Regulation Mechanism of Peptides Derived from Sea Cucumber (*Apostichopus japonicas*) for Modulation of Learning and Memory

Yanyan Li, Jing Shang, Zhenzhou Jiang, Luyong Zhang*, and Xiurong Su¹

Jiangsu Center for Drug Screening, Jiangsu Center for Pharmacodynamics Research and Evaluation, Key Laboratory of Drug Quality Control and Pharmacovigilance of the Ministry of Education, China Pharmaceutical University, Nanjing 210009, China

¹School of Marine Science, Ningbo University, Ningbo 315211, China

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*Corresponding Author
Tel: +86-25-83271023
Fax: +86-25-83271043
E-mail: lyzhangchina@hotmail.com

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Abstract Peptides derived from the sea cucumber *Apostichopus japonicas* have immunomodulatory and antioxidant activities. Three dosage levels of peptide hydrolysates were fed to Institute of Cancer Research (ICR) mice. Standardized Y and Morris water mazes were used to determine the effects of *A. japonicas* peptides on learning and memory. All 3 mouse test groups showed reductions in the latency period (LP), the number of times that a mouse swam over the location of a platform (NCP), the ratio of the distance from a target quadrant to the total swimming distance (DTQ), and the time spent in the target quadrant (TTQ). Microarray analysis was then carried out and 308 genes were differentially expressed between groups, of which 235 were up and 73 were down regulated in the brains of test group animals, compared with control group animals. Expressions of genes identified in microarrays were then analyzed using qRT-PCR.

Keywords: *Apostichopus japonicas*, peptide, Morris water maze, Gene Microarray, Quantitative Real Time- Polymerase Chain Reaction

Introduction

Peptides from marine animals have been reported to exert antioxidant, cytotoxic, and memory enhancement effects (1). Peptide hydrolysates derived from oysters, clams, and fish have all been reported to exert anti-inflammatory, anti-cancer, and anti-oxidant effects (2-4). The 3 common methods of solvent extraction, enzymatic hydrolysis, and microbial fermentation have been successfully used for biological production enrichment of marine peptides. Enzymatic hydrolysis is the preferred method due to lack of residual organic solvents and toxic chemicals in final products (5).

The sea cucumber *Apostichopus japonicas*, an echinoderm from Class Holothuroidea, is a traditional health food and medicinal resource in China, Japan, Korea, and other southeastern Asian countries (6,7). Previous studies have shown that this animal contains the bioactive materials collagen, sulfated polysaccharides, saponin, and glycosylsphingolipids (8-10). Peptides from *A. japonicas* have potential health benefits, including alteration of immunomodulatory pathways, inhibition of tumor cell proliferation, and antioxidant activities (11). Although the sea cucumber is considered to be a prospective source of medicinal compounds, few studies have evaluated the memory-modulation ability of sea cucumber hydrolysate

peptides. Increasing evidence has shown an association between brain development, long-term memory, and the MAPK signaling pathway based on regulation of gene expression during memory formation in both vertebrates and invertebrates (12). Several recent studies reported that MKP-1 (MAPK phosphatase) could dephosphorylate and inactivate specific members of the MAPK family, with a preference for p38 and JNK in the mouse brain (13-15). This study evaluated the function of sea cucumber hydrolyzes on learning and memory using a Y maze and a Morris water maze, and defined the underlying mechanism of action using DNA microarray technology. Expression patterns of identified candidates were further assessed using Quantitative Real Time- Polymerase Chain Reaction (qRT-PCR).

Materials and Methods

Sea cucumber hydrolysate peptides (SHP) Sea cucumbers (*A. japonicas*) were obtained from the Ningbo Bowang Aquaculture Company. The cultivation area is at the latitude of 29°25'-29°47', longitude of 121°03'- 121°46'. The body wall was sheared from anus to calcareous ring with scalpel, and rinsed under tap water 3 times. Enzymatic hydrolysates were produced as previously described (16).

Peptides of 1–3 kDa were separated using ultrafiltration membranes (PLBC03205; Millipore, Billerica, MA, USA), and lyophilized (Free Zone 6 Liter Console Freeze Dry System with Stoppering Tray Dryer; Labconco, Kansas, MO, USA) for experimental use.

Behavioral testing

Animal models: A total of 160 Institute of Cancer Research (ICR) mice (80 male and 80 female with an average weight of 20.7 ± 1.6 g) were obtained from Zhejiang Academy of Medical Sciences (Hangzhou, China), and were raised in the Ningbo University Laboratory Animal Center (Ningbo, China) (affiliated with the Zhejiang Laboratory Animal Common Service Platform), authorized under license No. SYXK (ZHE 2008-0110). Mice were divided into 4 groups with an equal gender distribution under controlled conditions of temperature ($24 \pm 2^\circ\text{C}$) and humidity (60–80%), and a 12 h light/dark cycle (lights on at 7 am). All animals were allowed to acclimatize for a week before interventions.

Three test groups (TB) were treated ig with SHP at dosages of 10 mg/kg/d (Al), 20 mg/kg/d (Am), and 30 mg/kg/d (Ah). A control group (CB) was treated with distilled water. The experimental period lasted 4 weeks. Before all operations, mice were anesthetized using 6.7 mg/mL mebumalatrium (dissolved in saline) by intraperitoneal injection at a final dose of 0.01 mL/g of body weight.

This study was approved by the Ethical Committee of China Pharmaceutical University, Nanjing University, the Laboratory Animal Management Committee of Jiangsu Province (Approval No: 2110748), the Ethical Committee of Ningbo University, and the Zhejiang Medical Animal Care Committee at Hangzhou (Approval No: 2008-0110). All animal experiments were conducted in compliance with standard ethical guidelines under control of the ethics committees mentioned above. All institutional and national guidelines for the care and use of laboratory animals were followed.

Y maze for assessing working memory: A Y maze (ZH0071.1; Lihua Electronic Technology Development Company, Xuzhou, China) consisting of 3 arms of $8 \times 30 \times 15$ cm with an angle of 120° between each arm was used. The Y maze (ZH0071.1; Lihua Electronic Technology Development Company) was placed in a darkened room and animals were placed at the intersection of the 3 arms. Mice were trained to enter the randomly bright arm illuminated using a 15 W lamp suspended at the end of the arm during the first 10 s. Choice of the dark arm was counted as a discrimination error. At the occurrence of an error, the animal received brief electric shocks from the floor (35 V AC, 45 mA, duration 1 s, every 5 s) until the bright arm was entered. Training was performed during an 8 day period and mice were subjected to 20 trials of 20–40 s random intervals every 24 h for comparison testing. The training procedure was increased to a 10-day period with 10 trials every day for behavioral testing. A latency period (LP) was used for quantification of training improvement. The learning criterion was a 90% correct rate (17).

The Morris water maze for spatial learning and memory: A stainless steel tank with a diameter of 1.2 m and a height of 100 cm was filled

with water to a depth of 50 cm and the temperature was maintained at $23 \pm 1^\circ\text{C}$. A plexiglass (1) platform with a diameter of 8 cm and a height of 25 cm was submerged 1 cm below the surface of the water. The Morris maze experimental assistant system (ZH; Huaibei Zhenghua Bioequipment Co., Ltd., Suixi, China) was used to record the number of times that a mouse swam over the location of the platform (NCP), the ratio of the distance to the target quadrant to the total distance swam (DTQ), and the time spent in the target quadrant (TTQ) for each mouse. For the place navigation task animals were tested over 6 days (18). On the first day, each mouse was released into the tank without the platform from 1 of 4 randomly chosen entry points (E, W, S, and N) with the animal's head towards the tank wall. Mice were allowed to swim for 2 min. From the second day onwards, the platform was located 1 cm below the surface of the water and each mouse was released from 1 of 4 entry points. If a mouse failed to reach the hidden platform within 120 s, the mouse was placed on the platform for 10 s then remove from the pool. Mice performed 4 trials per day with an interval of 60 s between trials and the average value of each parameter was calculated and recorded. After the place navigation task, spatial probe testing was performed. Each mouse was allowed to swim for 120 s after removal of the platform. The number of times the mouse crossed the platform former location and the time spent in the target area of the platform in each trial were recorded. NCP, DTQ, and TTQ were recorded (19).

Statistical analysis: All data for parametric testing were recorded as mean values $\pm$ standard deviation (SD). Data were analyzed using a one-way analysis of variance (ANOVA) with SPSS 16.0 statistical software for Windows (SPSS Inc., Chicago, IL, USA). Significant effects were determined using Duncan's multiple comparison test and differences at $p < 0.05$ considered to be significant. All experiments were performed in a completely randomized design with 3 replicates for each treatment.

Microarray analysis: Ten ICR mice (5 males and 5 females) from the control (CB) and the high dosage test group (TB, 30 mg/kg/d) were selected for gene expression analysis. Brains were rapidly removed, dissected and milled in liquid nitrogen for RNA extraction, and total RNA was isolated using TRIzol (Reagent; Invitrogen, Carlsbad, CA, USA). RNA from 2 biological replicates (1 male and 1 female) was pooled for a total of $n=5$ array replicates. The mRNA was then purified using a NucleoSpin[®] RNA cleanup kit (RNeasy Plus Universal Kits; Qiagen, Hilden, Germany). Double stranded cDNA was synthesized with 5 μg total RNA using T7-(Oligo)-dT promoter primer (CapitalBio Corporation, Beijing, China), then antisense RNA (aRNA) was amplified using a MEGAscript T7 Kit (AM1334; Ambion, Carlsbad, CA, USA). Amplified aRNA was then reverse-transcribed using PowerScript Reverse Transcriptase (K1811; Invitrogen). The cDNA was conjugated to both Cy3 and Cy5 reactive dye (PA 55021/PA53021; GE Healthcare, Little Chalfont, UK) following the manufacturer's instructions. Hybridization was performed using a 36k Mouse Genome Array (Oligo DNA Mouse Genome Version 4.0; CapitalBio Corporation) on a glass slide sinking in the hybridization solution, containing 0.2% SDS,

25% Formamide and 3×saline and sodium citrate (SSC, 20×; Sigma-Aldrich, St. Louis, MO, USA) buffer, at 60°C for 14 h. After hybridization, slides were washed 3x with solution, containing 0.2% SDS and 2×SSC, at 60°C, and dried using centrifugation (High-performance power in a bench top centrifuge Allegra 64R; Beckman Coulter, Inc., Atlanta, GA, USA) at 1,500×*g* for 3 min. Slides were then scanned using LuxScan dual-channel laser scanner (10KA; CapitalBio Corporation) at 532 nm (for Cy3) or 635 nm (for Cy5).

Extraction data were analyzed using Molecule Annotation System (MAS, Version 4.0; CapitalBio, Beijing, China). After intensity-dependent normalization, expression levels of different transcripts were calculated as a ratio relative to expression of genes from control group mice. Expression profiles of test and control group mice were then compared. Genes with an expression difference greater than 2.0x were used. One-way parametric ANOVA testing was performed, followed by a Duncan's multiple test correction with a false discovery rate of 1%. Each gene was classified according to gene ontology (GO) analysis using MAS software (Version 4.0; CapitalBio) in which genes were organized into hierarchical categories based on biological processes, molecular function, and cellular components. Intensity values around the 4 periphery of each spot were used to determine background levels. Genes that had an intensity value of <40,000 were excluded from subsequent analysis. Expression levels of each gene were represented as ratios of Cy3 to Cy5, and values were normalized using Locfit software. The purpose of normalization is to identify and remove systematic sources of variation in intensity values to allow between-array comparisons. Results were expressed as a magnitude change of test group mice genes compared with control mice genes.

Confirmation of differentially expressed genes using qRT-PCR

Expression levels of the PKC, CREB, caveolin-1, and AKT candidate genes from mouse brains were measured using qRT-PCR. A total of 5 gene samples (1 µL) was obtained from each treatment group. RNA extraction and complementary DNA (cDNA) synthesis were described previously (20). The mouse actin primers P1 and P2 (Table 1) were used to amplify a 202 bp fragment as an internal control for verification of successful reverse transcription, and for calibration of the cDNA template. Two specific primers for each gene (Table 1) were designed for amplification of the desired product. Real-time PCR amplification was performed using a Rotor-Gene 6000 real-time

Table 1. Quantitative RT-PCR primers

Primer	Sequence (5'-3')	Tm (°C)	Product size
actin-F	TGCTGTCCTGTATGCCTCT	59.85	202
actin-R	TGATGTCACGCACGATTTC	57.80	
PKC-F	CAGAGCAAGGGCATTATTACC	58.21	213
PKC-R	CCAACATTTCATACAGCAGGAC	58.21	
CREB-F	GGCGGAGGTGTAGTTTGAC	59.72	255
CREB-R	CTGGGCTAATGTGGCAATC	57.56	
CAV1-F	GCAGAACCAGAAGGGACACA	59.85	227
CAV1-R	ATCGCAGAAGGTATGGACG	57.56	
AKT-F	TGAAGCTACTGGGCAAGGG	59.72	219
AKT-R	CAAAGCAGAGGCGGTCTGT	59.58	

PCR detection system and reaction components and thermal profiles recommended by the manufacturer were followed. The $2^{-\Delta\Delta CT}$ method was used for analysis of candidate gene expression levels and the values obtained denoted the magnitude difference relative to control gene samples (21). Data were recorded as relative mRNA expression level means±SD with *n*=5 and analyzed using a paired *t*-test at *p*<0.05 for statistical significance.

Results and Discussion

Improvement of learning and memory in normal mice Changes in memory can be measured based on alteration in animal behavior after learning using elevated plus, Y, and Morris water mazes (22,23). Results of Y and Morris water maze tests after SHP administration are shown in Table 2. The 3 different SHP dosages of 10 mg/kg/d (Al), 20 mg/kg/d (Am), and 30 mg/kg/d (Ah) were used. In the Y maze experiment, compared with control group, all test groups exhibited a significant improvement in LP (*p*<0.01) in a dose-dependent manner. The LP value for the highest dosage group (Ah) was 7.15±1.21 seconds, compared with 15.61±1.14 seconds for the control group (CB). For Morris water maze testing, peptide-treated group mice exhibited a significant increase in NCP, DTQ, and TTQ values in the target quadrant, compared with control mice (*p*<0.05). Highest values were recorded for SHP group mice with NCP, DTQ, and TTQ values of 3.36±0.81 times, 1173.67±117.88 cm, and 41.08±3.88 min, respectively (Table 1). Thus, SHP appeared to be beneficial for memory improvement. Subsequent brain gene expression analysis comparisons of test and control group mice supported these results.

Table 2. Analysis of the behavior and memory capacity of control and peptide administered group mice using Y and Morris water maze testing

Group	LP ²⁾ (s)	NCP	DTQ (cm)	TTQ (min)
CB ¹⁾	15.61±1.14	1.64±0.81	805.1±14.35	29.95±4.7
Al	11.11±1.80 ^{aa}	2.46±0.78 ^{aa3)}	1046.06±114.25 ^a	35.91±4.66 ^a
Am	9.14±0.96 ^{aa}	2.82±0.98 ^a	1113.14±108.52 ^a	37.39±3.73 ^{aa}
Ah	7.15±1.21 ^{aa}	3.36±0.81 ^{aa}	1173.67±117.88 ^a	41.08±3.88 ^{aa}

¹⁾CB, Control group; Al, 10 mgkg⁻¹d⁻¹ peptide treatment; Am, 20 mgkg⁻¹d⁻¹ peptide treatment; Ah, 30 mgkg⁻¹d⁻¹ peptide treatment

²⁾LP, latency period; NCP, number of times a mouse swam over the platform location; DTQ, ratio of the distance to the target quadrant to the total swimming distance; TTQ, time spent in the target quadrant

³⁾a³⁾*p*<0.05; aa³⁾*p*<0.01 vs. control group

Fig. 1. Volcano plots of differences in gene expression in mouse brain tissue. Each dot represents an expressed gene that was detected during analysis. The horizontal line marks the threshold for defining an up-regulated (red) or down-regulated (green) gene with a change >2x as significant ($p < 0.05$). Unchanged genes are shown in black. Vertical lines represent 2x differences in expression. Control mice were not treated with peptide supplements. Test mice were treated with peptides at dosages of 30, 20, or 10 mg/kg/d.

Underlying mechanism of improved learning and memory

Identification of differentially expressed genes using microarray screening:

Analytical results for differentially expressed genes obtained from chips containing 36,000 genes from the mouse brain between control and Ah group mice are shown in Fig. 1 and 2. In a volcano plot graph (Fig. 1), each dot represents a detected gene. Up-regulated genes are shown in red, down-regulated genes are shown in green, and unchanged genes are shown in black. Further examination indicated that, out of a total of 5,051 candidates, 308 genes were differentially expressed, of which 235 were up and 73 were down regulated. The threshold for defining an up-regulated (red) or down-regulated (green) gene with a change >2x was identified as significant change ($p < 0.05$). These results were consistent with expression maps obtained from an unsupervised hierarchical cluster analysis. Hierarchical clustering analysis of brain gene expression profiles in control and treated mice was classified by the Cluster, Locfit and MAS software. Genes that showed significant changes (In this case, only genes that were over- and under-expressed > and <1.5-fold in SHP-treated mice versus controls were identified.). Data were presented using Tree-view (Fig. 2A, 2B, and 2C). Shades of red indicate increased expression and shades of green indicate decreased expression. Black indicates no change. Genes were assigned to a functional class based on previously reported functions.

Based on microarray analysis, 45 differentially expressed genes (DEGs) (43 up-regulated and 2 down-regulated) were identified between treatment group and control group mice using GO analysis. Up-regulated genes were involved in a wide array of processes, including neurotransmission, signal transduction, memory, cell recognition, the immune response, metabolism, ion channels, and cell structure. Conversely, genes down-regulated after peptide

Fig. 2. Cluster analysis of brain gene expression profiles in control and treated mice. 2A and 2B show different genes, and 2C shows that compared with CB, the statistically threshold change in gene expression (red represents an increase and green represents a decrease). Control mice were not treated with the peptide. Test mice were treated with peptides at dosages of 30 mg/kg/d. CB refers to the “Control group brain”. A, B, C, and D are 4 parallel samples of mice’s gene as blank controllers. TB refers to the “Treatment group brain”. A, B, C and D are 4 parallel samples of mice’s gene in experimental groups. Shades of red indicate increased expression and shades of green indicate decreased expression. Black indicates no change.

treatment have been linked to long-term depression and the biotin metabolism (Table 3). In order to determine peptide effects on gene expression more clearly, details were introduced based on pathways.

Gabrg1 and Grbra1, encoding subunits of GABAA receptors, were over-expressed in the neuroactive ligand-receptor pathway. The γ -aminobutyric acid subtype A (GABA_A) receptor, a transmembrane pentameric protein that is expressed in both the peripheral and central nervous systems (CNS), could transduce GABA signaling into a cascade of events, usually initiated by a Cl^- influx. The 5 families of GABA_A receptor subunits (α , β , γ , δ , ϵ) form ligand gated Cl^- channels. When the GABA_A receptor is activated, the Cl^- current increases and GABA_A receptor subtypes hyperpolarize the postsynaptic neuronal membrane. Therefore, GABA can depolarize the postsynaptic neuron. Modulation of brain GABA_A receptors by the sea cucumber peptide may form the basis of psycho-physiological phenomena, such as memory.

Pak7 and Cav2 were both over-expressed in the focal adhesion, axon guidance, T-cell receptor-signaling, and regulation of actin

Table 3. Gene ontology of differentially expressed genes between control and peptide treated group mice

Pathway	Gene	Magnitude Change	p value	Input Symbol
Neuroactive ligand-receptor	Gabrg1	8.28	0.001168	M300000171
	Gabra1	3.14	0.001168	M200002029
	Tac2	2.47	0.001168	M200000889
	Gria3	2.39	0.001168	M200013837
	Grin2b	2.29	0.001168	M400004037
	Ntsr2	2.21	0.001168	M400010857
Focal adhesion	Pak7	2.76	0.001476	M300012441
	Cav2	1.73	0.001476	M200007162
	Itgav	1.7	0.001476	M300005470
	Itgav	1.7	0.001476	M400014569
	Prkcb1	1.64	0.001476	M400004271
	Prkcb1	1.64	0.001476	M400004272
	Pak3	1.54	0.001476	M200001308
Calcium signaling pathway	Adcy2	2.44	0.004915	M300002815
	Prkacb	1.71	0.004915	M200003462
	Pde1a	1.66	0.004915	M200008305
	Prkcb1	1.64	0.004915	M400004271
	Prkcb1	1.64	0.004915	M400004272
Gap junction	Adcy2	2.44	0.005851	M300002815
	Prkacb	1.71	0.005851	M200003462
	Prkcb1	1.64	0.005851	M400004271
	Prkcb1	1.64	0.005851	M400004272
Regulation of actin cytoskeleton	Pak7	2.76	0.008342	M300012441
	Itgav	1.7	0.008342	M300005470
	Itgav	1.7	0.008342	M400014569
	Cyfp2	1.55	0.008342	M400012825
	Pak3	1.54	0.008342	M200001308
Axon guidance	Lrrc4c	3.1	0.016027	M300021276
	Pak7	2.76	0.016027	M300012441
	Pak3	1.54	0.016027	M200001308
Long-term potentiation	Grin2b	2.29	1.29E-4	M400004037
	Ppp1r1a	2.02	1.29E-4	M200009474
	Prkacb	1.71	1.29E-4	M200003462
	Prkcb1	1.64	1.29E-4	M400004271
	Prkcb1	1.64	1.29E-4	M400004272
MAPK signaling pathway	Cacna2d3	4.06	9.5E-5	M200013888
	Ptpr	1.97	9.5E-5	M200001445
	Prkacb	1.71	9.5E-5	M200003462
	Prkcb1	1.64	9.5E-5	M400004271
	Prkcb1	1.64	9.5E-5	M400004272
	Dusp6	1.62	9.5E-5	M200000698
	Mef2c	1.6	9.5E-5	M200004751
	Atf2	1.58	9.5E-5	M300005477
T cell receptor signaling pathway	Pak7	2.76	0.006978	M300012441
	Pak3	1.54	0.006978	M200001308
Lysine degradation	D17Rik	1.64	0.017055	M200008239
Biotin metabolism	D17Rik	1.64	0.035268	M200008239
Long-term depression	Gria3	0.39	0.035342	M200013837
Type I diabetes mellitus	H2-Aa	0.49	0.02629	M300010457

Fig. 3. Distribution of gene expression in brain tissues of control and test mice using qRT-PCR.

cytoskeleton pathways. PAK kinases were originally identified as serine/threonine kinases that bind to the Rho GTPases Rac and Cdc42, which suggests an important role for PAKs in the developing nervous system. PAK7 (P21-activated kinase 7) was shown to promote formation of filopodia. Filopodia formation is associated with outgrowth of neurons and formation of neurite-like extensions. A role in learning and formation of memory is strongly supported by high expression levels of both PAK5 and PAK6 in the cortex, hippocampus and striatum, which are structures that are critically involved in cognitive functions. In humans, the mutation in Pak3 resulted in mental retardation and expression of PAK3 was shown to be reduced in memory loss and Alzheimer's disease (24). Other studies have indicated the role of PAK3 in learning and memory processes (25).

In calcium signaling, long-term potentiation, and the MAPK and Gap junction pathways, the Prkacb gene is highly expressed. Prkacb encodes the cAMP-dependent protein kinase catalytic subunit beta protein. cAMP is a signaling molecule that is important for a variety of cellular functions. cAMP can be triggered by activation of cAMP-dependent protein kinase, which transduces the signal via phosphorylation of different target proteins, such as the cAMP response element binding protein (CREB) that is required for a variety of complex memory functions, including spatial and social learning. cAMP is apparently directly involved in control of synaptic strength and the molecular mechanism of short-term memory.

In the MAPK signaling pathway, the Cacna2d3 gene that encodes methylation of calcium channel regulatory- α -2- δ -3, was highly expressed. Because knockdown of straightjacket in *Drosophila* and knockout of α 2 δ 3 in mice resulted in impaired sensitivity to thermal pain, polymorphisms at the α 2 δ 3 (CACNA2D3) locus might be associated with heat pain variance in humans (26). The signaling proteins MAPK exhibited the largest difference in expression levels between treated and control mice in this study.

A wealth of evidence has suggested that memory plasticity depends on activation of MAPK signaling cascades, which ultimately leads to

de novo gene translation and, thus, to improved memory (27,28). Several molecules, including NMDA receptors, PKC, and MAPK, play key roles in this process. Jeon *et al.* (29) reported that oroxylin-A caused activation of synaptic NMDA receptors, which led to neuroprotective and memory enhancing effects. Calcium channel subunit α -2/delta-3 (*Cacna2d3*), protein tyrosine phosphatase receptor R (PTPRR), and N-methyl-D-aspartate receptor 2B (*Grin2b*) have also been shown to act on a common signal transduction pathway, affecting MAPK expression which, in turn, affects neuronal plasticity (30,31).

Characterization of mRNA expression: For confirmation of microarray results, expressions of PKC, CREB, caveolin-1, and AKT mRNA were analyzed using qRT-PCR (Fig. 3). PKC, CREB, caveolin-1, and AKT were all significantly ($p < 0.05$) up-regulated after peptide treatment, compared with controls, and were expressed at levels of 1.515x, 1.271x, 1.541x, and 1.453x higher than the control group level, respectively.

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