Roles of the $\mu$-opioid receptor and its related signaling pathways in the pathogenesis of premenstrual syndrome liver-qi stagnation

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Abstract. The present study aimed to investigate the roles of the $\mu$-opioid receptor (MOR) and its related signaling pathways in the pathogenesis of premenstrual syndrome (PMS) liver-qi stagnation, along with the therapeutic effects of the Shu-Yu capsule in treating the condition. A PMS liver-qi stagnation rat model was established using a chronic restraint stress method. The protein expression level of MOR within rat hippocampal tissue was detected via western blot analysis and cyclic adenosine monophosphate (cAMP) levels within the supernatant of a rat hippocampal cell culture were determined by ELISA. The western blot analysis indicated that the hippocampal expression level of MOR was significantly elevated in the PMS liver-qi stagnation model group. However, subsequent treatment with a Shu-Yu capsule was found to significantly decrease the level of MOR expression. In addition, in vitro experiments were performed, whereby primary hippocampal neurons were treated with model rat serum. It was observed that the level of MOR expression was significantly elevated, while brain-derived neurotrophic factor (BDNF) and cAMP levels in the culture supernatant were significantly decreased. These effects were reversed by treatment with serum from the Shu-Yu capsule-treated rats. Furthermore, when treated with the MOR activator DAMGO, the following were significantly decreased in the primary neurons: Phosphorylation levels of cAMP response element binding protein and extracellular signal-regulated protein kinases (ERK); BDNF expression; and cAMP content in the culture supernatant. These effects were reversed in primary neurons treated with DAMGO and Shu-Yu-containing rat serum. Collectively, the data suggest that increased MOR expression and activation of the cAMP/ERK signaling pathway in the hippocampus may be involved in the pathogenesis of PMS liver-qi stagnation. Furthermore, the efficacy of the Shu-Yu capsule in treating the condition may be via its regulation of MOR receptor signaling.

Introduction

Premenstrual syndrome (PMS) is a prevalent condition that occurs in women of a reproductive age, and is experienced in >75% of females aged 15–49 years old (1). In addition to the physical symptoms, including headaches, edema, lumbago, breast pain, and fatigue, cases of severe PMS cases are also associated with substantial mood changes, including anxiety, depression, suicidal ideation and aggressive behavior (2). However, the pathophysiological causes of PMS remain unclear. In traditional Chinese medicine, liver-qi stagnation is recognized as a major secondary condition of PMS, for which treatment with the Shu-Yu capsule is considered to be beneficial (3). PMS liver-qi stagnation is a syndrome induced by abnormal flow of liver qi, which is characterized by premenstrual depression, chest tightness and sighing. A previous study concerning 526 PMS females from the Shandong province, China, has indicated a liver-qi stagnation incidence of 27.5% (4). In traditional Chinese medicine, PMS liver-qi stagnation is mainly treated through relieving the depressed liver qi, and nourishing pi and dredging qi.

Increasing evidence indicates that the $\mu$-opioid receptor (MOR) serves a key role in the regulation of mood and emotional disorders (5–7). In particular, it has been observed that withdrawal of endogenous opioid peptides is associated with the development of PMS, and decreased levels of opioid peptides are considered as a marker of the condition (8–13). Furthermore, the effects of Vitex agnus-castus methanol extracts in the treatment of PMS seem to depend on the activation of MOR (14). Collectively, these findings suggest that MOR and its ligands may be associated with the pathophysiology of PMS.

Previous studies by our group have found that the level of serum endomorphin (EM), which is a highly agonistic and selective endogenous agonist for MOR, are significantly elevated in PMS rat models, indicating the involvement of MOR in PMS development (15–17). However, the role of MOR in PMS pathophysiology, along with the corresponding molecular mechanisms, it not well understood. In the present...
study, a PMS liver-qi stagnation rat model was established by a chronic restraint stress method. This rat model, along with a primary hippocampal neuron culture, were used to evaluate the pathophysiology of PMS and the potential involvement of MOR, whereby the expression levels of MOR and its related proteins were detected via ELISA and western blot analysis. Furthermore, the effects of the Shu-Yu capsule on PMS liver-qi stagnation and MOR signaling were investigated.

Materials and methods

Ethics statement. All animal experiments were conducted according to the ethical guidelines of the Ethics Committee of Shandong University of Traditional Chinese Medicine (Jinan, China).

Animal model establishment and grouping. A total of 50 non-pregnant, specific pathogen-free female Wistar rats, weighing 150-180 g and aged 5-6 weeks, were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; lot no. 2012052370409). These animals were maintained in cages, with a reversed 12-h light/dark cycle (lights on at 9:00 p.m.; lights off at 9:00 a.m.), with free access to water prior to the experiments. The rats were maintained at a temperature of 22-26°C and at a relative humidity of 50-70%.

The rats were randomly divided into control, PMS liver-qi stagnation model, and Shu-Yu treatment groups (n=10 per group). Shu-Yu capsules, which mainly consisted of white peony root, bupleurum, cyperus tuber and licorice root, were purchased from Yangguang Haichuan Pharmaceutical Science and Technology Development Co., Ltd. (investigational new drug approval no. 2008L11169; Qingdao, China). An in vivo model of PMS liver-qi stagnation was established in the PMS liver qi stagnation method rats and the Shu-Yu treatment group using the chronic restraint stress method, as previously described (18). In the treatment group, model rats were subjected to daily administration (9:00 a.m.), of a Shu-Yu capsule via gavage, at a dose of 0.41 g/kg body weight (~8 times the clinical dosage), over the modeling period. Rats in the control and model groups received sterile water at a dose of 10 ml/kg body weight over the same period. In order to assess behavioral alterations, rats were stimulated by glass rod pricking and behavior was judged subjectively.

Primary hippocampal neuron culture. Primary hippocampal neurons for culture were obtained from ~100 neonatal Wistar rats (Experimental Animal Center, Shandong University of Traditional Chinese Medicine; male:female, 1:1; weight, 5-7 g) within 24 h of birth. The rats were maintained in the conditions described above until they were sacrificed using decolloration and their brains were removed. Briefly, the hippocampi were separated under sterile conditions and digested with 0.25% trypsin at 37°C for 20 min. Following filtering with a 200-mesh filter, the cells were seeded into poly-lysine-coated 6-well plates at a density of 4x10^4/ml, then cultured in a 37°C, 5% CO₂ incubator. After 24 h, the cells were incubated at 37°C with serum-free Neurobasal-A medium (10888-022; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), which was replenished every 3 days. After 7 days, the cells were cultured with Neurobasal-A medium (10888-022; Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; 10099-133; Gibco; Thermo Fisher Scientific, Inc.) and B-27 supplement (17504-044; Gibco; Thermo Fisher Scientific, Inc.) for 24 h at 37°C.

Serum preparation and cell culture treatment. To prepare serum from the model rats, the rats were sacrificed via decapitation following modeling and blood serum samples were collected. The serum was inactivated in a 56°C water bath for 30 min and sterilized with a 0.45-mm membrane filter. Then, 200 µl doses of serum (10% V/V) from the control, model and treatment groups were added to each well to incubate the cultured primary hippocampal neurons for 24 h at 37°C. An additional blank group of cells received no treatment.

For the preparation of drug-containing serum, 30 normal male Wistar rats (Experimental Animal Center, Shandong University of Traditional Chinese Medicine), weighing 140-160 g, were administered with sterile water (10 ml/kg body weight/day), a Shu-Yu capsule (0.41 g/kg body weight/day) and naloxone (10 g/kg body weight/day; lot no. H20064789; Beijing Kawan Biotech, Beijing, China) for 5 days. The rats were maintained in the same conditions described above. Following the last drug administration (50 min), a 6-ml blood sample was collected via the inferior vena cava following an intraperitoneal injection of 1% sodium pentobarbital and the rats were then sacrificed by decolloration. Serum was obtained by centrifugation at 1,680 x g (4°C) for 15 min. Then, 200 µl serum (10% V/V) was added to each well for incubation of the primary neurons at 37°C for 24 h.

In addition, for the treatment of the MOR activator, DAMGO (E7384; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), primary neurons were incubated, in triplicates, with the above-mentioned drug-containing serum at 37°C for 24 h, then treated with 1 µmol/l DAMGO per well for 0, 5, 15, 30, 45 and 60 min.

Western blot analysis. The expression levels of MOR and brain-derived neurotrophic factor (BDNF) and the phosphorylation levels of extracellular signal-regulated kinases (ERK) and cyclic adenosine monophosphate response element-binding protein (CREB) were detected via western blot analysis. Total protein was obtained from the hippocampi of the neonatal rats following sacrifice and the primary neuron culture. Protein concentration was determined using an enhanced BCA protein concentration assessment kit (P0010; Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's instructions. A total of 150 µl pre-iced RIPA lysis buffer (P0013B) containing 1% PMSF (ST506; both Beyotime Institute of Biotechnology, Haimen, China) for 5 min. Then, 200 µl serum (10% V/V) from the model rats, the rats were sacrificed via decapitation following modeling and blood serum samples were collected. The serum was inactivated in a 56°C water bath for 30 min and sterilized with a 0.45-mm membrane filter. Then, 200 µl doses of serum (10% V/V) from the control, model and treatment groups were added to each well to incubate the cultured primary hippocampal neurons for 24 h at 37°C. An additional blank group of cells received no treatment.

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Shu-Yu capsule treatment reverses the altered hippocampal levels of MOR, BDNF and cAMP induced by model rat serum. The effects of model rat serum on the expression of MOR and BDNF in the primary hippocampal neurons and on cAMP levels in the culture supernatant were evaluated by western blot analysis (Fig. 3). Primary hippocampal neurons were treated with rat serum from the control, model and treatment groups, while cells in the blank group received no treatment. Results from western blotting indicated that there was no significant difference in MOR and BDNF expression between the blank cells and the cells treated with normal rat serum (P>0.05). For the primary neurons treated with model rat serum, levels of MOR were significantly elevated (P<0.05), while levels of BDNF were significantly lower (P<0.05) than in primary neurons treated with normal rat serum. These effects appeared to be reversed by treatment with the Shu-Yu capsule treatment group serum (P<0.05; Fig. 3A and B). Furthermore, results of the ELISA indicated that there was no significant difference in the cAMP level of culture supernatant between the blank cells and the cells treated with normal rat serum (P>0.05). Meanwhile, the level of cAMP in the culture supernatant from cells treated with model rat serum was significantly lower than that in cells treated with normal rat serum (P<0.05), with this level significantly elevated for cells treated with the Shu-Yu capsule treatment group serum relative to the model rat serum-treated rats (P<0.05; Fig. 3C). Collectively, these results suggest that, when cultured with the serum from model rats, MOR expression is increased and BDNF expression decreased in primary hippocampal neurons, while the cAMP level in the culture supernatant is decreased. However, these effects were reversed by incubation with the serum from the Shu-Yu capsule treatment group.

Effects of MOR activation on CREB and ERK phosphorylation, BDNF expression, and cAMP level are reversed by Shu-Yu treatment. To evaluate the role of MOR in PMS liver-qi stagnation pathophysiology, primary neurons were treated with the MOR activator, DAMGO. A western blot analysis was then performed to detect the phosphorylation of CREB and ERK, the expression of BDNF in the cells and the cAMP level in the culture supernatant. In order to determine the appropriate
treatment period, primary neurons were treated with 1 µmol/l DAMGO for 0, 5, 15, 30, 45 and 60 min, and the phosphorylation level of ERK was detected. The results indicated that 15-30 min was the most effective treatment duration for DAMGO (Fig. 4); therefore, a 20 min treatment duration was used in the following experiments. It was observed via western blotting that cells treated with the activator alone and the cells treated with activator plus normal rat serum had significantly decreased phosphorylation levels of CREB and ERK, and decreased levels of BDNF expression, relative to blank control cells (P<0.05). By contrast, in cells treated with the activator and Shu-Yu-containing serum, the phosphorylation levels of CREB and ERK, and BDNF expression, were significantly increased relative to blank control cells (P<0.05). By contrast, in cells treated with the activator and Shu-Yu-containing serum, the phosphorylation levels of CREB and ERK, and BDNF expression, were significantly increased relative to cells treated with the activator plus normal rat serum (P<0.05). Similar effects were observed in cells treated with the activator and naloxone-containing serum (P<0.05; Fig. 5A-C).

In the evaluation of cAMP level, it was observed that cAMP levels in the culture supernatant from cells treated with DAMGO or DAMGO plus normal rat serum was significantly decreased relative to the control cells (P<0.01). In turn, cAMP was significantly increased in cells treated with DAMGO plus Shu-Yu- or naloxone-containing serum (P<0.01 and P<0.05, respectively; Fig. 5D). Collectively, these results suggest that MOR activation may decrease hippocampal CREB and ERK phosphorylation, hippocampal BDNF expression and cAMP level in hippocampal culture supernatant, and that these effects were reversed by the administration of serum from Shu-Yu capsule-treated rats.

Discussion

In the present study, it was observed that MOR expression was significantly elevated within the hippocampal region of PMS liver-qi stagnation model rats, an effect that could be significantly inhibited by treatment with the Shu-Yu capsule. A previous study by the current authors in PMS liver-qi invasion rats has indicated that the level of MOR expression is decreased.
in the parietal and frontal cortex, while the receptor expression is increased in the hypothalamus and hippocampus (19). Collectively, these observations suggest that increased MOR expression within the hippocampus may be associated with the pathogenesis of PMS liver-qi stagnation.

The neurobiological factors involved in the development of PMS are considered to be similar to those involved in the development of severe depression (20,21). In rats, it has been observed that a non-selective opioid receptor antagonist was able to alter the time in suspension during forced swimming (22), which improved depressive-like behavior (17,23). In the present study, MOR and its related signaling pathways were investigated in rat primary hippocampal neurons. This was achieved via the treatment of cultured cells with DAMGO, followed by treatment with serum from Shu-Yu-treated and -untreated model rats. The results indicated that relative to the control group, MOR expression was increased, while BDNF expression was decreased, in the model serum treatments; an effect which could be reversed by administration of serum from the Shu-Yu treatment group. Furthermore, DAMGO was able to decrease hippocampal CREB and ERK phosphorylation, hippocampal BDNF expression and levels of cAMP in the hippocampal culture supernatant. These effects were reversed by administration of serum from the Shu-Yu treatment group.

As a potential mechanism, MOR may bind to and inactivate adenylate cyclase, by binding to the Gi/o and Gz subunits of the G-protein coupled receptor, thus inhibiting the production of cAMP and decreasing the expression of downstream proteins (24,25). Protein kinase A may be activated by cAMP, with active PKA then phosphorylating and activating CREB, leading to transcriptional regulation of the CREB target genes, including BDNF. This would result in increased BDNF binding to its receptor, tropomyosin receptor kinase B, and
initiation of the phospholipase Cγ, Ras-mitogen-activated protein kinase and phosphoinositide 3-kinase pathways (26). In addition, BDNF potentially exerts antidepressant effects through the Ras-Raf- mitogen-activated protein/extracellular signal-regulated kinase pathway (MEK)-ERK pathway (27). Under physiological conditions, ERK is the only downstream substrate of MEK to be identified, with activation of ERK potentially inducing the phosphorylation of various transcription factors and regulating the transcriptional expression of immediate early genes, including c-Fos, c-Myc, c-Jun and Egr1 (28).

The downstream effects of CREB also have implications for PMS. Phosphorylation and activation of CREB regulates the transcription of various cytokines, thereby modulating cell regeneration and survival (29). In addition, CREB serves an important role in the regulation of synaptic growth (30) and in the maintenance and modulation of learning, memory function and emotions (31,32). In the neurons of patients with depression, disruptions in the cAMP signaling pathway and decreased mRNA expression levels of CREB have been observed in the frontal lobe. This was alleviated by increasing the intracellular concentration of CREB with antidepressant drugs (33).

The present study found that BDNF expression was decreased in the PMS rat model. In previous studies, decreased BDNF levels have been found in the brains of depressed patients, with an increase in BNDF potentially exerting antidepressant effects (34,35). Furthermore, Liu et al (36) have observed that in rats with depression-like behavior, the level of BDNF mRNA was significantly decreased in the hippocampus, particularly in the CA1 and CA3 regions, indicating that BDNF may be a potential target for antidepressant treatment. It has been indicated that current antidepressants and mood stabilizers may act on the ERK signaling pathway, which regulates synaptic plasticity in the central nervous system (37).

In addition, the ERK signaling pathway is considered to be involved in the modulation of depression. Feng et al (38) found that in a mouse model of depression, levels of phospho-ERK1/2 and ERK2 were significantly reduced in the frontal cortex and hippocampus, indicating that the absence of the ERK signaling pathway may be associated with depression-like behavior in mice. These findings indicate that ERK signaling may be involved in the effects of Shu-Yu capsule treatment on PMS liver-qi stagnation in vivo and in vitro. However, further studies are necessary in order to determine which of the Shu-Yu capsule components have the observed therapeutic effects in PMS liver-qi stagnation.

The present study did have some limitations. In order to investigate the modulating mechanism of the Shu-Yu capsule on PMS liver-qi stagnation, the Shu-Yu capsule-containing serum was used to treat the cells. However, the individual effects of the active ingredient(s) in the capsule remain unclear. In future research, experimental groups should be treated with the active ingredient(s) of the Shu-Yu capsule in order to investigate the therapeutic effects of the active ingredient(s) on PMS liver-qi stagnation.

In conclusion, the present results indicated that Shu-Yu capsule treatment may decrease MOR expression within the hippocampi of PMS liver-qi stagnation model rats. Treatment with model rat serum was found to increase MOR and decrease BDNF expression in cultured primary hippocampal neurons, while also decreasing cAMP levels in the culture supernatant. Furthermore, the activation of MOR appeared to decrease hippocampal CREB and ERK phosphorylation, BDNF expression and culture supernatant cAMP levels. Such effects were reversed by administration of serum from Shu-Yu capsule-treated model rats. Collectively, these observations suggest that increased hippocampal MOR expression and cAMP/ERK signaling are involved in the pathogenesis of PMS liver-qi stagnation, with the Shu-Yu capsule exerting its therapeutic effects via the regulation of MOR signaling. These findings may aid in understanding the mechanisms behind PMS liver-qi stagnation therapeutics.

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References


