Environmental Enrichment Ameliorates Anxiety-Like Behavior in Rats without Altering Plasma Corticosterone Level

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Abstract

BACKGROUND: Anxiety disorder is one of the most common psychiatric problems. Prolonged stress gives rise to anxiety-like behavior in animals. Environmental interventions influence the outcome of anxiety treatment. Environmental enrichment (EE) can modulate brain's structure and function.

AIM: The objective of the study was to evaluate EE effects on anxiety-like behavior and corticosterone (CORT) level after unpredictable chronic mild stress (UCMS).

METHODS: A total of 28 rats were assigned into four groups randomly: Control, UCMS, UCMS+EE, and UCMS+fluoxetine. UCMS, EE, and fluoxetine were given for 21 days. Anxiety behavior was measured on day 22nd using Elevated Plus Maze. Behavioral measurement was based on the total time spent and total entries onto open and closed arms. CORT was measured using ELISA.

RESULTS: UCMS increased anxiety-like behavior as seen from reduced number of entries and time spent in open arms as well as increased number of entries and time spent in in closed arms in UCMS group than control. Rats in EE group spent more time and made more entries in the open arms than UCMS group (both p = 0.002). Anxiolytic effect of EE was stronger than fluoxetine. Plasma CORT level among groups did not differ significantly (p = 0.351).

CONCLUSION: EE can ameliorate stress-induced anxiety-like behavior without affecting CORT level.

Introduction

Stress is associated with the development of many diseases [1]. Chronic stress can dysregulate the hypothalamic-pituitary-adrenal (HPA) axis with subsequent negative implications on health including psychiatric problems [2]. Prolonged stress exposure can result in maladaptive behavioral changes such as depression and anxiety-like behavior that mimic mental disorders in human [3], [4]. Stress impact was prominent in two brain areas called the hippocampus and amygdala, structures that control the networks for mood regulation, suggesting the important role of stress response in emotional behavior [4].

Anxiety disorder is one of the most common psychiatric problems found in the current societies [5]. Anxiety research in preclinical setting has been focusing on the development of new anti-anxiety drugs [6] probably due to the fact that emotional behavior in human and animal especially rodents are relatively similar [7], [8]. There are three most popular assays that have been adopted in preclinical studies to assess anxiety behavior including the elevated plus maze (EPM) test, the open field (OF) test, and the light-dark (LD) box test [6]. A profound basis from preclinical research is crucial to direct further studies and to develop therapeutic interventions for anxiety disorders [9].

A number of studies suggested the involvement of certain factors such as genes, drugs, and environmental interventions in the management of anxiety [6]. Environmental enrichment (EE) was shown to induce not only morphological and molecular changes in the brain but also changes in the behavior [10], [11]. EE incorporates social and physical stimuli that help modulate brain's function and structure by affecting the expression of certain genes and neurotransmitter's activity [12], [13]. The previous studies showed that EE increased the animal's resilience to stress and inhibited anxiety-like behavior as well as fear induced by stress [14], [15] and restored memory impairment [16]. Despite the EE positive effects on behavior, the mechanism that underlies these effects is not clear yet. Considering these facts, we aimed to investigate whether EE can attenuate stress-induced

alteration in anxiety behavior and evaluate whether this alteration correlates with any changes in the level of a stress-related hormone, corticosterone (CORT).

Methods

Animal and housing condition

Twenty-eight male Wistar rats (*Rattus norvegicus*) aged approximately 6 weeks were used in this research. Rats were allocated randomly into four groups including control group (C), stress group (UCMS), stress plus EE group (UCMS+EE), and stress plus fluoxetine group (UCMS+Fluox). Each group consisted of seven rats. Rats in the UCMS+EE group were put in an enriched cage to facilitate more physical activity and social interaction. As compared to standard cage, the enriched one was larger (80 cm × 55 cm × 45 cm) and contained various kinds of playing tool such as a small ball, a slide, a running wheel, a plastic tube, bedding material, and a ladder step. These toys were re-positioned regularly to provide novel experience. All other rats were housed in standard cages with controlled lighting at 25–28°C. The rats could access food and drinking water freely.

Stress protocol

We applied a chronic unpredictable mild stress (UCMS) procedure in this study. A variety of stressors were given randomly at different time each day to ensure unpredictability as well as to avoid habituation. Stress exposure was given for 21 days to create a chronic state of the stress condition. Detailed protocol of the stress procedure is shown in Table 1.

Table 1: The protocol for chronic unpredictable mild stress

Day	Time I	Stressor I	Time II	Stressor II
1	08.00	Cold swimming (10°C, 4 min)	14.00	Predator noise (30 min)
2	10.00	Cage tilting at 45°C (4 h)	18.00	Overnight illumination
3	09.00	Cage darkened (3 h)	17.00	Continuous cage shaking (10 min)
4	08.00	Food deprivation (24 h)	15.00	Cold swimming (10°C, 4 min)
5	08.00	Cage darkened (3 h)	16.00	Tail pinch (2 min)
6	09.00	Predator noise (30 min)	18.00	Overnight illumination
7	07.30	Water deprivation (24 h)	16.00	Cage tilting at 45°c (4 h)
8	08.00	Damp sawdust (5 h)	13.00	Cold swimming (10°C, 4 min)
9	10.00	Cage darkened (3 h)	17.00	Predator noise (30 min)
10	06.30	Predator noise (30 min)	16.00	Cage tilting at 45°C (4 h)
11	11.00	Continuous cage shaking (10 min)	16.30	Tail pinch (2 min)
12	07.00	Cage darkened (3 h)	17.30	Overnight illumination
13	11.00	Cage tilting at 45°C (4 h)	17.00	Continuous cage shaking (10 min)
14	10.00	Damp sawdust (5 h)	15.30	Cold swimming (10°C, 4 min)
15	08.30	Cage darkened (3 h)	18.00	Overnight illumination
16	09.00	Cage tilting at 45°C (4 h)	14.00	Predator noise (30 min)
17	08.00	Tail pinch (2 min)	16.30	Cold swimming (10°C, 4 min)
18	06.30	Damp sawdust (5 h)	12.00	Cold swimming (10°C, 4 min)
19	10.30	Predator noise (30 min)	15.00	Continuous cage shaking (10 min)
20	09.00	Cage darkened (3 h)	18.00	Overnight illumination
21	08.00	Predator noise (30 min)	15.30	Cage tilting at 45°C (4 h)

Experimental procedure

After acclimation process, rats were assigned to four groups as previously mentioned. On the 1st day of the experiment, stress exposure as outlined in the UCMS protocol was given to all animals except those in the control group. Enrichment condition and fluoxetine administration were also started from day 1 along with the stress exposure in the UCMS+EE and UCMS+ Fluox group, respectively. Fluoxetine was administered once per day orally at 10 mg/kg [17]. Immediately before administration, fluoxetine was dissolved with saline 0.9%. Stress exposure, EE, and fluoxetine were given for 21 days. On day 22, the behavioral test was conducted. Blood sample was collected from the retro-orbital plexus on the following day at 9–10 a.m for the measurement of plasma CORT level. The study was conducted as per the guidelines of the Animal Care and Use of Universitas Sebelas Maret. Approval for the study protocol has been given by the Medical Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia (Ethical Clearance 244/UN27.6/KEPK/2018). Every effort was done to minimalize the animals' suffering.

Behavioral test

On the 22nd day of the experiment, anxiety-like behavior of the animals was measured with EPM test. The EPM test was conducted according to Park *et al*. previous work [18]. The task used an apparatus with a plus-shaped configuration comprising of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm) made from white opaque acrylic. The maze was raised 50 cm from the ground. A video camera placed directly above the middle of the apparatus was used to record the test. Sixty minutes before the test, rats were transported to the behavior testing room. At the start of the examination, the rat was put at the middle of the apparatus (5 cm × 5 cm) and was then given 5 min to explore the maze freely. After every session, 70% ethanol was applied to clean the maze. Before starting the following test, the maze must be clean and dry. An entry is counted when the rat puts all four paws onto the open or closed arm. EPM is a simple yet valid method to evaluate anxiety state in rats. It is developed in accordance with the innate behavior of rats to spontaneously explore novel environment as well as their natural aversion to be in elevated and open areas, thus, creating an exploration conflict. Increased anxiety level and fear is reflected in avoidance of the open arms of the EPM [19]. Anxiety behavior assessment was done by measuring the number of entrances and time spent in each arm. Increased number of entries and the time spent in open arms and rat's tendency to reside in closed arms display higher level of anxiety [20]. Total entries in closed arms are also used to measure the general locomotor activity [21].

Plasma CORT measurement

Immediately after collection, whole blood was processed to obtain the plasma by centrifugation (1500 rcf for fifteen minutes at 4°C). This plasma subsequently was kept in –20°C until ELISA test was performed. The concentration of plasma CORT was measured using ELISA kit (Fine Test, Wuhan Fine Biological Technology Co, Ltd) as per the manufacturer's instruction manual. Absorbance measurement was done at 450 nm using a spectrophotometer.

Statistical analysis

One-way analysis of variance (ANOVA) or Kruskal–Wallis test was used to analyze the data followed by Post hoc test for multiple comparisons using SPSS version 22.0. p < 0.05 was set as the level of significance.

Results

Behavioral response

Anxiety-like behavior was assessed based on several parameters including the total entries score and time spent in both the open and closed arms in the EPM test. UCMS significantly reduced entries onto open arms and tended to reduce the time spent in open arms. Rats housed in an enriched condition spent significantly more time in the open arms than rats in the UCMS group (p = 0.002) and so did the rats with fluoxetine treatment (p = 0.005). This response was stronger in the UCMS+EE group than the UCMS+Fluox group (p = 0.005). Similarly, the number of entries into open arms in both the environmentally enriched group and fluoxetine treated group was significantly higher as compared to UCMS group (p = 0.002 and p = 0.017, respectively). Result of the measurement on total time and entries onto the open arms is displayed in Figure 1.
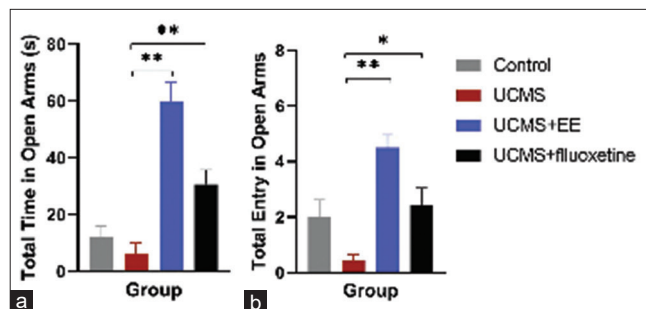

Figure 1: The average total time spent (a) and total entries (b) in open arms in each group depicted as mean + standard error of the mean (SEM). Data were analyzed with one-way analysis of variance followed by Post hoc least significant difference (LSD) test (Data A) and Kruskal–Wallis followed by Post hoc Mann–Whitney test (Data B). *p < 0.05 versus unpredictable chronic mild stress (UCMS) group; **p < 0.01 versus UCMS group

The number of entries in closed arms of UCMS group was significantly higher as compared to control (p = 0.040). The total time spent in closed arms was significantly higher in UCMS group than UCMS+EE group (p = 0.035). Effect of EE in decreasing the total time spent in closed arms was stronger than fluoxetine. The total entries into closed arms in UCMS+EE and UCMS+Fluox group did not differ significantly as compared to UCMS group (p = 0.659 and p = 0.067, respectively). Detailed result of the measurement on total time and entries onto the closed arms is displayed in Figure 2.
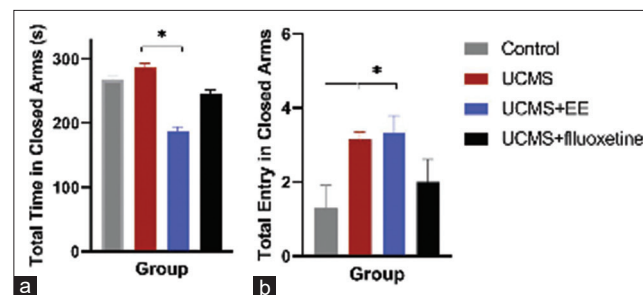

Figure 2: The average total time spent (a) and total entries (b) in Closed Arms in each group presented as mean + standard error of the mean (SEM). Data (a and b) were analyzed with Kruskal–Wallis followed by Post hoc Mann–Whitney test. *p < 0.05 versus unpredictable chronic mild stress group (a); * p < 0.05 versus control (b)

Plasma CORT level

We compared the level of plasma CORT between control, stress group, and treatment group. However, there was no significant difference in the concentration of plasma CORT among groups after we performed the one-way ANOVA analysis.

Discussion

Behavioral response

The previous works have suggested the correlation between stressful event and the development of several psychiatric disorders including affective disorders such as anxiety and depression [22], [23], [24]. This study showed that chronic exposure of unpredictable mild stress induced anxiety-like behavior in rodents as indicated by the propensity of the animals to enter the closed arms of the EPM apparatus and to spend most of the time in the closed arms during the test. Rat's preference to be in the closed arms suggests the need for a secure environment reflecting a sign of an anxious state [25]. Consistent with our finding, studies in animals showed that certain kinds of stress exposure resulted in the development of anxiety-like behavior [21], [25], [26]. In line with this result, clinical studies in human also revealed that stress exposure

increased the risk of developing anxiety disorder [27]. As a response to stress exposure, the HPA axis is stimulated. Stress causes the hypothalamus to secrete a neurohormone called corticotrophin releasing factor (CRF) which subsequently triggers the production of adrenocorticotropic hormone (ACTH) from anterior pituitary. ACTH is released into the bloodstream and then induces the production of glucocorticoid (called CORT in rodents and cortisol in human) from adrenal gland [28]. Stress-related elevation in CRF expression in the amygdala, a structure that regulates emotion, is considered to stimulate anxiety [29]. Moreover, rats with high anxiety state showed increased level of CRF-expressing neurons in the basolateral amygdala [30].

Rats naturally have the fear of height and open spaces as well as the drive to explore new environment. It is generally accepted that the closed arms and the open arms of EPM apparatus can induce similar exploratory drive. Thus, higher level of anxiety is characterized by the aversion of open arm exploration and the propensity to spend time in the closed arms [19]. This study showed that EE could attenuate anxiety-like behavior in rats assessed with EPM paradigm. EE significantly reduced the time spent in the closed arms and increased both the duration in open arms and total entries onto open arms. This change in anxiety behavior was not caused by altered general locomotor activity mainly due to the fact that we did not find any marked differences in the frequency of entries in closed arm between treated groups and UCMS group [21]. Similar to our result, a number of studies found that EE could reduce the level of anxiety in several kinds of animal models [31], [32], [33], [34], [35]. In addition, we found that the anxiolytic effect of EE was stronger than fluoxetine, an antidepressant drug that belongs to serotonine selective reuptake inhibitor (SSRI) group. The previous clinical studies demonstrated that antidepressant (Nutt DJ) could be used to treat anxiety disorders effectively [36] and SSRI was shown to have an anti-anxiety property [37], [38].

The previous research has shown that EE could modulate behavior [39], [40]. It improved the capacity to learn and store memory as well as lowered anxiety level [41], [14], [42]. However, there has been no conclusive evidence regarding the mechanism underlying the anxiolytic effect of EE. There has been no standardized protocol for EE. However, certain components are generally included in the procedure [43]. In this study, we put the UCMS+EE rats in a spacious cage to give them more opportunity to move and provided playing tools to stimulate more social, spatial, sensory, and physical activity. In particular, we put a running wheel to facilitate physical activity. The complexity of EE which incorporates various kinds of stimuli was proven to influence the brain's morphology and function by changing the expression of certain genes and the activity of neurotransmitter [12], [13] which may contribute to the anxiolytic property of EE.

CORT level

Increased anxiety-like behavior has been associated with elevation in CORT concentration [44]. In contrast, our study suggested that unpredictable chronic mild stress induced an increased anxiety state without any effect on plasma CORT concentration. There was no significant difference in the plasma concentration of CORT among groups (p = 0.351). Neither EE nor fluoxetine administration affected CORT level as compared to both UCMS group and control. This result was somewhat surprising because the previous works indicated that stress exposure elevates the concentration of CORT [45], [46]. Stress-induced emotional disorders in animals also correlated with elevated level of CORT [3], [47], [48]. However, it is noteworthy to see that our findings corroborate previous research showing that rats assigned to EE condition have high resting plasma CORT level as compared to control [49]. Other studies in rats also revealed that neither the basic nor the response level of plasma CORT was affected by EE treatment [50], [51]. In addition, both blockade of CORT secretion and CORT replacement did not normalize the stress-induced alteration in OF behavior following chronic HPA axis disruption [2]. Therefore, it is plausible that CORT regulates behavioral disorders related to stress but may not involve in amelioration of anxiety-like behavior stimulated by EE.

A number of published works showed that many systems plays an important role in the pathophysiology of anxiety disorders including monoaminergic system (serotonergic, dopaminergic, and noradrenergic system) [52] and neuroimmune system [14], [15]. A large cohort study in human also reported an association between immune dysregulation with anxiety disorder [54]. Stress exposure was reported to alter these systems [1], [55], [56], [57]. Thus, it is likely that these systems contributed to alterations in anxiety-like behavior in the EPM test.

Strength and weaknesses

The stress-induced anxiety model that we used in this study has been widely used in neuroscience research. In addition, anxiety measurement was done using EPM test that has become an established procedure to test anxiety level. It has been validated for usage in both mice and rats [58]. However, we realize that there have been several limitations in this study including the fact that we only measured CORT level at one time point. Thus, we cannot evaluate any changes in the baseline and response CORT level. Additional data from baseline CORT measurement and other stress-related hormone such as CRF will give more support to our study. Further research could investigate the role of other factors that may contribute to the anxiolytic effect of EE such as the neurotransmitter, inflammatory cytokines, and neurotrophic factors

to gain more understanding on the mechanism that underlies EE effect on anxiety behavior.

Conclusion

Enriched environment can ameliorate stress-induced anxiety like behavior. This anxiolytic effect is not associated with alteration in stress hormone level.

Author's Contribution

MM contributed in designing the research, data collecting and analysis, and drafting and revising of the manuscript. WAS was involved in the study conception and design, data analysis and conception as well as providing technical support. NW and DAG contributed to the concept and design of the study; provided statistical expertise and help data interpretation. RDY's expertise in statistic was used in data analysis and interpretation. RDY also critically revised the content of the article and obtained funding. BW's contribution includes study conception and design, statistical expertise, obtaining research funding, and critical revision of the manuscript. Final approval for the manuscript has been given by all authors.

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