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EPAC2 knockout causes abnormal tau pathology through calpain-mediated CDK5 activation

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Abstract

Tau pathology, including aberrant tau hyperphosphorylation, aggregation, and mislocalization, is implicated in many neurodegenerative disorders, including Alzheimer’s disease (AD), which is the most prevalent dementia among the elderly. A better understanding of the molecular mechanisms underlying tau pathology should help advance therapies for neurodegenerative diseases. Perturbations in cyclic adenosine monophosphate (cAMP)-dependent signaling play an important role in the pathophysiology of numerous neurological diseases. EPAC2 is an intracellular cAMP receptor whose expression is downregulated in AD. However, the involvement and role of EPAC2 in tau pathology remain unclear. In this study, we report for the 1st time that EPAC2 is downregulated in the hippocampus of Tg2576 mice, a widely used transgenic mouse model of familial AD. Furthermore, genetic deletion of EPAC2 resulted in abnormal hyperphosphorylation at multiple sites on tau. Aberrant tau aggregation and abnormal neuronal morphology were also detected in these EPAC2−/− mice. Administration of inhibitors of CDK5 or calpain effectively rescued the tau pathology in EPAC2−/− mice. This suggests that the activation of CDK5 by calpain plays an important role in the development of tau pathology in these EPAC2−/− mice. Collectively, our findings demonstrate a direct link between EPAC2 and tau pathology, and suggest that the EPAC2 and calpain/CDK5 signaling pathways may have potential as therapeutic targets for AD.

Keywords: CDK5; Tau pathology; EPAC2

1. Introduction

Alzheimer’s disease (AD) is one of the most common neurodegenerative disorders in the elderly. AD is clinically characterized by progressive cognitive decline accompanied by other psychiatric disorders. An autopsy study showed that intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques (SPs) in neurons are the two major pathological hallmarks of AD brains². NFTs are composed of abnormally hyperphosphorylated microtubule-associated protein tau. Normal tau promotes the aggregation of tubulins and maintains the stability of microtubules. In contrast, hyperphosphorylated tau loses these normal functions and acquires the ability to form paired helical filaments that induce synaptic degeneration and neuronal loss. Studies suggest that NFTs are more closely related to cognitive decline in the progression of
AD than SPs. Therefore, it is important to understand the mechanisms underlying the formation of NFTs in AD\cite{2,3}.

Cyclic adenosine monophosphate (cAMP)-dependent signals have been implicated in the regulation of the expression of numerous genes and are essential for normal neuronal function and memory. There are two ubiquitously expressed intracellular cAMP receptors in eukaryotic cells; namely, protein kinase A and cAMP/cAMP-regulated guanine nucleotide exchange factor (EPAC/cAMP-GEF)\cite{4,5}. To date, two EPAC isoforms have been identified in mammals – EPAC1 and EPAC2. Although EPAC1 and EPAC2 are present in most tissues, EPAC2 is primarily expressed in the central nervous system. A number of studies have implicated EPAC proteins in neurological disorders, including AD\cite{6,7}. For example, postmortem quantitative polymerase chain reaction (PCR) analyses have shown that AD patients had increased EPAC1 mRNA and decreased EPAC2 mRNA in the frontal cortex and hippocampus compared with age-matched healthy controls\cite{8,9}. EPAC activates, through crosstalk between Ras and Rho small G-protein subfamilies, the \(\alpha\)-secretase pathway to cleave amyloid precursor protein (APP) in its extracellular domain, thereby releasing the soluble APP\(\alpha\) domain into the extracellular space\cite{10,11}. Together, these lines of evidence support a critical role for EPAC proteins in AD pathogenesis. However, the underlying mechanisms remain unclear, and it is not known whether abnormal EPAC2 signaling contributes to the aberrant tau pathology.

In the present study, we report for the 1\textsuperscript{st} time that EPAC2 null mice (EPAC2\textsuperscript{−/−}) display abnormal tau hyperphosphorylation at multiple sites, and that these changes are also detected in EPAC2 siRNA transfected neurons. The hyperphosphorylation of tau in EPAC2\textsuperscript{−/−} mice was age-dependent and formed aggregates. Moreover, using Bielschowsky silver staining, we found that the intracellular accumulation of argyrophilic substances was more apparent in the EPAC2\textsuperscript{−/−} mice. We also screened upstream tau kinases/phosphatases and found that the activation of CDK5 might play an important role in the tau hyperphosphorylation\cite{14,15}. Administration of roscovitine, a specific inhibitor of CDK5, effectively attenuated the tau hyperphosphorylation and aggregation in EPAC2\textsuperscript{−/−} mice\cite{16,17}. We further explored the possible reasons for aberrant CDK5 activation and found that calpain activity was upregulated in EPAC2\textsuperscript{−/−} mice. Application of a calpain inhibitor, calpain inhibitor IV, also reduced tau hyperphosphorylation and aggregation\cite{18,19}.

2. Materials and methods

2.1. Mice and experimental procedure

The EPAC2\textsuperscript{−/−} mice used in the present study have been previously described\cite{6}. The EPAC2\textsuperscript{−/−} mice (S129sv) were purchased from Vital River Laboratory Animal Technology (Beijing, China). Wild-type C57/BL6J was purchased from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Hubei, China.

Tg2576 mice were purchased from Jackson Laboratory (Bar Harbor, USA). The mice were fed in a room under a 12/12 h light-dark cycle and a temperature of 25 ± 2°C, with free access to food and water. All animal care and experimental procedures complied with local and international guidelines on the ethical use of animals, and were approved by The University Animal Welfare Committee, Tongji Medical College, Huazhong University of Science and Technology.

2.2. Cell culture and treatments

In 6-well plates filled with DMEM and 10% fetal bovine serum, we seeded mouse neuroblastoma 2a (N2a) cells (friendly gift of Dr. Huaxi Xu at Xiamen University). Cells were cultured in a humidified atmosphere of 5% CO\textsubscript{2}, at 37°C. When 60–80% confluent, the culture medium was replaced with serum- and antibiotic-free DMEM before treatment. Plasmids used for transfection were amplified and purified using EndoFree Plasmid Kits (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. For transfection, N2a cells were seeded in 6-well plates, grown to 60–70% confluence, and then cultured in serum- and antibiotic-free OPTI-MEM media for 4 h. According to the manufacturer’s instructions, plasmids were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Cells transfected with green fluorescent protein (GFP) constructs were visualized at 48 h after transfection under an Olympus IX70 microscope with a 209LCPlanF1 lens (Olympus Corporation, Matsue, Japan).

2.3. Chemicals and antibodies

The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Company (Rockford, IL, USA). The immunohistochemistry kit (Histostain-SP) was purchased from ZEMED Company (San Francisco, CA, USA), and the DAB kit was from ZSGB-Bio (Beijing, China). The Odyssey two-color infrared fluorescence imaging system reagent was purchased from LI-COR Biosciences (Lincoln, NE, USA). The primary antibodies employed in this study are listed below (Table 1). The secondary antibodies for Western blot analysis were purchased from Amersham Pharmacia Biotech (Little Chalfont, England).

2.4. Reverse transcription PCR (RT-PCR)

Total RNA from brain tissue was extracted using TRIzol reagent (Invitrogen). A 6 \(\mu\)g aliquot of the RNA was
Table 1. Primary antibodies used for Western blot analysis and immunohistochemistry

| Antibody | Specificity         | Type  | Dilution (WB) | Dilution (IHC) | Source    |
|----------|---------------------|-------|---------------|----------------|-----------|
| pT205    | P-tau at Thr205     | pAb   | 1:1000        | 1:200          | Biosource |
| pT231    | P-tau at Ser231     | pAb   | 1:1000        |                | Biosource |
| pS396    | P-tau at Ser396     | pAb   | 1:1500        | 1:200          | Biosource |
| pS404    | P-tau at Ser404     | pAb   | 1:1500        |                | Biosource |
| P35/25   | Total P25/25        | pAb   | 1:1000        |                | Cell signaling |
| CDK5     | Total CDK5          | mAb   | 1:1000        |                | Abcam     |
| P35      | P35                 | pAb   | 1:500         |                | Santa Crus |
| p35/p25  | P35 and P25         | mAb   | 1:500         |                | Cell signaling |
| TAU-1    | Non-phosphorylated tau | mAb   | 1:500         |                | Millipore |
| TAU-46   | Total tau           | mAb   | 1:1000        |                | Millipore |
| EPAC2    | Epac2               | pAb   | 1:500         |                | Santa Crus |
| DM1A     | β-tubulin           | mAb   | 1:1000        |                | Sigma     |

WB: Western blot; IHC: Immunohistochemistry; pAb: Polyclonal antibody; mAb: Monoclonal antibody.

The primers used in this experiment were produced by Tsingke Biotechnology (Beijing, China).

The primer sequences for EPAC2 were as follows: (Forward primer) 5'-AACTGGTAGCTGTGCTCCTGGC-3' and (reverse primer) 5'-TAGGGAGAGCCAGAAGTCC-3'. The primer sequences for β-actin were as follows: (Forward primer) 5'-AGCCCTCTTCCTTGGGAT-3' and (reverse primer) 5'-GTCAGAACATCGCCGCTA-3'. The primers used in this experiment were produced by Tsingke Biotechnology (Beijing, China).

RT-PCR was performed on a PCR thermocycler (Bio-Rad, New York, USA). Reactions were prepared in a total volume of 50 μL containing 0.5 μg cDNA, 2 μL of each 10 μM primer, and 25 μL of Taq MasterMix (Cwbio, Beijing, China).

2.5. Calpain activity assay

Analysis of calpain activity in the hippocampus was carried out, according to the manufacturer’s instructions.

2.6. Preparation of the sarkosyl-insoluble fraction

The detection of soluble and insoluble tau was performed as described previously[20]. In brief, the hippocampi were homogenized in radio immunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl, 150 mM NaCl, 20 mM EDTA, 1% Nonidet-P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaF, and 0.25% sodium deoxycholate, and centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected, and after evaporation of the formic acid in a speed vacuum, the pellet was resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (240 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, and 0.06% bromophenol blue). The RIPA fraction and the formic acid fraction contained relatively soluble tau and detergent-insoluble tau, respectively, and were sonicated extensively before immunoblotting.

2.7. Western blotting

The animals were decapitated under chloral hydrate (600 mg/kg, i.p.) anesthesia, and the hippocampi were rapidly removed and homogenized at 4°C using a Teflon glass homogenizer in a reagent containing 50 mM Tris-HCl (pH 7.4), 1 mM NaVO₄, 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 2 mM benzamidine, 1 mM PMSF, and proteinase inhibitor cocktail (1:100 dilution). Three volumes of the homogenized tissue were added to one volume of extraction buffer containing 200 mM Tris-HCl, pH 7.6, 8% SDS, 40% glycerol, and 40 mM dithiothreitol, and then the mixture was placed in a boiling water bath for 10 min. Next, the lysates were sonicated briefly and centrifuged at 12,000 × g for 5 min. The supernatant was stored at −80°C for Western blotting. The protein concentration of supernatants was measured using the Pierce BCA Protein Assay Kit, according to the manufacture’s instructions. The proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking in 3% bovine serum albumin for 1 h at 25°C, the membranes were incubated first with primary antibodies at 4°C overnight and then with anti-mouse or anti-rabbit IgG conjugated to IRDye 800CW as secondary antibody for 1 h at 25°C. Protein expression was then visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.8. Immunohistochemistry and Bielschowsky silver staining

The mice were anesthetized with chloral hydrate (600 mg/kg, i.p.) and immediately perfused with 200 mL normal saline followed by 200 ml phosphate buffer (4°C) containing 4% paraformaldehyde. The brains were then post-fixed in perfusate at 4°C for 24 h. Paraffin-embedded brain sections were prepared for immunostaining after xylene treatment and progressive rehydration with 70–100% ethanol. Sections were blocked and then incubated with...
primary antibody overnight at 4°C in phosphate-buffered saline containing 0.3% Triton X-100. The sections were incubated with secondary antibody for 1 h at 25°C in the dark. The immunoreaction was detected by incubation with horseradish peroxidase-labeled antibodies for 1 h at 37°C and visualized with the DAB-tetrachloride system (Solelybio, Beijing, China). For each primary antibody, 3–5 consecutive sections from each brain were used. The images were observed under a microscope (Olympus BX60). The primary antibodies employed in this study are listed below (Table 1).

2.9. Imaging and statistical analysis

ImageJ (NIH, Bethesda, MD, USA) was used to measure the immunohistochemical optical density. All the data in the study were expressed as mean ± standard error of the mean and analyzed with SPSS 16.0 statistical software (SPSS, Chicago, IL, USA). One-way analysis of variance, followed by Student–Newman–Keuls test, was used to assess the statistical significance of differences among the means for multigroup comparisons. A difference with $P < 0.05$ was considered statistically significant.

3. Results

3.1. Downregulation of EPAC2 induces age-dependent tau hyperphosphorylation

As EPAC2 is downregulated in the AD brain, we first evaluated tau phosphorylation in the hippocampus of EPAC2$^{-/-}$ mice on the 6th month. Immunoreactivities for the pT205, pT231, pS396, and pS404 epitopes and decreased staining for Tau-1 were observed in the EPAC2$^{-/-}$ mice, suggesting hyperphosphorylation of tau at the Ser396/404 (pS396, pS404), Thr231 (pT231), Thr205 (pT205), and Ser199/202 (Tau-1) sites (Figure 1H and I). We also examined the distribution of hyperphosphorylated tau at Thr205 and Ser396 in the hippocampus of EPAC2$^{-/-}$ mice. The staining for pT205 and pS396 was much stronger in the

![Figure 1. Abnormal tau hyperphosphorylation in EPAC2$^{-/-}$ mice.](image)

(A) The mRNA levels of EPAC2 in the Tg2576 mice and the age-matched wild-type mice (c57) were examined by PCR. (B) Quantification of (A); n = 3; **P < 0.01. (C) The protein levels of EPAC2 in the Tg2576 mice and the age-matched wild-type mice (c57) were examined by Western blot. (D) Quantification of (C); n = 4, *P < 0.05. (E) Genomic DNA was extracted to identify the proper knockout of the EPAC2 gene in EPAC2$^{-/-}$ mice. (F) The hippocampal extracts were prepared from 6-month-old EPAC2$^{-/-}$ null mice and wild-type EPAC2$^{+/+}$ littermates. The relative levels of EPAC2 were examined. (G) Quantification of (F); n = 3–6; **P < 0.01. (H) Hippocampal extracts were prepared from 6-month-old EPAC2$^{-/-}$ mice and wild-type EPAC2$^{+/+}$ littermates. The relative levels of EPAC2, total tau (TAU-46), non-phosphorylated tau (TAU-1), and phosphorylated tau at Thr205 (pT205), Thr231 (pT231), Ser396 (pS396), and Ser404 (pS404) sites were examined by Western blot. (I) Quantification of (H); n = 5 or 6; *P < 0.05. (J) The N2a cells were transfected with siRNA targeting mouse EPAC2 (si-EPAC2) or the control plasmid (vector). After 48 h, cell lysates were made for western blot. The relative levels of EPAC2, total tau (TAU-46), non-phosphorylated tau (TAU-1), and phosphorylated tau at Thr205 (pT205), Thr231 (pT231), Ser396 (pS396), and Ser404 (pS404) sites were examined by Western blot. (K) Quantification of (J); n = 4 or 5; *P < 0.05. (L–O) The brain slices were taken from 9-month-old EPAC2$^{-/-}$ mice and wild-type EPAC2$^{+/+}$ littermates. The relative levels of EPAC2, total tau (TAU-46), non-phosphorylated tau (TAU-1), and phosphorylated tau at Thr205 (pT205), Thr231 (pT231), Ser396 (pS396), and Ser404 (pS404) sites were examined by Western blot. (L) Quantification of (M); n = 3–6; **P < 0.01.
somatodendritic compartment of CA1 and CA3 neurons in EPAC2−/− mice (Figure 1L-O). To exclude the involvement of developmental factors in tau phosphorylation, we transfected short hairpin RNA (shRNA) toward mouse EPAC2 into N2a cells and examined tau phosphorylation at these sites. In line with our in vivo data, silencing of EPAC2 led to the hyperphosphorylation of tau at multiple epitopes (Figure 1J and K). We next examined whether EPAC2 expression was reduced in the transgenic AD mouse model. By RT-PCR and Western blot, both the mRNA and protein levels of EPAC2 were reduced in the hippocampus of Tg2576 mice on the 12th month (Figure 1A-D). Moreover, the levels of tau hyperphosphorylation in the hippocampus of EPAC2−/− mice gradually increased with age (Figure 2A-G). Thus, the downregulation of EPAC2 in AD can induce the age-dependent hyperphosphorylation of tau in the brain.

3.2. Downregulation of EPAC2 induces tau insolubility and somatodendritic abnormalities

The hyperphosphorylation of tau induces conformational changes that result in the aggregation of the protein[21]. Therefore, we next examined whether the absence of EPAC2 impacts the soluble and insoluble pools of tau using a previously reported protocol[20]. In the RIPA fraction (soluble tau), only slight increases in pT231 and pT205/pT231 were found in the EPAC2−/− mice on the 6th and 18th months, respectively (Figure 3A-D). However, immunoactivity toward phosphorylated tau (pT205, pT231, pS396, and pS404) in the 70% FA fraction (insoluble tau) was dramatically increased in EPAC2−/− mice, especially in the 18-month-old mice (Figure 3E-H). Thus, loss of EPAC2 decreases the solubility of tau and promotes its aggregation. Because the aggregation of tau is one of the key steps in the formation of NFTs, we then performed Bielschowsky silver staining to visualize the morphology of neurons. We found that the cortical neurons in the EPAC2−/− mice (9th month) displayed more argyrophilic aggregates and more dystrophic neurites than the control mice (Figure 3I). Thus, the absence of EPAC2 leads to tau aggregation and somatodendritic abnormalities.

3.3. Downregulation of EPAC2 activates CDK5 by increasing calpain activity

An imbalance of tau kinases and phosphatases is implicated in the abnormal tau hyperphosphorylation in AD[22]. We, therefore, examined the levels and activities of PP2a, ERK,

Figure 2. Age-dependent tau hyperphosphorylation in EPAC2−/− mice. (A) The hippocampal homogenates were prepared from EPAC2−/− mice and wild-type EPAC2+/+ littermates on the 3rd, 6th, 9th, and 18th months. The relative levels of EPAC2, total tau (TAU-46), non-phosphorylated tau (TAU-1), and phosphorylated tau at Thr205 (pT205), Thr231 (pT231), Ser396 (pS396), and Ser404 (pS404) sites were examined by Western blot. (B–G) Quantification of the immunoreactivities of the different antibodies indicated in (A); n = 3, *P < 0.05.
GSK-3β, JNK, and CDK5, all of which are critical regulators of tau hyperphosphorylation. We found that the absence of EPAC2 did not induce any changes in the protein levels of the catalytic subunit of PP2a (PP2ac), β subunit of PP2a (PP2ab), total ERK (t-ERK), phosphorylated Thr202/Tyr204 in ERK (p-ERK), total GSK-3β (t-GSK-3β), phosphorylated serine 9 in GSK-3β (pS9-GSK-3β), total JNK (t-JNK), or phosphorylated Thr183/185 in JNK (p-JNK) (Figure 4A and B). However, CDK5 levels and the p25/p35 ratio were dramatically increased in EPAC2−/− mice (Figure 4C and D), suggesting that CDK5 activation might be involved in EPAC2 loss-induced tau pathology. Consistent with these observations, silencing of EPAC2 in the N2a cells also resulted in the activation of CDK5,

Figure 3. Abnormal tau aggregation and neuronal morphology in EPAC2−/− mice. (A–H) Hippocampus extracts from 6-month-old and 18-month-old EPAC2−/− mice and wild-type EPAC2+/+ littermates. (A and B) Representative blot images for soluble tau. (E and F) Representative blot images for insoluble tau. The densitometric analyses of the immunoreactivities to the antibodies indicated in (A and B) and (E and F) are shown in (C and D) and (G and H), respectively; n = 3; *P < 0.05; **P < 0.01. (I) Cortical slices from 9-month-old EPAC2−/− mice and wild-type EPAC2+/+ littermates were subjected to Bielschowsky silver staining. Representative images with magnifications are shown.
indicated by the increases in CDK5 protein levels and the p25/p35 ratio (Figure 4E and F). To further clarify the role of CDK5 in the tau hyperphosphorylation in EPAC2 null mice, we infused roscovitine, a specific inhibitor of
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CDK5, into the hippocampus of EPAC2−/− mice and then examined tau phosphorylation and aggregation 48 h later. We found that the administration of roscovitine reduced tau hyperphosphorylation at multiple sites and reduced insoluble phosphorylated tau levels (Figure 5A-F). These results strongly suggest that downregulation of EPAC2 induces tau hyperphosphorylation through CDK5 activation.

Given that an aberrant p25/p35 ratio was also detected in the EPAC2−/− mice, and that calpain activation leads to cleavage of p35 to p25\(^{23}\), we hypothesized that calpain might be involved in CDK5 activation in EPAC2−/− mice. Indeed, calpain activity was increased in the hippocampus of EPAC2 null mice (Figure 6A). Importantly, the application of calpain inhibitor IV not only decreased the activity of CDK5 (Figure 6B and C) but also reduced tau hyperphosphorylation and aggregation in the EPAC2−/− mice (Figure 6B-G).

4. Discussion

In the present study, we demonstrate for the 1st time that abnormal tau hyperphosphorylation is present in the hippocampus of EPAC2−/− mice, suggesting a critical role of EPAC2 downregulation in the pathogenesis of AD.
A single-nucleotide polymorphism in the EPAC2 gene, rs17746510, which is adjacent to the catalytic domain sequence, is associated with cognitive decline, apathy, and mood disturbance in Chinese AD patients[7]. Consistent with this finding, EPAC2−/− mice exhibit emotional perturbations, including anxiety, and depression[7], as seen in the early stages of AD in humans[24]. The reduction in EPAC2 is particularly notable in the frontal cortex and hippocampus, the two most severely affected brain areas in AD, but not in resistant regions such as the cerebellum[8]. In our previous study, we found that EPAC2 was decreased in rats with scopolamine-induced short-term amnesia, an animal model of dementia[25]. Loss of EPAC2 results in the over-stabilization of excitatory synapses and an increase in inhibitory input onto neurons, leading to an imbalance in excitatory/inhibitory synapses[26], which is an important synaptic phenotype in AD[27]. Moreover, a rare missense mutation in EPAC2, V646F-EPAC2, mimics the effect of EPAC2 knockdown, with reduced GEF activity and perturbed dendritic spines[28], which is one of the most important post-synaptic compartments severely affected in AD[29]. In this study, we identified aberrant tau hyperphosphorylation at multiple sites. It is known that tau hyperphosphorylation is one of the key pathological hallmarks of AD[2]. Hyperphosphorylated tau is highly correlated with the synaptic disorder in AD. For example, hyperphosphorylation of tau reduces its binding affinity for microtubules, causing it to detach and mislocalize to the dendritic compartment[11,30]. Furthermore, hyperphosphorylated tau can immobilize synaptic vesicles by preventing their release from F-actin[31]. In many tau mutant and transgenic models, dysregulation of the synaptic proteome, impaired synaptic plasticity, and a reduction in synapses and dendritic spines are found[32]. Thus, it is possible that the EPAC2 loss-induced tau hyperphosphorylation plays an important role in the synaptic dysfunction in AD.

We also found here that CDK5 activation is the principal event involved in tau hyperphosphorylation in EPAC2−/− mice. It is known that CDK5 activation induces the phosphorylation of tau at Thr181, Ser199, Ser202, Thr205, Thr212, Ser214, Thr217, Thr231, Ser235, Ser396, and Ser404, which are sites that are critical in the formation of NFTs in AD[13,31]. CDK5 is upregulated in neurons containing NFTs[34]. Numerous studies have revealed a positive correlation between CDK5 activation and NFTs in AD brains[33,35]. Directly blocking CDK5 activity or the cleavage of p35 to p25 in cultured neurons effectively suppresses tau hyperphosphorylation[36,37]. In our present study, administration of roscovitine, a specific inhibitor of CDK5, not only attenuated the tau hyperphosphorylation but also reduced tau aggregation. These findings suggest an important role of CDK5 in tau pathology (Figure 7). Notably, CDK5 is a pro-apoptotic factor, especially in the contexts of neurotoxicity, oxidative stress, and Aβ oligomer stimulation[38]. Moreover, activation of CDK5 leads to the inactivation of peroxiredoxins I and II, two important reactive oxygen species (ROS) scavengers, resulting in the accumulation of ROS in neurons and mitochondrial damage. In line with this, hyperphosphorylated tau also induced mitochondrial dysfunction both in vitro and in vivo. In addition, CDK5 can directly phosphorylate APP at Thr668 to increase Aβ secretion[39], and indirectly facilitate Aβ production by promoting Golgi fragmentation[40]. Finally, CDK5 activation can cause disruption to the dendritic spines and dendritic morphology by impacting numerous downstream substrates. Therefore, targeting CDK5 might be a promising therapeutic approach for AD.

We noticed that for the activation of CDK5 in EPAC2−/− mice, calpain activity was crucial. As reported, an upregulation of calpain activity is found in both AD animal models and human postmortem brains[41,42]. In addition, calpain activation plays a very important role in neuronal loss (necrosis and apoptosis) in AD, as shown by many in vitro and in vivo studies[43]. Moreover, calpain is activated by Aβ exposure, resulting in the dysregulation of brain-derived neurotrophic factor signaling, which might lead to the synaptic disorder in AD[44]. Furthermore, in an AD mouse model, calpain activation is suggested to promote Aβ generation, which may form a vicious cycle[45]. Here, blocking the activation of calpain in the EPAC2−/− mice robustly reduced tau hyperphosphorylation and CDK5 activation. In some previous reports, the upregulation of calpain activity was found to generate a toxic tau fragment in hippocampal neurons[46]. Together, the lines of evidence strongly suggest a critical role of calpain in tau pathology in AD. However, further study is needed to elucidate whether calpain activation plays a direct or indirect role in tau hyperphosphorylation in EPAC2−/− mice.

5. Conclusion

The current findings collectively provide strong evidence for a critical role of EPAC2 in tau pathology and provide insight into the underlying signaling pathways.

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Conflict of interest
The authors have no conflicts of interest to declare.

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