

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

XIANGYU ZHOU<sup>1\*</sup>, ZHENDONG YANG<sup>2\*</sup>, LI HAN<sup>3</sup>, XIAOYONG LI<sup>4</sup>, MEINA FENG<sup>5</sup>, TENG ZHANG<sup>3</sup>,  
HONGBIN LUO<sup>6</sup>, LIPING ZHU<sup>7</sup>, JIWEI ZHANG<sup>7</sup>, QI ZHANG<sup>3,8</sup> and QINGHUA HU<sup>7</sup>

<sup>1</sup>Department of Neurology, The Affiliated First Hospital of Shizuishan, Ningxia Medical University,

Shizuishan, Ningxia 753200; <sup>2</sup>Department of Neurology, Wuhan No. 1 Hospital, Tongji Medical College,

Huazhong University of Science and Technology, Wuhan, Hubei 430022; <sup>3</sup>Department of Neurology, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430070; <sup>4</sup>Department of Pathology,

Medical College, Hubei Polytechnic University, Huangshi, Hubei 435003; <sup>5</sup>Department of Neurology, Wuhan Brain Hospital, Wuhan, Hubei 430010; <sup>6</sup>Department of Biological Chemistry, Medical College, Hubei University for Nationalities, Enshi,

Hubei 445000; <sup>7</sup>Department of Pathophysiology, Key Laboratory for Pulmonary Diseases of The Health Ministry of China,

Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030;

<sup>8</sup>Department of Pathophysiology, Key Laboratory for Neurological Diseases of The Education Ministry of China,

Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430030, P.R. China

Received July 28, 2014; Accepted June 23, 2015

DOI: 10.3892/mmr.2015.4191

**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.

## 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

*Correspondence to:* Professor Qi Zhang, Department of Neurology, Liyuan Hospital, Tongji Medical College, Huazhong University of Science and Technology, 39 Lake Avenue, Wuchang, Wuhan, Hubei 430070, P.R. China

E-mail: zhangqi@mails.tjmu.edu.cn

Professor Qinghua Hu, Department of Pathophysiology, Key Laboratory for Pulmonary Diseases of The Health Ministry of China, Tongji Medical College, Huazhong University of Science and Technology, 13 Hangkong Road, Jiangnan, Wuhan, Hubei 430030, P.R. China

E-mail: qinghuaa@yahoo.com.cn

\*Contributed equally

**Key words:** calcium oscillations, memory-associated proteins, glutamate, raloxifene

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  $\mu$ g/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

$1 \times 10^5$  cells/cm<sup>2</sup> and were incubated in the dark with 5  $\mu$ M Fura-2 AM for 30 min at 37°C in Krebs-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer [containing: NaCl (137  $\mu$ M), KCl (4.9  $\mu$ M),  $CaCl_2$  (2  $\mu$ M),  $MgSO_4$  (1.2  $\mu$ M), *D*-glucose (10  $\mu$ M) and HEPES (10  $\mu$ M)]. 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 ( $1 \times 10^4$  cells/100  $\mu$ l/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  $\mu$ M glutamate (Sigma-Aldrich), 300  $\mu$ M glutamate + raloxifene (300 nM–10  $\mu$ M; Sigma-Aldrich), or culture media based on the previous experimental design for 48 h. The cells were treated with 20  $\mu$ l MTT (5 mg/ml; Sigma-Aldrich) for 4 h, the culture media were discarded, 150  $\mu$ l 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  $\pm$  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  $\mu$ M to 1 mM ( $n=30$ –40 for each calculation; 73–89% of neurons showed oscillation waves after being stimulated with 30  $\mu$ M to 100  $\mu$ M glutamate; each experiment was repeated three times) (Fig. 1A). At the 10  $\mu$ M 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  $\mu$ M 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  $\mu$ M, 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  $\mu$ M, 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  $\mu$ M glutamate for 5 min, the spontaneous calcium oscillations were inhibited ( $n=30$ –40 for each calculation;  $\sim 90\%$  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  $\mu$ M (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  $\mu$ M, the oscillation wave appeared regularly after 15 min, and the frequency was  $\sim 0.7$ /min and persisted for 52 sec (Fig. 1Bb). When 3  $\mu$ M 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  $\mu$ M, 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  $\mu$ M glutamate did not significantly affect neuronal survival; however, when the concentration was increased to 100  $\mu$ M, the neuronal survival markedly declined as indicated by the reduced OD values. In addition, 300  $\mu$ M glutamate also impaired neuronal survival to a similar degree to that of 100  $\mu$ M 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  $\mu$ M raloxifene, the neuronal survival did not significantly change; however, when the concentration of raloxifene was increased to 3  $\mu$ M, neuronal survival significantly improved. In addition, 10  $\mu$ M raloxifene had a similar effect on neuronal survival as that of 3  $\mu$ M 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  $\mu$ M and 100  $\mu$ M glutamate significantly increased the expression of Cav1.2, NR1, NR2B, PSD95 and pCREB. 10  $\mu$ M 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  $\mu$ M 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  $\mu$ M) stimulated the release of calcium and increased the expression of several calcium memory-associated proteins, while high concentrations

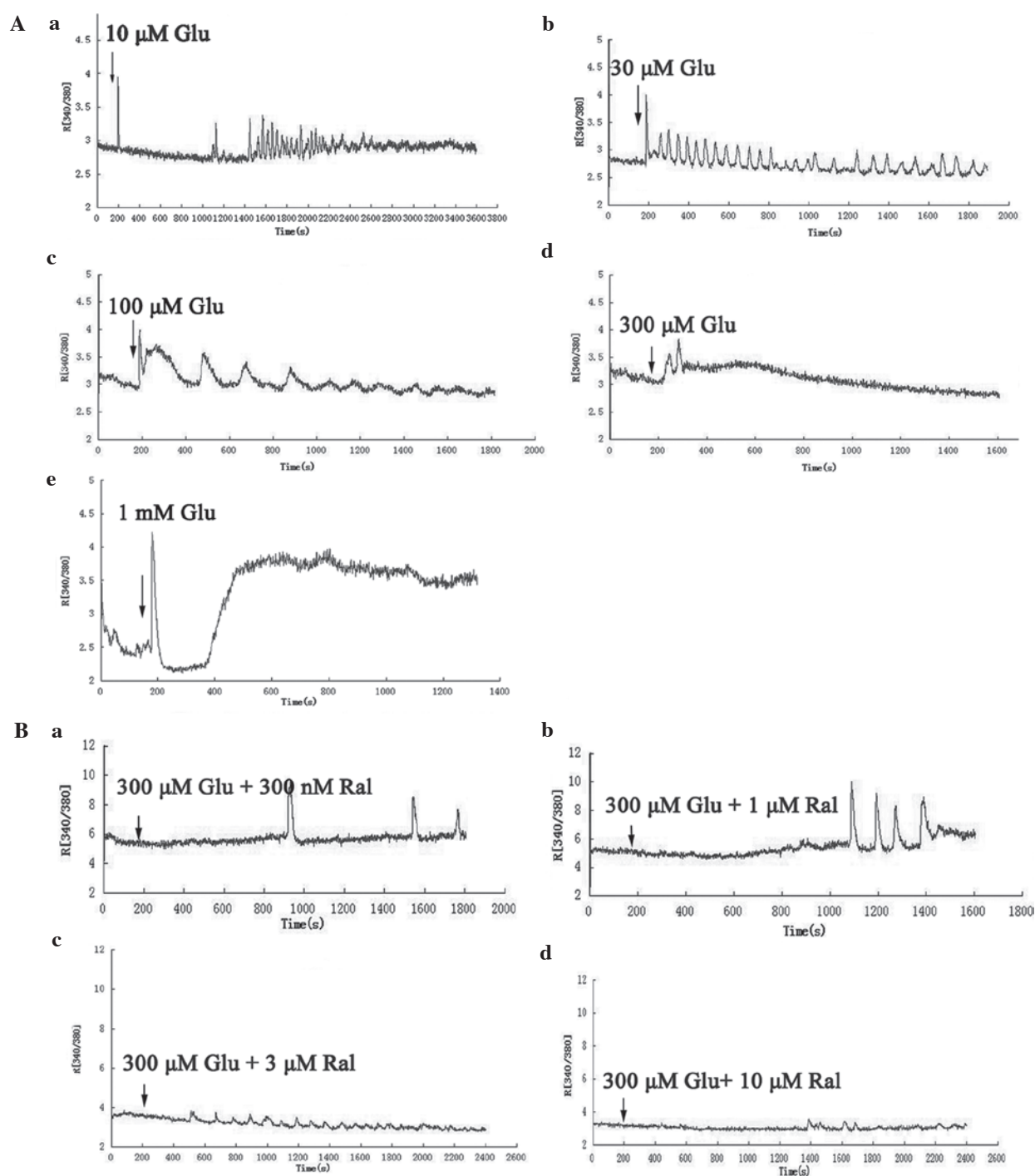


Figure 1. Influence of calcium dyshomeostasis on calcium oscillations and the neutralizing effect of Ral. (A) Influence of Glu on calcium oscillations. Neurons were stimulated with (a) 10  $\mu$ M, (b) 30  $\mu$ M, (c) 100  $\mu$ M, (d) 300  $\mu$ M or (e) 1 mM Glu. (B) Neutralizing effect of Ral on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

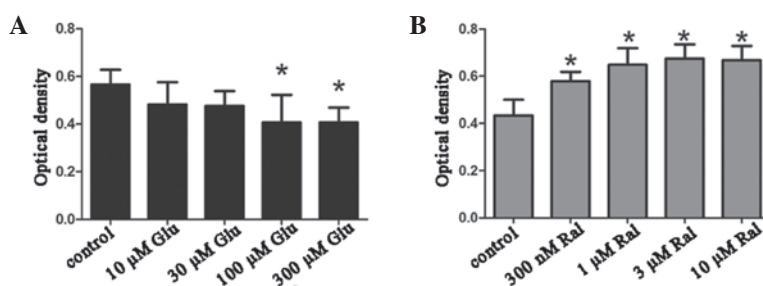


Figure 2. Effects of Glu and Ral on neuronal survival assessed using an MTT assay. (A) Effect of Glu on neuronal survival. (B) Neutralizing effect of Ral after neurons were stimulated with 300  $\mu$ M Glu. Optical density values are expressed as the mean  $\pm$  standard deviation. \* $P$ <0.05 vs.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



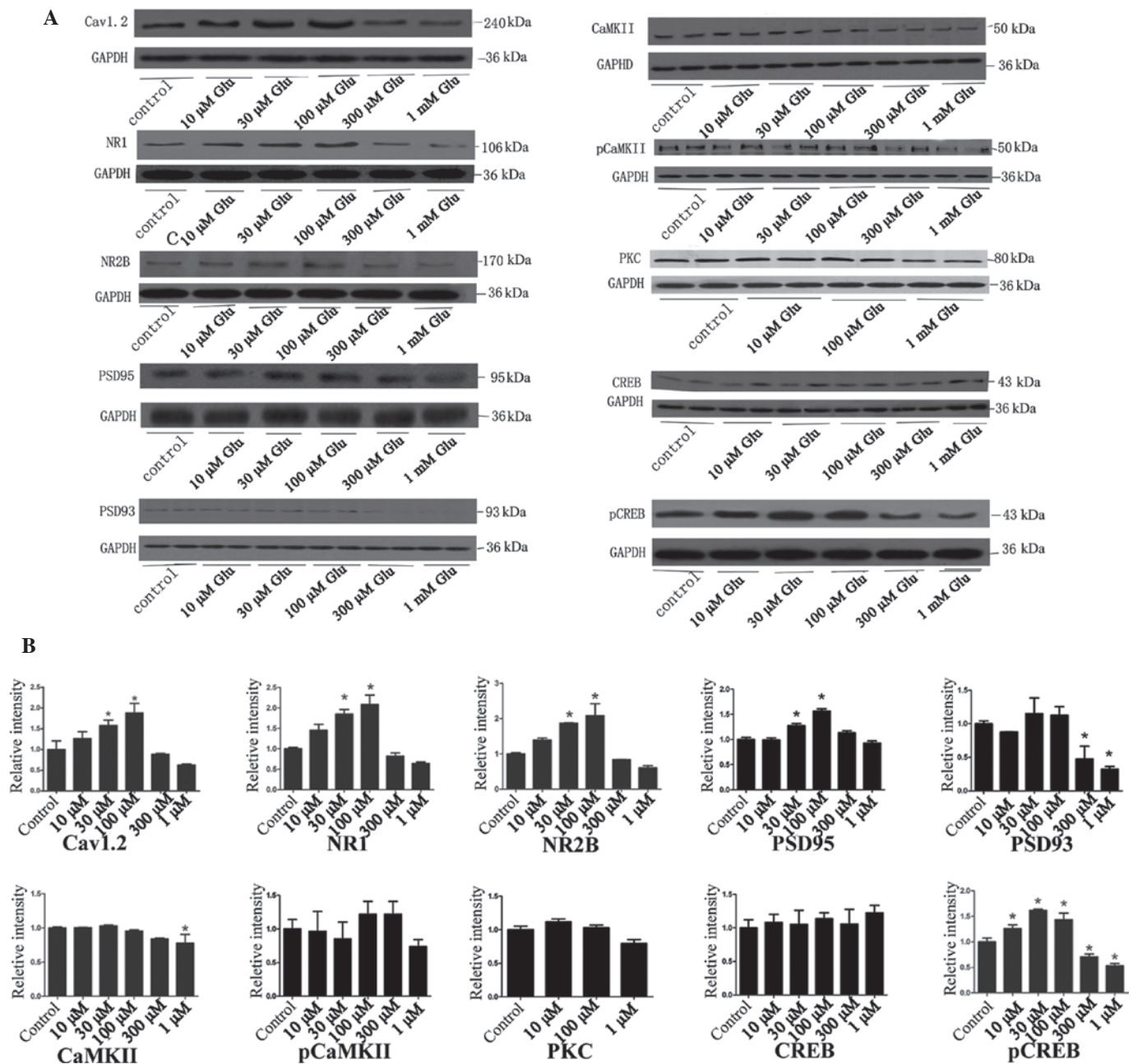


Figure 3. Western blot analysis of calcium memory-associated proteins following stimulation of neurons with various concentrations of Glu. (A) Representative western blots and (B) column diagrams showing the quantified protein levels obtained by grey value analysis of A. Grey values are expressed as the mean  $\pm$  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  $\mu$ M 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  $\mu$ M glutamate.** To examine the reversal effect of raloxifene on the expression of calcium memory-associated proteins after pre-treatment with 300  $\mu$ M glutamate, the neuronal cells were subjected to western blot analysis after the calcium oscillation tests. The results showed that 100  $\mu$ M raloxifene significantly increased the expression of Cav1.2. The expression of NR2B was significantly elevated in cells treated with raloxifene at 100 nM-10  $\mu$ M, but not at 3  $\mu$ M. The PKC levels significantly

increased when the concentration of raloxifene was 1  $\mu$ M, and raloxifene concentrations ranging from 1-10  $\mu$ M significantly promoted the expression of CREB. 100 nM-1  $\mu$ M raloxifene significantly increased the expression of pCREB in a concentration-dependent manner, while 3 and 10  $\mu$ M 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

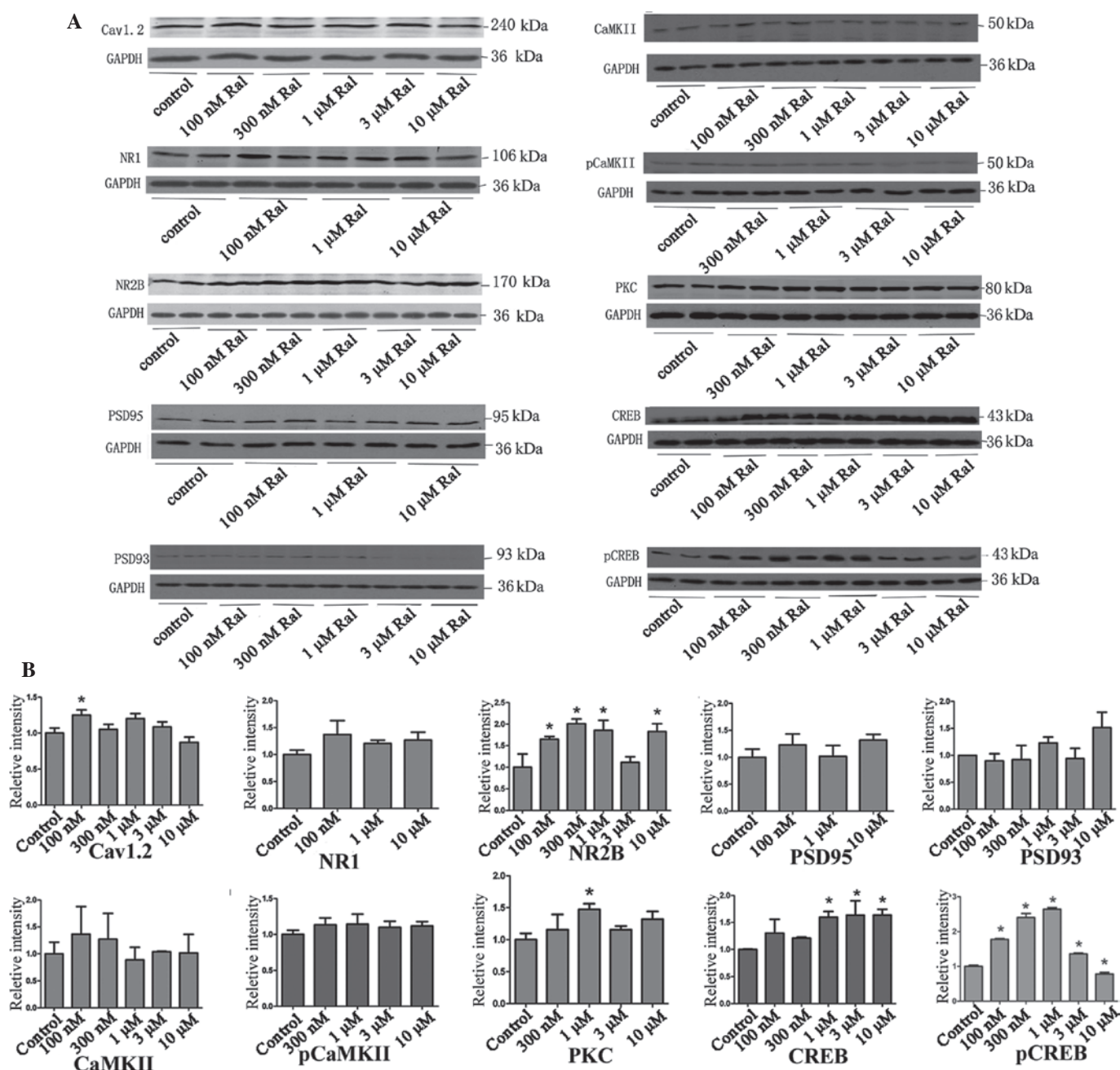


Figure 4. Neutralizing effect of Ral treatment after pre-treatment with 300  $\mu$ M Glu. (A) Representative western blots and (B) column diagrams showing the quantified protein levels obtained by grey value analysis of A. Grey values are expressed as the mean  $\pm$  standard deviation. \* $P$  < 0.05 vs. control. Ral, raloxifene; Glu, glutamate; p, phosphorylated; poly, rabbit polyclonal antibody; CaMK, calcium/calmodulin-dependent protein kinase; CREB, cyclic adenosine mono-phosphate 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 APP<sup>swe</sup> 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 $\beta$ <sub>25-35</sub> 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  $\mu$ M) 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  $\mu$ M 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  $\mu$ M (moderate concentration) and 300  $\mu$ M (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 against 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.

## Acknowledgements

This study was funded by the National Natural Science Fund of China (grant nos. 30971008 and 81260172).

## References

- Kawamoto EM, Vivar C and Camandola S: Physiology and pathology of calcium signaling in the brain. *Front Pharmacol* 3: 61, 2012.
- Oliveira AM and Bading H: Calcium signaling in cognition and aging-dependent cognitive decline. *Biofactors* 37: 168-174, 2011.
- Carew TJ: Molecular enhancement of memory formation. *Neuron* 16: 5-8, 1996.
- Lucchesi W, Mizuno K and Giese KP: Novel insights into CaMKII function and regulation during memory formation. *Brain Res Bull* 85: 2-8, 2011.
- Berridge MJ: Calcium signalling and Alzheimer's disease. *Neurochem Res* 36: 1149-1156, 2011.
- Garwood C, Faizullahoy A, Wharton SB, Ince PG, Heath PR, Shaw PJ, Baxter L, Gelsthorpe C, Forster G, Matthews FE, *et al*: MRC Cognitive Function and Ageing Neuropathology Study Group: Calcium dysregulation in relation to Alzheimer-type pathology in the ageing brain. *Neuropathol Appl Neurobiol* 39: 788-799, 2013.
- Pelizzoni I, Maccò R, Morini MF, Zacchetti D, Grohovaz F and Codazzi F: Iron handling in hippocampal neurons: Activity-dependent iron entry and mitochondria-mediated neurotoxicity. *Aging Cell* 10: 172-183, 2011.
- Huang Y, Huang YL, Lai B, Zheng P, Zhu YC and Yao T: Raloxifene acutely reduces glutamate-induced intracellular calcium increase in cultured rat cortical neurons via inhibition of high-voltage-activated calcium current. *Neuroscience* 147: 334-341, 2007.
- Wang X, Dykens JA, Perez E, Liu R, Yang S, Covey DF and Simpkins JW: Neuroprotective effects of 17 $\beta$ -estradiol and nonfeminizing estrogens against H<sub>2</sub>O<sub>2</sub> toxicity in human neuroblastoma SK-N-SH cells. *Mol Pharmacol* 70: 395-404, 2006.
- Zhu L, Luo Y, Chen T, Chen F, Wang T and Hu Q: Ca<sup>2+</sup> oscillation frequency regulates agonist-stimulated gene expression in vascular endothelial cells. *J Cell Sci* 121: 2511-2518, 2008.
- Hu Q, Deshpande S, Irani K and Ziegelstein RC: [Ca<sup>2+</sup>]<sub>i</sub> oscillation frequency regulates agonist-stimulated NF- $\kappa$ B transcriptional activity. *J Biol Chem* 274: 33995-33998, 1999.
- Dolmetsch RE, Xu K and Lewis RS: Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 392: 933-936, 1998.
- Xu F, Hennessy DA, Lee TK and Syed NI: Trophic factor-induced intracellular calcium oscillations are required for the expression of postsynaptic acetylcholine receptors during synapse formation between *Lymnaea* neurons. *J Neurosci* 29: 2167-2176, 2009.
- Kaech S and Banker G: Culturing hippocampal neurons. *Nat Protoc* 1: 2406-2415, 2006.
- Tsuruta F, Green EM, Rousset M and Dolmetsch RE: PIKfyve regulates CaV1.2 degradation and prevents excitotoxic cell death. *J Cell Biol* 187: 279-294, 2009.
- Rosenegger D and Lukowiak K: The participation of NMDA receptors, PKC, and MAPK in the formation of memory following operant conditioning in *Lymnaea*. *Mol Brain* 3: 24, 2010.
- Michel M, Green CL and Lyons LC: PKA and PKC are required for long-term but not short-term *in vivo* operant memory in *Aplysia*. *Learn Mem* 18: 19-23, 2011.
- Kornhauser JM, Cowan CW, Shaywitz AJ, Dolmetsch RE, Griffith EC, Hu LS, Haddad C, Xia Z and Greenberg ME: CREB transcriptional activity in neurons is regulated by multiple, calcium-specific phosphorylation events. *Neuron* 34: 221-233, 2002.
- Bito H, Deisseroth K and Tsien RW: CREB phosphorylation and dephosphorylation: A Ca(2+) and stimulus duration-dependent switch for hippocampal gene expression. *Cell* 87: 1203-1214, 1996.
- Kloskowska E, Malkiewicz K, Winblad B, Benedikz E and Bruton JD: APP<sup>swe</sup> mutation increases the frequency of spontaneous Ca<sup>2+</sup> oscillations in rat hippocampal neurons. *Neurosci Lett* 436: 250-254, 2008.
- Flint AC, Dammernan RS and Kriegstein AR: Endogenous activation of metabotropic glutamate receptors in neocortical development causes neuronal calcium oscillations. *Proc Natl Acad Sci USA* 96: 12144-12149, 1999.
- Santos SF, Tasiaux B, Sindic C and Octave JN: Inhibition of neuronal calcium oscillations by cell surface APP phosphorylated on T668. *Neurobiol Aging* 32: 2308-2313, 2011.
- Santos SF, Pierrot N, Morel N, Gailly P, Sindic C and Octave JN: Expression of human amyloid precursor protein in rat cortical neurons inhibits calcium oscillations. *J Neurosci* 29: 4708-4718, 2009.
- Rui Y, Li R, Liu Y, Zhu S, Yu X, Sheng Z and Xie Z: Acute effect of beta amyloid on synchronized spontaneous Ca<sup>2+</sup> oscillations in cultured hippocampal networks. *Cell Biol Int* 30: 733-740, 2006.
- De Koninck P and Schulman H: Sensitivity of CaM kinase II to the frequency of Ca<sup>2+</sup> oscillations. *Science* 279: 227-230, 1998.

26. Söderblom T, Laestadius A, Oxhamre C, Aperia A and Richter-Dahlfors A: Toxin-induced calcium oscillations: A novel strategy to affect gene regulation in target cells. *Int J Med Microbiol* 291: 511-515, 2002.
27. Doody RS, Tariot PN, Pfeiffer E, Olin JT and Graham SM: Meta-analysis of six-month memantine trials in Alzheimer's disease. *Alzheimers Dement* 3: 7-17, 2007.
28. Liljelund P, Netzeband JG and Gruol DL: L-type calcium channels mediate calcium oscillations in early postnatal purkinje neurons. *J Neurosci* 20: 7394-7403, 2000.
29. Biewenga E, Cabell L and Audesirk T: Estradiol and raloxifene protect cultured SN4741 neurons against oxidative stress. *Neurosci Lett* 373: 179-183, 2005.
30. Kawagoe J, Ohmichi M, Takahashi T, Ohshima C, Mabuchi S, Takahashi K, Igarashi H, Mori-Abe A, Saitoh M, Du B, *et al*: Raloxifene inhibits estrogen-induced up-regulation of telomerase activity in a human breast cancer cell line. *J Biol Chem* 278: 43363-43372, 2003.
31. Engdahl C, Jochems C, Gustafsson JA, van der Saag PT, Carlsten H and Lagerquist MK: *In vivo* activation of gene transcription via oestrogen response elements by a raloxifene analogue. *J Endocrinol* 203: 349-56, 2009.
32. Legault C, Maki PM, Resnick SM, Coker L, Hogan P, Bevers TB and Shumaker SA: Effects of tamoxifen and raloxifene on memory and other cognitive abilities: Cognition in the study of tamoxifen and raloxifene. *J Clin Oncol* 27: 5144-5152, 2009.