The Influences of Chrysin on Stress-Induced Changes of Melanin-Concentrating Hormone and Orexin Gene Expression in Rats

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Abstract

Introduction: Chrysin is a bioactive component of herbal medicines such as Passiflora incarnate, Passiflora caerulea, and Oroxylum indicum. Although evidence has demonstrated the neuroprotective, anti-inflammatory, and pain-relieving effects of chrysin, the intra-hypothalamic molecular mechanisms underlying the anxiolytic effects of chrysin are still unclear. This study aimed to explore the effects of chrysin on hypothalamic orexin and melanin-concentrating hormone (MCH) gene expression in a rat model of stress.

Methods: Twenty male Wistar rats (200 ± 10 g) were segregated into four groups (n = 5). For the induction of stress, the animals were placed in the restraint cage for 2 hours. The intact and stressed groups received saline. Thirty minutes before the induction of stress, chrysin was injected into the other two groups of the stress model at a dosage of 20 or 40 µg via the third cerebral ventricle. Hypothalamic samples were removed and frozen, and the relative gene expression of orexin and MCH was measured using the real-time polymerase chain reaction technique.

Results: The induction of stress significantly increased mRNA levels of orexin and MCH compared to the control rats. The mRNA levels of MCH and orexin significantly declined in rats receiving chrysin compared to the stress group.

Conclusion: The inhibition of the hypothalamic MCH and orexin neuronal circuits may be involved in the preventive effects of chrysin against stressful situations. Chrysin may be a potential target to manage anxiogenic behaviors due to the down-regulation of MCH and orexin gene expression upstream the hypothalamic corticotropin-releasing hormone.

Keywords: Chrysin, MCH, Orexin, Stress

Introduction

Stress is a neuroendocrine disorder that may result in mental health conditions, including anxiety and depression.1,2 In addition to the stimulation of the hypothalamus-pituitary-adrenal (HPA) axis in exposure to psychological and physical stressors,3 several neuropeptides and neurotransmitters are also implicated in the control or mediation of anxiogenic or anxiolytic responses.

Orexins, also called hypocretins, consist of two neuropeptides, including orexin A (33 amino acids (and orexin B )28 amino acids). Orexin neurons are predominantly concentrated in the lateral hypothalamus and interact with various other neuropeptides within the whole parts of the central nervous system.4 In addition to the regulation of sleep and metabolism, animal and human studies have shown that orexins and their receptors are associated with stress-related responses. The injection of orexins induces anxiety, especially by activating the HPA axis and up-regulating the corticotrophin-releasing hormone. In fact, the studies demonstrated that the activation of orexin neurons is a stress-coping mechanism for animals in stressful situations.4,5 Melanin-concentrating hormone (MCH), which contains 19 amino acids, exerts its physiological activity via binding to MCH-1R in rodents, while in humans, its effects are mediated via two receptors, including MCH-1R and MCH-2R. These receptors are widely distributed in the brain, but MCH is predominantly expressed in the lateral hypothalamus and zona increta. The MCH system’s role in managing anxiety is a contentious issue based on anxiogenic factors6,7 or anxiolytic effects.8 However, most studies have demonstrated the anxiogenic effects of MCH.6,7 In fact, MCH-1R has been introduced as a potent target for the treatment of anxiety and depression. Several previous studies established that the injection of
different MCH-1R antagonists leads to anxiolytic and antidepressant influences.\textsuperscript{5,10} In addition, as orexins, the MCH signalling pathways interact with other neurotransmitters and the HPA axis to manage stress-related responses.\textsuperscript{6,7}

Chrysin, from the flavonoid group, is a bioactive constituent of herbal medicines such as Passiflora incarnate, Passiflora caerulea, and Oroxyllum indicum.\textsuperscript{14} Previous studies demonstrated the neuroprotective, anti-inflammatory, antioxidant, and pain reliever effects of chrysin.\textsuperscript{11,12} It has been shown that chrysin may suppress stressful behaviors in rats by affecting the functions of dopaminergic, noradrenergic, serotoninergic, or GABAergic signalling pathways.\textsuperscript{13} In rodents, the anxiolytic influences of chrysin have been established by affecting the amygdala, hippocampus, and prefrontal cortex.\textsuperscript{13} However, intra-hypothalamic molecular mechanisms for the anti-stress effects of chrysin have not been studied so far. Therefore, the current study aimed to explore the impact of chrysin on the hypothalamic MCH and orexin gene expression in a stress-induced rat model.

**Materials and Methods**

**Animals**

Male Wistar rats weighing 200±10 g were used in this experimental study. The animals were maintained in the standard laboratory environment (12-hour light/12-hour dark cycle and temperature at 22±2 °C) with access to water and food. Twenty rats were assigned to four groups (n=5 in each group). Group I, as the control rats, or group II, as stress model rats, received saline. Groups III and IV were stress groups that received 20 or 40 µg (per rat) of chrysin.

**Third Cerebral Ventricular (Intracerebroventricular) Cannulation, Acute Restraint Stress Induction, and Drug Administration**

The anesthetization was achieved through the administration of ketamine (80 mg/kg) and xylazine (10 mg/kg). The cannula was implanted in the skull based on the coordinates of the Paxinos and Watson Atlas (AP=0.84 mm, ML=0, DV=6.5 mm).\textsuperscript{14} The animals were kept in the laboratory. After the one-week recovery period, acute restraint stress was induced by placing the rats in a well-ventilated plastic tube (18 cm long and 5 cm wide) for 2 hours.\textsuperscript{15} All injections were given 30 minutes before stress induction via the third cerebral ventricle using a Hamilton syringe attached to a polyethylene tube 20 . After the end of the experiment, the rats were sacrificed, and the hypothalamic samples were promptly frozen using liquid nitrogen after being removed.

**Real-Time Polymerase Chain Reaction**

The total RNA was extracted from the samples using the acid guanidinium thiocyanate-phenol-chloroform extraction method. First, the hypothalamus tissue was homogenized using 1 mL of TRIzol. Chloroform was added to the homogenized solution and centrifuged. The obtained supernatant contained RNA. Then, the RNA solution was washed by adding isopropanol and 70% ethanol. RNA precipitation was dissolved in diethyl pyrocarbonate-treated water and stored at -80 °C. The cDNA was synthesized using 1 µg of RNA according to the kit instructions (Biotech rabbit, German). The synthesized cDNA was subjected to the RT-PCR approach, which was performed with the SYBR Green I kit (Takara Bio Inc., Japan). For the RT-PCR, a time cycle was defined at 95 °C for 15 minutes of one cycle, followed by 40 cycles of denaturation at 95 °C for 20 seconds, annealing at 60 °C for 15 seconds, and extension at 72 °C for 10 seconds. Specific oligonucleotide sequences for forward and reverse primers are summarized in Table 1. The orexin-, MCH-, and GAPDH-amplified products were 87, 195, and 120 base pairs, respectively. Equation 2\textsuperscript{ΔΔCT} was used to calculate the fold change of gene expression.

**Statistical Analysis**

The data were analyzed using SPSS software (version 23) and the one-way analysis of variance. A post-hoc Tukey’s test was performed to compare the significance between groups, and a P≤0.05 was considered statistically significant. The results are presented as means ± standard errors.

**Results**

**Hypothalamic Melanin-concentrating Hormone and Orexin Gene Expression**

The induction of stress (group II) caused a significant increase in hypothalamic MCH and orexin gene expression in comparison to control group I (Figures 1 and 2, P≤0.05). The third cerebral ventricular injection of chrysin at both doses 20 µg (group III) or 40 µg (group IV) significantly declined the hypothalamic MCH and orexin gene expression compared to the stress group (Figures 1 and 2, P≤0.05). However, there was no significant difference in the effects of 40 or 20 µg chrysin on the gene expression of MCH or orexin among the rats of each group (Figures 1 and 2).

**Discussion**

Stress is an endocrine disorder that leads to health...
threats, including insomnia, mental diseases, metabolic disorders, anxiety, and depression.\textsuperscript{1,2} Therefore, finding a suitable treatment to reduce stress is crucial. Plants and their derivatives are considered suitable medicinal options due to their less side effects and easy access. The neuroprotective and anti-stress effects of chrysin have been investigated in a number of studies.\textsuperscript{11-13} However, the molecular mechanism of its anti-stress effects remains unknown. Thus, this study explored the molecular mechanisms that contribute to the anti-anxiety properties of chrysin.

Our findings elucidated that acute stress causes a significant increase in hypothalamic orexin mRNA levels. These results align with the findings of previous research, suggesting that acute psychological stressors such as restraint stress activate the orexin neurons and increase the mRNA levels of orexin.\textsuperscript{4} Most studies have shown that orexin neurons or their axons are located in the brain areas associated with stress-induced responses. Orexin neurons are densely located in the bed nucleus of the stria terminalis, lateral hypothalamus, paraventricular nucleus of the hypothalamus, and brainstem monoaminergic systems. The central injection of orexin induces stress by activating the HPA axis and sympathetic system.\textsuperscript{4,16} Based on the present results, the central injection of chrysin attenuated the increased mRNA levels of orexin in the hypothalamus of a rat model of stress.

Glutamatergic systems have been considered possible intermediary pathways to activate the HPA axis.\textsuperscript{35} The interactions of orexin with glutamatergic neurons are involved in the induction of anxiety.\textsuperscript{16} As previously reported, glutamate is co-released with orexin in the terminals of orexin neurons, and the administration of the antagonists of orexin or N-Methyl-D-aspartate (glutamate receptor) receptors suppresses the anxiety responses induced by orexin.\textsuperscript{16}

In addition to glutamatergic systems, the function of orexin neurons interacts with that of GABAergic neurons. Orexin neurons express GABA\textsubscript{A} receptors, which are associated with anxiety behavior. Furthermore, research has shown that removing inhibitory GABAergic tone in the hypothalamus activates the orexin neuron.\textsuperscript{37}

The involvement of \textit{Passiflora incarnata} and its derivative, chrysin, has been proven in the regulation of the brain's glutamatergic and GABAergic systems.\textsuperscript{18,19} Chrysin was able to reduce glutamate levels in the cerebral and hippocampus of hypothryoidism-induced mice. In addition, the stimulation of the brain's GABAergic system has been demonstrated by chrysin via activating GABA\textsubscript{A} receptors.\textsuperscript{16-20} The antagonism of the GABA\textsubscript{A} receptor can block the anxiolytic effect of chrysin completely.\textsuperscript{16} Accordingly, the inhibitory effects of chrysin on hypothalamic orexin mRNA levels may be partly mediated via activating the GABAergic or inhibiting glutamatergic neurons, which are both directly associated with the action of orexin neurons and the subsequent decrease of stressful behaviors.

Based on the present data, restraint stress also upregulated hypothalamic MCH gene expression. This finding is consistent with that of previous research, indicating that lateral hypothalamic neurons expressing MCH are involved in the control of stress-related behaviors in animals.\textsuperscript{20} In fact, the stress or corticosterone injection can activate MCH neurons, and blocking the MCH receptor has anti-anxiety effects.\textsuperscript{6,21} It has been established that MCH stimulates the HPA axis predominantly through the corticotrophin-releasing hormone neurons of the paraventricular nucleus\textsuperscript{3}; thus, this study attempted to investigate the impact of chrysin on MCH mRNA levels. The IVC injection of chrysin significantly down-regulated hypothalamic MCH gene expression in stressed rats. It has been shown that the majority of MCH neurons in the lateral hypothalamus are glutamatergic, and a subset of MCH neurons are GABAergic.\textsuperscript{22,23} Thus, the down-regulation of hypothalamic MCH due to the GABAergic and anti-glutamatergic functions of chrysin may be...
another possible mechanism to inhibit hypothalamic MCH gene expression in stressed rats.

**Conclusion**

Our findings confirmed that acute stress causes a significant increase in the hypothalamic orexin and MCH mRNA levels. The third cerebral ventricular injection of chrysin down-regulated the hypothalamic orexin and MCH mRNA levels in a rat model of stress. Chrysin may be suggested as a drug target for the treatment of stress-related disorders via the down-regulation of neuropeptides upstream HPA axis.

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**Authors’ Contribution**

**Conceptualization:** Fariba Mahmoudi.

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**Formal analysis:** Khadijeh Haghighat.

**Writing–review & editing:** Fariba Mahmoudi.

**Competing Interests**

There is no conflict of interests in this article.

**Ethical Approval**

The study was conducted under the supervision of the Research Ethics Committee of the University of Mohaghegh Ardabili (code: IR.UMA.REC.1400.029).

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