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• BRIEF REPORTS •

Changes of mRNA expression of enkephalin and prodynorphin in hippocampus of rats with chronic immobilization stress

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Abstract

AIM: To observe the changes of enkephalin mRNA and prodynorphin mRNA in hippocampus of rats induced by chronic immobilization stress.

METHODS: Thirty rats were randomly divided into three groups of 10 each: the normal control group (group A), the group induced by chronic immobilization stress for 7 d (group B) and the group induced by chronic immobilization stress for 21 d (group C). The changes of the enkephalin mRNA and prodynorphin mRNA in the rat hippocampus were detected by reverse transcription-polymerase chain reaction (RT-PCR).

RESULTS: Expression levels of enkephalin mRNA and prodynorphin mRNA in rat hippocampus were significantly increased under chronic immobilization stress, and the expression of prodynorphin mRNA in the rat hippocampus in group C was remarkably higher than that in group B (0.624±0.026; $n = 5$; $P<0.01$).

CONCLUSION: The increased enkephalin mRNA and prodynorphin mRNA gene expressions in rat hippocampus were involved in chronic stress.

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INTRODUCTION

It is known that exposure to a variety of stressors can lead to the increase of plasma β-endorphin up to 10-15 times higher than normal. β-endorphin, enkephalin and dynorphin are termed as opioid peptides that are likely to exert influence on emotional and psychological state. Endogenous opioid peptides are extensively involved in the modulation of stress[1] and regulation between central nervous system and immune system. We found that immune function was changed markedly (IL-1_β in serum was increased, while IL-2 and IL-6 in serum were decreased) in rats under chronic immobilization stress[2]. The average optical density of glucocorticoid receptor (GR) in hippocampus CA_1 and parietal lobe cortex was markedly increased after 7 d immobilization stress (180 min daily) exposure in comparison with normal control, but it was declined to the normal level after 21 d of stress exposure; the level of plasma adrenocorticotropin (ACTH) and serum cortisol had similar changes to that of GR[3]. Are the opioid peptides in hippocampus implicated for the regulation of immune and internal secretion function under restrain stress? Here, we used animal models induced by chronic immobilization stress to observe the characteristic changes of expression of enkephalin mRNA and prodynorphin mRNA with reverse transcription-polymerase chain reaction (RT-PCR) in rat hippocampus.

MATERIALS AND METHODS

Subjects

Thirty male, Sprague-Dawley rats, weighing 180-220 g, were supplied by the Research Center of Experimental Animals "Weitong Lihua" in Beijing, with qualified certification No: SCXK-BJ2002-0003. They were randomly divided into three groups of 10 each: the normal control group (group A), the model group of 7 d (group B) and the model group of 21 d (group C). The rats were housed in groups of 5 rats in each cage and provided free access to food and water (20-24 °C, relative humidity of 30-40%).

All experiments conformed to the guidelines of NIH on the ethical use of animals. All efforts were made to minimize the animal suffering and maintain the number of animals necessary to produce reliable data.

Modeling methods

A T-form bound brace (self-made) was used: A frame with length of 20 cm and width of 10 cm and thickness of 2.8 cm was prepared. Its upper platform was of 22 cm in length and 6.6 cm in its widest part. A small frame for fixing the rat's head at the front end and grooves for rat's limbs were adapted. In the upper platform three adaptable soft bands, of which two are wide and one is thin, for fixing the rat in the head and neck, chest and loins, loins and back respectively were added. Means of chronic binding were applied in order to create stress models of rats. The rats were immobilized in the above-noted binding brace for 3 h daily and for either 7 or 21 consecutive days.

Extraction and measurement of total RNA

Extraction of total RNA of rat hippocampus: Rats were decapitated at the end of modeling and hippocampus tissues were rapidly taken bilaterally out and placed on the ice-plate. The tissues of 50-100 mg were mixed with 1 mL pre-cold TRIzol (Gibcol) and were sufficiently ground in the appliance for grinding tissue. The homogenates produced were thereafter placed in ice for 5 min. Homogenates of hippocampus were added into 0.2 mL chloroform and drastically agitated for 15 s and placed in ice for 2-3 min. The resulting solution was then centrifuged at 12 000 *g* for 15 min. Supernatant was taken out and mixed with 0.5 mL isopropanol and placed for 5-10 min at room temperature. The resulting solution was then centrifuged at 12 000 *g* for 10 min at 4 °C and the supernatant produced was then removed. The

sediment was rinsed with 0.5 mL of 750 mL/L freshly made alcohol solution and centrifuged at 7 500 r/min for 5 min at 4 °C and the resulting supernatant was then removed. The sediment was dried at room temperature and then dissolved by 1 g/L diethyl pyrocarbonate (Sigma), in order to produce a concentration at which 1 μg RNA was resolved in 1 mL water.

Total RNA detection by electrophoresis

A 2-μL sample of the total RNA extracted was demonstrated by electrophoresis on 10 g/L agarose gel under constant voltage for 20 min. The strips of 28S, 18S and 5S were observed in the gel image system.

Quantification and purity identification of RNA

Certain amount of RNA sample was diluted (10- or 20- fold) and its concentration and purity were measured by ultraviolet spectrophotometer. Measurement of absorbance A_{260} was chosen to count the concentration of RNA (μg/μL) and ratios of A_{260}/A_{280} were chosen for its purity which was required as 1.8-2.0. Therefore, RNA with ratios less than 1.7 was deserted.

PCR primer design

Primers of β-actin, enkephalin and prodynorphin, of which β-actin was taken as inner-control of quasi-quantification PCR, were respectively designed by the software of Primer 5.0 according to the sequence of GenBank and synthesized by Sanboyuanzhi Company. Sequences of primers were as follows: β-actin: Primer of the upper stream: 5'-CATCTCTTGCTCGAAG TCCA-3', Primer of the down stream: 5'-ATCATGTTTGAGAC CTTCAACA-3'. Enkephalin: Primer of the upper stream: 5'-ATGGCGTTCCTGAGACTTTGA-3', Primer of the down stream: 5'-TAGAGTTTTGGCGTATTTCGGAGGC-3'. Prodynorphin: Primer of the upper stream: 5'-ATGGCGTGGTCCAGGCTGATGC-3', Primer of the down stream: 5'-AGTTTGTAGATTTA GAAGCCTTATCC-3'. Genes of enkephalin (810 bp) and prodynorphin (747 bp) were respectively amplified.

Reverse transcription reaction

A sample of RNA was kept at constant temperature of 70 °C for 10 min, centrifuged for several seconds and then placed in a water-bath. The following reaction (20 μL) happened in the centrifugal tube of PCR of 0.5 mL: 1 μL random primer 50 ng/μL; 1 μL dNTP (10 mmol/L) (Promega); 4 μL 5×RT buffer; 1 μL mixed solution of reverse transcriptase; 2 μL RNA sample; 1 μL DEPC-ddH_2O. The above-noted reaction system was placed at room temperature for 10 min and kept at constant temperature of 37 °C for 30 min, at 95 °C for 5 min, and then at 4 °C for 3 min prior to next procedures.

Amplification of target DNA by PCR

Enkephalin and prodynorphin were respectively amplified by PCR (PCT-100TM Programmable Thermal Controller, product of MJ RESEARCH, INC, America). The following materials were included in a 50 μL reaction system: 6 μL RT reaction product; 4.5 μL 10×PCR buffer; 1 μL dNTP (10 mmol/L); 1 μL (50 pmoL) sense primer (enkephalin or prodynorphin); 1 μL (50 pmoL) antisense primer (enkephalin or prodynorphin); 1 μL *Taq* DNA polymerase (Promega); 35.5 μL DEPC-ddH2O. In the meantime, primer of enkephalin or prodynorphin was replaced by β-actin to perform PCR reaction with the rest reaction system noted above. The above systems were sufficiently mixed and centrifuged, then PCR reactions performed after adding 50 μL light mineral oil.

Temperatures of 46, 52 and 58 °C were respectively chosen as annealing temperature, so that three of them would be able to be used for amplification, while non-specific DNA segments would not occur. The last condition of optimized reaction was as follows: pre-degeneration at 94 °C for 3 min, tempering at 52 °C and extension at 72 °C, for 30 cycles, thereafter extension at 72 °C for another 15 min.

Amplified product was detected with 10 g/L agarose gel electrophoresis and analyzed with gel image analysis system FIT-5000 (Sweden). Ratio of optical density of the target genes and ratio of optical density of β-actin, which can be expressed as a stable value in the tissue (and it was therefore taken as interior control strip), were employed as data for quasi-quantified analysis.

Statistical analysis

Data were expressed as mean±SD and software of SPSS was applied for statistical analysis. ANOVA was performed for comparison among groups and followed by a post-hoc test. Significance was accepted at $P<0.05$.

RESULTS

General state of rats

Rats manifested symptoms such as lassitude, fewer activities, no gloss in hair, decreased food intake and loose stools.

Electrophoresis of total RNA

Three strips of 28S, 18S and 5S were observed after the extraction of the total RNA from the rat hippocampus with TRIzol RNA and electrophoresis.

Results of PCR amplification

Corresponding strips of target DNA were obtained via reverse transcription and PCR. The length of the strip of β-actin DNA was 300 bp, that of the enkephalin was 810 bp and that of the prodynorphin was 747 bp.

The expression of enkephalin mRNA was mildly increased in the rat hippocampus of group A, and significantly increased in the rat hippocampus of group C ($P<0.01$, $P<0.05$) when compared with rats in group A and B, respectively. The expression of prodynorphin mRNA was significantly increased in the rat hippocampus of group B and C when compared with rats in group A ($P<0.01$). The expression of prodynorphin mRNA in the rat hippocampus in group C was remarkably higher than that in group B ($P<0.01$). The results are shown in Table 1.

Table 1 Expression of enkephalin mRNA and prodynorphin mRNA in rat hippocampus (n = 5, mean ±SD)

Group	Enkephalin mRNA	Prodynorphin mRNA
A	0.284±0.013	0.360±0.017
B	0.308±0.018	0.492±0.036[b]
C	0.352±0.028[bc]	0.624±0.026[bd]

Statistical analyses were performed by analysis of variance and post-hoc test. [b]$P<0.01$ *vs* group A; [c]$P<0.05$, [d]$P<0.01$ *vs* group B.

DISCUSSION

Endogenous opioid peptides, which mainly include enkephalin family (methionine enkephalin and leucine enkephalin), endorphin family (α-endorphin, β-endorphin and γ-endorphin) and dynorphin family (dynorphin A and dynorphin B), are one class of matters with opioid action, naturally generated in the brain of mammals. It has been reported that endogenous opioid peptides are extensively involved in the regulation of stress and exert important effect on mediating the central nervous system and immune system[4-7].

The increased expression of opioid peptides including enkephalin mRNA and prodynorphin mRNA in our study, may influence hippocampal function and facilitate long-term depression (LTD) of the Schaffer collateral input to CA1 pyramidal neurons[8]. Chronic stress increased the synthesis

of opioid peptides in the nervous system and increased plasma glucocorticoid[9] .

Enkephalin is extensively distributed inside the brain as a kind of endogenous opioid peptides. It plays several roles in analgesia, cardiovascular, respiratory and body temperature regulation via receptors of endogenous opioid peptides including widespread immunity modulation after it is generated and released. In addition, enkephalin can exert its effect on regulating the immune functions of body under both normal and stress conditions. Both methionine enkephalin and leucine enkephalin can influence the activity and the formation rate of rosettes of T lymphocyte, as well as the activity of natural killer cells in the body under physiological conditions[6,10]. The mechanism of enkephalin involved in regulating immunity under physiological conditions has yet to be clarified. More researches have focused on the mechanism of enkephalin in regulating immunity in different stress situations. Researches suggested that methionine enkephalin and leucine enkephalin could markedly inhibit the cytotoxic action that can be stopped by naloxone, a substance that is produced by the natural killer cells, under the stress induced by electric shock in rats. Therefore, endogenous opioid peptides played an important role in immune regulation stimulated by stress[11,12].

Hippocampus is a crucial structure is which enkephalin can regulate immune function via IL-1α. A spleen lymphocyte proliferation reaction stimulated by Canavalia protein A could be markedly reinforced by microinjection of methionine enkephalin and leucine enkephalin into rat hippocampus (similar result obtained in the experiment of mice)[13]. The inhibitive effect of enkephalin on IL-1 gene expression of the glia cell in hippocampus may shed lights on the mechanisms involved in the immunological amplification by microinjection of enkephalin, which inhibits gene expression of IL-1α via opioid receptors in glia cells of hippocampus or in certain nerve cell membranes that possess the capacity of generating IL-1α, and result in decreased activating effect of IL-1α on the hypothalamus-pituitary-adrenal axis together with decreased synthesis of IL-1α inside the brain, and simultaneous strengthening of the immune function of body by decreasing the blood plasma corticoid[14-16].

It was demonstrated in the present study that expression of enkephalin mRNA was mildly increased after 7 d and significantly increased after 21 d under the conditions of chronic immobilization. In general, increase of enkephalin will result in poorer immune function. In line with our former research[2], the substantial decrease of the transforming function of the spleen lymphocytes in rats most likely originated from the indirect inhibitive action of enkephalin. It means that the increase of enkephalin would inhibit gene expression of central IL-1α and is likely to result in lower IL-1α inside the brain.

Prodynorphin, containing the sequence of leucine enkephalin at N-terminal which was considered as its precursor at first, is another member of the endogenous opioid peptides with analogous distribution inside the brain in striatum, hippocampus and hypothalamus.It also exists in adrenal glands and genital organs. The splitting process of prodynorphin is complicated and its main products include dynorphin A_{1-17}, dynorphin B_{1-29}, dynorphin A_{1-8} and neo-endorphin. In addition, generation of prodynorphin mRNA is affected by multiple factors in many regions of brain. For instance, gamma-aminobutyric acid (GABA) can decrease its generation in neo-striatum.

With dual action, dynorphin can not only protect, but also damage the nerve cells in some cases. It was indicated that the expression of prodynorphin in rat hippocampus was significantly increased and its change was likely to be earlier than that of enkephalin under the condition of chronic immobilization stress. Taken together our previous and current experiments, increase of dynorphin is likely to result in certain nerve damage as fewer numbers of brain-derived neurotrophic factors (BDNF) and neurotrophin 3 (NT_3) might exert fewer protective effects on the nervous system under chronic immobilization stress. Whether and how dynorphin correlates with neurotrophins have yet to be established.

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