Mannotriose regulates learning and memory signal transduction in the hippocampus

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Research Highlights
(1) The main component of the Chinese herb Rehmannia, mannotriose, was investigated here in a broader attempt to analyze the expression of serum/glucocorticoid-regulated protein kinase in models of hippocampal neuron damage. The influence of mannotriose on these models was determined, providing new signal transduction molecular mechanisms of mannotriose in learning and memory.
(2) In vitro cytological experiments showed that mannotriose had a neuroprotective effect in models of hippocampal neuron damage, and positively regulated intracellular protein gene expression associated with learning and memory signal transduction.
(3) Similar to donepezil and RU38486, mannotriose could protect hippocampal neurons from high-concentration corticosterone injury.

Abstract
Rehmannia is a commonly used Chinese herb, which improves learning and memory. However, the crucial components of the signal transduction pathway associated with this effect remain elusive. Primary hippocampal neurons were cultured in vitro, insulted with high-concentration (1 × 10⁻⁴ mol/L) corticosterone, and treated with 1 × 10⁻⁴ mol/L mannotriose. Thiazolyl blue tetrazolium bromide assay and western blot analysis showed that hippocampal neuron survival rates and protein levels of glucocorticoid receptor, serum and glucocorticoid-regulated protein kinase, and brain-derived neurotrophic factor were all dramatically decreased after high-concentration corticosterone-induced injury. This effect was reversed by mannotriose, to a similar level as RU38486 and donepezil. Our findings indicate that mannotriose could protect hippocampal neurons from high-concentration corticosterone-induced injury. The mechanism by which this occurred was associated with levels of glucocorticoid receptor protein, serum and glucocorticoid-regulated protein kinase, and brain-derived neurotrophic factor.

Key Words
neural regeneration; traditional Chinese medicine; Rehmannia; mannotriose; corticosterone; hippocampus; neurons; learning and memory; grants-supported paper; neuroregeneration

INTRODUCTION
The hypothalamus-pituitary-adrenal axis in elderly rats is hyperactive, leading to high corticosterone hyperlipidemia and long-term potentiation in the hippocampus[1]. The hippocampus is part of the limbic system and is vulnerable to high concentrations of corticosterone given the high glucocorticoid receptor expression at this site. The hippocampus is highly involved in learning and memory[2]. Therefore, hippocampal toxic damage by high concentrations of glucocorticoid might be a fundamental reason for learning and memory degradation.

Serum and glucocorticoid-regulated protein
kinase has an apparent, positive impact on the cognition of animals\cite{3}. Brain-derived neurotrophic factor is regarded as a key protein in the formation of memories, and allows signal transmission from cytoplasm to nuclei, leading to an altered pattern of gene expression, influencing learning and memory.

Rehmannia is a commonly used Chinese herb, which supplies blood and tonifies the kidney. It also has anti-aging functions and improves memory\cite{4}. For instance, Rehmannia improved learning and memory abilities in dementia model mice or rats induced by AlCl_3\cite{5}. Mannotriose is the active component of Rehmannia\cite{6,7}. In this study, cultured hippocampal neurons were treated with high concentrations of corticosterone to mimic corticosterone injury \textit{in vivo}, in a broader attempt to observe whether mannotriose can affect the protein expression of learning and memory-related signaling molecules, such as the glucocorticoid receptor, serum and glucocorticoid-regulated protein kinase, and brain-derived neurotrophic factor. Furthermore donepezil (Aricept) and the glucocorticoid receptor antagonist RU38486\cite{8,9} were selected as positive controls for this study.

**RESULTS**

**Identification of neurons**

After hippocampal neurons were cultured for 24 hours, cells grew vigorously with many protrusions. At 2 days, cell bodies enlarged and extended, with dipolar or multipolar protrusions and halation, and some cells connected into networks. Subsequently cell protrusions increased and became thicker, and the majority of cells connected into the network through dendrites and axons. At 8 days, hippocampal neurons grew well and a neural network was visible. Immunocytochemical staining showed that the cultured hippocampal cells were positive for neuronal specific enolase (Figure 1).

Nissl staining showed that hippocampal neurons cultured for 8 days displayed a definite structure, with cell bodies, dendrites and axons being clearly visible (Figure 2).

**Mannotriose increased the survival rate of injured hippocampal neurons**

SYTO13-propidium iodide double fluorescence staining showed that the survival rate of hippocampal neurons in the model group significantly decreased compared with the control group. The cell survival rate in the mannotriose, RU38486 and donepezil groups was significantly increased compared with the model group. RU38486 can reverse the glucocorticoid effect, indicating that high-concentration corticosterone injury in hippocampal neurons is mediated by the glucocorticoid receptor (Figure 3).

Thiazolyl blue tetrazolium bromide (MTT) assay results showed that cell viability in the model group decreased compared with control ($P < 0.05$). Cell viability in the mannotriose, RU38486 and donepezil groups was higher than that in the model group ($P < 0.05$). These findings suggest that $1 \times 10^{-4}$ mol/L corticosterone had injured the hippocampal neurons. RU38486 increased hippocampal cell viability. This evidence indicates that the injury induced by corticosterone was mediated by the glucocorticoid receptor. Mannotriose and donepezil significantly increased cell viability (Figure 4).

**Mannotriose increased learning and memory-related signal transduction protein expression in injured hippocampal neurons**

Western blot analysis showed that expression levels of glucocorticoid receptor, brain-derived neurotrophic factor, and serum and glucocorticoid-regulated protein kinase were significantly lower in the model group than in the control group ($P < 0.05$). In the mannotriose, RU38486 and donepezil groups, the expression of the above markers were significantly increased compared with the model group ($P < 0.05$; Figure 5, Table 1).

**DISCUSSION**

The glucocorticoid receptor is a nuclear receptor, and is abundant in hippocampal neurons\cite{10}. Growing evidence\cite{11,12} has shown that glucocorticoids negatively regul-
ate glucocorticoid receptor mRNA levels. Our findings also showed that high concentration of corticosterone led to a decrease in glucocorticoid receptor protein expression. The decrease in glucocorticoid receptor in hippocampus could destroy the structure and functions of hippocampal neurons, thereby causing abnormal hypothalamus-pituitary-adrenal axis hyperactivity, resulting in the dysfunction of learning and memory\cite{13, 14}. This response may be self-defense as neurons attempt to reduce target cell response to hormone and prevent cell damage caused by continuous hormonal stimulation.

Brain-derived neurotrophic factor is considered to be one of the key proteins in the memory formation process\cite{15}. It can transduce signals from the cytoplasm to the nucleus, leading to changes in related molecular gene expression\cite{16, 17, 18}. Learning and memory in rats have been positively correlated with brain-derived neurotrophic factor expression\cite{19, 20, 21}. Glucocorticoid and glucocorticoid receptor can change brain-derived neurotrophic factor gene expression. In this study, the protein expression of brain-derived neurotrophic factor in the corticosterone group decreased, which was consistent with previous reports.

Serum and glucocorticoid-regulated kinase was also found in rat hippocampal neurons. There is a glucocorticoid response element in the promoter of serum and glucocorticoid-regulated protein kinase. Glucocorticoids can act on this element to regulate serum and glucocorticoid-regulated protein kinase gene expression\cite{22}. In 2002, the observation that serum and glucocorticoid-regulated protein kinase could promote cognitive behavior in animals was first published\cite{23}.
This finding shows that high concentrations of corticosterone in the glucocorticoid receptor antagonist group.

The expressions of GCR, BDNF and SGK were significantly increased in the mannnotriose, RU38486 and donepezil groups compared with the control group.

Table 1 Effect of mannnotriose on glucocorticoid receptor (GCR), brain-derived neurotrophic factor (BDNF), and serum and glucocorticoid-regulated protein kinase (SGK) expression (absorbance ratio of target protein to β-actin) in hippocampal neurons (western blot analysis)

| Group     | SGK       | BDNF      | GCR       |
|-----------|-----------|-----------|-----------|
| Control   | 0.200±0.028 | 0.107±0.021 | 0.224±0.035 |
| Model     | 0.117±0.027a | 0.041±0.014a | 0.049±0.011a |
| RU38486   | 0.155±0.022b | 0.055±0.009a | 0.193±0.027b |
| Mannnotriose | 0.194±0.033c | 0.047±0.013d | 0.289±0.022ab |
| Donepezil | 0.122±0.032b | 0.058±0.018b | 0.204±0.033b |

Data are expressed as mean ±SD. There were ten wells used in each group. Independent t-test was applied to compare two groups of data, and one-way analysis of variance for comparison of several groups. *P < 0.05, vs. control group; **P < 0.05, vs. model group.

Our study showed that serum and glucocorticoid-regulated protein kinase and brain-derived neurotrophic factor protein expression, and cell viability of hippocampal neurons stimulated by high concentrations of corticosterone decreased significantly compared with the control group. This evidence suggests that high concentrations of corticosterone injures hippocampal cells and may negatively impact on learning and memory abilities through reducing serum and glucocorticoid-regulated protein kinase and brain-derived neurotrophic factor protein expression. Western blot results revealed that the decrease in serum and glucocorticoid-regulated protein kinase and brain-derived neurotrophic factor protein expression is reversed in the glucocorticoid receptor antagonist group. This finding shows that high concentrations of corticosterone exert an influence on serum and glucocorticoid-regulated protein kinase and brain-derived neurotrophic factor protein expression via the glucocorticoid receptor. Maiyar et al. [24] also suggested that dexamethasone acted on the glucocorticoid responsive element in the promoter region of serum and glucocorticoid-regulated protein kinase after binding to glucocorticoid receptor in the cytoplasm to regulate the expression of serum and glucocorticoid-regulated protein kinase.

Rehmannia is a commonly used Chinese herbal medicine, functioning to improve memory [5]. The two main active components of Rehmannia are mannnotriose and 5-hydroxymethyl furfural [6-7]. Mannnotriose and positive control donepezil could significantly enhance cell viability and correct the reduction in glucocorticoid receptor, brain-derived neurotrophic factor and serum and glucocorticoid-regulated protein kinase expression in the model group to varying degrees. The regulatory mechanism associated with the signal transduction pathway mediated by the glucocorticoid receptor and mannnotriose interaction prevents the damage caused by corticosterone in hippocampal neurons. This action resulted in increased learning and memory-related signaling molecules, such as brain-derived neurotrophic factor and serum and glucocorticoid-regulated protein kinase protein expression. These observations may reveal a role for mannnotriose in the prevention and treatment of learning and memory impairment. Further studies are still needed to elucidate the underlying mechanism by which the active ingredients in Rehmannia exert these effects and to explore the responses of associated signaling pathways.

MATERIALS AND METHODS

Design
A randomized, controlled, neurobiochemical study.

Time and setting
Experiments were performed at the Scientific Experimental Center of Shanghai University of Traditional Chinese Medicine, China from July 2009 to November 2010.

Materials

Animals
Newborn (24 hours after birth) Sprague-Dawley rats, of specific pathogen free grade, irrespective of gender, were purchased from the Animal Laboratories in Shanghai University of Traditional Chinese Medicine, China (license No. SCXK 2008-0016). Rats were handled in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China [25].

Drugs
Corticosterone (U.S. Biological Co., Massachusetts, MA,
USA; purity > 99%) was used in this study.

Mannotriose (purity > 98%) was provided by Megazyme International Ireland Ltd. (Bray, Ireland); its structural formula is shown as follows:

![Mannotriose Structural Formula](image)

RU38486 (purity > 98%) was provided by Sigma-Aldrich Co., St. Louis, MO, USA; its structural formula is shown as follows:

![RU38486 Structural Formula](image)

Donepezil (purity > 99%) was provided by Shandong Hongfuda Company (Jinan, Shandong Province, China).

Methods

Culture of hippocampal neurons

Newborn rats were decapitated and hippocampi were removed from cerebral hemispheres. The meninges were then removed and the hippocampi were placed into a tube with Dulbecco’s modified Eagle medium (DMEM; Hyclone Co., Logan, UT, USA). The tissue was then cut into small pieces, treated with 0.25% trypsin (Invitrogen, Carlsbad, CA, USA) for 30 minutes at 37°C, and gently shaken every 10 minutes. The reaction was terminated by the addition of 20% fetal bovine serum (Hyclone) added into DMEM. The tube was left standing for 3 minutes, whereupon the supernatant was removed. Cells were resuspended in DMEM containing 20% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. This step was then repeated. Medium was then entirely replaced with the same volume of Neurobasal medium containing 2% B27 (Invitrogen) after 24 hours of incubation in a humidified 37°C incubator with a 5% CO₂ atmosphere. Half the volume of medium was replaced every 2 or 3 days in the next 7 days. On the eighth day, neuron specific enolase immunocytochemical staining assay, Nissl staining and grouping was performed. The cultures were performed 21 times for the study.

Identification of hippocampal neurons using immunohistochemical staining

Neuron specific enolase immunocytochemical staining assay is a classic method to identify neurons[26]. For this assay, Nissl staining and double-fluorescence staining, 2.0 × 10^5 cells were plated onto 6-well plates. After being fixed with 70% ethyl alcohol, cultures were preincubated in PBS supplemented with 0.01% Triton X-100 for 20 minutes at room temperature, followed by incubation in rabbit anti-rat neuron specific enolase monoclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour at room temperature. Cultures were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2 000; Santa Cruz Biotechnology) for 10 minutes at room temperature according to the manufacturer instructions on the SP immunohistochemical kit. 3,3-Diaminobenzidine tetrahydrochloride was used as the substrate. The neuron specific enolase immunostained cultures were then visualized and analyzed microscopically (XDS-1B inverted phase contrast microscope, Olympus, Tokyo, Japan).

Morphology of hippocampal neurons using Nissl staining

Tigroid bodies are characteristic neuronal structures, being present in cell bodies or dendrites, and can be dyed purple by Nissl dye[27]. Cells were plated onto 6-well culture plates. After being fixed with 4% paraformaldehyde, cultures were washed, stained with 1% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C, dehydrated and then analyzed microscopically.

Cell intervention

Hippocampal neurons were prepared from newborn (24 hours after birth) rats. After 8 days of incubation, the cultured neurons were divided into five groups according to drug administration: blank group; model group (1 × 10⁻⁴ mol/L corticosterone); RU38486 group (1 × 10⁻⁴ mol/L corticosterone + 3 nmol/L RU38486; Sigma-Aldrich); mannotriose group (1 × 10⁻⁴ mol/L corticosterone + 0.1 g/L mannotriose); positive control group (1 × 10⁻⁴ mol/L corticosterone + 40 μmol/L Donepezil; Shandong Hongfuda, China). All drugs were given simultaneously.

Cell survival as detected by SYTO13-propidium iodide double-label fluorescent immunohistochemistry

At 24 hours after intervention, cells were plated onto a 6-well plate, with four wells in each group. SYTO-13 in HEPES buffer (2 μL of 2.5 μmol/L; Invitrogen) was added to each well. The plate was then covered with aluminum foil. Fifteen minutes later, propidium iodide dye (2 μL;
Amresco Inc, Solon, OH, USA) was added to each well, and covered with aluminum foil. After the supernatant was carefully removed, cells were gently washed twice with HEPES buffer. Cells were then examined using microscopy and imaged (XDS-1B Fluorescence microscope, Olympus).

**Cell viability as detected with MTT assay**
At 24 hours after intervention, 2 × 10^5 cells were plated onto 96-well plates, with ten wells in each group. Cell viability was measured by MTT assay. An aliquot (20 μL) of MTT solution (5 mg/mL; Sigma-Aldrich) in PBS was directly added to each well with hippocampal neurons. Cultures were then incubated for 4 hours allowing deoxidize MTT to form formazan. The supernatant was discarded. Dimethyl sulfoxide (150 μL) was added to dissolve the formazan and the culture plate was shaken for 10 minutes. Absorbance values were measured with an automated spectrophotometric plate reader (Bio-Tech Co, Washington, DC, USA) at 570 nm. Cell viability was expressed as relative percentages in comparison with untreated controls.

**Protein expression as detected with western blot analysis**
At 24 hours after intervention, 2.0 × 10^6 cells were plated onto 6-well plates, with six wells in each group. Hippocampal neurons were lysed with cell lysis buffer in the presence of protease and phosphatase inhibitors, and the protein content was quantified using the BCA method (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). Protein sample (30 × g per well) was separated onto a 10% gradient sodium dodecyl sulfate polyacrylamide gel using electrophoresis and transferred to a nitrocellulose membrane (Millipore Corporation, Billerica, MA, USA). Membranes were then blocked with PBS containing 5% skim milk for 1 hour. After three washes, membranes were immunolabeled with mouse anti-rat glucocorticoid receptor monoclonal antibody (1:150; Cell Signaling Technology Inc., Beverly, MA, USA), rabbit anti-brain-derived neurotrophic factor polyclonal antibody (1:150; Cell Signaling Technology), and rabbit anti-serum and glucocorticoid-regulated protein kinase polyclonal antibody (1:100; Cell Signaling Technology) in PBS containing 5% skim milk overnight at 4°C. Subsequently, membranes were incubated in peroxidase conjugated goat anti-mouse/rabbit IgG (1:2,000; Santa Cruz Biotechnology) for 1.5 hours at 37°C. Membranes were stripped and reprobed with rabbit anti-β-actin antibody (1:2 500; Cell Signaling Technology) as a positive control. Immunoreactivity was detected using enhanced chemiluminescence detection reagent (Pierce, Rockford, IL, USA). Bands were quantified by absorbance ratio of target protein and β-actin (FR-200 ultraviolet and visible analysis instrument, Shanghai FURI Science and Technology Co., Ltd., Shanghai, China) and normalized to controls.

**Statistical analysis**
Data were analyzed using SPSS 11.0 software (SPSS, Chicago, IL, USA) and were expressed as mean ± SD. The independent t-test was applied to compare two groups of data, and one-way analysis of variance for the data comparison of several groups. For all statistical comparisons, a value of P < 0.05 was considered statistically significant.

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