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RESEARCH ARTICLE

Andrographolide exerts an anxiolytic-like effect possibly via regulation of the hypothalamic-orexinergic system

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Abstract

Background: Anxiety is a psychiatric disorder that causes many problems in life such as poor concentration, sleep disturbance, and feelings of unease to anticipate future threats. The hypothalamus is a part of the limbic system that regulates stress. The orexinergic system, naturally produced in the hypothalamus, is associated with anxiety and depression which is induced by stress. Andrographolide has antioxidants and anti-inflammation properties that are possibly effective in preventing stress-induced anxiety. **Objective:** This study was designed to investigate the effect of andrographolide on anxiety-like behaviour and orexinergic systems. **Method:** The ddY strain mice were exposed to electrical footshock stress for five days. The light was used as a contextual fear conditioning during stress exposure. Exactly 50 mg/kg of Andrographolide was given intraperitoneally, along with stress induction. The anxiety-like behaviour was investigated using two approaches: the duration of the freezing behaviour and the mice's natural aversion to exploring open space. The hypothalamus was collected to examine the orexinergic systems. **Result:** Stress induction developed an anxiety-like behaviour in mice while administering andrographolide attenuated this effect. Additionally, andrographolide administration stimulated the hypothalamic orexinergic system. **Conclusion:** Andrographolide exerts an anxiolytic effect possibly via regulation of the hypothalamic orexinergic system.

Introduction

Stress is an important factor in developing mental disorders such as anxiety, depression, and post-traumatic stress disorder (Daviu *et al.*, 2019; Biltz *et al.*, 2022). Anxiety disorder, due to stress or traumatic events, has caused many problems for humans of all ages, including excessive worry, negative problem orientation, difficulty concentrating, sleep disturbance, restlessness, chronic fatigue, irritability, and muscle tension (Boi & Llera, 2023). In addition, anxiety also

disrupts decision-making, working memory, and lower self-esteem, which alters social interaction and results in detrimental behaviour, especially during the developmental period. This evidence was reflected in the reduced learning ability of students at school (Charpentier *et al.*, 2016; Isiogugu *et al.*, 2022; Rahem *et al.*, 2022). Even further, anxiety disorder is associated with poor work productivity (de Oliveira *et al.*, 2023). Considering the increasing level of prevalence of anxiety disorders following the COVID-19 pandemic, it deserves special attention from health

workers, governments, and researchers worldwide (COVID-19 Mental Disorders Collaborators, 2021).

The hypothalamus is a part of the limbic system in the brain, which is associated with several stress-related diseases, including anxiety disorder and depression. This linkage of the hypothalamus and anxiety is based on the ability of the hypothalamus to regulate stress hormones through the hypothalamic-pituitary-adrenal (HPA) axis modulation. The HPA axis affects systemic body systems and certain brain parts, like the hippocampus, by activating the glucocorticoid receptors (Russell & Lightman, 2019). Interestingly, in addition to the HPA axis, the hypothalamus also has an important role in the activation of the orexinergic system. This orexin system regulates several aspects, such as feeding behaviour, the sleep-wake cycle, and autonomic functions.

Furthermore, several studies have suggested the association of this system with changes in anxiety and depression states. The hypothalamus naturally produces the orexin A and B neurotransmitters from pre-pro-orexin (PPO) mRNA. Orexin A and B positive neurons in the hypothalamus are projected to various brain areas. Orexin A and B neurotransmitters bind specifically to the orexin 1 receptor (OX1R) or orexin 2 receptor (OX2R) and affect the downstream signalling pathway in the amygdala, nucleus accumbens, and hypothalamus which are strongly connected with stress and anxiety (Sargin, 2019; Katzman & Katzman, 2022).

Andrographolide is a secondary metabolite found in *Andrographis paniculata* (Burm. f.). Andrographolide is known to exhibit various pharmacological activities such as antipyretic, anti-inflammatory, analgesic, antiviral, antibacterial, antitumor, immune regulatory, and antidiabetic (Hartini *et al.*, 2021; Li *et al.*, 2022). Furthermore, andrographolide has been widely studied on depression, stroke, and Alzheimer's disease (Zhang *et al.*, 2019; Abedi *et al.*, 2021; Gong *et al.*, 2022). This evidence shows the potential of andrographolide to ameliorate diseases related to the central nervous system. Unfortunately, there is a lack of evidence demonstrating andrographolide's potential in modulating anxiety disorders. Thus, this study aimed to find scientific evidence regarding the potential of andrographolide in regulating anxiety disorders. The study further elaborated on the involvement of the orexinergic system modulation in the hypothalamus.

Methods

Experimental design

All research procedures have been approved by the Faculty of Veterinary Medicine, Airlangga University

ethical commission (No: 2.KEH.139.08.2023). A total of 18 ddY-strain mice were used in the study and were divided into three groups: "control", "Stress", and "Stress + andrographolide 50 mg/Kg". The mice were acclimatised for seven days in a cage with a temperature of 25±2°C under a 12-hour light/dark cycle and freely accessing food and water. The stress induction procedure for this study refers to previous research by Ardianto *et al.* (2021). Mice were induced by using an electric footshock of one ampere for one second with an interval of nine seconds. Electric footshock induction was carried out for five minutes within five days. The stress induction was carried out 30 minutes after the animals were given vehicle or andrographolide 50 mg/Kg. Andrographolide (TCI Chemical, Japan) was dispersed using a vehicle containing 10% DMSO, 10% tween, and 80% saline. The behavioural test was conducted on days zero (pre-stress induction) and six (post-stress induction). The animals were tested using the elevated plus maze test, open field test, and freezing behaviour. Every behavioural data was presented as a difference value between behavioural tests on days zero and six. On the seventh day, the animals were sacrificed, and the hypothalamus tissues were obtained.

Open field test

This test was conducted to observe the mice's anxiety-like behaviour. The open field apparatus uses a box with a huge wall so that it cannot escape the test. The floor of the open field apparatus is divided into 16 parts in a square shape. This test was conducted for five minutes. The mice were returned to their natural cage after the test was done. The duration of mice spent in the centre of the open field apparatus was recorded.

Elevated plus maze test

This test was carried out to measure anxiety-like behaviour in mice. This maze is designed to be at a certain height so that animals feel afraid of exploring the maze's open arm. The maze consists of both an open and a closed-arm area. The mice were placed between the open and closed arms and allowed to explore the arm for five minutes. The spending time of the mice in the open arm was recorded.

Freezing behaviour

Freezing behaviour is a natural response of animals when faced with a threat. In this study, the freezing behaviour is provided from the memory of a painful event due to a light clue in stress induction. The mice were placed under the same conditions as a stress

induction but without footshock. The duration of the freezing behaviour was recorded for five minutes.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Biomolecular testing was carried out using the RT-PCR technique. Following the research timeline, on the last day, the hypothalamus was obtained and stored at -80°C . Total RNA was extracted using an RNA purification kit (Jena Bioscience, Germany), followed by equalising the total RNA concentration. The first strand cDNA synthesis was carried out from each total RNA using the Goscript reverse transcription (Promega, USA). The PCR was performed according to standard cycling conditions from the Gotaq qPCR master mix (Promega, USA) including a polymerase activation at 95°C for two minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for one minute. Specific primers used in this study included: PPO (5'-TGT TCC TGC CGT CTC TAC GAA-3'; 5'-TGG TTA CCG TTG GCC TGA A-3'), OX1R (5'-TTG GTG CGG AAC TGG AAA C-3'; 5'-CCA TCA GCA TCT TAG CCG TCT-3'), OX2R (5'-TTC CCG GAA CTT CTT CTG TGG-3'; 5'-TCA GCA GCA ACA GCG CTA ATC-3'), B-actin (5'-TTC TTG GGT ATG GAA TCC TGT-3'; 5'-AGC ACT GTG TTG GCA TAG AG-3'). The PCR results were validated using the melting temperature analysis. PCR cycle thresholds were further analysed using the $2^{-\Delta\Delta\text{CT}}$ formula.

Data analysis

All data are presented as mean \pm S.E.M. All data were analysed using a one-way ANOVA followed by a post hoc Tukey test and t-test when appropriate.

Results

Andrographolide exerts an anxiolytic-like effect in stressed mice

The present study results (Figure 1) show that stress induction significantly increases the duration of freezing behaviour, while administration of andrographolide 50 mg/Kg significantly reduces the duration of freezing behaviour. This result demonstrates that the administration of andrographolide can reduce anxiety-like behaviour induced by stress.

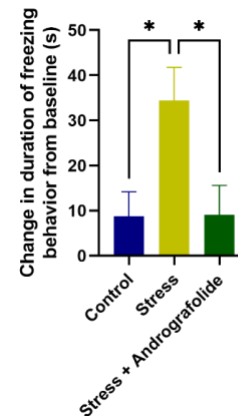


Figure 1: Effect of andrographolide on changes in the duration of freezing behaviour. Data are presented as mean \pm SEM, n = 6. * $p < 0.05$

Figure 2 shows that stress induction tends to reduce the duration of time in the centre, indicating that stress induction produced anxiety-like behaviour in mice (Ardianto *et al.*, 2021; Knight *et al.*, 2021). Furthermore, the administration of andrographolide 50 mg/Kg did not change the duration of time in the centre compared to the stress group.

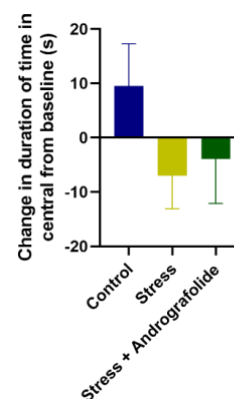


Figure 2: Effect of andrographolide on changes in the duration of time in the centre in open field test. Data are shown as mean \pm SEM, n = 6

Figure 3 shows that stress induction significantly reduces the duration of time in the open arm. This result indicates that stress induction successfully produced anxiety-like behaviour in mice (Anggreini *et al.*, 2019; Knight *et al.*, 2021). Furthermore, the administration of andrographolide 50 mg/Kg tended to increase the duration of time in the open arm compared to the stress group. This result demonstrates that the administration of andrographolide reduces anxiety-like behaviour induced by stress.

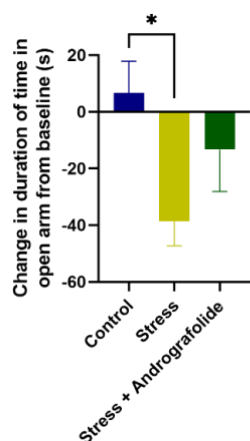


Figure 3: The effect of andrographolide on changes in the duration of time in open arms in an elevated plus maze test. Data are shown as mean \pm SEM, $n = 6$, $*p < 0.05$

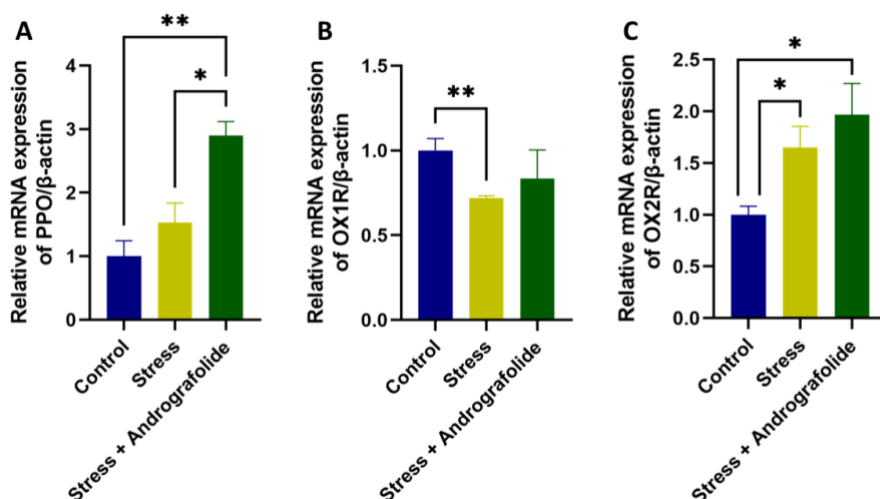


Figure 4: Effect of andrographolide on the orexinergic system in the hypothalamus. (A) PPO mRNA expression in the hypothalamus. (B) OX1R mRNA expression in the hypothalamus. (C) OX2R mRNA expression in the hypothalamus. PPO: Pre-pro orexin; OX1R: Orexin 1 Receptor; OX2R: Orexin 2 Receptor. Data are presented as mean \pm SEM, $n = 4$. $*p < 0.05$, $**p < 0.01$

Discussion

Anxiety-like behaviour in this study was carried out using three tests, including changes in the duration of freezing behaviour to conditioning behaviour, changes in the duration of time in the centre using an open field test, and changes in the duration of time in the open arm using an elevated plus maze test to assess the mice's non-conditioning behaviour. All the behavioural tests in this study were not invasive, so it was possible to use all three tests. In addition, using various tests with various approaches certainly strengthens the research findings (Liu *et al.*, 2021).

The effect of andrographolide on the hypothalamic orexinergic system in stressed mice

The present study found that stress induction tends to increase PPO and significantly increase OX2R mRNA expression in the hypothalamus, but the opposite occurred in the expression of OX1R mRNA, which decreased compared to the normal group. Surprisingly, the administration of andrographolide 50 mg/Kg caused a further increase in the hypothalamic mRNA expression of PPO and OX2R and did not affect OX1R mRNA expression (Figure 4).

Freezing behaviour is a form of animal defensive behaviour against perceived threats. Animals may be threatened by actual dangers like the presence of predators nearby or the presence of clues of a stressful event (Verbitsky *et al.*, 2020). In this study, the duration of the freezing behaviour test was carried out using the fear conditioning test using lights that remind animals memory about stressful events that happened in the past. The present study shows that stress induction significantly increased the duration of the freezing behaviour. This result indicates that stress induction successfully produced fear memory for the freezing

behaviour test situation. The present study also found that stress causes decreases in the time in the centre and time in open arms. Taken together, these data indicated that the stress model used in this study successfully develops an anxiety-like behaviour in mice (Prajapati & Krishnamurthy, 2021; Han *et al.*, 2022). Furthermore, the present revealed that andrographolide administration exhibits an anxiogenic effect by reducing the freezing behaviour and has a tendency to normalise the duration of mice to explore an open arm that should be decreased due to stress induction. This result suggests that andrographolide has a beneficial effect in improving anxiety-like behaviour.

The present study found that stress induction tends to increase the hypothalamic mRNA expression of PPO. This finding is consistent with previous research which states that there is an increasing activity of the hypothalamic orexin-positive neurons due to stress (Chen *et al.*, 2014). The overactivation of the orexin circuit is associated with the disruption of various excitatory neurons in the hypothalamus related to stress, including the activity of corticotropin-releasing hormone neurons, norepinephrine, and serotonin. Previous reports have shown that the increase in orexin activity may be implicated with the activation of the HPA axis system. Thus, the present finding suggests that increasing orexin production may reflect the brain response to the stress induction and anxiety state.

Local circuits of the hypothalamus are activated by stimulating the neurons containing OX1R and OX2R (Sargin, 2019). In the present study, the increase in OX2R mRNA expression, but not OX1R mRNA expression, marks the specific role of OX2R in the anxiety state. It is known that the dominant local projection of orexin B neurons in the hypothalamus binds specifically to OX2R. The OX2R is coupled with multiple types of G-protein (Tang *et al.*, 2008; Mitsukawa & Kimura, 2022). The intracerebroventricular injection of OX2R agonist exerts an anxiolytic effect in the stress model, suggesting the overall activation of OX2R in the brain, including in the hypothalamus, possibly produces a defence mechanism to stress (Staton *et al.*, 2018; Li *et al.*, 2021). Taken together, the present study suggests that the increase of OX2R expression in the hypothalamus is part of the response to reduced neuronal disruption in the hypothalamus due to orexin overactivation.

It is reported that in certain conditions such as during the disruption of dynein light chain protein, the orexin release may lower the OX1R expression (Duguay *et al.*, 2011). Since the OX1R is coupled with the Gq protein receptor, activating this receptor resulted increase in

neuron's downstream signalling and transcription factors. Thus, the downregulation of the OX1R is hypothetically occurred to preserve the balance in orexinergic signalling.

The administration of andrographolide caused an increase in the mRNA of the orexin precursor while reducing anxiety-like behaviour. In agreement with the above discussion, the increase in orexin signalling throughout the brain, particularly the increase in orexin precursor and OX2R expression in the hypothalamus, may reflect the mechanism of andrographolide to maintain resilience to stress induction. Further research is needed to determine the exact mechanism of how the andrographolide modulates the orexinergic activity in the hypothalamus in producing an anxiolytic effect.

Conclusion

Stress induction developed an anxiety-like behaviour in mice while administering andrographolide 50 mg/kg attenuated this effect. Additionally, andrographolide administration stimulates the hypothalamic orexinergic system.

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