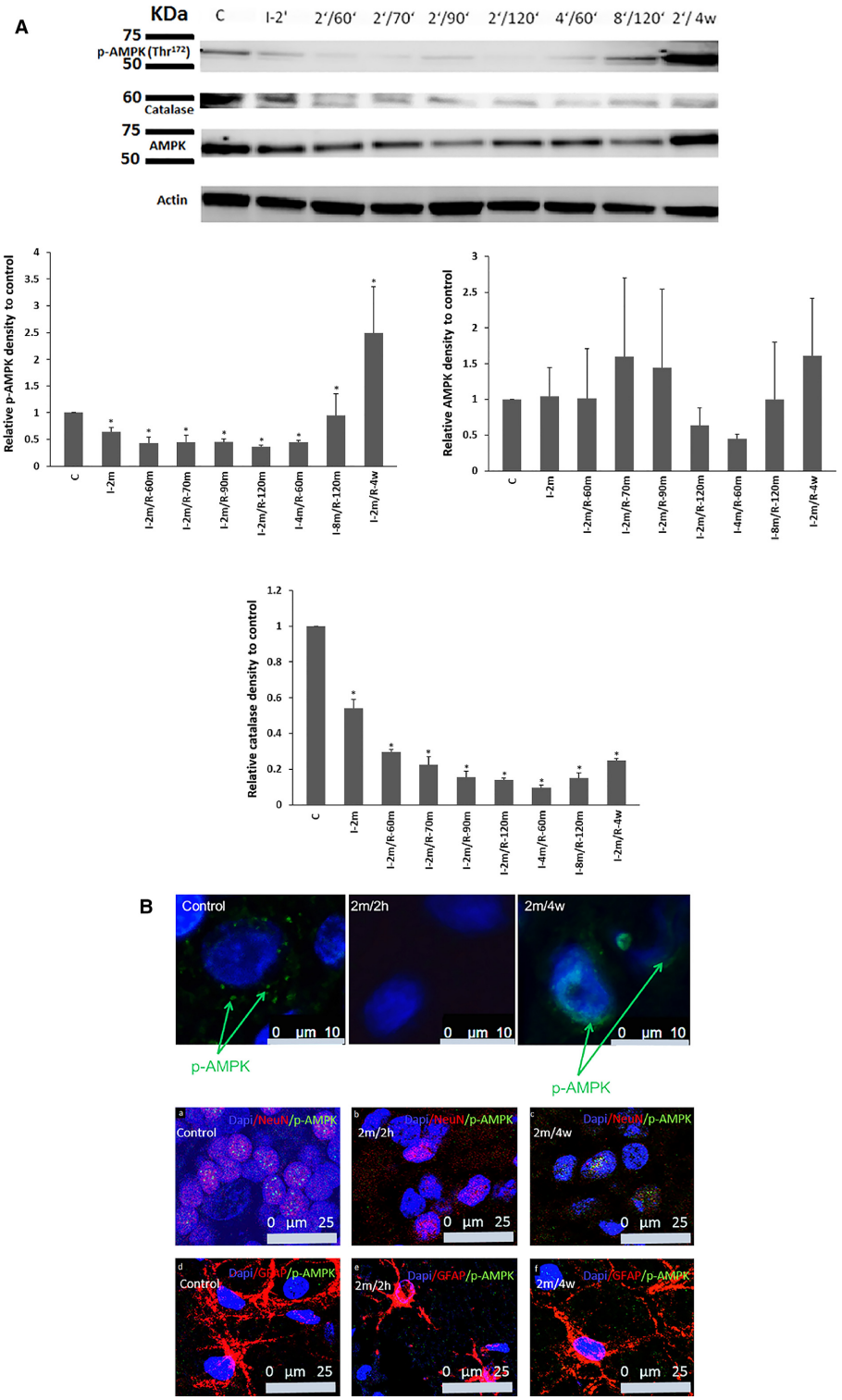

FIG. 6. (A) Western blot of protein levels of p-AMPK, AMPK and catalase have been analysed using the brain samples from control group and during ischaemia and reperfusion and after 4 weeks of recovery. I-2': 2 min ischaemia, 2'/60': 2 min ischaemia followed by 60 min reperfusion, 2'/70': 2 min ischaemia followed by 70 min reperfusion, 2'/90': 2 min ischaemia followed by 90 min reperfusion, 2'/120': 2 min ischaemia followed by 120 min reperfusion, 4'/60': 4 min ischaemia followed by 60 min reperfusion, 8'/120': 8 min ischaemia followed by 120 min reperfusion, 2'/4 w: 2 min ischaemia followed by 4 weeks recovery period. *P < 0.005 for p-AMPK in I-2', 2'/60', 2'/70' and 2'/90' vs. control, *P < 0.001, for p-AMPK in 2'/120' and 4'/60' versus control, also for 8'/120' and 2'/4 w versus control (one-way ANOVA). For catalase, *P < 0.001 for all experimental groups versus control group (one-way ANOVA). Error bars depict the SD. All values are expressed as percent change relative to control group and were corrected by the Actin level. (B) Evaluating the of AMPK activity through analysing p-AMPK (green) presence and distribution within the neurons that were co-stained with NeuN (Red, a–c) or GFAP (Red, d–f) from the parietal cortex and hippocampus of control, 2 min isachemia followed by 2 h reperfusion or 4 weeks recovery groups.

an underlying mechanism for tau hyperphosphorylation in AD (Liu *et al.*, 2005a; Rudrabhatla & Pant, 2011). To investigate the role of tau kinase-phosphatase balance break down in creating the early pattern of tau dephophorylation, which persisted during reperfusion, we examined the level of GSK-3β and PP2A activities. Our hypothesis was based on GSK-3β inactivation in early ischaemia and reperfusion. Our observations however showed no obvious change in phosphorylated GSK-3β at Ser^{9} (inactive form) and the total GSK-3β within very short periods of ischaemia (2–8 min) as well as reperfusion (60, 70, 90 and 120 min). Additionally, both PP2A (active form) and p-PP2A at Tyr^{307} (inactive form) remained at an almost constant level.

We suggest that that changes in GSK-3β and PP2A activities on tau protein are not the initial mechanism of reducing tau phosphorylation in the very early stages of ischaemia. The unchanged phosphorylation status of Ser^{202}/Thr^{205}, residues that are directly affected by GSK-3β activity supports our hypothesis. Theoretically, the dephosphorylation of tau could be due to an up-regulation or down-regulation of tau kinases and phosphatases, respectively. PP2A has been proposed as the major tau phosphatase, it has been shown that tau phosphorylation is not always affected by its activity (Gong *et al.*, 2000; Kins *et al.*, 2001), suggesting a role for other phosphatases such as Protein Phosphatase 5 in addition to PP2A (Liu *et al.*, 2005b). On the other hand, tau could be phosphorylated/hyperphosphorylated at more than 40 sites of Ser/Thr residues (Hanger *et al.*, 2007), by other kinases such as calmodulin-dependent protein kinase (CAMK) (Iijima-Ando *et al.*, 2010; Thornton *et al.*, 2011), the AGC kinase subfamily (including PKA and PKC) (Pei *et al.*, 2006; Virdee *et al.*, 2007), and AMP-activated protein kinase (AMPK)-related kinase (Mailliot *et al.*, 2000; Thornton *et al.*, 2011; Yoshida & Goedert, 2012).

Previous studies have reported an increased in p-AMPK in a rat model of middle cerebral artery occlusion MCAO for 2 h, starting after 90 min of ischaemia (McCullough *et al.*, 2005). Our results showed a very early dephosphorylation of AMPK starting as early as two minutes after onset of ischaemia, which did not show rephosphorylation during reperfusion times of 60, 70, 90 and 120 min. The global decrease in p-AMPK levels within the first minutes of ischaemia suggested that AMPK activation reported after longer periods of ischaemia does not occur in the immediate stage of ischaemia. We submit that this reduced AMPK activation could be considered as the first mechanism of neuronal protection against ischaemic insult. A previous study showed a neuro-protective effect of AMPK inhibition in stroke that supports our hypothesis (Li *et al.*, 2007). After 8 min of ischaemia p-AMPK levels began to rise and remained at a very high level in the long-term recovery group after 4 weeks. This second stage of AMPK phosphorylation is consistent with the role of AMPK in modulating energy metabolism of cells triggered by hypoxia/anoxia, adjusting the intracellular ATP by stimulating the catabolic process producing ATP, and blocking the anabolic process consuming ATP (Culmsee *et al.*, 2001; Russell *et al.*, 2004; Ramamurthy & Ronnett, 2006). Tau phosphorylation is also an indicator of altered neuronal metabolism (van der Harg *et al.*, 2014). Thus, a number of metabolic-related events of ischaemia could explain tau dephosphorylation, with the short-term and hyperphosphorylation during the long-term recovery.

We found that the level of p-AMPK was reduced within the first min of ischaemia and remained reduced during the reperfusion time, with the same pattern of reduction in p-tau. Further evidence to support the AMPK activity alteration immediately after ischaemia, and during reperfusion comes from the results of testing one of the other main downstream substances of AMPK, which is catalase. There are several schools of thought, showing a direct link between activation of AMPK during hypoxia and increasing the level of cellular catalase (Awad *et al.*, 2014). Sengupta *et al.* (Sengupta *et al.*, 2011) found that hypoxia/reperfusion increased the cardiomyocytes catalase through activation of AMPK. Our data showed that the catalase level reduced significantly immediately after ischaemia and during reperfusion in parallel with a reduction in AMPK activity. These findings support our hypothesis of the involvement of the AMPK pathway in early tau dephosphorylation, suggesting the dominant role for this kinase in changing tau phosphorylation status as one of the immediate brain response to ischaemic situations.

Further examination of the long-term effect of ischaemia after 4 weeks recovery showed rephosphorylation of AMPK in parallel with tau hyperphosphorylation. This effect could be consistent with the hypothesis that AMPK activation is an important component of cell adaptive mechanisms to ischaemic insult protecting cells in response to a persistent stressful situation (Russell *et al.*, 2004). Considering AMPK as one of the kinases responsible for the phosphorylation of tau (Thornton *et al.*, 2011; Yoshida & Goedert, 2012), we showed the pattern of tau phosphorylation following short episodes of ischaemia was similar to that of p-AMPK, without dramatic changes in GSK-3β and/or PP2A activity during short-term and long-term recovery. This suggests a possible role for AMPK either as the main sensor of cellular energy balance taking the leading role in defining the phosphorylation status of tau or closely linked to that sensor. We believe that the global situation of reduced oxygen supply leads to a very early decrease in phosphorylation of AMPK, with a consequent reduction in p-tau. This reduced phosphorylation of two main proteins could be a possible cellular mechanism saving energy for more essential cellular functions.

In conclusion, this study suggests an early mechanism for dephosphorylation of the tau protein during global cerebral ischaemia/reperfusion linked to reduced AMPK activity. While GSK-3β and PP2A, the usual tau kinase and phosphatase, are considered to have central roles in phosphorylation/dephosphorylation of tau in the longer term response to brain ischaemia (Song *et al.*, 2013), we suggest AMPK as either the most likely sensor of metabolic stress in the very early stages of ischemia or closely linked to that sensor. In the early stages of ischemia reduction in AMPK activity plays a dominant role in reducing tau phosphorylation, with the possible benefit of saving more ATP for neuronal survival until energy production can be restored. This proposed neuroprotective event seems to be temporary and the full activation of AMPK at later stages of ischaemia eventually takes place, switching on the entire ATP catabolic pathways while switching off the ATP-consuming anabolic ones.

Conflict of interests

There are no conflicts of interest to disclose in this study.

Acknowledgements

This study was supported by the Australian Resuscitation Council and Flinders University Establishment grant. The authors thank the Biomedical Engineering Department of Flinders University specially Mark McEwen and Noel Kitto for their technical support, the Proteomics Facility of Flinders University specially Dr. Tim Chataway, the Animal Facility of Flinders University, Professor Neil Sims from Medical Biochemistry Department and from the Alzheimer's and Parkinson diseases' lab especially Fariba Chegini for their enthusiastic discussions and support. All authors involved in the study design and development of the model. SM and HG were responsible for completing the experiments. The initial draft was written by SM and reviewed by HG and JP.

Abbreviations

AMPK, Adenosine Monophosphate Kinase; CA, cardiac arrest; CAMK, calmodulin-dependent protein kinase; CPR, cardiopulmonary resuscitation; GSK, Glycogen Synthase Kinase; NFTs, neurofibrillary tangles; PBS, Phosphate Buffered Saline; PVDF, Polyvinylidene Difluoride; PP2A, Protein Phosphatase 2A; GSK-3β, Glycogen Synthase Kinase-3.

References

Awad, H., Nolette, N., Hinton, M. & Dakshinamurti, S. (2014) AMPK and FoxO1 regulate catalase expression in hypoxic pulmonary arterial smooth muscle. *Pediatr. Pulm.*, **49**, 885–897.

Ballatore, C., Lee, V.M. & Trojanowski, J.Q. (2007) Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat. Rev. Neurosci.*, **8**, 663–672.

Bright, N.J., Carling, D. & Thornton, C. (2008) Investigating the regulation of brain-specific kinases 1 and 2 by phosphorylation. *J. Biol. Chem.*, **283**, 14946–14954.

Brywe, K.G., Mallard, C., Gustavsson, M., Hedtjarn, M., Leverin, A.L., Wang, X., Blomgren, K., Isgaard, J. *et al.* (2005) IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3-beta? *Eur. J. Neuorsci.*, **21**, 1489–1502.

Castro-Alvarez, J.F., Gutierrez-Vargas, J., Darnaudery, M. & Cardona-Gomez, G.P. (2011) ROCK inhibition prevents tau hyperphosphorylation and p25/CDK5 increase after global cerebral ischemia. *Behav. Neurosci.*, **125**, 465–472.

Chen, J., Martin, B.L. & Brautigan, D.L. (1992) Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation. *Science*, **257**, 1261–1264.

Chen, M.H., Liu, T.W., Xie, L., Song, F.Q., He, T., Zeng, Z.Y. & Mo, S.R. (2007) A simpler cardiac arrest model in rats. *Am. J. Emerg. Med.*, **25**, 623–630.

Culmsee, C., Monnig, J., Kemp, B.E. & Mattson, M.P. (2001) AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and promotes neuronal survival following glucose deprivation. *J. Mol. Neurosci.*, **17**, 45–58.

Dewar, D. & Dawson, D. (1995) Tau protein is altered by focal cerebral ischaemia in the rat: an immunohistochemical and immunoblotting study. *Brain Res.*, **684**, 70–78.

Eldar-Finkelman, H. (2002) Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol. Med.*, **8**, 126–132.

Endo, H., Nito, C., Kamada, H., Nishi, T. & Chan, P.H. (2006) Activation of the Akt/GSK3beta signaling pathway mediates survival of vulnerable hippocampal neurons after transient global cerebral ischemia in rats. *J. Cerebr. Blood F. Met.*, **26**, 1479–1489.

Ferrer, I., Barrachina, M. & Puig, B. (2002) Glycogen synthase kinase-3 is associated with neuronal and glial hyperphosphorylated tau deposits in Alzheimer's disease, Pick's disease, progressive supranuclear palsy and corticobasal degeneration. *Acta Neuropathol.*, **104**, 583–591.

Geddes, J.W., Schwab, C., Craddock, S., Wilson, J.L. & Pettigrew, L.C. (1994) Alterations in tau immunostaining in the rat hippocampus following transient cerebral ischemia. *J. Cerebr. Blood F. Met.*, **14**, 554–564.

Gong, C.X., Shaikh, S., Wang, J.Z., Zaidi, T., Grundke-Iqbal, I. & Iqbal, K. (1995) Phosphatase activity toward abnormally phosphorylated tau: decrease in Alzheimer disease brain. *J. Neurochem.*, **65**, 732–738.

Gong, C.X., Lidsky, T., Wegiel, J., Zuck, L., Grundke-Iqbal, I. & Iqbal, K. (2000) Phosphorylation of microtubule-associated protein tau is regulated by protein phosphatase 2A in mammalian brain. Implications for neurofibrillary degeneration in Alzheimer's disease. *J. Biol. Chem.*, **275**, 5535–5544.

Guerra-Araiza, C., Amorim, M.A., Camacho-Arroyo, I. & Garcia-Segura, L.M. (2007) Effects of progesterone and its reduced metabolites, dihydroprogesterone and tetrahydroprogesterone, on the expression and phosphorylation of glycogen synthase kinase-3 and the microtubule-associated protein tau in the rat cerebellum. *Dev. Neurobiol.*, **67**, 510–520.

Hanger, D.P. & Noble, W. (2011) Functional implications of glycogen synthase kinase-3-mediated tau phosphorylation. *Int. J. Alzheimer Dis.*, **2011**, 352805.

Hanger, D.P., Byers, H.L., Wray, S., Leung, K.Y., Saxton, M.J., Seereeram, A., Reynolds, C.H., Ward, M.A. *et al.* (2007) Novel phosphorylation sites in tau from Alzheimer brain support a role for casein kinase 1 in disease pathogenesis. *J. Biol. Chem.*, **282**, 23645–23654.

Hanger, D.P., Anderton, B.H. & Noble, W. (2009) Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. *Trends Mol. Med.*, **15**, 112–119.

van der Harg, J.M., Nolle, A., Zwart, R., Boerema, A.S., van Haastert, E.S., Strijkstra, A.M., Hoozemans, J.J. & Scheper, W. (2014) The unfolded protein response mediates reversible tau phosphorylation induced by metabolic stress. *Cell Death Dis.*, **5**, e1393.

Hernandez, F., Lucas, J.J. & Avila, J. (2013) GSK3 and tau: two convergence points in Alzheimer's disease. *J. Alzheimers Dis.*, **33**(Suppl 1), S141–144.

Iijima, K., Gatt, A. & Iijima-Ando, K. (2010) Tau Ser262 phosphorylation is critical for Abeta42-induced tau toxicity in a transgenic Drosophila model of Alzheimer's disease. *Hum. Mol. Genet.*, **19**, 2947–2957.

Iijima-Ando, K., Zhao, L., Gatt, A., Shenton, C. & Iijima, K. (2010) A DNA damage-activated checkpoint kinase phosphorylates tau and enhances tau-induced neurodegeneration. *Hum. Mol. Genet.*, **19**, 1930–1938.

Iqbal, K., Alonso, A.C., Gong, C.X., Khatoon, S., Pei, J.J., Wang, J.Z. & Grundke-Iqbal, I. (1998) Mechanisms of neurofibrillary degeneration and the formation of neurofibrillary tangles. *J. Neural. Transm.*, **53**, 169–180.

Kins, S., Crameri, A., Evans, D.R., Hemmings, B.A., Nitsch, R.M. & Gotz, J. (2001) Reduced protein phosphatase 2A activity induces hyperphosphorylation and altered compartmentalization of tau in transgenic mice. *J. Biol. Chem.*, **276**, 38193–38200.

Koh, P.O. (2011) Focal cerebral ischemia reduces protein Phosphatase 2A Subunit B expression in brain tissue and HT22 Cells. *Lab. Anim. Res.*, **27**, 73–76.

Li, J., Zeng, Z., Viollet, B., Ronnett, G.V. & McCullough, L.D. (2007) Neuroprotective effects of adenosine monophosphate-activated protein kinase inhibition and gene deletion in stroke. *Stroke*, **38**, 2992–2999.

Liu, F., Grundke-Iqbal, I., Iqbal, K. & Gong, C.X. (2005a) Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *Eur. J. Neuorsci.*, **22**, 1942–1950.

Liu, F., Iqbal, K., Grundke-Iqbal, I., Rossie, S. & Gong, C.X. (2005b) Dephosphorylation of tau by protein phosphatase 5: impairment in Alzheimer's disease. *J. Biol. Chem.*, **280**, 1790–1796.

Liu, R., Zhou, X.W., Tanila, H., Bjorkdahl, C., Wang, J.Z., Guan, Z.Z., Cao, Y., Gustafsson, J.A. *et al.* (2008) Phosphorylated PP2A (tyrosine 307) is associated with Alzheimer neurofibrillary pathology. *J. Cell Mol. Med.*, **12**, 241–257.

Lovell, MA, Xiong, S, Xie, C, Davies, P & Markesbery, WR (2004) Induction of hyperphosphorylated tau in primary rat cortical neuron cultures mediated by oxidative stress and glycogen synthase kinase-3. *J. Alzheimers Dis.*, **6**, 659–671; discussion 673-681.

Lucas, J.J., Hernandez, F., Gomez-Ramos, P., Moran, M.A., Hen, R. & Avila, J. (2001) Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J.*, **20**, 27–39.

Madureira, S., Guerreiro, M. & Ferro, J.M. (2001) Dementia and cognitive impairment three months after stroke. *Eur. J. Neurol.*, **8**, 621–627.

Mailliot, C., Podevin-Dimster, V., Rosenthal, R.E., Sergeant, N., Delacourte, A., Fiskum, G. & Buee, L. (2000) Rapid tau protein dephosphorylation and differential rephosphorylation during cardiac arrest-induced cerebral ischemia and reperfusion. *J. Cerebr. Blood F. Met.*, **20**, 543–549.

Martin, L., Latypova, X., Wilson, C.M., Magnaudeix, A., Perrin, M.L. & Terro, F. (2013) Tau protein phosphatases in Alzheimer's disease: the leading role of PP2A. *Ageing Res. Rev.*, **12**, 39–49.

Mateen, F.J., Josephs, K.A., Trenerry, M.R., Felmlee-Devine, M.D., Weaver, A.L., Carone, M. & White, R.D. (2011) Long-term cognitive outcomes following out-of-hospital cardiac arrest: a population-based study. *Neurology*, **77**, 1438–1445.

McCullough, L.D., Zeng, Z., Li, H., Landree, L.E., McFadden, J. & Ronnett, G.V. (2005) Pharmacological inhibition of AMP-activated protein kinase provides neuroprotection in stroke. *J. Biol. Chem.*, **280**, 20493–20502.

Noble, W., Pooler, A.M. & Hanger, D.P. (2011) Advances in tau-based drug discovery. *Expert Opin. Drug Dis.*, **6**, 797–810.

Pei, J.J., Braak, E., Braak, H., Grundke-Iqbal, I., Iqbal, K., Winblad, B. & Cowburn, R.F. (1999) Distribution of active glycogen synthase kinase 3-beta (GSK-3beta) in brains staged for Alzheimer disease neurofibrillary changes. *J. Neuropath. Exp. Neur.*, **58**, 1010–1019.

Pei, J.J., An, W.L., Zhou, X.W., Nishimura, T., Norberg, J., Benedikz, E., Gotz, J. & Winblad, B. (2006) P70 S6 kinase mediates tau phosphorylation and synthesis. *FEBS Lett.*, **580**, 107–114.

Pohjasvaara, T., Erkinjuntti, T., Ylikoski, R., Hietanen, M., Vataja, R. & Kaste, M. (1998) Clinical determinants of poststroke dementia. *Stroke*, **29**, 75–81.

Qian, W., Shi, J., Yin, X., Iqbal, K., Grundke-Iqbal, I., Gong, C.X. & Liu, F. (2010) PP2A regulates tau phosphorylation directly and also indirectly via activating GSK-3beta. *J. Alzheimers Dis.*, **19**, 1221–1229.

Ramamurthy, S. & Ronnett, G.V. (2006) Developing a head for energy sensing: AMP-activated protein kinase as a multifunctional metabolic sensor in the brain. *J. Physiol.*, **574**, 85–93.

Rudrabhatla, P. & Pant, H.C. (2011) Role of protein phosphatase 2A in Alzheimer's disease. *Curr. Alzheimer Res.*, **8**, 623–632.

Russell, R.R. 3rd, Li, J., Coven, D.L., Pypaert, M., Zechner, C., Palmeri, M., Giordano, F.J., Mu, J. *et al.* (2004) AMP-activated protein kinase mediates ischemic glucose uptake and prevents postischemic cardiac dysfunction, apoptosis, and injury. *J. Clin. Invest.*, **114**, 495–503.

Sengupta, A., Molkentin, J.D., Paik, J.H., DePinho, R.A. & Yutzey, K.E. (2011) FoxO transcription factors promote cardiomyocyte survival upon induction of oxidative stress. *J. Biol. Chem.*, **286**, 7468–7478.

Shackelford, D.A. & Yeh, R.Y. (1998) Dephosphorylation of tau during transient forebrain ischemia in the rat. *Mol. Chem. Neuropathol.*, **34**, 103–120.

Song, B., Ao, Q., Wang, Z., Liu, W., Niu, Y., Shen, Q., Zuo, H., Zhang, X. *et al.* (2013) Phosphorylation of tau protein over time in rats subjected to transient brain ischemia. *Neural Regen. Res.*, **8**, 3173–3182.

Sontag, E., Luangpirom, A., Hladik, C., Mudrak, I., Ogris, E., Speciale, S. & White, C.L. 3rd (2004) Altered expression levels of the protein phosphatase 2A ABalphaC enzyme are associated with Alzheimer disease pathology. *J. Neuropath. Exp. Neur.*, **63**, 287–301.

Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruynseels, K., Lasrado, R., Vandezande, K. *et al.* (2000) Glycogen synthase kinase-3beta phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice. *J. Biol. Chem.*, **275**, 41340–41349.

Takashima, A. (2006) GSK-3 is essential in the pathogenesis of Alzheimer's disease. *J. Alzheimers Dis.*, **9**, 309–317.

Tatemichi, T.K., Desmond, D.W., Mayeux, R., Paik, M., Stern, Y., Sano, M., Remien, R.H., Williams, J.B. *et al.* (1992) Dementia after stroke: baseline frequency, risks, and clinical features in a hospitalized cohort. *Neurology*, **42**, 1185–1193.

Thornton, C., Bright, N.J., Sastre, M., Muckett, P.J. & Carling, D. (2011) AMP-activated protein kinase (AMPK) is a tau kinase, activated in response to amyloid beta-peptide exposure. *Biochem. J.*, **434**, 503–512.

Trinczek, B., Brajenovic, M., Ebneth, A. & Drewes, G. (2004) MARK4 is a novel microtubule-associated proteins/microtubule affinity-regulating kinase that binds to the cellular microtubule network and to centrosomes. *J. Biol. Chem.*, **279**, 5915–5923.

Virdee, K., Yoshida, H., Peak-Chew, S. & Goedert, M. (2007) Phosphorylation of human microtubule-associated protein tau by protein kinases of the AGC subfamily. *FEBS Lett.*, **581**, 2657–2662.

Yoshida, H. & Goedert, M. (2012) Phosphorylation of microtubule-associated protein tau by AMPK-related kinases. *J. Neurochem.*, **120**, 165–176.