Conditional inactivation of Akt three isoforms causes tau hyperphosphorylation in the brain

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Abstract

Background: Tau hyperphosphorylation plays a critical role in neurodegenerative diseases [EMBO Mol Med. 6:1142-60, 2014; Annu Rev Neurosci. 24:1121-59, 2001]. Recent evidence has shown that Akt is down-regulated in AD [J Pathol. 225:54-62, 2011]. However, it remained unknown which pathological process, e.g. tau pathology or neuron death, Akt may contribute to. In this study, Cre-loxP technique was employed to generate a viable Akt three isoforms conditional knockout (Akt cTKO) mouse in which total Akt levels were dramatically reduced in the adult brain.

Results: Significantly increased levels of tau phosphorylated (p-tau) at various sites were observed in Akt cTKO mice as compared to age-matched littermate controls. Increased levels for phosphorylated GSK3α and phosphorylated PKA substrates were detected in Akt cTKO brains. In contrast, no significant changes on p-tau levels were found in Akt1−/−, Akt2−/− or Akt3−/− mice.

Conclusions: Akt may regulate tau phosphorylation in the adult brain by affecting activities for PKA and GSK3α.

Keywords: Akt, Tau phosphorylation, Tau kinases, GSK3 and PKA

Findings

Mice with global deletion of Akt three isoforms (Akt TKO or Akt1−/−;Akt2−/−;Akt3−/−) were early embryonic lethal [1], precluding the possibility to study its in vivo function. To obtain viable Akt conditional TKO (cTKO) mouse, we generated floxed Akt1 (Additional file 1: Figure S1A). After several steps of crossing, Akt1f/f;Akt2−/−;Akt3−/−;CAG-CreER mice were obtained and became Akt cTKO after the treatment with tamoxifen. To examine Cre-mediated recombination efficiency in the brain, biochemical analyses were conducted. Dramatic reduction on levels for total Akt (t-Akt) was observed in the brain of cTKO mice (Additional file 1: Figure S1B). Levels of p-AktSer473, an activated form of Akt, were markedly decreased in Akt cTKO (Additional file 1: Figure S1B). Immunohistochemistry (IHC) results showed very weak immuno-reactivity of t-Akt in the brain of Akt cTKO mice (Additional file 1: Figure S1C-d, a-c for controls).

Increased levels of tau phosphorylated (p-tau) at the Thr205, Thr231 and Ser396 epitopes have been widely reported in Alzheimer’s brain and mouse models [2]. Our Western results showed highly increased levels for p-tauT205, p-tauT231 and p-tauS396 in Akt cTKO mice (Additional file 1: Figure S1C-d, a-c for controls). Levels of p-AktSer473, an activated form of Akt, were markedly decreased in Akt cTKO (Additional file 1: Figure S1B). Increased levels of tau phosphorylated (p-tau) at the Thr205, Thr231 and Ser396 epitopes have been widely reported in Alzheimer’s brain and mouse models [2]. We conducted additional control experiments and used two groups of Akt2/3 double KO mice, Akt1f/f;Akt2−/−;Akt3−/−;CAG-CreER and Akt1f/f;Akt2−/−;Akt3−/−, which were age-matched littermates to Akt cTKOs. We found that Akt DKO mice did not show significant changes on p-tau levels (Additional file
1: Figure S2). Strong p-tau\textsuperscript{Thr205} immuno-reactivity was clearly seen in the cytoplasm of neurons in various brain sub-areas of Akt cTKOs but not controls (Fig. 1b). Increased p-tau\textsuperscript{Ser396} immuno-reactivity was largely detected in glial-like cells in the brain of Akt cTKOs (Additional file 1: Figure S3). Immunostaining on p-tau\textsuperscript{Thr231} showed strong signals in neuronal branches of the cTKO mice (Additional file 1: Figure S4), and double-staining with MAP2 suggested dendritic localization for p-tau\textsuperscript{Thr231} (Additional file 1: Figure S4C: a-d).

TUNEL assay was performed to examine cell death. No difference in the total number of TUNEL+ cells was observed in brains of Akt cTKO and control mice (Additional file 1: Figure S5), suggesting no significant change in apoptosis. Markers for neurons, astrocytes and microglia were used for biochemical and morphological analyses. First, there was no significant reduction on cortical levels for NeuN, GFAP and Iba1 in Akt cTKO mice (Additional file 1: Figure S6A). Second, there was comparable immuno-reactivity for NeuN, GFAP or Iba1 (Additional file 1: Figure S6B-C) in control and Akt cTKO mice. Therefore, deletion of Akt does not significantly affect the survival of cortical cells.

To determine the role of Akt single isoform in tau phosphorylation in the brain, we analyzed p-tau levels using cortical lysates from three lines of Akt single isoform KO mice including Akt\textsuperscript{1−/−} [3], Akt\textsuperscript{2−/−} [4] and Akt\textsuperscript{3−/−} [5]. Nissl staining revealed comparable brain structure between Akt\textsuperscript{1−/−}, Akt\textsuperscript{2−/−}, Akt\textsuperscript{3−/−} and their age-matched WT littermates (Fig. 2). Consistent with previously published observations [5, 6], Akt\textsuperscript{3−/−} mice displayed smaller brain than WT (Fig. 2e). However, no significant changes in p-tau levels were observed in Akt\textsuperscript{1−/−}, Akt\textsuperscript{2−/−} and Akt\textsuperscript{3−/−} mice (Fig. 2b,d,f: ps > 0.05), suggesting that tau hyperphosphorylation shown in Akt cTKO mice is unlikely to be caused by loss of a single Akt isoform.

To dissect molecular pathways involved, we conducted Western blotting using an antibody against phosphorylated Akt substrates. We found increased intensities for several bands including those for GSK3α/3β in Akt cTKO mice (Additional file 1: Figure S7A). Since GSK3 is a substrate of Akt, it is expected that deletion of Akt may cause reduced levels for phosphorylated GSK3α/3β [7]. However, we found that relative levels for p-GSK3β\textsuperscript{Ser9} were significantly increased in Akt cTKO mice (Additional file 1: Figure S7B) and those for p-GSK3α\textsuperscript{Tyr279} were also
increased (Fig. 3a), suggesting inhibition of GSK3β but activation of GSK3α. Since β-catenin is a well-known substrate of GSK3 [8, 9], p-β-cateninSer33/Ser37/Thr41 was examined and showed significantly increased levels in Akt cTKOs (Additional file 1: Figure S7C), suggesting elevated GSK3α activity. Moreover, we examined several other tau kinases. First, although levels for p-Cdk5Ser159 were increased, those for p25 were decreased (Fig. 3b, p < 0.05). Second, no significant changes on levels for p-Erk1/2 and p-p38 were detected in Akt cTKO mice (Fig. 3c-d, ps > 0.05). Overall, activities for GSK3β, Cdk5, Erk and MAPK p38 were not enhanced.

Fig. 2 Unchanged levels of p-tau in Akt single isoform KO mice. a Nissl staining for the brain of Akt1−/− mice. Normal brain structure was observed. b Western analyses on p-tau levels using cortical samples of Akt1−/− mice. There was no significant difference on relative levels of p-tauThr205, p-tauThr231 and p-tauSer396 in Akt1−/− mice. c Nissl staining for the brain of Akt2−/− mice. No abnormal brain structure was detected. d Western analyses on p-tau levels using cortical samples of Akt2−/− mice revealed no significant difference. e Nissl staining for the brain of Akt3−/− mice. There was a smaller brain in Akt3−/− mice than in WT animals. f Western analyses on p-tau levels showed no significant difference in Akt3−/− mice (NS = not significant)
It has been shown that GSK3β is phosphorylated at the Ser9 site not only by Akt but also by PKA [10]. The tyrosine phosphorylation of GSK3 requires the cAMP-PKA signaling [11, 12]. Moreover, it is known that PKA is a kinase to phosphorylate tau at the sites of Thr205, Thr231 and Ser396 [2, 13]. To test the possibility that PKA is involved, we performed the following experiments. First, an antibody against phosphorylated PKA substrates was used to conduct Western blotting. Increased levels for several bands with a wide range of molecular weights were observed (Fig. 4a), suggesting increased PKA activity. Second, we examined VASP, a well-known PKA substrate [14, 15], and observed highly increased p-VASP<sup>Ser157</sup> levels (Fig. 4b). In contrast, expression levels of PKA regulatory subunits, 1α and 1β, were unchanged (Fig. 4b). Third, since the Ser214 [16] and Ser356 [17, 18] sites of tau are phosphorylated by PKA as well, we analyzed p-tau<sup>Ser214</sup> and p-tau<sup>Ser356</sup>, which exhibited increased levels in Akt cTKO mice (Fig. 4c).

**Discussion**

Akt levels are decreased in Alzheimer’s brain [19]. However, it remains unknown exactly how Akt is involved in pathophysiological processes of AD, e.g. tau pathology and neurodegeneration. In this study, a novel Akt cTKO mouse model was generated and displayed tau hyperphosphorylation in the brain. We demonstrated that Akt cTKO mice exhibited significantly increased levels for several PKA downstream substrates. We observed increased levels for an activated form of GSK3α in Akt cTKO mice. We reported unchanged levels for activated forms of GSK3β, Cdk5, Erk and MAPK p38 in Akt cTKO mice.

It had been difficult to study normal physiological functions of Akt in the adult brain. First, while there are...
three Akt isoforms in mammals [20], a recent study did not identify which Akt isoform plays important role in neurodegenerative disease [19]. Second, since global deletion of Akt three isoforms causes early embryonic death in mice [1], a viable Akt cTKO mouse model was generated to overcome developmental problems in this study. Interestingly, Akt cTKO animals but not Akt single isoform KO mice exhibited increased p-tau levels in the brain, suggesting that total Akt but not a single isoform plays a key role in tau phosphorylation. Immuno-staining using AT8 or AT100 antibodies revealed no NFTs-like structure in Akt cTKO mice (data not shown). No significant changes on the total number of TUNEL+ cells or NeuN+ cells were detected in Akt cTKO mice. These findings are consistent with the concept that tau hyperphosphorylation is an early event during neurodegenerative process [21].

Tau phosphorylation status is determined by activities of multiple protein kinases [22]. Since there were unchanged levels for activated forms of GSK3β, Cdk5, Erk and p38, it is unlikely that they could account for abnor- mal tau phosphorylation in Akt cTKO mice. Although it is believed that inactivation of Akt may cause reduced p-GSK3α<sup>S21</sup>/3β<sup>S9</sup> [7] and thus lifts the inhibition on GSK3, levels for p-GSK3β<sup>S9</sup> were actually increased in Akt cTKO mice. Since GSK3β is a substrate for both Akt and PKA [10, 12], in the absence of Akt, it can be phosphorylated by PKA. The findings on phosphorylated PKA substrates, p-VASP and p-tau (Fig. 4) suggest enhanced PKA activity, which helps explain why there was tau hyperphosphorylation in Akt cTKO mice. There are two possible mechanisms by which Akt regulates PKA activity. The first possibility is a compensatory mechanism. It is known that the PI3K-Akt [23] and PKA pathways [24] can be activated by extracellular signal molecules such as cytokines. In the absence of the PI3K-Akt pathway, such molecules may over-activate the cAMP-PKA signaling as compensation. The second mechanism is that phosphodiesterases (PDEs) might be involved in the regulation of PAK activity. PDEs are Akt substrates [25] and can degrade cAMP, the second messenger molecule [26]. Therefore, deletion of Akt may inhibit PDEs and cAMP levels may get increased. Upon activation by cAMP, PKA can act on various downstream targets including tau, GSK3 and VASP.

**Methods**

**Generation of Akt<sup>f/f</sup> and Akt cTKO mice**

We used a strategy shown in Additional file 1: Figure S1A to generate floxed Akt1 mice. The detailed breeding plan for the generation of Akt cTKO mice was described in the Additional file 1.

**Treatment of mice with tamoxifen**

Tamoxifen was used to treat Akt1<sup>f/f</sup>;Akt2<sup>−/−</sup>;Akt3<sup>−/−</sup>;CAG-CreER and age-matched littermate controls for 5 consecutive days. Detailed information was described in the Additional file 1.

**Brain lysates preparation**

Procedures were described previously [27]. Immunoblotting methods and antibody information were described in the Additional file 1.
Statistical analysis
Data were presented as the mean ± SEM. Two-tailed Student’s t-test was performed to examine the difference between control and cKO mice. P < 0.05 (*) was considered statistically significant.

Ethical approval
Mouse breeding was conducted under IACUC approved protocols at the MARC (Model Animal Research Center). All the experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the MARC at Nanjing University.

Additional file
Additional file 1: Conditional inactivation of Akt three isoforms causes tau hyperphosphorylation in the brain. (PDF 3519 kb)

Abbreviations
AD: Alzheimer’s disease; Akt: Protein kinase B; PKA: Protein kinase A; cAMP: Cyclic adenosine 3’5’ monophosphate; Akt cTKO: Akt three isoforms conditional knockout; P3K: Phosphatidylinositol-3 kinase; PDE: Phosphodiesterase; tau: Microtubule-associated protein; p-tau: Phosphorylated tau; GSK3: Glycogen synthase kinase-3; cdk5: Cyclin-dependent kinase 5; MAPK: Mitogen-activated protein kinase; Etk: Extracellular signal-regulated kinase; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; NFTs: Neurofibrillary tangles; MAP2: Microtubule-associated protein; GFAP: Glial fibrillary acidic protein; Iba1: Ionized calcium-binding adapter molecule 1; VASP: Vasodilator stimulated phosphoprotein.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
LW, SC, JH, ZY, CX, SL, XX, TY, XZ and GC performed experiments. LW and GC analyzed the data. GC, ZY and YY designed the research. GC wrote the manuscript. GC, YP and YX edited the manuscript. All authors read and approved the final manuscript.

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