Raloxifene neutralizes the adverse effects of glutamate on cultured neurons by regulation of calcium oscillations

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Abstract. Calcium dyshomeostasis is an important pathology of memory impairment. However, the mechanism of how calcium dyshomeostasis impairs neurons has remained elusive. The aim of the present study was to reveal the influence of calcium dyshomeostasis on the expression of calcium memory-associated proteins and the ability of raloxifene to neutralize the adverse effects of glutamate on cultured neurons by regulation of calcium oscillations. After neurons were treated with various concentrations of glutamate alone or with raloxifene, the expression of calcium memory-associated proteins and the influence on calcium dyshomeostasis was assessed. The results indicated that glutamate regulated calcium oscillation waves and expression of calcium memory-associated protein in a concentration-dependent manner. Raloxifene increased the expression of these proteins as well as neuronal survival. It is therefore concluded that glutamate regulated calcium oscillations in a dose-dependent manner, while raloxifene protected neurons from destruction through glutamate exposure and at the same time neutralized the decrease in expression of the memory-associated proteins.

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Introduction

Calcium is an important second messenger involved in signal transduction pathways that regulate memory formation and consolidation (1,2). In combination with calcium/calmodulin-dependent protein kinase (CaMK), calcium triggers autophosphorylation and maintains short-term memory for several minutes. The CaMK-calcium complex travels into the nucleus, where it activates the cyclic adenosine monophosphate response element binding protein (CREB), promotes the synthesis of memory-associated proteins and aids in the formation of permanent memory that lasts from several hours to numerous years (3,4). If the intracellular calcium concentration decreases to a level that is insufficient to trigger the autophosphorylation of CaMKII and the synthesis of memory-associated protein, memory is impaired. Calcium dyshomeostasis has been observed in Alzheimer's disease (5,6), and numerous studies have determined that excess calcium promotes neurotoxicity and oxidative stress, impairs mitochondrial activity and causes apoptosis (7-9); therefore, the calcium concentration must be at a suitable level for calcium signaling transduction. Calcium oscillations, which are periodic fluctuations of the intracellular calcium concentration, are a common form of information coding, and the expression levels of various proteins are determined by the frequency of oscillation waves as well as their amplitude and cumulative wave width (10,11). Our group and others have

Table I. Primary antibodies used for western blot analysis in the present study.

Antibody	Dilution	Source
CaMKII (poly)	1:1,000	Cell Signaling Technology, Inc. (Boston, MA, USA)
p-CaMKII (mAb)	1:500	Cell Signaling Technology, Inc.
NR1 (poly)	1:500	Alomone (Jerusalem, Israel)
NR2B (poly)	1:1,000	Abcam (Cambridge, England)
Cav1.2 (poly)	1:200	Alomone
PKC (mAb)	1:200	Santa Cruz Biotechnology (Dallas, TX, USA)
PSD95 (mAb)	1:200	Santa Cruz Biotechnology
PSD93 (poly)	1:1,000	Abcam
CREB (poly)	1:1,000	Cell Signaling Technology, Inc.
p-CREB (poly)	1:500	Cell Signaling Technology, Inc.

mAb, mouse monoclonal antibody; p, phosphorylated; poly, rabbit polyclonal antibody; CaMK, calcium/calmodulin-dependent protein kinase; CREB, cyclic adenosine monophosphate response element binding protein; NR2B, *N*-methyl D-aspartate receptor subtype 2B; NR1, NR1 subunit of the *N*-methyl-D-aspartate receptor; PKC, protein kinase C; Cav1.2, calcium channel, voltage-dependent, L type, alpha 1C subunit; PSD95, postsynaptic density protein 95.

previously reported that calcium oscillations regulate RNA transcription and protein expression (12,13). The synthesis of memory-associated proteins is regulated by calmodulin, signal transducer protein and calcium channel proteins, which are referred to as calcium memory-associated proteins. Whether the calcium oscillations regulate calcium memory-associated protein synthesis has remained to be demonstrated, and the present study aimed to clarify this issue.

The present study assessed the effect of a selective estrogen receptor modulator, raloxifene, on calcium oscillations and the expression of calcium memory-associated proteins during calcium overload.

Materials and methods

Neuron cultures. Primary hippocampal neuron cultures were prepared from 17-18 day-old Wistar rat embryos as previously described (14). The neurons were plated on 24-mm round coverslips in six-well plates coated with 20 μg/ml poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA) and cultured for 10 days *in vitro* in neurobasal medium supplemented with 2% (v/v) B-27 medium (Gibco Life Technologies, Carlsbad, CA, USA); half of the medium was changed every four days. One pregnant Wistar rat was ordered from the animal center affiliated to Wuhan University (Wuhan, China). The present study was conducted in strict accordance with the recommendations set out in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (eighth edition, 2011). The protocol involving animals was reviewed and approved by the Institutional Animal Care and Use Committee of the Tongji Medical college of Huazhong Science and Technology (Wuhan, China).

Calcium measurements. Changes in the cytosolic free calcium concentration, $[Ca^{2+}]i$, were measured using the $[Ca^{2+}]i$ indicator Fura-2 AM (Dojingo Molecular Technologies, Inc., Kumamoto, Japan). The neurons were cultured for 10 days on coated 24-mm round glass coverslips at a density of $1x10^5$ cells/cm^2 and were incubated in the dark with 5 μM Fura-2 AM for 30 min at 37˚C in Krebs-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer [containing: NaCl (137 μM), KCl (4.9 μM), $CaCl_2$ (2 μM), $MgSO_4$ (1.2 μM), D-glucose (10 μM) and HEPES (10 μM)]. The coverslips were then washed, and the cells were maintained for at least 30 min prior to experimentation in indicator-free Krebs-HEPES buffer. The emitted Fura-2 fluorescence was recorded from neurons on the coverslips in a perfusion chamber mounted on the stage of a modified Olympus inverted epifluorescence microscope (IX-30; Olympus, Tokyo, Japan) after excitation at 340±10 and 380±10 nm using a xenon short-arc lamp (Ushio, Tokyo, Japan), corresponding to the Ca^{2+}-bound and Ca^{2+}-free forms of the indicator, respectively. Bandpass interference filters (Omega Optical, Brattleboro, VT, USA) selected wavelength bands of emitted fluorescence at 510±10 nm. Emitted Fura-2 fluorescence was collected and measured using a spectrofluorometer (PTI Deltascan; Photon Technology International, Inc., Monmouth Junction, NJ, USA). Autofluorescence created by unloaded neurons was generally <5% of Fura-2-loaded neurons and was subtracted automatically from Fura-2-fluorescence recordings. The baseline mean ratio value (R mean) was the mean ratio value after a 3-min recording taken at the beginning of the experiment. Calcium oscillations, expressed as the ratio (R) of fluorescence intensities at 340/380 nm, were defined as variations of 10% from the mean R, occurring synchronously in several cells of the field.

Western blot analysis. Following the abovementioned treatments, the neurons were collected after culturing for 10 days and lysed with 1X loading buffer (containing 1% v/v phenylmethanesulfonylfluoride), and total protein was extracted. The cell extracts were mixed with sample buffer containing 50 mM Tris-HCl (pH 7.6), 2% SDS, 10% glycerol, 10 mM dithiothreitol and 0.2% bromophenol blue and boiled for 5 min. Boiled samples were subjected to 10% SDS-PAGE (Sigma-Aldrich) and the separated proteins were transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Maidstone, UK).

The membranes were then incubated with primary antibodies (see Table I) that were detected using anti-rabbit or anti-mouse immunoglobulin G conjugated to IRDye (800CW; LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature and visualized using the Odyssey infrared Imaging System (no. 9120; LI-COR Biosciences, Lincoln, NE, USA).

MTT assay. Hippocampal neurons ($1x10^4$ cells/100 μl/well) were plated on 96-well plates, and the neurons were cultured for 10 days prior to the experiment. After the neurons matured, they were treated with 10-300 μM glutamate (Sigma-Aldrich), 300 μM glutamate + raloxifene (300 nM-10 μM; Sigma-Aldrich), or culture media based on the previous experimental design for 48 h. The cells were treated with 20 μl MTT (5 mg/ml; Sigma-Aldrich) for 4 h, the culture media were discarded, 150 μl dimethylsulfoxide (Sigma-Aldrich) was added, and the plates were agitated using a micro-oscillator at a low speed for 5 min. The optical density (OD) was tested using a standard ELISA microplate reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. Values are expressed as the mean ± standard deviation and were analyzed using Graph Pad Prism 5 (Graph Pad Inc., La Jolla, CA, USA). A one-way analysis of variance procedure followed by least significant difference post-hoc tests as well as Student's t-tests were used to determine differences between the groups. $P<0.05$ was considered to indicate a statistically significant difference between values.

Results

Glutamate regulates calcium oscillations in a concentration-dependent manner. After the cells were scanned for 3 min, glutamate was added to the buffer to reach a working concentration of 10 μM to 1 mM (n=30-40 for each calculation; 73-89% of neurons showed oscillation waves after being stimulated with 30 μM to 100 μM glutamate; each experiment was repeated three times) (Fig. 1A). At the 10 μM concentration, the calcium oscillations waves appeared within 15 min, whereas the oscillation wave frequency was low at the beginning (Fig. 1Aa). At the 28th minute, the frequency increased to 2/min, and the oscillation waves persisted for 18 sec. For the 30 μM concentration, the oscillation wave promptly appeared when glutamate was added to the buffer, the frequency was 1.2/min, and the oscillation waves persisted for 31 sec at the beginning (Fig. 1Ab). The frequency then declined to 0.8/min, but the oscillation waves persisted for 44 sec. When the glutamate concentration was 100 μM, the oscillation appeared when the drug was added to the buffer, the frequency was 0.3/min, and the oscillation waves persisted for 121 sec (Fig. 1Ac). At a concentration of 300 μM, the single oscillation wave appeared immediately, then disappeared, and the R[340/380] increased (Fig. 1Ad). When the glutamate concentration was 1 mM, the R[340/380] declined at first and then increased to a very high level (Fig. 1Ae).

Raloxifene reverses glutamate-induced calcium oscillations. 10-day-old neurons were pre-treated with 300 μM glutamate for 5 min, the spontaneous calcium oscillations were inhibited (n=30-40 for each calculation; ~90% of neurons showed spontaneous calcium oscillations after being cultured for 10 days; each experiment was repeated three times), and raloxifene was then added to the buffer at working concentrations of 300 nM to 10 μM (Fig. 1B). When the concentration of raloxifene was 300 nM, the calcium oscillation waves irregularly re-appeared after 10 min and continued at a low frequency (Fig. 1Ba). When the concentration was increased to 1 μM, the oscillation wave appeared regularly after 15 min, and the frequency was ~0.7/min and persisted for 52 sec (Fig. 1Bb). When 3 μM raloxifene was used, the calcium oscillations appeared after 5-7 min, the frequency was 0.6/min, and the oscillation waves persisted for 63 sec (Fig. 1Bc). When the concentration of raloxifene was 10 μM, the oscillation wave appeared intermittently (Fig. 1Bd).

Calcium dyshomeostasis reduces neuronal survival and is reversed by raloxifene treatment. The present study analyzed neuronal survival using the MTT assay. Overall, 10-30 μM glutamate did not significantly affect neuronal survival; however, when the concentration was increased to 100 μM, the neuronal survival markedly declined as indicated by the reduced OD values. In addition, 300 μM glutamate also impaired neuronal survival to a similar degree to that of 100 μM glutamate. When raloxifene was added, neuronal survival improved as the concentration increased, and all groups exhibited increased neuronal survival compared with that in the control group. When the neurons were treated with 300 nM and 1 μM raloxifene, the neuronal survival did not significantly change; however, when the concentration of raloxifene was increased to 3 μM, neuronal survival significantly improved. In addition, 10 μM raloxifene had a similar effect on neuronal survival as that of 3 μM raloxifene (Fig. 2).

Glutamate regulates expression of calcium memory-associated proteins in a concentration-dependent manner. After the intracellular calcium concentrations were measured, the neurons were collected and subjected to western blot analysis of calcium memory-associated proteins. The present study selectively assessed the following proteins: Calcium channel, voltage-dependent, L type (L-VGCC), alpha 1C subunit (Cav1.2), which is the primary subunit of L-VGCC in the brain (15); the NMDA receptor (the important post-synaptic calcium channel) subunits NR1 and NR2B; two important proteins distributed in postsynaptic density, post-synaptic density protein (PSD) 95 and PSD 93; the calcium-sensitized proteins CaMKII and phosphorylated (p) CaMKII; protein kinase C (PKC), which mediates the conversion of short-term memory into permanent memory (16,17); and key molecules that control transcription, CREB and pCREB (18,19). The results showed that 30 μM and 100 μM glutamate significantly increased the expression of Cav1.2, NR1, NR2B, PSD95 and pCREB. 10 μM glutamate non-significantly increased the expression of Cav1.2, NR1, NR2B, PKC and CREB. However, none of the test concentrations of glutamate significantly affected the expression of pCaMKII, PKC and CREB. 300 μM and 1 mM glutamate significantly decreased the expression of PSD93 and pCREB, and 1 mM glutamate significantly decreased the expression of CaMKII. These results suggested that low concentrations of glutamate (10-100 μM) stimulated the release of calcium and increased the expression of several calcium memory-associated proteins, while high concentrations

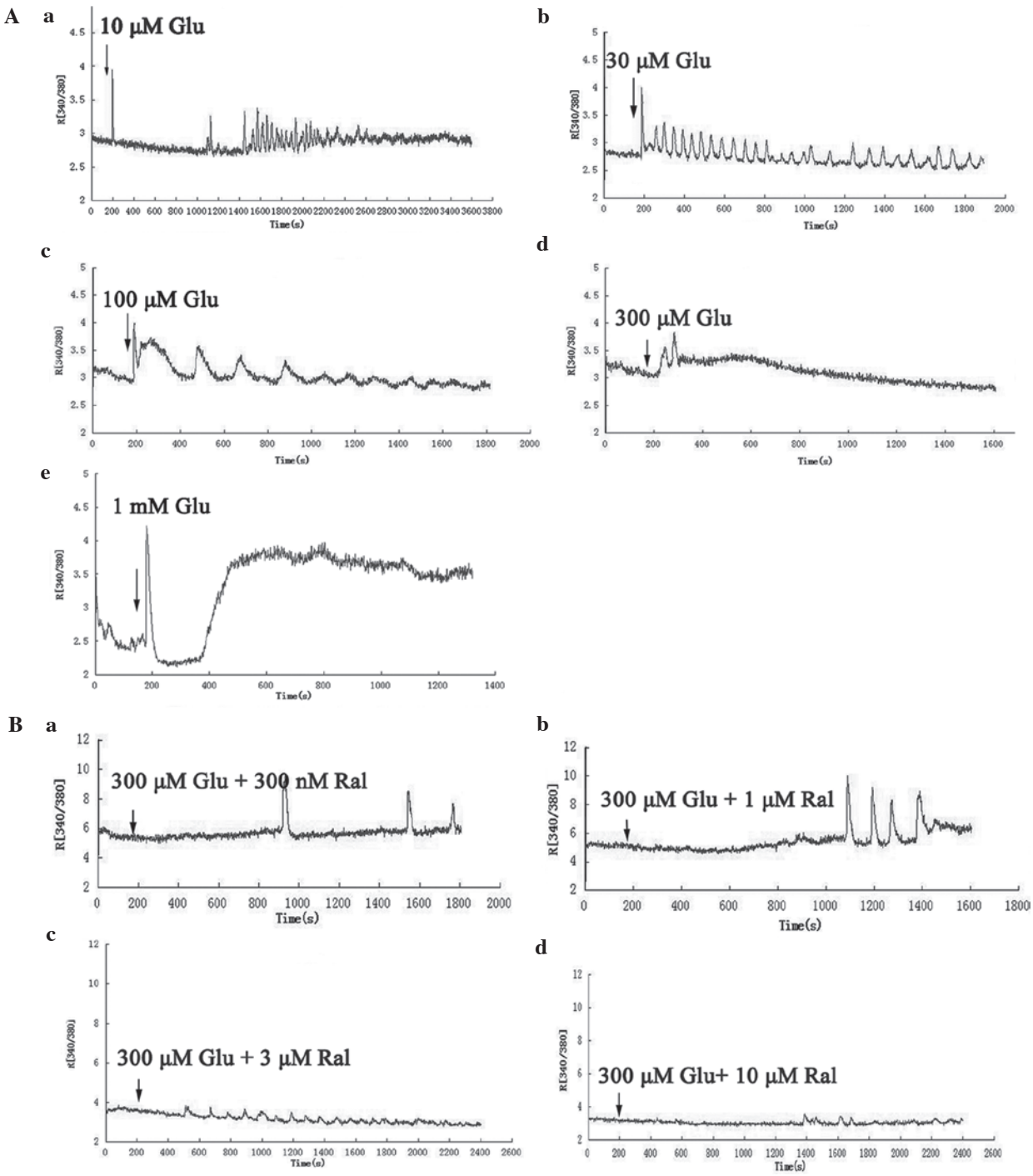

Figure 1. Influence of calcium dyshomeostasis on calcium oscillations and the neutralizing effect of Ral. (A) Influence of Glu on calcium oscillations. Neurons were stimulated with (a) 10 μM, (b) 30 μM, (c) 100 μM, (d) 300 μM or (e) 1 mM Glu. (B) Neutralizing effect of Ral on neuron calcium oscillations after treatment with 300 μM Glu. Neurons treated with (a) 300 nM, (b) 1 μM, (c) 3 μM or (d) 10 μM Ral. Ral, raloxifene; Glu, glutamate.

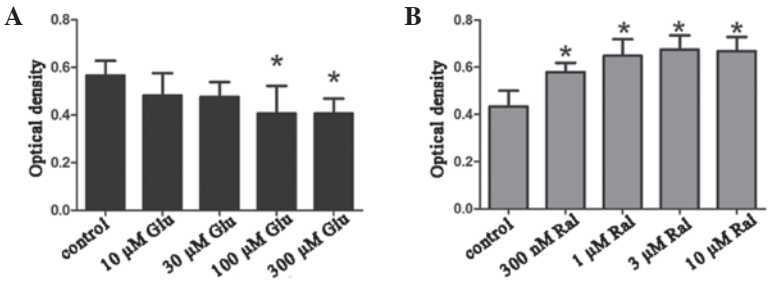

Figure 2. Effects of Glu and Ral on neuronal survival assessed using an MTT assay. (A) Effect of Glu on neuronal survival. (B) Neutralizing effect of Ral after neurons were stimulated with 300 μM Glu. Optical density values are expressed as the mean ± standard deviation. *P<0.05 vs. control. Ral, raloxifene; Glu, glutamate.

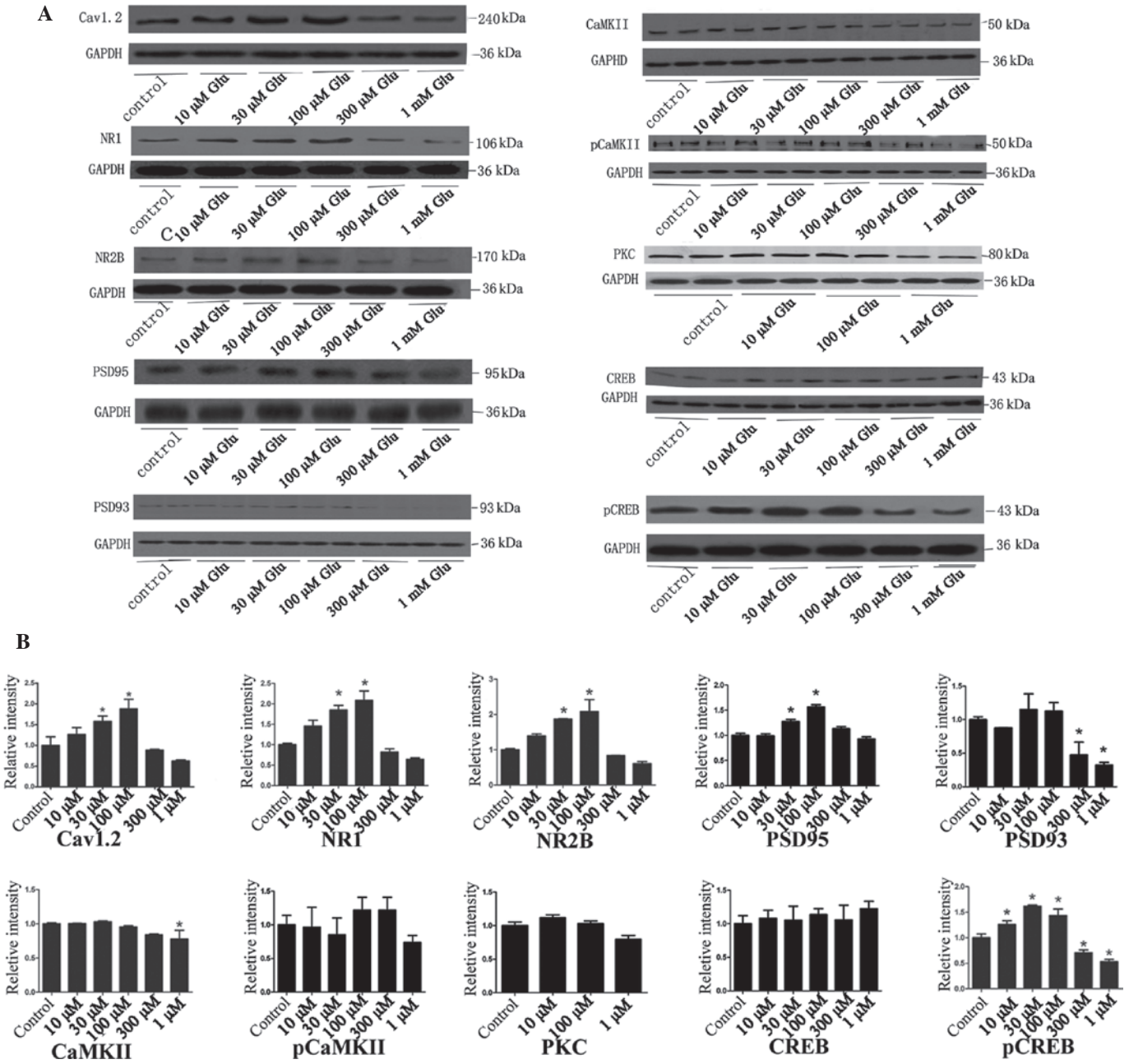

Figure 3. Western blot analysis of calcium memory-associated proteins following stimulation of neurons with various concentrations of Glu. (A) Representative western blots and (B) column diagrams showing the quantified protein levels obtained by grey value analysis of A. Grey values are expressed as the mean ± standard deviation. *P<0.05 vs. control. Glu, glutamate; p, phosphorylated; poly, rabbit polyclonal antibody; CaMK, calcium/calmodulin-dependent protein kinase; CREB, cyclic adenosine monophosphate response element binding protein; NR2B, *N*-methyl D-aspartate receptor subtype 2B; NR1, NR1 subunit of the *N*-methyl-D-aspartate receptor; PKC, protein kinase C; Cav1.2, calcium channel, voltage-dependent, L type, alpha 1C subunit; PSD95, postsynaptic density protein 95.

(300 μM or higher) of glutamate decreased the expression of several calcium memory-associated proteins (Fig. 3).

Raloxifene increases the expression of calcium memory-associated proteins in cells pre-treated with 300 μM glutamate. To examine the reversal effect of raloxifene on the expression of calcium memory-associated proteins after pre-treatment with 300 μM glutamate, the neuronal cells were subjected to western blot analysis after the calcium oscillation tests. The results showed that 100 μM raloxifene significantly increased the expression of Cav1.2. The expression of NR2B was significantly elevated in cells treated with raloxifene at 100 nM-10 μM, but not at 3 μM. The PKC levels significantly increased when the concentration of raloxifene was 1 μM, and raloxifene concentrations ranging from 1-10 μM significantly promoted the expression of CREB. 100 nM-1 μM raloxifene significantly increased the expression of pCREB in a concentration-dependent manner, while 3 and 10 μM pCREB significantly decreased its expression. The expression levels of NR1, PSD95, PSD93, CaMKII, pCaMKII were not significantly altered at the tested concentrations (Fig. 4).

Discussion

Calcium signaling in the cell is highly regulated and is generated by an ion influx through voltage and/or ligand-gated

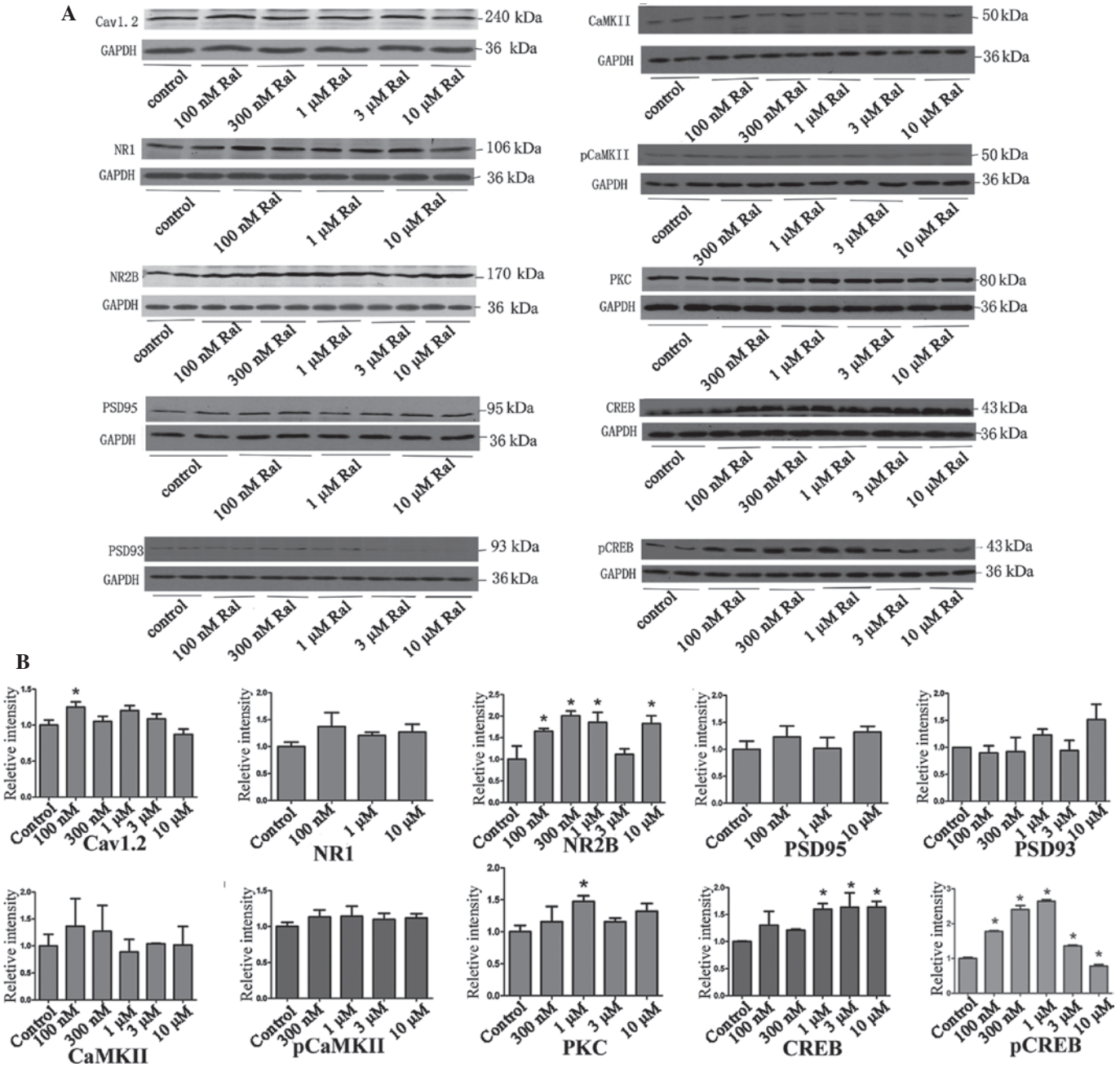

Figure 4. Neutralizing effect of Ral treatment after pre-treatment with 300 μM Glu. (A) Representative western blots and (B) column diagrams showing the quantified protein levels obtained by grey value analysis of A. Grey values are expressed as the mean ± standard deviation. *P<0.05 vs. control. Ral, raloxifene, Glu, glutamate; p, phosphorylated; poly, rabbit polyclonal antibody; CaMK, calcium/calmodulin-dependent protein kinase; CREB, cyclic adenosine monophosphate response element binding protein; NR2B, *N*-methyl D-aspartate receptor subtype 2B; NR1, NR1 subunit of the *N*-methyl-D-aspartate receptor; PKC, protein kinase C; Cav1.2, calcium channel, voltage-dependent, L type, alpha 1C subunit; PSD95, postsynaptic density protein 95.

calcium-permeable ion channels, or ion release from internal stores. Furthermore, calcium signaling is sequestered or cleared by calcium pumps and exchangers (1). Numerous pathogenic changes influence calcium oscillations. For example, the APPswe mutation was shown to increase the frequency of spontaneous calcium oscillations in rat hippocampal neurons (20). Furthermore, the endogenous activation of metabotropic glutamate receptors during neocortical development causes neuronal calcium oscillations (21). The phosphorylation of T668, the expression of the human amyloid precursor protein, treatment with $A\beta_{25-35}$ or isoflurane-induced ischemic tolerance all inhibit calcium oscillations (22-24).

Glutamate is an important excitatory amino acid in the brain. In the present study, glutamate was used to induce calcium oscillations. It is a ligand of the NMDA receptor and induces calcium influx. If the glutamate concentration is too high, calcium flows into the cell and cannot be eliminated in a timely manner, which results in excess calcium build-up in the cell. This accumulation inversely inhibits calcium oscillations. The present study found that low concentrations of glutamate (10-100 μM) increased the duration of a single peak (18-44 sec) but decreased the frequency of the waves (2-0.8/min). Furthermore, high concentrations of glutamate (300 μM or higher) inhibited calcium oscillations. These

results suggested that glutamate is necessary to maintain neural excitation at specific concentrations, but it terminates signal transduction if the concentration is too high.

Calcium oscillations increase the efficiency and specificity of gene expression. CaMKII is sensitive to the frequency of oscillations modulated by several factors, such as the amplitude and duration of individual peaks (25). Calcium oscillations at a specific periodicity of 12 min were found to affect gene expression in target epithelial cells. For example, calcium oscillations specifically induced the pro-inflammatory cytokine interleukin (IL)-6 and chemokine IL-8 (26). Trophic factor-induced intracellular calcium oscillations are required for the expression of post-synaptic acetylcholine receptors during synapse formation between Lymnaea neurons (13). The results of the present study showed that an increase in the duration of calcium oscillations promotes the expression of several calcium memory-associated proteins, and inhibition of calcium oscillations decreased the expression of several calcium memory-associated proteins. The MTT analysis showed that glutamate at 100 μM (moderate concentration) and 300 μM (high concentration) decreased neuronal survival. These results suggested that memory formation and consolidation were enhanced when the concentration of intracellular calcium was suitable but weakened due to the decline of calcium memory-associated proteins induced by calcium overload.

Several medicines have been used to regulate calcium dyshomeostasis, including nimodipine and memantine, inhibit calcium flux (27,28). Of note, the inhibition of calcium oscillations, neuronal survival and calcium memory-associated protein synthesis were reversed in the present study when the neurons were treated with raloxifene, which is a selective estrogen receptor modulator that induces calcium oscillations when the calcium concentration is very high or low. There are three mechanisms to explain the restorative effects of raloxifene on calcium oscillations: Its activity agains oxidative stress (29), up-regulation of telomerase activity (30) and activation of gene transcription and expression (31). Raloxifene may decrease the risk of mild cognitive impairment by 33% and slightly lowers the risk of Alzheimer's disease (32). Therefore, this drug has the potential to be used clinically to regulate calcium dyshomeostasis.

In conclusion, glutamate regulates calcium oscillations or the expression of calcium memory-associated proteins and neuronal survival in a dose-dependent manner, which may be an important mechanism of memory impairment. Raloxifene, which is a selective estrogen receptor modulator, effectively reversed these effects, and it may therefore be used as an alternative drug to regulate calcium dyshomeostasis for treating memory impairment diseases such as Alzheimer's disease.

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