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Research Article

Acute Immobilization Stress-Induced Anxiety and its Impact on Hippocampal 5-HT1A Signaling in Long-Term Coffee-Treated Rats

Sadia Gul[1]*, Hira Ahmed[2], Atia Gohar[1], Rushda Afroz[2] and Darakhshan Jabeen Haleem[2]

[1]Neuroscience Research Laboratory, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan

[2]Neuroscience Research Laboratory, Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan

***Corresponding Author:** Sadia Gul, Neuroscience Research Laboratory, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Science (ICCBS), University of Karachi, Karachi, Pakistan.

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Abstract

Caffeine, a legal psychostimulant, present in coffee, plays a significant role in reversing anxiety-like behavior following exposure to stress. The present study aimed to investigate the effect of acute immobilization stress in long-term coffee-treated rats drinking coffee as a sole source of water, altered locomotor activity, and anxiety in rats using behavioral and receptor expression studies. Forty-eight male Albino Wistar rats (180 - 200g) were randomly divided into 3 groups (n = 16); Water-treated; Coffee low-dose (10 mg/mL); and Coffee high-dose (20 mg/mL) rat groups. The animals received their respective coffee treatment for 14 days, while the animals of the control group were on drinking water. On day 14, animals were further subdivided into 3 groups (n = 8); (i) Water-treated unstressed; (ii) Water-treated stressed; (iii) Coffee low-dose (10 mg/mL) unstressed; (iv) Coffee low-dose (10 mg/mL) stressed; (v) Coffee high-dose (20 mg/mL) unstressed; (vi) Coffee high-dose (20 mg/mL) stressed rat groups. On day 16, 24h after the immobilization stress, activities in the open field (OF) and elevated plus maze (EPM) paradigms were used to assess locomotor and anxiety-like behavior, then animals were subjected to decapitation. Another similar group of rats was used for 14 days of coffee treatment. On day 16, 24h after the immobilization stress, all stressed and unstressed groups were decapitated and the brain region hippocampus was isolated to determine the 5-HT1A receptors expression by using qRT-PCR. The results showed that acute immobilization stress in coffee-treated rats exhibited a motor-depressant effect. Anxiety-like behavior was found both in long-term coffee-treated unstressed and stressed rats. The levels of hippocampal 5-HT1A receptors were downregulated in both unstressed and stressed coffee-treated rats compared to respective controls. The present findings showed that acute immobilization stress in long-term coffee-treated rats potentially induced anxiogenic effects by downregulating 5-HT1A signaling in the hippocampus.

Keywords: *Coffee; 5-HT1A Receptor; Anxiety-Like Behavior; Hippocampus; Immobilization Stress*

Introduction

Stress, characterized as a psychological and physiological response to a perceived threat [1], disrupts the homeostasis and equilibrium of an organism. It serves as a primary etiological factor contributing to a spectrum of diseases, including anxiety and depression [2]. Acute

immobilization stress exposure has been reported to decrease locomotor activity [3] and induce anxiety in the elevated-plus maze (EPM) test [4]. It represents a restraint-type stress model, commonly employed for its effective simulation of both physical and psychological stress [5].

Research showed that the most common source of caffeine is coffee, the second most consumed drink after water [5]. Caffeine is a widely consumed legal psychostimulant [7], found naturally in coffee beans, tea leaves, and cocoa beans. In addition to its natural occurrence, it is used as an additive in various foods such as soft drinks, candy, chocolates, and different medicines [8]. Caffeine can easily cross the blood-brain barrier and produces a stimulant effect by enhancing arousal and alertness [9], improving locomotor activity [10], concentration, and focus [11], and reducing sleep and appetite [12]. It has been reported that long-term caffeine consumption in the form of coffee produces an anxiogenic effect [13], while some have shown caffeine produces an anxiolytic effect during stress [14].

5-HT1A receptor is a G-protein coupled receptor, highly effective during stress [15], and involved in stress-induced anxiety and depression-like behaviors. In rats, regular mild to moderate stress suppressed hippocampal 5-HT1A receptor expression [16]. Reduced 5-HT1A expression reduced locomotor activity [15] and anxiety-like behavior [17]. Reports showed that stress responses varied among individuals consuming caffeine or not [18]. It has been found that chronic stress is involved in altering behavior and monoamine levels in both short and long-term coffee treatments (14), whereas, studies on chronic stress in long-term coffee-treated rats have shown that the chronically unpredictable stress model has anxiolytic effects by increasing serotonin (5-HT) and dopamine levels in the hippocampus [19] but the effect of acute immobilization stress in long-term coffee-treated rats on anxiety-like behavior, through 5-HT1A receptor signaling is not clear.

Aim of the Study

In this study, we aimed to investigate the effect of acute immobilization stress in long-term coffee-treated rats at doses of 10 mg/mL and 20 mg/mL on anxiety-like behavior in the open field and Elevated plus maze (EPM) test, as well as its effect on 5-HT1A receptor expression in the hippocampus.

Materials and Methods

Animals

Male Albino Wistar rats (weighing between 180 - 200g) were individually housed in plastic cages under controlled laboratory conditions, maintaining a room temperature of 24 ± 2°C and humidity at 50 ± 5%, with a 12-hour light and dark cycle. During the one-week acclimatization period, the animals had free access to water *ad libitum*, and standard food in the hopper of each cage. The animals underwent various handling procedures during this period to minimize potential psychological stress from their surroundings. Housing and handling procedures followed the guidelines outlined in the 'Guide for the Care and Use of Laboratory Animals,' published by The National Academies Press, Washington D.C, USA, and were approved by the Institutional Animal Ethics Committee (IAEC) under Animal Study protocol no. 2019-010. All treatments, behavioral activities, and molecular studies were conducted in a balanced design to reduce sequence and time effects.

Chemicals and drugs

Coffee (Instant Classic) was purchased from METRO Safari in Karachi, Pakistan. It was dissolved in tap water before use, and the measured quantity was then transferred to the rats' water bottles at doses of 10 mg/mL and 20 mg/mL. Other chemicals were procured from Sigma Aldrich Chemicals, USA. Specific primers were obtained from Penicon Pharmaceuticals, while cDNA synthesis kits, TRIzol Reagent, and SYBR Green Master Mix for Real-time qPCR were acquired from Thermo Fisher Scientific (Life Technologies and Fermentas).

Experimental protocol

For behavioral activities, forty-eight animals were divided into three equal groups, each consisting of 16 individuals: Water-treated, Coffee low-dose (10 mg/mL), and Coffee high-dose (20 mg/mL) groups. Freshly prepared coffee was given as a sole source of drinking water to the treated groups for 14 days, while the control group received regular drinking water, between 9:00 and 9:30 am. Subsequently, the animals were divided into six subgroups, with eight in each group designated as Water-treated unstressed and stressed, Coffee low-dose (10 mg/mL) unstressed and stressed, and Coffee high-dose (20 mg/mL) unstressed and stressed rats. On day 15, stressed animals were individually placed in a room for immobilization stress for 2 hours, between 10:30 and 12:30 pm. After 24 hours of immobilization stress, behavioral activities (open field and EPM tests with a 2-hour lapse) were monitored, and then the animals were subjected to decapitation.

For receptor expression studies, another group of rats received similar coffee treatment for 14 days, divided into six subgroups, with eight in each group designated as Water-treated unstressed and stressed, Coffee low-dose (10 mg/mL) unstressed and stressed, and Coffee high-dose (20 mg/mL) unstressed and stressed rats. On day 15, the stressed group of animals underwent 2 hours of immobilization stress in a separate room. On day 16, after 24 hours of immobilization stress, all unstressed and stressed rats were decapitated, and their hippocampus was isolated. All brain samples were immediately frozen at -80°C for further analysis.

Immobilization procedure

The animals underwent an immobilization procedure as previously described [20]. A metal wire grid measuring 10" × 9", properly framed with a 9" × 6.5" Perspex plate, was utilized for this purpose. Immobilization was achieved by inserting the forelegs of the rats through the grid gaps and taping them together with Zinc Oxide plaster tape. The hind limbs were also taped, and the animal's head was positioned on the Perspex plate. After 2 hours of immobilization stress, the animals were released and returned to their cages.

Open field

An open square arena measuring 76" x 76" with walls of 42 cm in height was used to assess activity in the open field. The floor was divided by lines into 25 equal-sized squares. To evaluate activity in this field, an animal was placed in the center of the arena, and the number of squares crossed with all four paws was counted for a cut-off time of 5 minutes. The rat was removed from the open field arena and returned to its cage. The open field arena was cleaned with 70% ethanol before exposure to another rat.

Elevated plus maze activity

The EPM test is employed in animals to investigate anxiety-like behavior, screening the treatment effects for either anxiolytic or anxiogenic outcomes. The apparatus consists of four equal arms (50 cm long and 10 cm high), forming the shape of a 'plus (+)' sign and elevated 60 cm above the floor. Two arms have no side walls, while the other two have side walls (14 cm high). Both closed and open arms intersect at a central square (10 x 10 cm). Rats are placed in the central square facing the closed arm and allowed to explore all arms for a cut-off time of 5 minutes. During this period, the time spent and the number of entries into the open arm are recorded. Subsequently, the animal is returned to its cage, and the maze is thoroughly cleaned with 70% ethanol before exposure to another rat.

Gene expression analysis

Initially, in the process of RNA extraction and cDNA synthesis, the isolated brain region was homogenized using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. mRNA in the samples was measured using a NANODROP™ 2000 spectrophotometer, and the samples were stored at -80°C for subsequent analysis. cDNA was synthesized with the Revert Aid first-strand cDNA synthesis kit (Thermo Fisher Scientific, US) and stored at -80°C for qRT-PCR studies. For qRT-PCR, the beta-actin gene was used as a housekeeping gene control. mRNA levels of the 5-HT1A receptor were normalized to beta-actin mRNA. PCR studies were conducted using the following primers: for

beta-actin (forward 5'-ACCCACACTGTGCCCATCTA-3' and reverse 5'-CGGAACCGCTCATTGCC-3'), and for the 5-HT1A receptor (forward 5'-CCCCCCAAGAAGAGCCTGAA-3' and reverse 5'-GGCAGCCAGCAGAGGATGAA). qRT-PCR expression analysis was performed using AriaMx G8830A (Agilent Technologies, USA) with Maxima SYBR Green/ROX qPCR Master Mix 2X (Cat # K0221-Thermo Scientific) according to the manufacturer's protocol.

Statistical analysis

Statistical analysis was conducted by using IBM SPSS statistical software v.21.0. The results are expressed as means ± S.D. Data on open field and EPM test results and 5-HT1A receptor expression were analyzed by using two-way ANOVA. Post-hoc comparisons were performed using Tukey's test. p-values less than 0.05 were considered significant.

Results

Figure 1 depicts the effect of acute immobilization stress on motor behavior in an open field in rats drinking coffee for 14 days, monitored after 24 hours of stress. Data analyzed by two-way ANOVA revealed a significant stress effect ($F = 105.355$, $df = 1, (30)$, $p < 0.01$) and a significant interaction of stress x treatment ($F = 11.800$, $df = 2, (30)$, $p < 0.01$) effect. The treatment effect on the number of squares crossed in an open arena ($F = 1.421$, $df = 2, (30)$, $p > 0.05$) was not significant. Tukey's test showed no significant difference in locomotor activity in coffee-treated unstressed groups. The stressed group data exhibited a significant motor depressant effect in an open field at both doses (10 mg/mL and 20 mg/mL) of coffee compared to the unstressed group. The stressed low-dose coffee treatment group significantly lowered the motor activity of the control stressed group in an open field. The results suggest that long-term coffee consumption in stressed animals reduces motor activity in an open field.

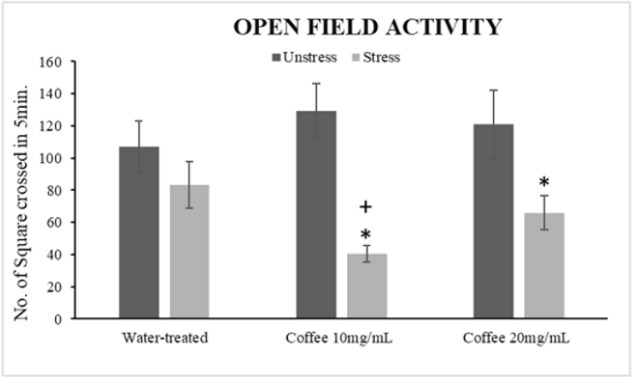

Figure 1: *Effect of 2h immobilization stress on open field activity in rats drinking water or coffee as sole source of water for 14 days, monitored 24h of stress. Values are mean ± SD (n = 8). Significant difference by Tukey's test: *p < 0.01 from similarly treated normal unstressed rats; +p < 0.01 from respective water-treated (control) rats, following two-way ANOVA.*

Figure 2 illustrates the effect of acute immobilization stress on anxiety-like behavior in an EPM test in rats drinking coffee for 14 days, monitored after 24 hours of stress.

For figure 2A, data on time spent in an open arm analyzed by two-way ANOVA showed a significant treatment effect ($F = 54.551$, $df = 2, (30)$, $p < 0.01$), stress effect ($F = 37.693$, $df = 1, (30)$, $p < 0.01$), and a significant interaction of stress x treatment ($F = 81.970$, $df = 2, (30)$, $p < 0.01$). Tukey's test indicated that rats spent less time in the open arms with coffee treatment. Time spent in an open arm in the

water-treated control, and the low and high-dose (10 mg/mL and 20 mg/mL) coffee-treated stressed group showed less time spent in the open arm than the unstressed group.

For figure 2B, data on the number of entries in an open arm analyzed by two-way ANOVA displayed a significant treatment effect ($F = 38.217$, $df = 2, (30)$, $p < 0.01$), stress effect ($F = 35.186$, $df = 1, (30)$, $p < 0.01$), and a significant interaction of stress x treatment ($F = 24.497$, $df = 2, (30)$, $p < 0.01$). Tukey's test revealed that unstressed coffee-treated animals reduced their entries in an open arm compared to water-treated unstressed animals. The controls and low-dose coffee-treated (10 mg/mL) stressed animals showed fewer entries in the open arm than similar unstressed animals.

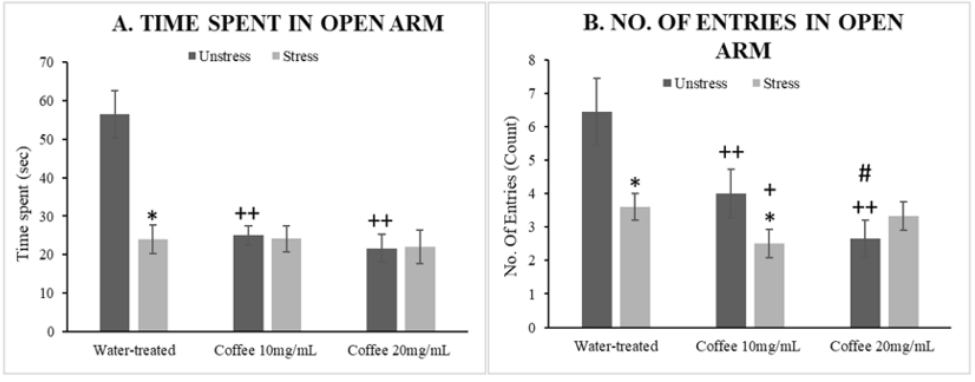

Figure 2: *Effect of 2h immobilization stress on elevated plus maze (EPM) activity in rats drinking water or coffee as sole source of water for 14 days, monitored 24h of stress. Time spent (A) and No. of entries in an open arm (B). Values are mean ± SD (n = 8). Significant difference by Tukey's test: *p < 0.01 from similarly treated stressed rats; ++p < 0.01, +p < 0.05 from respective water-treated controls; #p < 0.01 from respective LD (10 mg/mL) coffee-treated rats, following two-way ANOVA.*

Figure 3 illustrates the effect of acute immobilization stress on hippocampal 5-HT1A receptor expression in rats drinking coffee for 14 days, monitored after 24 hours of stress. Data analyzed by two-way ANOVA revealed a significant treatment effect ($F = 32.180$, $df = 2, (30)$, $p < 0.01$) and stress effect ($F = 8.782$, $df = 1, (30)$, $p < 0.01$), while the interaction between treatment x stress ($F = 0.656$, $df = 2, (30)$, $p > 0.05$) was not significant. Tukey's test results showed that in the hippocampus, 5-HT1A receptor expression significantly decreased at both doses (10 mg/mL and 20 mg/mL) of coffee in both unstressed and stressed animals compared to respective controls.

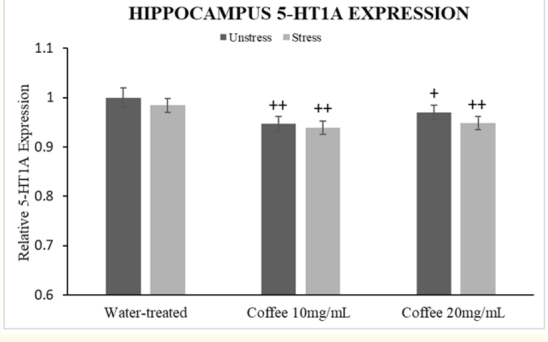

Figure 3: *Effect of 2h immobilization stress on 5-HT1A receptor expression in the hippocampus by quantitative RT-PCR, in rats drinking water or coffee as sole source of water for 14 days, monitored 24h of stress. Values are mean ± SD (n = 8). Significant difference calculated by Tukey's test: +p < 0.05, ++p < 0.01 from the respective water-treated group following two-way ANOVA. Values of the treatment group were normalized with the water-treated group of unstressed and stressed groups.*

Discussion

The primary objective of this study was to investigate the effect of acute immobilization stress on locomotor, and anxiety-like behavior in long-term coffee-treated rats. The results showed that acute immobilization stress reduced locomotor activity in water-treated controls and at both doses of coffee. Prior research has documented that acute immobilization stress causes a reduction in locomotor activity in the open field compared to unstressed rats, monitored 24 hours of stress [21], indicating depressive-like behavior [22]. Also, research has reported that caffeine, a psychostimulant present in coffee, stimulates locomotor activity during stress [19] also found that long-term caffeine intake reverses the chronic unpredictable stress model-induced depressive-like symptoms in rats [14]. In contrast, our findings revealed that acute immobilization stress decreases locomotor activity in long-term coffee-treated rats and produces motor-depressant effects. Various literature reported that repeated long-term coffee treatment tends to have no noticeable effect on locomotor activity in the open field, likely due to the development of tolerance in both male and female rats [23,24]. In line with these findings, our study similarly observed that rats subjected to repeated coffee treatment exhibited no significant alterations in locomotor activity within the open field, indicating a state of tolerance.

Furthermore, our results revealed the anxiogenic effects of acute immobilization stress in the EPM test for long-term coffee-treated rats. The time spent and number of entries in the open arm were reduced in the EPM test at both coffee doses (10 mg/mL and 20 mg/mL) for both unstressed and stressed rats. The extensive literature supports the notion that 2-hour immobilization stress is associated with the induction of anxiety-like behavior in normal rats [25].

A study by Noschang and colleagues reported that caffeine administered for both short and long-term induced anxiety-like behavior monitored in the EPM test [23]. A prior study reported that the chronic unpredictable stress model helps to decrease the anxiety-like behavior of caffeine [26]. Conversely, another study reported that in chronically restrained stress-exposed animals consuming chronic caffeine (0.3 mg/mL and 1 mg/mL) increases anxiety in the EPM test [23]. Inconsistent effects of caffeine have been observed with different chronic stress models. However, using an acute immobilization stress model, no impact on coffee intake, resembling the human coffee consumption model, has been reported in rats. Therefore, our findings, based on exposure to short-duration stress, indicate that the acute immobilization stress model is not effective in reversing the anxiogenic effect of coffee at doses of 10 mg/mL and 20 mg/mL in rats.

Moreover, the hippocampus is the main brain region critically linked with stress-related pathologies such as anxiety and depression [27]. Among the multiple serotonergic receptors in the brain, post-synaptic 5-HT1A receptor signaling in the hippocampus plays a significant role in antidepressant-like effects [28]. In rats, moderate stress suppressed hippocampal 5-HT1A receptor expression [17]. The suppressed serotonergic 5-HT1A expression decreases locomotor activity [15] and causes anxiety-like behavior [16]. Therefore, as a secondary objective of this study, this region was selected to assess the effect of acute immobilization stress in long-term coffee-treated rats on 5-HT1A receptor expression. Our results revealed that acute immobilization stress downregulated 5-HT1A receptor expression in the hippocampus of water-treated animals. Additionally, hippocampal downregulation was more pronounced in both unstressed and stressed coffee-treated animals than in the water-treated controls. Downregulated serotonergic 5-HT1A receptor signaling indicates decreased serotonin (5-HT) neurotransmission, suggesting decreased locomotor activity and increased anxiety-like behavior. These findings suggest that the effect of acute immobilization stress in long-term coffee-treated rats indicates an anxiogenic effect, as evidenced by the decreased expression of the 5-HT1A receptor in the hippocampus.

To the best of our literature study survey, this study is the first to explore the potential link between acute immobilization stress in long-term coffee-treated rats and hippocampal 5-HT1A receptor signaling in rats.

Conclusion

In conclusion, we evaluated the anxiogenic effect of coffee following acute immobilization stress in rats. The results revealed more pronounced behavioral deficits induced by acute immobilization stress in long-term coffee-treated rats as compared to water-treated control rats. Additionally, coffee is implicated in the regulation of hippocampal 5-HT1A receptor signaling, and its downregulation is involved in producing anxiogenic effects. The present study therefore emphasizes that dietary sources rich in caffeine contents and/or supplementation of caffeine should be cautiously consumed in the diet during stressful life events.

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Conflict of Interest

There is no conflict of interest.

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