

# Inhibition of Lactate-GPR81-PI3K/Akt Pathway May Exacerbate A $\beta$ Aggregation in 3-Month-Old APP/PS1 Mice

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## Research Article

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# Abstract

Lactate is not only an energy metabolite for neurons, but also serves as a molecule regulator affecting neuronal activity through its receptor, G protein-coupled receptor 81 (GPR81). This receptor can trigger cellular signaling pathways, such as phosphatidylinositol 3 kinase /protein kinase B (PI3K/Akt) pathway. Particularly, lactate deficit and inhibition of PI3K/Akt pathway were observed to be related with early synaptic dysfunction in Alzheimer's disease (AD). In addition, amyloid beta ( $A\beta$ ) is toxic to neurons, while in vitro lactate administration of neurons can resist against this toxicity. Hence, this work focuses on the effect of lactate deficiency on  $A\beta$  production, suggesting that lactate decrease can inhibit GPR81-PI3K/Akt pathway, and then reduce deoxyribonucleic acid methyltransferase 1 (DNMT1) expression, further resulting in increase of beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) and  $A\beta$  production. Based on the analysis of results, this study primarily proves that lactate reduction suppresses its downstream GPR81-PI3K/Akt pathway, which decreases the expression of DNMT1 through regulating cyclic-adenosine-monophosphate response element-binding protein (CREB)/P300. Then, it is proved that DNMT1 reduction can lead to the increase of BACE1 and  $A\beta$  accumulation in AD. At last, in vitro experiment recognizes that lactate directly activates GPR81-PI3K/Akt pathway. Thus, this study provides a novel insight in  $A\beta$  production in relation with lactate deficit at early stage of AD. Particularly, it is suggested that extra addition of lactate might be protective for neurons targeting  $A\beta$  clearance in early treatment of AD.

## Introduction

Alzheimer disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline and dementia [1]. About 95% of AD is sporadic form, which is characterized by a late onset, and is the consequence of the failure to clear the amyloid beta ( $A\beta$ ) peptide from the interstices of the brain[2]. Genetic risk factors for sporadic AD are inherited mutations in genes that affect processing of  $A\beta$  and develop the disease at a much younger age (mean age of about 45 years)[3]. Continuous production of  $A\beta$  leads to aggregation of  $A\beta$ -containing amyloid plaques and accelerates tau-derived neurofibrillary tangles (NFTs), which ultimately leads to AD dementia. [4]. Hence, cerebral accumulation of  $A\beta$  peptide is not merely an important molecular hallmark of AD, but a promising therapeutic target[5].

$A\beta$  clearance can decrease progressive axonal degeneration [6]. Unfortunately, two key questions remain unanswered: How to effectively lower  $A\beta$  production and promote  $A\beta$  clearance? Which stage of AD would be efficacy in an  $A\beta$ -directed therapeutic approach[7]? beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is an aspartic protease which functions in the first step of the pathway leading to the production and deposition of  $A\beta$ [8]. Therefore, increase of BACE1 expression in AD at early stage can accelerate  $A\beta$  accumulation and quicken AD progression. BACE1 expression can be regulated by deoxyribonucleic acid methyltransferase 1 (DNMT1), DNMT1 reduction leads to hypomethylation of specific loci within the BACE1 gene promoter, which further enhances BACE1 expression[9]. Hence, decrease of DNMT1 can upregulate BACE1 expression, which may further lead to  $A\beta$  production during

AD progression. Otherwise, the change of DNMT1 expression in relation with the levels of BACE1 and A $\beta$  in AD brain at early stage hasn't been recognized.

The aggregation of A $\beta$  plaques is associated with inflammation, oxidative stress, and energy deficit[10]. Especially, suppression of cerebral glucose utilization is identified in individuals of familial AD before the manifestation of A $\beta$  plaques[11, 12]. At this moment, glycolytic pathway and its product, lactate, become the main energetic resource of neurons, which can further defend against A $\beta$ -mediated impairment of mitochondrial respiration[13]. In vitro experiment has proved that nerve cells favoring the glycolytic pathway resist against A $\beta$  toxicity[14]. Even, lactate administration rescues the death of cultured neurons[15]. Further, it is discussed that lactate is necessary for long-term memory formation and improvement of cognitive function[16]. Hence, lactate may play a protective role in neuronal damage at early stage of AD. Otherwise, in our previous work, it is recognized that cerebral lactate content is decreased in 3-month-old double-transgenic amyloid precursor protein/presenilin 1 (APP/PS1) mice[17]. the observed lactate deficiency is proved to be associated with early neuronal damage in AD brain[17, 18]. Here, this study aims to explore underlying mechanism of lactate deficit in relation with the production of neuronal A $\beta$  in AD.

Lactate, an intermediate metabolite of glucose, transports from bloodstream to blood brain barrier via monocarboxylate transporters (MCTs)[19]. In the brain, lactate is temporarily stored in glial cells. During specific periods, such as brain development and AD, lactate can be quickly transported from glia to neurons and metabolized to sustain neuronal activity [20, 21]. Therefore, lactate is normally recognized as a quickly energetic substrate of neurons. Actually, lactate is not merely an energy resource, but also recognized as a signaling molecule[22]. Lactate can bind to its receptor, G protein-coupled receptor 81 (GPR81), which activates several downstreams, including phosphatidylinositol 3 kinase /protein kinase B (PI3K/Akt), extracellular regulated protein kinases (ERK1/2) pathway, Nod-like receptor family pyrin domain-containing 3/nuclear factor- $\kappa$ B (NLRP3/NF- $\kappa$ B) inflammatory signaling pathway and so on[23]. In PI3K/Akt pathway, its activation promotes phosphorylation of cyclic-adenosine-monophosphate response element-binding protein (CREB), and then CREB binds to P300 which regulates gene expression via histone acetylation[24]. In addition, the recognized dysfunction of PI3K/Akt signaling pathway results in tau hyperphosphorylation and A $\beta$  deposition[25]. Suppression of PI3K/Akt pathway leads to the downregulation of DNMT1[26]. Thus, lactate, as a molecule, may regulate A $\beta$  production through PI3K/Akt-CREB/P300-DNMT1 pathway.

Here, this study hypothesizes that lactate reduction results in A $\beta$  production, its underlying mechanism is that lactate deficit leads to the inhibition of GPR81-PI3K/Akt pathway, and then downregulating DNMT1 expression through CREB/P300. Moreover, DNMT1 decrease induces the hypomethylation of specific loci within the BACE1 gene promoter, which upregulates BACE1 expression, further aggravating A $\beta$  production in AD, especially at early stage. The results illustrate that lactate reduction inhibits GPR81-PI3K/Akt-CREB-DNMT1-BACE1 signaling pathway, which exacerbates A $\beta$  aggregation in AD. Hence, this study provides a novel insight into the mechanism of A $\beta$  accumulation and provides a novel target in anti-A $\beta$  therapy for early treatment of AD.

# Materials And Methods

## Animals

3-month-old heterozygous APP/PS1 mice (n = 10) and their nontransgenic littermates (wild type, n = 10) were used in this study. Animals were housed in individual cages in a controlled environment (temperature, 22 ± 1 °C; humidity, 50% ± 10%; 12-hour light/12-hour dark cycle). Food and water were available ad libitum. Animals were grouped and named as Wild Type and APP/PS1.

## Primary Neuron Culture and Grouping

Adult C57BL/6 mice were obtained from the Experimental Animal Center of Army Medical University (Chongqing, China). The primary culture of cerebral cortical neurons from embryos of C57BL/6 mice was performed as previously described [27]. In brief, cerebral cortex of 18-day-old embryos was dissected from brain and then cut into slices. The slices were mechanically dissociated by trituration. The dissociated cells were suspended in Eagle's minimal essential medium supplemented with 3% B27 Minus AO, 10 µg/ml insulin, 0.25 µM glutamine, 1 mM β-hydroxybutyrate, 1 mM fumarate, and 50 ng/ml sodium selenite. The cell suspension was plated onto 96-well culture plate previously coated with poly-D-lysine and fibronectin. Cells were incubated at 37 °C in a humidified atmosphere of 95 % O<sub>2</sub>/5 % CO<sub>2</sub> for 14 days. To detect the impact of lactate on neurons, the obtained neurons were given lactate for 24 hours in a dose-dependence (5mM, 10mM, 15mM), and lactate inhibitor (sodium oxamate) administration. Further, these cells were grouped and named as Control, 5mM LAC, 10mM LAC, 15mM LAC, and Inhibitor.

## Reagents and Antibodies

Radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and phenylmethyl sulfonyl fluoride ([PMSF] Beyotime, Shanghai, China) were used to prepare homogenates of brain tissues. The BCA protein assay kit (Beyotime, Shanghai, China) was used to determine protein concentrations. Lactate levels were detected using a lactate assay kit (Nanjing Jiancheng, Jiangsu, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), polyvinylidene difluoride (PVDF) filtermembranes (Bio-Rad, Hercules, CA), tris-buffered saline (TBS), tris-buffered saline containing 0.1% tween 20 ([TBST] Beyotime), and an enhanced chemiluminescence (ECL) kit (Invitrogen, Carlsbad, CA) were used for western blotting (WB) analysis.

Primary antibodies used in this study included mouse anti-beta actin ([anti-β-actin], Beyotime), mouse anti-amyloid beta ([anti-Aβ] Abcam, Cambridge, MA), mouse anti-neuronal nuclei ([anti-NeuN] Merck Millipore, Darmstadt, Germany), rabbit anti-GPR81 (Abcam, Cambridge, MA), rabbit anti-DNMT1 (Abcam, Cambridge, MA), rabbit anti-BACE1 (Abcam, Cambridge, MA), mouse anti-p-PI3K (Abcam, Cambridge, MA), mouse anti-p85/p55 (Abcam, Cambridge, MA), mouse anti-PI3Kp85 (Abcam, Cambridge, MA), mouse anti-pAkt (Abcam, Cambridge, MA), mouse anti-Akt (Abcam, Cambridge, MA), mouse anti-pCREB (Abcam, Cambridge, MA), mouse anti-CREB (Abcam, Cambridge, MA). Anti-mouse fluorescein isothiocyanate (FITC) and anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC) secondary

antibodies (Beyotime, Shanghai, China) were used in immunofluorescence staining. 4,6-diamidino-2-phenylindole ([DAPI] Sigma, St. Louis, MO) was applied to label nuclei. In addition, horseradish peroxidase conjugated secondary anti-mouse antibody (Beyotime, Shanghai, China) was used in WB.

## **Tissue Processing**

Following perfusion with phosphate buffer solution (PBS), pH 7.4, the left hemispheres of brains from APP/PS1 mice and wild type mice (n = 5, respectively) were collected and stored in  $-20^{\circ}\text{C}$  for lactate measurement. Meanwhile, the right hemispheres of brains were homogenized by RIPA lysis buffer containing 1mM PMSF. Homogenates were then centrifuged for 20minutes at 14000rpm and concentrations of the supernatants were assayed using the BCA protein assay kit and adjusted to 1.5mg/mL. Finally, the samples were stored at  $-20^{\circ}\text{C}$  for WB analysis. For immunohistochemical assays, APP/PS1 mice and wild type mice (n = 5, respectively) under anesthesia were perfused with saline followed by 4% paraformaldehyde in PBS and the brains were extracted and postfixed with fresh 4% paraformaldehyde at  $4^{\circ}\text{C}$ . Tissues were transferred to 30% sucrose solution for 2-3 days and subsequently cut into 20  $\mu\text{m}$  slices.

## **Measurement of Lactate Concentration**

Determinations of lactate concentration were performed with a lactate assay kit. The cortex and hippocampus were homogenized in saline at  $4^{\circ}\text{C}$  for 10 minutes, then the homogenates were centrifuged for 15 minutes at a speed of 2500 rpm. Assay buffers were added to the supernatants and incubated for 10 minutes at  $37^{\circ}\text{C}$ . Finally, optical density (OD) values were recorded at 530 nm after the reaction of lactate and assay buffers. Lactate content was calculated using the following formula:

$$\text{Lactate content (mmol/g)} = (\text{OD measured value} - \text{OD blank value}) / (\text{OD standard value} - \text{OD blank value}) \times \text{standard substance content (3 mmol/L)} / \text{protein content (g/L)}$$

## **WB Analysis**

Protein samples were subjected to SDS-PAGE and transferred to PVDF filter membranes. The membranes were blocked with 5% nonfat milk for 1 hour at  $37^{\circ}\text{C}$  and incubated with primary antibodies for 12 hours at  $4^{\circ}\text{C}$ , including mouse anti-A $\beta$ , mouse anti-p-PI3K, mouse anti-p85/p55, mouse anti-PI3Kp85, mouse anti-pAkt, mouse anti-Akt, mouse anti-pCREB, mouse anti-CREB. After washing with TBST, the membranes were incubated for secondary antibodies for 1 hour at  $37^{\circ}\text{C}$  and detected using ECL kit. Finally, the blots were qualified by Image J software (NIH, Bethesda, MD).

## **Immunofluorescence Staining**

Brain sections were probed with mouse anti-A $\beta$ , rabbit anti-GPR81, rabbit anti-DNMT1, rabbit anti-BACE1 primary antibodies, respectively. Brain sections were incubated overnight with primary antibodies in a humidified chamber at  $4^{\circ}\text{C}$ . Sections were then washed 3 times with PBS for 5minutes each, followed by incubation with anti-mouse FITC secondary antibody. For double-labeling immunofluorescence, sections

were incubated with the mixture of 2 primary antibodies overnight at 4°C as follows: Mouse anti-neuronal nuclei (Neu N) and rabbit anti-GPR81. Fluorescent secondary antibodies, raised in different species (FITC with green signal against mouse and TRITC with red signal against rabbit) were used to locate complexes of antigen/primary antibody. Nuclei were counterstained with DAPI for 5 minutes. Images were obtained using a fluorescence microscope (Olympus, Tokyo, Japan) at 400× magnifications. Positive expressions of staining pictures were analyzed by OD. OD values were calculated using Image-Pro Plus 6.0 (IPP6.0) software according to manufacturer's instructions.

## Statistical Analysis

All data are expressed as mean ± standard deviation (SD). All statistical analyses were performed by Statistical Product and Service Solution (SPSS, IBM version 21) and Prism 6 (GraphPad Prism Software Inc., La Jolla, CA). Results of WB and immunofluorescence were analyzed using one-way analysis of variance (ANOVA) and, correlation analysis, and the least significant difference (LSD) test. Differences with \* $P < 0.05$  and \*\* $P < 0.01$  were considered statistically significant.

## Results

### Reduction of lactate and GPR81 expression in the cortex and hippocampus of 3-month-old APP/PS1 mice

Lactate content and GPR81 expressions in cortex and hippocampus of 3-month-old APP/PS1 mice and wild type mice were assessed by immunofluorescence staining and WB. Lactate levels in the cortex and hippocampus of APP/PS1 mice are  $5.1 \pm 1.28$  mmol/g and  $5.29 \pm 1.76$  mmol/g, which are lower than lactate contents in cortex ( $14.8 \pm 1.13$  mmol/g) and hippocampus ( $15.91 \pm 1.03$  mmol/g) of wild type mice ( $n = 5$ , \* $p < 0.05$ ; Fig. 1a). As arrowheads pointed, Positive staining of GPR81 in cortex and hippocampus of APP/PS1 mice is lower than that of wild type mice (Fig. 1b). In statistic, OD values of GPR81 in cortex and hippocampus of APP/PS1 are  $21196 \pm 2187$  and  $18503 \pm 1895$ , which are decreased in comparison with wild type mice ( $32741 \pm 3012$  in cortex and  $27332.3 \pm 2198$  in hippocampus) ( $n = 5$ , \* $p < 0.05$ ; Fig. 1c).

### PI3K-Akt-CREB signaling pathway is suppressed in APP/PS1 mice

To assess the activity of PI3K-Akt-CREB pathway, the downstream of GPR81, WB was used to detect levels of p-PI3K, p-Akt and p-CREB in the brains of APP/PS1 mice and wild type mice. Positive protein bands of p-PI3K, p-Akt and p-CREB in APP/PS1 mice and wild type mice are recognized (Fig. 2a). There are significant differences between APP/PS1 mice and wild type mice in the ratios of p-PI3K/PI3K, p-Akt/Akt, and p-CREB/CREB. Respectively, ratios of p-PI3K/PI3K, p-Akt/Akt, and p-CREB/CREB are  $0.43 \pm 0.07$ ,  $0.18 \pm 0.04$  and  $0.587 \pm 0.01$  in APP/PS1 mice. ratios of p-PI3K/PI3K, p-Akt/Akt, and p-CREB/CREB are  $1.04 \pm 0.02$ ,  $0.38 \pm 0.03$  and  $0.97 \pm 0.05$  in wild type mice ( $n = 5$ , \* $p < 0.05$ ; Fig. 2b).

### DNMT1 expression is reduced in the cortex and hippocampus of 3-month-old APP/PS1 mice

DNMT1 expressions in cortex and hippocampus were evaluated by WB and immunostaining. Positive protein bands of DNMT1 are identified in cortex and hippocampus of APP/PS1 mice and wild type mice. In comparison with wild type mice, relative expressions of DNMT1 in cortex and hippocampus of APP/PS1 mice are reduced. Specifically, relative levels of DNMT1 in cortex and hippocampus of APP/PS1 mice are  $0.14 \pm 0.02$  and  $0.08 \pm 0.01$ . In wild type, relative expressions of DNMT1 are  $0.53 \pm 0.04$  in cortex and  $0.38 \pm 0.02$  in hippocampus ( $n = 5$ ,  $**p < 0.01$ ; Fig. 3a). In cortex and hippocampus of APP/PS1 mice and wild type mice, arrowheads points out positive expressions of DNMT1 which mainly localizes in cellular nuclei (Fig. 3b). Statistically, OD values of DNMT1 in cortex and hippocampus of APP/PS1 mice are  $10057 \pm 428$  and  $9900 \pm 568$ . In wild type, OD values of DNMT1 are  $16863 \pm 1486$  in cortex and  $14157 \pm 1521$  in hippocampus. Positive expressions of DNMT1 in cortex and hippocampus of APP/PS1 mice are lower than than thoes of wild type mice ( $n = 5$ ,  $*p < 0.05$ ; Fig. 3c).

### **BACE1 and A $\beta$ expression is increased in the cortex and hippocampus of 3-month-old APP/PS1 mice**

Expressions of BACE1 and A $\beta$  were checked by immunostaining and WB. BACE1 is postively labelled and spreads in cortex and hippocampus of APP/PS1 mice and wild type mie, pointed by arrowheads (Fig. 4a). Statistically, positive expressions of BACE1 in cortex and hippocampus of APP/PS1 mice are increased in comparison with wild type mice. In specific, OD values of BACE1 in cortex and hippocampus of APP/PS1 mice are  $38036 \pm 1299$  and  $21917 \pm 2229$ . In wild type mice, OD values of BACE1 are  $21896 \pm 1595$  in cortex and  $15333 \pm 1052$  in hippocampus ( $n = 5$ ,  $*p < 0.05$ ; Fig. 4b). BACE1 expressions were futher assessed by WB. There are positive protein bands of BACE1 in cortex and hippocampus of APP/PS1 mice and wild type mice (Fig. 4c). Compared with wild type mice, relative levels of BACE1 in cortex and hippocampus of APP/PS1 mice are increased. Relative expressions of BACE1 in cortex and hippocampus of APP/PS1 mice are  $1.2 \pm 0.05$  and  $0.59 \pm 0.02$ . In cortex and hippocampus of wild type mice, relative quantities of BACE1 are  $0.67 \pm 0.02$  and  $0.19 \pm 0.04$  respectively ( $n = 5$ ,  $*p < 0.05$ ; Fig. 4c). Moreover, A $\beta$  contents were evaluated by WB, positive bands of A $\beta$  are mainly found in cortex and hippocampus of APP/PS1 mice (Fig. 4d). In comparison with wild type mice, A $\beta$  levels in cortex and hippocampus of APP/PS1 mice are increased. Relative quantities of A $\beta$  in cortex and hippocampus of APP/PS1 mice are  $0.93 \pm 0.05$  and  $0.19 \pm 0.02$ . In wild type mice, relative levels of A $\beta$  are  $0.3 \pm 0.02$  in cortex and  $0.02 \pm 0.01$  in hippocampus respectively ( $n = 5$ ,  $*p < 0.05$ ; Fig. 4d).

### **Lactate activates GPR81-PI3K/Akt signaling pathway in neurons**

Cultured neurons were divided into 5 groups and given different administrations. Then, expressions of GPR81, p-PI3K, p-Akt, p-CREB were assessed to identify whether lactate can directly activate GPR81-PI3K/Akt pathway. As results showed, in comparison with Control group, 10 mmol/L lactate effectively upregulates relative levels of GPR81, p-PI3K, p-Akt and p-CREB expressions. Otherwise, expressions of GPR81, p-PI3K, p-Akt and p-CREB are suppressed by 15 mmol/L lactate or sodium oxamate, an inhibitor of lactate (Fig. 5 and Fig. 6).

The cells were double stained by GPR81 (red) and Neu N (green) (Fig. 5a). In statistic, OD values of GPR81 (red) are  $46525 \pm 1983$ ,  $55061 \pm 1823$ ,  $61165 \pm 2330$ ,  $52608 \pm 1390$  and  $36800 \pm 1711$  in Control,

5 mM, 10 mM, 15mM and Inhibitor groups respectively. OD values of Neu N (green) are  $35717 \pm 3019$ ,  $35334 \pm 1558$ ,  $33931 \pm 1758$ ,  $34275 \pm 1693$  and  $35161 \pm 593$  in each group. There is an increase of GPR81 expression in 10 mM group, and a reduction in GPR81 expression in Inhibitor group ( $n = 5$ ,  $*p < 0.05$ ). No differences of Neu N expressions can be found in each group (Fig. 5b). Through WB analysis, it is further identified that 10 mM lactate obviously upregulates GPR81 level, while lactate inhibitor reduces GPR81 expression. Relative levels of GPR81 are  $0.1 \pm 0.02$ ,  $0.096 \pm 0.05$ ,  $0.313 \pm 0.01$ ,  $0.076 \pm 0.01$  and  $0.017 \pm 0.003$  in each group ( $n = 5$ ,  $**p < 0.01$ ;  $n = 5$ ,  $*p < 0.05$ ; Fig. 5c).

WB was used to detect relative expressions of p-PI3K, p-Akt, p-CREB in Control, 5mM, 10mM, 15mM and Inhibitor groups (Fig. 6a). In each group, relative levels of p-PI3K are  $0.15 \pm 0.02$ ,  $0.16 \pm 0.01$ ,  $0.94 \pm 0.02$ ,  $0.24 \pm 0.04$ ,  $0.08 \pm 0.04$ , relative quantities of p-Akt are  $0.62 \pm 0.04$ ,  $0.85 \pm 0.01$ ,  $1.62 \pm 0.05$ ,  $0.95 \pm 0.01$ ,  $0.75 \pm 0.03$  and relative expressions of p-CREB are  $0.27 \pm 0.02$ ,  $0.24 \pm 0.02$ ,  $0.65 \pm 0.04$ ,  $0.31 \pm 0.05$ ,  $0.12 \pm 0.03$  respectively. Statistically, Compared with Control group, 10 mM lactate increases the expressions of p-PI3K, p-Akt and p-CREB, while lactate inhibitor decreases relative levels of p-PI3K, p-Akt and p-CREB ( $n = 5$ ,  $**p < 0.01$ ;  $n = 5$ ,  $*p < 0.05$ ; Fig. 6b).

## Discussion

In 1930s, lactate is considered as a way to clear unwanted or toxic metabolite rather than as a process through which lactate could produce energy[28]. Until 1950s, lactate is considered as an alternative energy substrate to glucose in the case of glucose deprivation[29]. Even, in the presence of adequate glucose, lactate is a preferred substrate to sustain neuronal activity[30]. Recently, studies signify that lactate is not merely an energy resource of neurons, but plays as a molecule to regulate activities of neural cells[31]. As a signaling regulator, lactate can bind to its receptor, GPR81, which can activate PI3K/Akt, ERK1/2 and NLRP3/NF- $\kappa$ B pathways[23]. Also, lactate can signal by being transported into neurons through MCTs, which further modulates redox-dependent and energy-dependent mechanism[32]. Here, this study discusses lactate, as a signaling molecule, effects on the regulation of A $\beta$  production through GPR81.

During AD progression, A $\beta$  deposition starts with the production of insoluble A $\beta$  fibrils. Hence, anti-A $\beta$  production at early stage of AD is thought to be a promising therapeutic strategy[34]. BACE1 is the rate-limiting enzyme of insoluble A $\beta$  cleavage, which cleaves amyloid precursor protein to produce insoluble A $\beta$ [34]. Meanwhile, promoter of BACE1 gene in AD subjects is hypomethylation, which is related with the reduction of DNMT1[9, 35]. In addition, inhibition of PI3K/Akt pathway was proved to aggravate A $\beta$  accumulation[36]. Therefore, this work proposes that at early stage of AD, lactate deficit leads to the suppression of GPR81-PI3K/Akt pathway, which downregulates DNMT1 level, further promoting BACE1 expression and A $\beta$  production.

Based on the assessment of 3-month-old APP/PS1 mice and wild type mice, this study primarily identifies that lactate content and GPR81 expression are reduced in cortex and hippocampus of APP/PS1 mice. It is recognized that the activity of GPR81 downstream PI3K/Akt pathway is inhibited in APP/PS1

mice. Hence, it is proved that lactate and its downstream GPR81-PI3K/Akt are suppressed at early period of AD. Otherwise, what's the direct effector of this pathway? It is showed that the inhibition of GPR81-PI3K/Akt downregulates DNMT1 expressions through CREB/P300. Moreover, DNMT1 reduction leads to hypomethylation of BACE1 promoter[9]. this is in accordance with the observed increase of BACE1 and A $\beta$  levels in 3-month-old APP/PS1 mice. Thus, it is proves that at early stage of AD, lactate is an important molecule regulator, its reduction leads to the suppression of GPR81-PI3K/Akt pathway, which aggravates A $\beta$  production through decreasing DNMT1 and increasing BACE1 levels.

These evidences illustrate that lactate decrease promotes A $\beta$  production through inhibiting PI3K/Akt pathway in AD at early stage. Otherwise, the direct regulation of lactate in GPR81-PI3K/Akt pathway hasn't been recognized. Therefore, we further checked the effect of lactate and lactate inhibitor on the activation of GPR81-PI3K/Akt pathway. As results showed, lactate directly triggers GPR81 and activate PI3K/Akt pathway in the cultured neurons. Hence, this work discovers a novel mechanism of early A $\beta$  accumulation in AD that is dominated by lactate-GPR81-PI3K/Akt signaling pathway. Meaningfully, it is suggested that lactate supplement might be a novel therapeutic strategy targeting early A $\beta$  production of AD.

## Abbreviations

AD: Alzheimer disease; ANOVA: one-way analysis of variance; APP/PS1: double-transgenic amyloid precursor protein/presenilin 1; A $\beta$ : amyloid beta; BACE1: beta-site amyloid precursor protein cleaving enzyme 1; CREB: cyclic-adenosine-monophosphate response element-binding protein; DAPI: 4,6-diamidino-2-phenylindole; DNMT1: deoxyribonucleic acid methyltransferase 1; ECL: enhanced chemiluminescence; ERK: extracellular regulated protein kinases; FITC: isothiocyanate; GPR81: G protein-coupled receptor 81; IPP: Image-Pro Plus; LSD: least significant difference; MCTs: monocarboxylate transporters; Neu N: neuronal nuclei; NFTs: tau-derived neurofibrillary tangles; NLRP3/NF- $\kappa$ B: Nod-like receptor family pyrin domain-containing 3/nuclear factor- $\kappa$ B; OD: optical density; PBS: phosphate buffer solution; PI3K/Akt: phosphatidylinositol 3 kinase /protein kinase B; PMSF: phenylmethyl sulfonylfluoride; PVDF: polyvinylidene fluoride; RIPA: radio-immunoprecipitation assay; SD: standard deviation; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPSS: Statistical Product and Service Solution; TBS: tris-buffered saline; TBST: tris-buffered saline containing 0.1% tween 20; TRITC: tetramethyl rhodamine isothiocyanate; WB: western blotting

## Declarations

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### ***Contributions***

MZ funded, designed and performed experiments, analyzed data, prepared the figures, and drafted the manuscript; YYW prepared the neuronal cultures and reviewed the manuscript; XDC assisted with WB analysis; LMD assisted with immunostaining; YB reviewed and revised the manuscript; ZXY assisted with the design of the experiments; RRL assisted with the preparation of materials; HG designed, and oversaw the whole project including experimental design, data analysis, drafting and reviewing the manuscript.

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### **Ethics Declarations**

#### ***Ethics Approval***

All procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All animal experiments were carried out in accordance with “Army Medical University Committee on the Care and Use of Laboratory Animals”.

#### ***Consent for Publication***

All authors have reviewed and approved the manuscript for publication in this journal.

#### ***Consent to Participate***

Not applicable.

#### ***Competing Interests***

The authors declare no competing interests.

### **Data Availability**

All data generated or analyzed during this study are included in this manuscript.

### **Research involving Animals**

3-month-old APP/PS1 mice and 3-month-old wild type mice were used in this study.

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## Figures

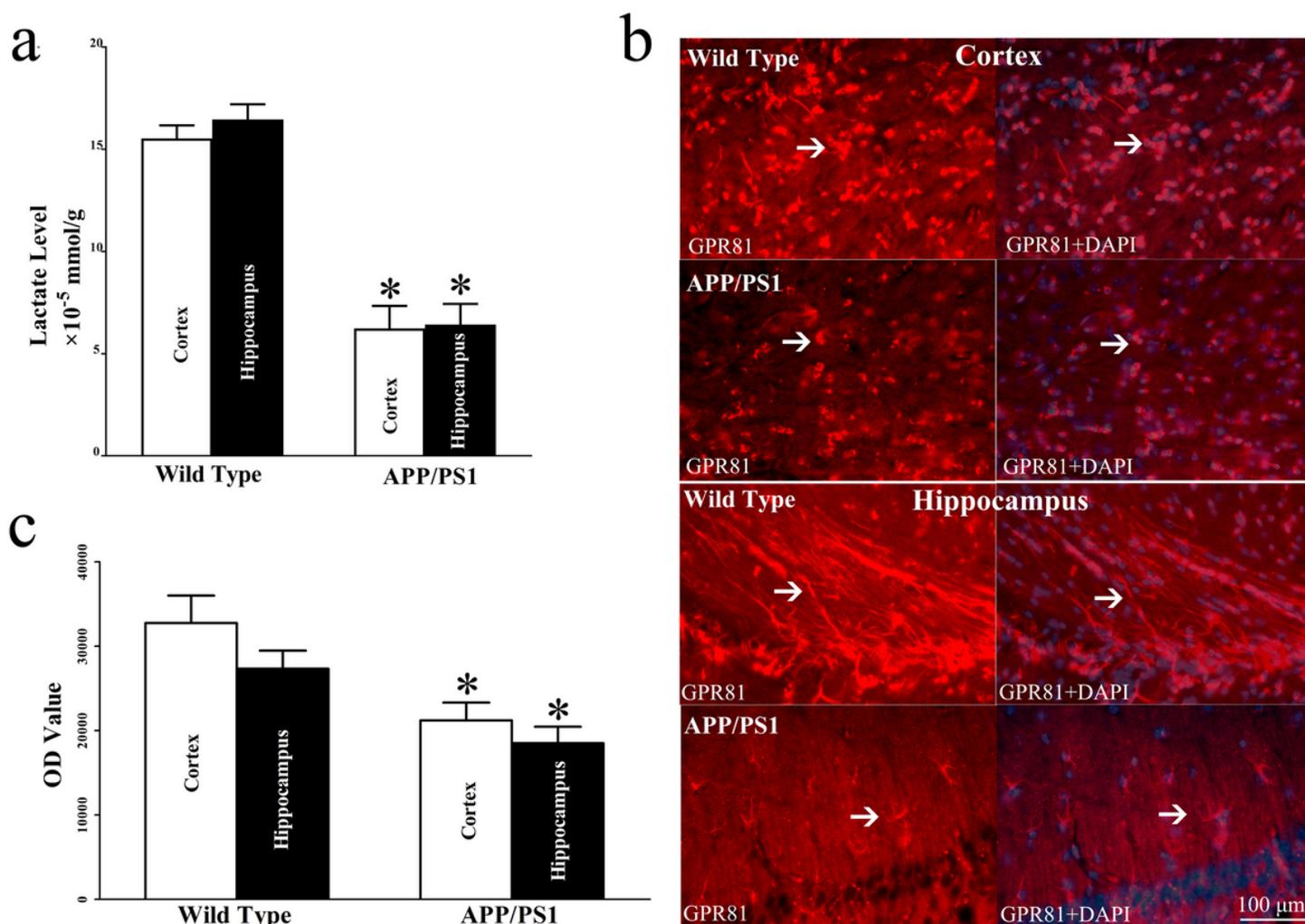
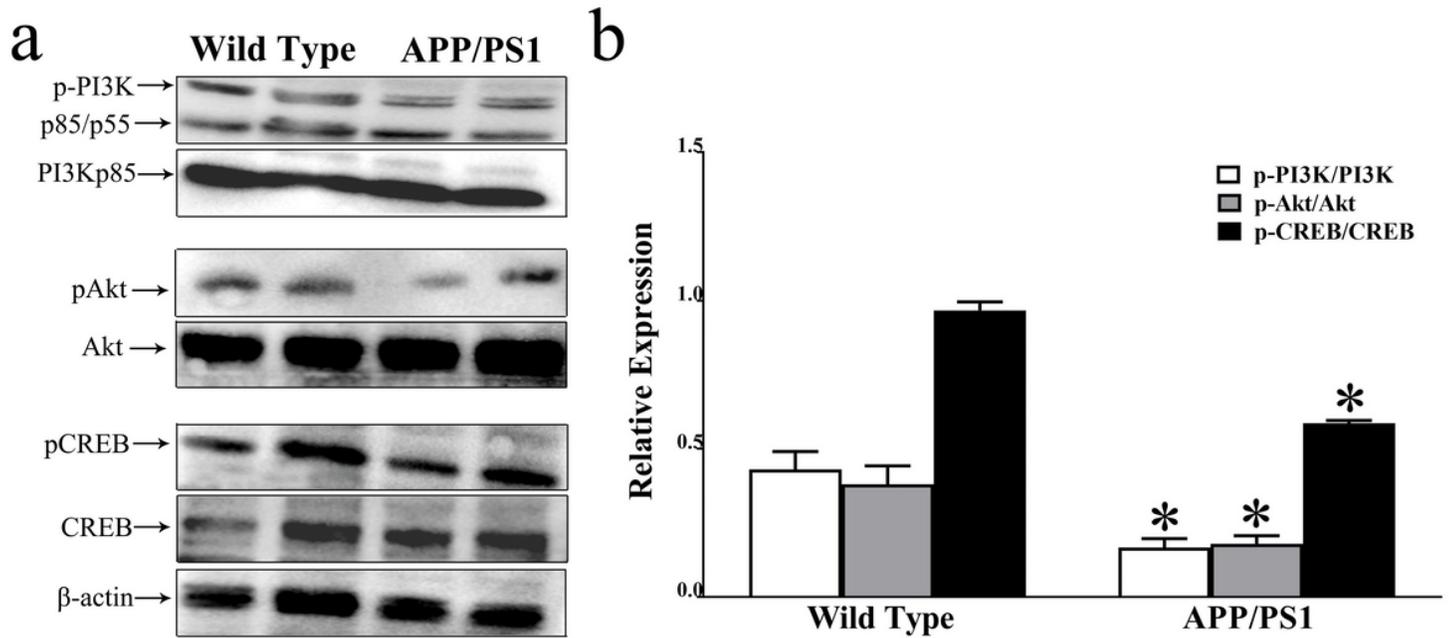


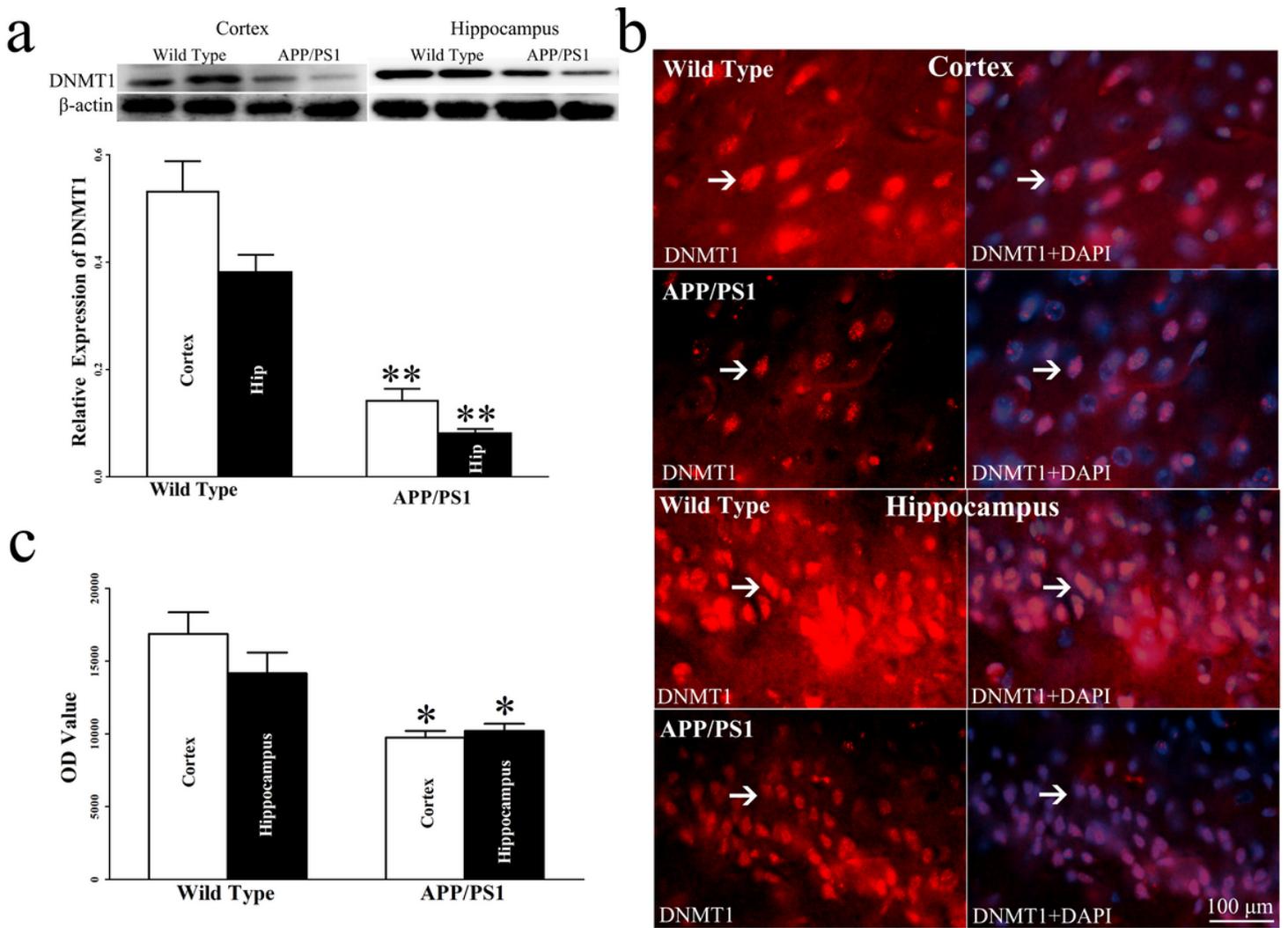
Figure 1

Lactate content and GPR81 expression are reduced in the cortex and hippocampus of 3-month-old APP/PS1 mice. (a) Lactate levels are decreased in the cortex and hippocampus of APP/PS1 mice compared with wild type mice (n = 5, \*p < 0.05). (b) As arrowheads pointed, GPR81 is expressed in the cortex and hippocampus of APP/PS1 mice and wild type mice. (c) In comparison with wild type mice, relative expressions of GPR81 in the cortex and hippocampus are reduced (n = 5, \*p < 0.05).



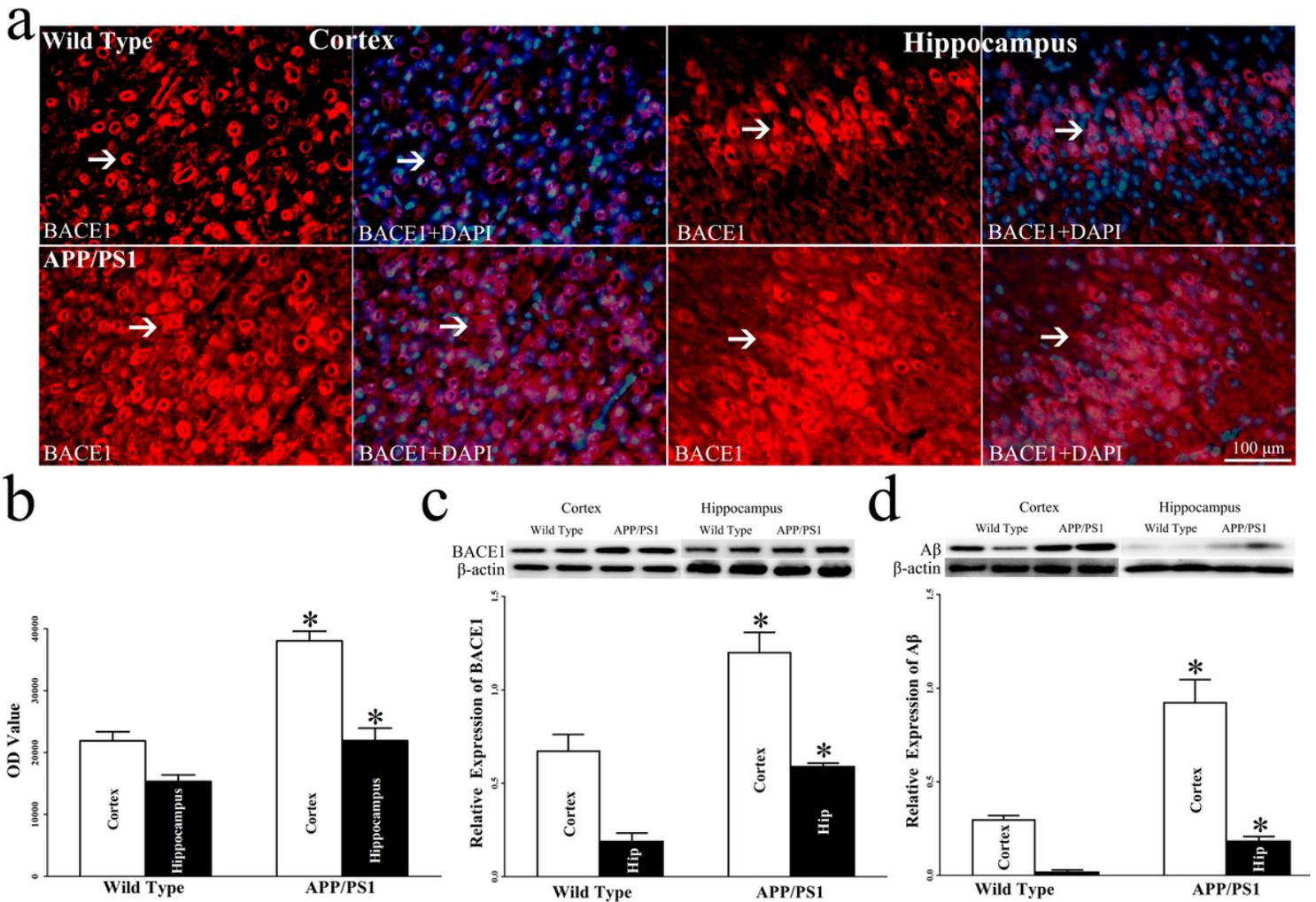
**Figure 2**

Relative levels of p-PI3K, p-Akt and p-CREB are reduced in 3-month-old APP/PS1 mice. (a) Through WB analysis, positive protein bands of p-PI3K, p-Akt and p-CREB can be found in 3-month-old APP/PS1 mice and wild type mice. (b) Statistically, compared with wild type mice, relative expressions of p-PI3K, p-Akt, p-CREB are decreased in APP/PS1 mice (n = 5, \*p < 0.05).



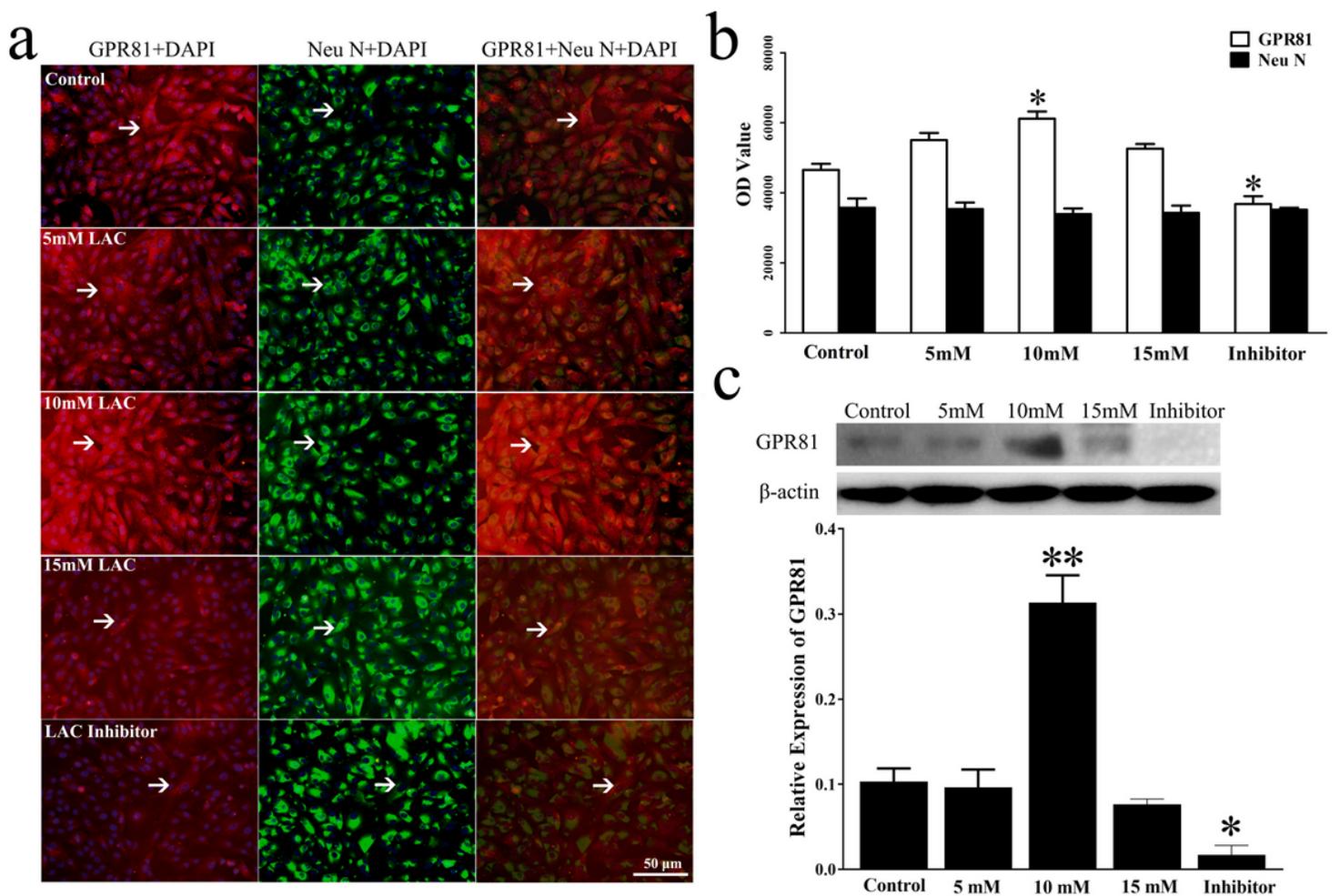
**Figure 3**

DNMT1 expressions are decreased in the cortex and hippocampus of 3-month-old APP/PS1. (a) WB was used to assess DNMT1 expressions in cortex and hippocampus of APP/PS1 mice and wild type mice. Relative levels of DNMT1 in cortex and hippocampus of APP/PS1 mice are lower than those of wild type mice (n = 5, \*\*p < 0.01). (b) DNMT1 is expressed in the cortex and hippocampus of APP/PS1 mice and wild type mice, pointed by arrowheads. (c) OD values of DNMT1 expressions are decreased in cortex and hippocampus of APP/PS1 mice in comparison with wild type mice (n = 5, \*p < 0.05).



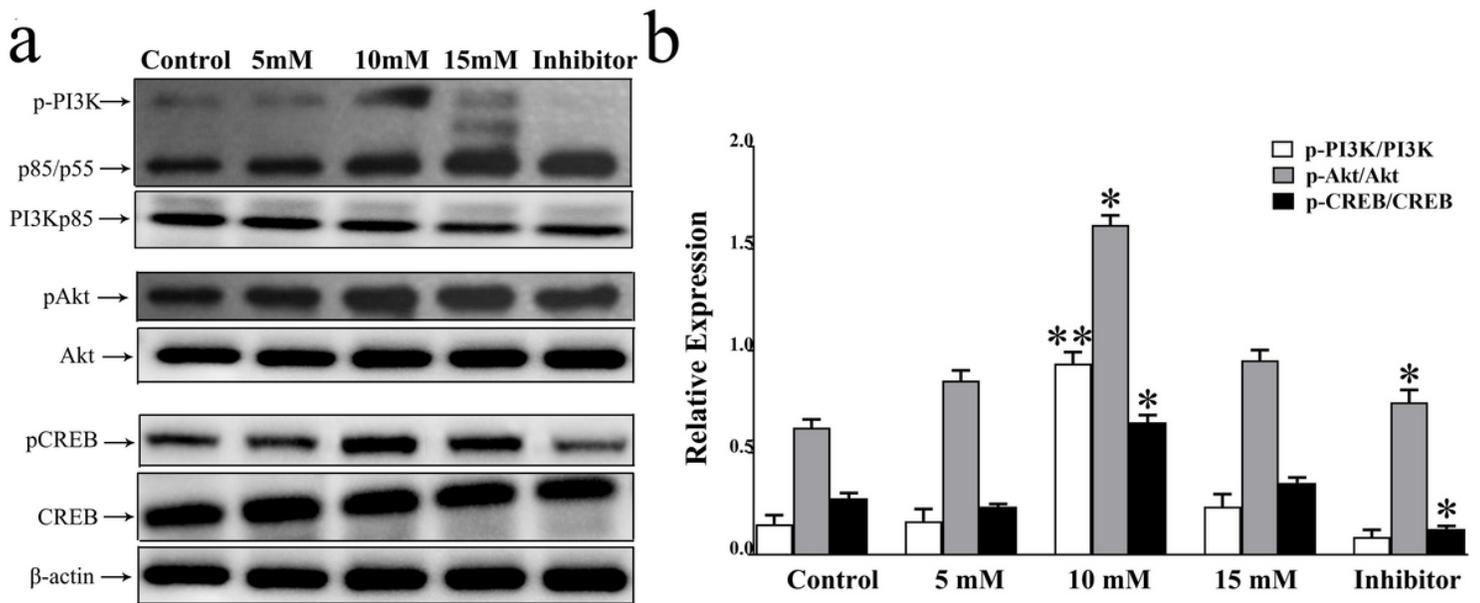
**Figure 4**

BACE1 expression and A $\beta$  content are decreased in the cortex and hippocampus of APP/PS1 mice. (a) It shows that BACE1 is expressed in the cortex and hippocampus of APP/PS1 mice and wild type mice. (b) OD values of BACE1 expressions are reduced in the cortex and hippocampus of APP/PS1 mice compared with wild type mice (n = 5, \*p < 0.05). (c) Positive protein bands of BACE1 can be found in cortex and hippocampus of APP/PS1 mice and wild type mice. Particularly, relative quantities of BACE1 are upregulated in the cortex and hippocampus of APP/PS1 mice in comparison with wild type mice (n = 5, \*p < 0.05). (d) Positive bands of A $\beta$  are dominantly found in the cortex and hippocampus of APP/PS1 mice. In statistic, relative contents of A $\beta$  in the cortex and hippocampus of APP/PS1 mice are higher than thoes of wild type mice (n = 5, \*p < 0.05).



**Figure 5**

Lactate directly upregulates GPR81 expression in cultured neurons. (a) GPR81 (red) and Neu N (green) were double labelled by immunostaining. GPR81 is localized to neurons, pointed by arrowheads. (b) In comparison with Control group, neuronal GPR81 expression is upregulated by 10 mM lactate, while downregulated by lactate inhibitor ( $n = 5$ ,  $*p < 0.05$ ). (c) Relative level of GPR81 was further assessed by WB. Compared with Control group, 10 mM lactate evidently increases neuronal GPR81 expression ( $n = 5$ ,  $**p < 0.01$ ). Otherwise, lactate inhibitor reduces GPR81 level in neurons ( $n = 5$ ,  $*p < 0.05$ ).



**Figure 6**

Lactate activates PI3K/Akt pathway. (a) Relative expressions of p-PI3K, p-Akt and p-CREB in different groups of neurons were checked by WB. (b) Statistically, 10 mM lactate upregulates relative levels of p-PI3K, p-Akt and p-CREB in comparison with Control group ( $n = 5$ ,  $**p < 0.01$ ;  $n = 5$ ,  $*p < 0.05$ ). There are no significant differences in p-PI3K expression between Control group and group of lactate inhibitor ( $n = 5$ ,  $p > 0.05$ ). Otherwise, lactate inhibitor downregulates p-Akt and p-CREB expressions ( $n = 5$ ,  $*p < 0.05$ ).