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The alterations of Ca^{2+}/calmodulin/CaMKII/$Ca_V1.2$ signaling in experimental models of Alzheimer's disease and vascular dementia

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HIGHLIGHTS

- CaM was up-regulated in the hippocampus of both APP/PS1 mice and VD gerbils.
- The expression of $Ca_V1.2$ protein was increased in VD gerbils and in cultured neurons but decreased in APP/PS1 mice.
- The number of CaMKII and $Ca_V1.2$ co-localization positive neurons was decreased in AD and VD models.

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ABSTRACT

The two critical forms of dementia are Alzheimer's disease (AD) and vascular dementia (VD). The alterations of Ca^{2+}/calmodulin/CaMKII/$Ca_V1.2$ signaling in AD and VD have not been well elucidated. Here we have demonstrated changes in the levels of $Ca_V1.2$, calmodulin, p-CaMKII, p-CREB and BDNF proteins by Western blot analysis and the co-localization of p-CaMKII/$Ca_V1.2$ by double-labeling immunofluorescence in the hippocampus of APP/PS1 mice and VD gerbils. Additionally, expression of these proteins and intracellular calcium levels were examined in cultured neurons treated with $A\beta_{1-42}$. The expression of $Ca_V1.2$ protein was increased in VD gerbils and in cultured neurons but decreased in APP/PS1 mice; the expression of calmodulin protein was increased in APP/PS1 mice and VD gerbils; levels of p-CaMKII, p-CREB and BDNF proteins were decreased in AD and VD models. The number of neurons in which p-CaMKII and $Ca_V1.2$ were co-localized, was decreased in the CA1 and CA3 regions in two models. Intracellular calcium was increased in the cultured neurons treated with $A\beta_{1-42}$. Collectively, our results suggest that the alterations in $Ca_V1.2$, calmodulin, p-CaMKII, p-CREB and BDNF can be reflective of an involvement in the impairment in memory and cognition in AD and VD models.

1. Introduction

Progressive impairment in memory and cognition is a key clinical feature of senile dementia and is reflective of degeneration within the central nervous system (CNS). The two major forms of dementia are Alzheimer's disease (AD) and vascular dementia (VD).

Although the exact causative mechanisms of senile dementia remain unknown until now, there is a growing body of evidence supporting the hypothesis that Ca^{2+} dysregulation contributes in part to AD and VD [3,4]. The activity of the L-type calcium channel (LTCC) is linked to neuronal survival and death *via* transduction of calcium-regulated signaling events to the nucleus. The $Ca_V1.2$ channel accounts for 80% of the LTCCs in the brain, which is necessary to effectively activate cAMP response element-binding (CREB) and CREB-mediated gene transcription [1].

Calmodulin (CaM), the major intracellular calcium ion binding protein in the mammalian brain [24], has been implicated in many basic neuronal functions [12]. Calcium/calmodulin-dependent protein kinase II (CaMKII) is a Ca^{2+}-activated enzyme, which plays an important role in learning and memory as a

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modulator of excitation-transcription coupling in neurons [11]. It is also critical for synaptic plasticity and behavioral training that are activated by Ca^{2+}/CaM binding [5,12]. Additionally, tethering of CaMKII to the COOH terminus of the $Ca_V1.2\alpha$ subunit is an essential molecular feature of Ca^{2+}-dependent facilitation [7].

Brain-derived neurotrophic factor (BDNF) is a critical protein that supports the development, differentiation, maintenance and plasticity of brain function. The activation of CREB through phosphorylation of Ser133 controls the induction of many genes, including BDNF [18].

However, until now, the changes in Ca^{2+}/calmodulin/CaMKII/$Ca_V1.2$ signaling in APP/PS1 mice, in cultured neurons treated with $A\beta_{1-42}$, and in ischemia-induced VD gerbils, have not been well documented. We hypothesize that these proteins might be involved in the impairment of learning and memory in these models. Thus, the present study was performed to examine the alterations in $Ca_V1.2$, CaM, p-CaMKII, p-CREB and BDNF levels and the relationships between p-CaMKII and $Ca_V1.2$ in AD and VD models.

2. Materials and methods

2.1. Ethical approval

All experimental procedures were conducted in strict accordance with the guidelines established by the Ministry of Health of China and were approved by the Animal Care Committee of China Medical University.

2.2. Animal husbandry

Male 9-month-old APP/PS1-Tg mice and age-matched wild-type C57BL/6 mice were obtained from the Jackson Laboratory (West Grove, PA, USA).

2.3. Induction of global cerebral ischemia

Transient global ischemia was induced according to the method described previously [20]. Briefly, gerbils were anesthetized with 10% chloral hydrate (350 mg/kg) and the bilateral common carotid arteries were occluded using artery clips. After 10 min, the clips were removed to restore cerebral blood flow. A sham group underwent the same surgical procedure without ligation of the common carotid arteries.

2.4. The Morris water maze test

A modified Morris water maze was used on the 21st day [13]. Seven gerbils were in each group. An acrylic platform (8 cm in diameter) was placed in the center of the third quadrant. On days 1–3, gerbils were trained individually to find the platform in a maximum time of 60 s. On days 4–7, the latency to climb onto the hidden platform was recorded. On the 8th day, the hidden platform was removed and the number of times the gerbils crossed the center of the third quadrant was recorded.

2.5. Nissl staining

Following the Morris water maze test, the entire brains were removed and post-fixed for one day. They were then placed in a 30% sucrose solution at 4 °C. Representative coronal sections (7 μm-thick) were obtained using a rotary microtome and stained with Nissl staining solution.

2.6. Primary neuronal cell cultures and cell viability analysis

Primary cultures of hippocampal neurons were prepared according to a previous method [9]. Hippocampal neuronal cells from one day-old rats were dissociated in Hanks' balanced salt solution containing 0.25% trypsin and then plated onto dishes in Dulbecco's modified Eagle's medium (DMEM) including 15% fetal bovine serum. After plating for 24 h, the medium was replaced with neurobasal medium supplemented with 2% B27. $A\beta_{1-42}$ was prepared according to a previous method [21]. On the 9th day, the culture medium was replaced with the DMEM without phenol red and containing different concentrations of $A\beta_{1-42}$ (0, 1, 2, 4, 8, and 16 μM) and culturing was continued for a further 24 h. The cells were treated with MTT (0.5 mg/mL) per well [14]. After 4 h incubation, the MTT was replaced with 150 μL of DMSO and the absorption of the medium at 570 nm was determined.

2.7. Measurement of intracellular calcium concentration

On the 9th day, neurons of the control and $A\beta_{1-42}$ (4 μM) pretreated for 24 h were loaded with 5 μM Fluo-3/AM dissolved in DMSO and 0.5% pluronic F-127 in serum-free growth medium for 30 min at 37 °C. Confocal imaging was performed on three separate fields of cells for each group. The concentration of intracellular Ca^{2+} was expressed as fluorescent intensity by the software (EZ-C1 3.70 FreeViewer NIKON) and the average intensity of each neuron was calculated.

2.8. Western blot analysis

The hippocampal tissues of four gerbils, four mice and the cultured neurons treated with $A\beta_{1-42}$ for 24 h were used. Total protein levels were determined using a BCA protein assay kit. The primary antibodies: rabbit anti-p-CREB (1:1000, Abcam), rabbit anti-CREB (1:500, SAB), rabbit anti-BDNF (1:200, Santa Cruz), mouse anti-$Ca_V1.2$ (1:400, Abcam), rabbit anti-CaMKII (1:400, Santa Cruz), rabbit anti-p-CaMKII (1:400, Santa Cruz), mouse anti-CaM (1:400, Santa Cruz) and β-actin (1:5000, Santa Cruz) were prepared for use as per manufacturers instructions. Membranes were incubated with HRP-conjugated secondary antibodies (1:5000, Santa Cruz) for 1 h at room temperature. Immunodetection was performed with enhanced chemiluminescence (ECL; Applygen) followed by exposure to X-ray film. All data were analyzed by Quantity One software (BioRad).

2.9. Double-labeling immunofluorescence

APP/PS1 and C57BL/6 mice were anesthetized and perfused intracardially with 4% paraformaldehyde. Coronal brain sections of 7 μm thickness were cut with a cryostat (Leica CM1900 UV). Nonspecific binding was blocked by 3% normal goat serum diluted in 5% BSA with 0.25% Triton X-100 for 1 h and then incubated overnight in a mixture of primary antibodies, mouse anti-$Ca_V1.2$ (1:50) and rabbit anti-CaMKII (1:100; Santa Cruz). The sections were incubated with FITC-and Cy3-conjugated goat anti-mouse or anti-rabbit antibodies (Beyotime) for 2 h at room temperature. Nuclear counter-staining was achieved with DAPI (1:500). Sections were examined using a confocal laser scanning microscope (C1, Nikon, Japan). Confocal imaging was performed on four separate fields for each group. The cells in which antigens were co-localized were clearly yellow and their numbers per 100 cells were counted.