Research Article

Different Types of Environmental Stressors Could Have Disruptive or Constructive Effects on Vestibular Compensation

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Highlights

- The process of vestibular compensation after unilateral lesion was assessed
- Two types of stressors were applied: calorie restriction and maternal separation
- IF loses its beneficial outcomes if the animal has received another source of stress

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ABSTRACT

Background and Aim: Stress could play either helpful or harmful roles in vestibular compensation, the process of recovery after vestibular system lesions. Herein, we examined the effect of two stressor types on vestibular compensation: chronic anxiety disorder induced by early maternal separation (MS), and caloric restriction by an intermittent fasting (IF) diet.

Methods: Male Wistar rats (n=56) received maternal separation (the MS group), intermittent fasting (IF group), unilateral vestibular deafferentation (UVD group), or a mixture of these interventions (UVD+IF, UVD+MS, and UVD+IF+MS). All the groups were compared with control animals. The animals' balance, motor coordination, anxiety, locomotor activity, and serum cortisol levels were evaluated by rotarod, open field, and enzyme-linked immunosorbent assay methods, respectively. The data were compared with those of the healthy control (HC) group.

Results: The UVD animals did not show a significant change in the time on the rod, except for the IF+UVD group ($p=0.04$). There was no significant difference between the experimental groups on the open field indices, except for the MS+IF+UVD group which traveled a significantly less total distance ($p=0.02$). Serum cortisol levels were significantly higher than HCs for all the groups except for the sham saline and IF+UVD group ($p<0.05$).

Conclusion: IF seems to promote compensation after UVD, while MS may disrupt it. However, IF loses its beneficial outcomes if the animal has received another source of stress, i.e. MS.

Keywords: Anxiety; fasting; vestibular; compensation

Introduction

Today, we encounter the words stress and anxiety repeatedly in our daily lives. Stress and its management pose a serious challenge to our lives, and stress is known to affect many vital functions of the human brain, including spatial orientation and navigation.

Maintaining balance and correct posture depends on the integrity and proper functioning of visual, proprioception, and vestibular organs. To remain balanced in difficult or novel situations, the sensory integrity and proper functioning of these three organs are essential. However, in case of injury to any of these organs, the defect will be compensated by the other two organs after a while; hence, it does not lead to major disruption of daily activities. Environmental vestibular injury in the early stage of acute lesions leads to the emergence of symptoms such as dizziness, imbalance, eyesight problems, and nausea, which will be recovered over time to some degree. This recovery process is known as compensation, and its success varies depending on the patient's condition [1].

The changes leading to the compensatory process include adaptive changes to gamma-aminobutyric acid (GABA) and glycine neurotransmitter's effect on vestibular neurons; an electrophysiologic change in neuron excitability; cerebellar inhibitory control on vestibular networks of the brain stem; neurogenesis and gliosis in vestibular nucleus neighboring the injury site; and activity-dependent rearrangement of the synaptic activity of vestibular pathways [2].

Animal studies indicated the presence of neural pathways between the vestibular nucleus and parts of the limbic system, including the hypothalamus. Thus, the stress response triggered by vestibular signs can improve synaptic and neural plasticity through the vestibular system [3, 4]. Clinical evidence suggests that environmental stressors can play either a constructive or disruptive role in the process of vestibular compensation. Therefore, if we promote our knowledge about the mutual interaction of stress and compensation, we may be able to manage the process. This would be especially invaluable in the case of recovery of patients with vestibular lesions.

The effectiveness of steroids in the treatment of acute vestibular symptoms manifests the importance of acute stress response management in facilitating vestibular compensation [1].

The stress response is complex and involves a group of chemical mediators such as neuropeptides, steroidal, and monoamine hormones. The hypothalamus-hypophysis axis (HPA) is a mediator of the canonical stress response. Corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) release from the paraventricular nucleus (PVN) and cause adrenocorticotropic hormone (ACTH) secretion from the pituitary gland which, in turn, triggers glucocorticoids' release from the adrenal cortex [1].

Neural connections between vestibular system and HPA have been identified in anatomical studies. For example, viral retrograde tracing in the rat brain has shown a direct vestibular-paraventricular route as well as a PVN-vestibular route [5].

There is a strong correlation between vestibular and psychiatric symptoms (anxiety, stress, and a history of mental illness) [6]. In addition, several studies have demonstrated that consuming antipsychotic and antidepressant drugs in the acute phase of vestibular symptoms accelerates and improves the degree of compensation [7]. An animal study, however, revealed contradictory results: in contrast with the study that found imbalance improved in mice by using anxiolytic drugs [4], another study did not report any beneficial effects of antipsychotic drugs in promoting imbalance, or even any change in the animal's anxiety level or locomotion after bilateral vestibular loss [8].

Caloric restriction in food diet or fasting leads to a mild stress induction in the neural system, possibly due to the reduction of available energy (glucose). Calorie reduction (CR) can be applied in the form of reducing daily calorie intake (e.g. 60%) or fasting by not receiving food (intermittent fasting or prolonged fasting) [5]. Intermittent fasting (IF) is administered as a normal diet with intermittent fasting (e.g. fasting every two days) [9, 10]. According to clinical observations, fasting increases the level of vigilance and improvement of mood, well-being and, in some cases, euphoria, which has been confirmed by several studies [11-13]. In contrast, the long-term or acute calorie restriction can also lead to depression and anxiety behaviors [14].

Another type of known stressor is deprivation from maternal care at different stages of life. Early maternal separation (MS) is a well-studied, well-known type of environmental stressor, which could seriously affect people's well-being and physical and mental health [2].

Stress can act as a double-edged sword in the process of vestibular compensation: In contrast with potentially beneficial stressors such as IF, some types of stressors could be harmful and destructive, with life-long consequences. Human studies are very scarce and contradictory in this area; however, some reports supported the possible harmful cumulative effects of multiple stressor types [1, 15-17]. Due to the above-mentioned link between mental health, cognitive deficits, and vestibular compensation, it would be valuable to conduct more investigations in this domain. Nevertheless, almost no research was conducted on this topic. More studies in this area can pave the way for better treatments and more efficient therapeutic strategies to promote the process of compensation.

Herein, we aimed to evaluate the vestibular compensation process in rats by inducing unilateral vestibular loss by intra-tympanic injection of gentamycin in chronically stressed rats, with stress being induced by maternal separation and intermittent fasting.

Methods

Animals

Animal preparation

Fifty-six male Wistar rats (seven rats per group) were held in a temperature-controlled environment with an artificial light/dark cycle (12 h). All the groups except for those receiving a fasting diet had free access to a standard pellet diet and water ad libitum. The groups included:

1) Healthy controls (HC)

2) The sham-saline group

3) Unilateral vestibular de-afferentiated rats (UVD)

4) The intermittent fasting group (IF)

5) Early maternally separated group (MS)

6) The group receiving MS and UVD (UVD+MS)

7) The group receiving IF and UVD (UVD+IF)

8) The group receiving all the interventions (UVD+MS+IF)

Treatments

Maternal separation

Every mother was held with her offspring in a separate cage. From day one after birth, at 9 AM, the pups were isolated from their mother for three hours (They were transferred to another cage in the same room, but the mother stayed at the home cage). After three hours, the pups were returned to their mother. During separation time, the offspring of every mother were held with their cage-mate brothers/sisters. The process of separation was continued for two weeks [2].

Intermittent fasting diet

After the breastfeeding period (from birth to day 21), the offspring were weaned off and separated from their mother (Only the male animals were preserved). From postnatal day (PND) = 26 for eight weeks, the animals received a fasting diet: One day, they received normal food, and every other day, they were deprived of that. The animals had free access to water all the time [9].

Intra-tympanic gentamycin injection

At the age of three months (PND=90), the animals received gentamycin (27 mg/ml, about 0.5 ml for each rat), directly injected to the left middle ear cavity by an insulin syringe passing tympanic membrane layers. The rats were maintained in the injection position (left ear upside) until the end of anesthesia. The injected drug was infiltrated into the inner ear from the round window, causing the ablation of vestibular hair cells and their afferent nerve endings [3, 18, 19].

In the sham-saline group, an equal amount of normal saline (0.5 ml) was injected into the middle ear cavity, exactly in the same way as gentamycin, to exclude the possible effects of injection on middle ear function or afferent's physiology [3].

Evaluations

Serum cortisol evaluation

The blood samples were gathered from the posterior-orbital and orbital venous sinus of the animal. We had to apply this method since the serum cortisol level had to be measured before and after UVD.

Sampling was performed by capillary heparinized tubes, under general anesthesia applied by ketamine/

xylazine injection (0 mg/kg–5 mg/kg) [20]. The samples were centrifuged and refrigerated for later analyses.

Sampling was conducted on PND=91 for healthy control, sham-saline, MS, and IF groups (These groups were sacrificed subsequently). For the UVD-treated groups (UVD, UVD+IF, UVD+MS, and UVD+MS+IF), the blood samples were taken three months after injection (PND=180). However, based on previous studies, significant changes in corticosterone levels were observed only in aged rats (eight months or older) [21]; hence, it did not seem necessary to torture the UVD-treated animals by repeated blood sampling. Therefore, the corticosterone levels of UVD-treated groups were compared with the data from the healthy control group.

Balance function evaluation

The rotarod apparatus was used for balance function measurement. To assess the effect of interventions on balance and motor function and their recovery after UVD, an accelerating rotarod (Hugo Sachs Electronic, Hamburg, and Germany) was employed. The rotarod started from a speed of 10 rounds per min (RPM) to the maximum speed of 60 RPM. Three trials were performed for each rat (inter-trial interval=30 min), with each trial lasting for a total maximum of 300 s. The total duration that each animal spent on the rod while maintaining its balance without falling was recorded as a measure of motor coordination and balance.

Locomotor activity and anxiety-like behavior evaluation

The open field apparatus consisted of a square arena [90×90×50 cm] made of Plexiglas; its floor was divided into 16 squares, and the field was divided into central and peripheral squares. Twenty-four hours and seven days after separation the rats were placed in the middle of an open field chamber and their behaviors were recorded and analyzed by an automated video tracking system (Ethovision; Noldus Technology, Wageningen, the Netherlands) during a 5-min period. The following parameters were recorded for each animal: total distance moved (cm), the number of groomings and rearings, and the time spent in the periphery (outer zone) and center (inner zone). After each test, the animal was removed from the box, and the chamber was cleaned with a cotton cloth and 10% ethanol.

Statistical analysis

The data were analyzed in SPSS 20. All the data were expressed as mean±SEM.

The level of statistical significance was considered $p<0.05$. An analysis of variance (ANOVA) followed by Tukey's post-hoc analysis and t-test were used to compare the differences between groups. Additionally, the Spearman correlation coefficient analysis was performed to study the relationship between anxiety level and the balance function.

Results

Serum cortisol evaluations

Serum cortisol measurements were performed either on PND 91 (for HC, sham-saline, MS, and IF group) or three months after UVD (in the case of UVD-treated groups) (Figure 1). Significant changes in serum cortisol levels were observed in all the groups, except for the sham-saline and the IF+UVD group ($p<0.05$).

Behavioral measures

The effect of unilateral vestibular deafferentiation and intermittent fasting on balance function

Figure 2 depicts the results of rotarod evaluations. Compared with sham-saline rats, all the groups (UVD, UVD+MS, UVD+IF, UVD+MS+IF, and the sham-saline group) showed a reduction of time spent on the rod two weeks after gentamycin injection ($p<0.05$). Due to the goal of this evaluation (studying the interactive effect of interventions UVD, IF, and MS), the ELISA results in IF and MS groups are not shown.

Additionally, to study the process of balance recovery over time, the rotarod evaluations were repeated three months after UVD. Except for the sham-saline group ($p=0.2$), in the other groups, the durability of animals on the rod was still significantly less than the healthy control group (all $p<0.05$), indicating that the reduction of time on the rod was continued up to three months after injection.

However, the main finding of the rotarod evaluations was the time that the animals stayed on the rod two weeks after the lesion compared with three months after that. Only the UVD+IF group stayed significantly longer on the rod ($p=0.04$). The other groups, however, had better function (more time) on the rod three months after

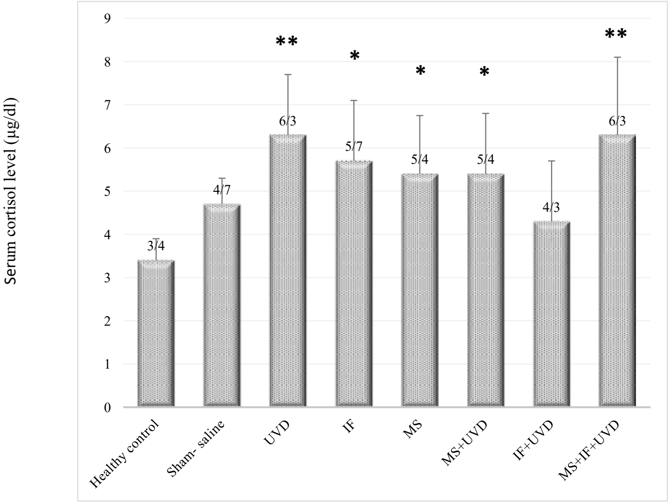

Figure 1. Serum cortisol means±SD levels (μg/dl) (n=7); all experimental groups differed significantly from the healthy control group, except for the sham-saline and the IF+UVD group. UVD; unilateral vestibular de-afferentiation, IF; intermittent fasting, ms; maternal separation

*p≤0.05, **p≤0.01

the lesion, but the difference did not reach the level of significance.

Locomotor activity and anxiety-like behavior evaluation

The open field test was performed to study the effect of interventions on anxiety and locomotion. The total distance traveled by the animals significantly differed across the groups (p=0.02). Post-hoc analysis showed that the MS+IF+UVD group traveled significantly less distance compared with healthy control animals (p=0.02). However, no significant difference was observed in the covered distance between controls and other groups.

No significant difference was seen between groups on the other parameters of open field evaluation (all p>0.05). Hence, UVD, MS, or IF did not exert a significant effect on rearing, grooming, and the frequency of entrance to the inner and outer zones of the apparatus.

Discussion

Previous studies on the process of vestibular compensation proved that it could be modified by environmental stressors. Thus, the question that arises is whether the type of stressor matters in these modifications. In the present study, we aimed to investigate the effect of two

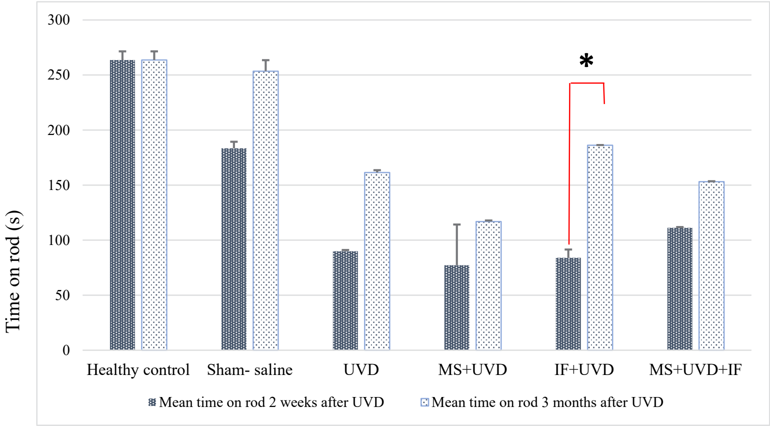

Figure 2. Rotarod test results, two weeks and three months after UVD. Only the IF + UVD group stayed significantly longer on the rod three months after UVD (in comparison with 2 weeks after that). UVD; unilateral vestibular de-afferentiation, MS; maternal separation, IF; intermittent fasting

*p ≤ 0.05

well-known types of good and bad stressors, the intermittent fasting diet and maternal separation, respectively, on the vestibular compensation process.

Based on our results, except for the sham-saline and the IF+UVD groups, the cortisol levels were significantly higher in all the groups compared to the healthy control group.

Since all the animals were treated identically before the UVD intervention, it could be supposed that the hormone levels of UVD-treated animals before the injection were equal to the healthy control group. Consequently, cortisol levels showed a significant increment in all UVD-treated groups, except for IF+UVD, three months after UVD. Thus, the corticosterone levels subsided to normal limits only in the UVD+IF group.

Serum cortisol is among the most studied stress-mediated hormones. Corticosterone levels increase after unilateral vestibular loss [1]. However, this increment is modified via a magnificent feedback mechanism, so that the cortisol levels return to normal limits in healthy animals [22]. Turning HPA back to its activity is essential for adequate compensation, resulting in the recovery of annoying symptoms of imbalance and dizziness.

In the sham-saline group, two hypotheses could be imagined. First, the cortisol level may be returned to the normal limits after 90 days, which implies that the saline-injected rats passed the process of compensation normally. Second, they might not develop any injury within the vestibular system. Since they have shown symptoms of imbalance and motor dysfunction on the rotarod test, this hypothesis has less strength than the former one.

Balance function and its recovery were evaluated by rotarod testing. Upon rotarod evaluations:

1) All the animals had less durability on the rod two weeks after the injection. Compared with the healthy controls, the capability of the animals to stay on the rod was significantly reduced for all groups two weeks after injection. Hence, it seems that the intratympanic gentamycin injection procedure successfully induced symptoms of vestibular dysfunction.

2) Three months after that, all of the groups (except for the sham-saline group) remained less on the rod. Three months after the injection, the durability of all animals on the rod (except for the sham-saline group) was still significantly less than the healthy control group. Therefore, it seems they could not fully recover from the symptoms of vestibular disorder, at least up to three months after UVD.

3) Three months after the injection, although all the animals had better durability on the rod in comparison with two weeks after injection, only the IF+UVD group showed significantly better results. Although the findings of rotarod evaluations indicated an upgrade of motor function and balance for all the study groups, this promotion reached the significance level solely for the IF+UVD group. In other words, only the IF+UVD animals could significantly recover 90 days after the injection.

The adaptive response to reduced calorie intake resulted from IF may involve the complex interaction between the hormones controlling energy balance, appetite, cell proliferation and apoptosis, metabolic rate, inflammation, stress response, and repair systems [23]. Glucocorticoids and insulin, as the main mediators of energy balance, play reciprocal roles in homeostasis in mammals. Normally, hypoglycemia increases the serum cortisol levels, which raises the blood glucose levels (due to the inhibition of glucose transporter into the cells).

Fasting, starvation, or insulin-induced hypoglycemia elevates glucocorticoid levels [24]. Although it was once proposed that physiologic levels of endogenous glucocorticoids stimulate the inflammatory response as part of the general adaptation to stress [25], it now appears that their physiologic role during stress is to protect the organism from an overstimulated inflammatory response.

It is now generally accepted that many of the anti-inflammatory and antimitogenic effects of glucocorticoids are mediated by the glucocorticoid-inducible protein lipocortin 1 [25]. Lipocortin 1 (also known as annexin 1) is a glycosylated 37-kDa $Ca2^+$ dependent phospholipid-binding protein that inhibits phospholipase A2, a key enzyme in the synthesis of inflammatory prostaglandins and leukotrienes from arachidonic acid [25].

To study the effect of stressors on anxiety and locomotor activity, an open field evaluation was performed. Despite some reports of alterations in locomotor activity and showing anxiety after UVD [1], herein, we did not observe any change. A possible cause of this observation could be related to the dose of the drug used for UVD induction, as we did not use very high doses of gentamycin (e.g. 80 mg/Kg). Heavy doses of the drug could completely ablate the vestibular organ, which leads to intense symptoms of vestibular syndrome. In cases of massive lesions of the vestibular end organ, the process of compensation may be potentially incomplete (even unmeasurable). Therefore, selecting very high doses of gentamycin could blur the slight modifications resulting from compensation at the behavioral or hormonal level.

Conclusion

For the first time, the present study demonstrated that the type of environmental stressor could strongly alter the vestibular compensation after peripheral lesion. As shown in the previous works, the IF diet managed to improve the recovery of the nervous system after lesion. In contrast, maternal separation (MS) degraded the process of recovery. A very interesting finding was the combined effect of these two types of stressors; although intermittent fasting (IF) seemed to play a helpful role in compensation, it lost its beneficial effects when accompanied by another type of stressor (MS). Generally, the findings support the beneficial effects of IF on the process of recovery after sensory vestibular damage; however, caution should be exercised when applying it in the presence of other potential stressors.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Animal Care and Ethics Committee of Kerman University of Medical Sciences (Ethics Code: EC/KMU/940196).

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Authors' contributions

SS: Study design, data collection, analysis of the results and writing the manuscript; MAS: Study design, stimulus preparation, data collection, analysis of the data, interpretation and writing the manuscript; MK: Study design, IA: Data collection; FD: Data collection; ZV: Data collection.

Conflict of interest

The authors of the present study declare no conflict of interest.

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