Forced swimming stress does not affect monoamine levels and neurodegeneration in rats

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Abstract: Objective The current study was aimed to investigate the correlations between immobility time in the forced swimming test (FST, a behavioral indicator of stress level) and hippocampal monoamine levels (markers of depression), plasma adrenalin level (a peripheral marker of stress) as well as fluoro-jade C staining (a marker of neurodegeneration). **Methods** Male Sprague-Dawley rats were subjected to acute, sub-chronic (7 d) or chronic (14 d) FSTs and immobility time was recorded. Levels of noradrenalin, serotonin and dopamine in the hippocampus, and adrenalin level in the plasma were quantified by high-performance liquid chromatography with electrochemical detection. Brain sections from rats after chronic forced swimming or rotenone treatment (3 mg/kg subcutaneously for 4 d) were stained with fluoro-jade C. **Results** The rats subjected to swimming stress (acute, sub-chronic and chronic) showed long immobility times [(214 ± 5), (220 ± 4) and (231 ± 7) s, respectively], indicating that the animals were under stress. However, the rats did not exhibit significant declines in hippocampal monoamine levels, and the plasma adrenalin level was not significantly increased compared to that in unstressed rats. The rats that underwent chronic swimming stress did not manifest fluoro-jade C staining in brain sections, while degenerating neurons were evident after rotenone treatment. **Conclusion** The immobility time in the FST does not correlate with markers of depression (monoamine levels) and internal stress (adrenalin levels and neurodegeneration), hence this parameter may not be a true indicator of stress level.

Keywords: forced swimming test; immobility time; noradrenalin; serotonin; dopamine; adrenalin; neurodegeneration

1 Introduction

Depression is a prevailing neuropsychiatric disorder that affects approximately 20% of the world population. In terms of the global burden of disease, it is estimated

to rank third, and is expected to lead by the year 2030 (The global burden of disease, WHO 2004). According to McKinney and Bunney^[1], an animal model should fulfill the criteria of resemblance in etiology, symptomatology and treatment of the respective disease. It is extremely difficult to mimic the symptoms of depression in laboratory animals, such as suicidal thoughts and feelings of guilt. Nevertheless, various animal models have been developed to assess the antidepressant potential of substances.

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Because of its simplicity, high specificity and reliability, the forced swimming test (FST) remains the most widely used rodent model of depression^[2,3]. In FST, the animal is subjected to an inescapable situation, i.e. a water-filled tank. Following initial escape-oriented behavioral movements (jumping, swimming and diving), the animal exerts immobility (a sign of behavioral despair) which is reduced by antidepressants^[4]. Hence the immobility time (the time of immobility or only making movements required to keep the head above water) is the parameter noted in the FST and serves as an indicator of stress level.

The monoamine hypothesis of depression states that reduction in the levels of monoamines [serotonin (5-HT), noradrenalin (NA) and dopamine (DA)] in the brain underlies the pathophysiology of depression, and that antidepressants act by returning their levels to normal^[5]. High-performance liquid chromatography (HPLC) with electrochemical detection (ECD) is the most widely used method for the measurement of monoamine levels in biological samples^[6]. In rat brain, the hippocampus is the most studied region because it is a target for stress hormones^[7] and is associated with 3 major hypotheses of depression i.e. those based on monoamines, neurogenic mechanisms, and the hypothalamic-pituitary-adrenal (HPA) axis^[8,9]. Stress increases the level of circulating adrenalin, a hormone secreted by the adrenal medulla that helps to cope with stress^[10].

Stress may also lead to neurodegeneration, most likely via elevated cortisol^[11,12], whereas neurogenesis is considered to be a compensatory phenomenon, leading to the formation of new neurons to replace those that degenerated^[13]. Hematoxylin and eosin staining (hematoxylin for nuclei, eosin for cytoplasm) is a conventional method for studying neurodegeneration, but is prone to produce “false-positive” results. However, a novel fluorochrome, fluoro-jade, is considered to be a highly sensitive and reliable marker of degenerating nerve cell bodies, dendrites, axons and terminals^[14]. It is an anionic dye that incorporates into degenerating neurons independent of the nature of the insult (chemical, environmental or physical). Specifically, fluoro-jade C has a greater signal-to-noise ratio and a higher

resolution than its predecessors fluoro-jade and fluoro-jade B^[15].

Reports vary regarding the effect of stress on monoamine levels in rat hippocampus. Our preliminary studies (unpublished data) showed no effect of acute FST on monoamine levels. With this in mind, we set out to address the question of whether animals are under stress during FST, by monitoring hippocampal monoamine levels (markers of depression), plasma adrenalin level (a peripheral marker of stress) and fluoro-jade C staining (a marker of neurodegeneration), and correlating them with measured immobility time.

2 Materials and methods

2.1 Animals Male Sprague-Dawley rats (weighting 150–250 g) were obtained from the Animal Care Facility of the International Center for Chemical and Biological Sciences (ICCBS), University of Karachi. The animals were housed under standard environmental conditions at $(25 \pm 1)^\circ\text{C}$ with a 12:12 h light/dark cycle. Food and water were available *ad libitum*. All experiments were performed in accordance with the guidelines of the National Institute of Health (NIH publication No. 85-23, revised in 1985) for the care and use of laboratory animals.

2.2 Chemicals Adrenaline hydrochloride, ethylenediaminetetraacetic acid (EDTA) disodium salt and sodium chloride were all from Sigma, USA. Fluoro-jade® C, acetic acid, hydrochloric acid and rotenone were from Sigma-Aldrich, Germany. Acetonitrile, methanol and 1-octane sulfonic acid were from Fisher Chemicals, UK. Chromium (III) potassium sulfate and paraformaldehyde were from Merck, Germany. Other chemicals were sodium dihydrogen citrate anhydrous (Fluka, Germany), xylene (Scharlau, Spain), isopropyl alcohol (Tedia Co., USA) and deionized water (Millipore simplicity 185 deionizer).

2.3 Animal grouping and FST For measurement of monoamines (noradrenalin, adrenalin, serotonin and dopamine), the rats were divided into unstressed, acute FST stress (FST conducted once) and sub-chronic FST stress (FST conducted for 7 consecutive days) groups ($n = 6$ –8 per group). For fluoro-jade C staining, the animals were di-

vided into unstressed, chronic FST stress (FST conducted for 14 consecutive days) and rotenone treatment (3 mg/kg subcutaneously for 4 d without FST) groups ($n = 3$ per group).

In all FST stress groups, rats were subjected to FST for 6 min, of which the first minute was for acclimatization. The immobility time was recorded manually using digital count-down and count-up timers. Briefly, after the animal was placed in the FST tank, the count-down timer (set at 6 min) began timing. After 1 min, immobility time recording was started with a count-up timer (set at 0 min) within the following 5 min. During the 5 min, the time of each immobility was added to the last reading.

2.4 Measurement of monoamines For measurement of NA, 5-HT and DA, animals were decapitated using guillotine and the hippocampi were collected and stored at -80°C until analysis. The tissues were weighed and homogenized in 1 mol/L perchloric acid (100 mg/mL), followed by centrifugation at 15 000 rpm for 20 min at 4°C . The supernatant was collected, filtered using a syringe-driven filter unit (0.22 μm , Millex-GV, Millipore, Bedford, USA) and immediately subjected (20 μL) to monoamine analysis by HPLC-ECD (Shimadzu, Kyoto, Japan).

For adrenalin measurement, blood was collected by decapitation and centrifuged at 3 000 rpm for 15 min. Plasma was collected and stored at -80°C for analysis. Plasma (100 μL) was purified by ultrafiltration^[16,17] (centrifugal filters with a molecular weight cut-off at 10 000; Microcon, Ultracel YM-10, Millipore) at 14 000 g for 30 min at 4°C . The resulting filtered plasma (20 μL) was immediately subjected to HPLC-ECD for adrenalin measurement. The citrate buffer (0.1 mol/L sodium dihydrogen citrate anhydrous, 1 mmol/L EDTA and 2.5 mmol/L 1-octane sulfonic acid, pH 3.4) with 10 % acetonitrile at a flow rate of 0.5 mL/min and a reversed phase nucleosil column (C18, 250/4.6, 5 μm , Macherey Nagel) were used.

2.5 Fluoro-jade C staining The animals were anesthetized by thiopental sodium (60 mg/kg) and blood was cleared by intracardial administration (peristaltic pump Eyela, Japan) of phosphate buffer saline (0.05 mol/L) along with cutting the auricle. After clearing (indicated by

de-coloration of liver), rats were perfused with 4% paraformaldehyde (PFA). The brain was dissected and post-fixed in PFA for 3 d followed by dehydration, xylene infiltration and paraffin embedding. Sagittal brain sections cut at 6 μm were stained with fluoro-jade C (Millipore, USA) as described previously^[14] and observed under a Nikon Eclipse TE2000-E fluorescence microscope.

2.6 Statistical analysis The immobility time and hippocampal and plasma levels of monoamines were expressed as mean $\pm$ SEM ($n = 6-8$). Comparison between various means was made by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) using SPSS 11.0.

3 Results

The acute, sub-chronic and chronic FST resulted in statistically similar immobility times [(214 $\pm$ 5), (220 $\pm$ 4) and (231 $\pm$ 7) s, respectively] ($n = 6-8$).

As shown in Table 1, acute FST stress did not induce significant changes in NA or DA levels but a significant increase in 5-HT level in the hippocampus compared with unstressed rats. Furthermore, sub-chronic FST stress did not induce significant changes in any of the monitored monoamines compared with unstressed rats.

In addition, neither acute nor sub-chronic FST stress induced any significant changes in plasma adrenalin levels compared to unstressed rats [(8 $\pm$ 1), (11 $\pm$ 2) and (9 $\pm$ 2) ng/mL respectively].

The brain sections from unstressed and chronic FST stressed (FST conducted for 14 d) animals did not exhibit positive fluoro-jade C staining, whereas the stained degen-

Table 1. Effects of acute and sub-chronic forced swimming stress on monoamine levels in rat hippocampus (ng/g tissue)

Groups	NA	5-HT	DA
Unstressed	598 $\pm$ 34	246 $\pm$ 23	11 $\pm$ 1.5
Acute forced swimming stress	564 $\pm$ 11	333 $\pm$ 18*	12 $\pm$ 0.7
Sub-chronic forced swimming stress	512 $\pm$ 29	261 $\pm$ 25	15 $\pm$ 2.4

Data are presented as mean $\pm$ SEM. $n = 6-8$. * $P < 0.05$ compared to unstressed group. NA: noradrenalin; 5-HT: serotonin; DA: dopamine.

Fig. 1 Effects of chronic forced swimming stress for 14 d (A) and rotenone treatment (3 mg/kg for 4 d) (B) on fluoro-jade C staining of neurons. Scale bars: 80 μ m.

erating neurons were evident in the rotenone-treated group (Fig. 1).

4 Discussion

FST is the most commonly used tool to assess the antidepressant potential of various substances. Long immobility time is a sign of behavioral despair which matches the symptom of depression. Likewise, substances that reduce the immobility time are considered as antidepressants. So the immobility time indicates the state of animals i.e. depressed or not depressed / stressed or unstressed. The rats subjected to acute, sub-chronic and chronic FST exhibit long immobility time, the magnitudes of which are similar to those exhibited by stressed animals^[18,19], hence reflecting a depressed state. This also implies that the maximal stress is induced on the first day of FST and does not increase upon repetitive exposure. Thus, the behavioral outcome in rats due to the FST is independent of the duration of stress.

A decrease in the levels of monoamines is considered to be an underlying cause of depression^[5] and animal models should fulfill the criteria of resemblance in etiology, symptomatology and treatment of the respective disease^[1]. However, in the present investigation, neither acute nor sub-chronic FST induced any significant reduction in the levels of NA, DA or 5-HT except 5-HT of acute FST rats, as compared to unstressed (Table 1), which is incompatible with the well-known monoamine hypothesis of depression.

Notably, after acute FST stress, the 5-HT level was significantly elevated which is suggestive of its enhanced synthesis and can possibly be linked with homeostatic response in the brain. However, this unusual observation is worthy of detailed investigation in order to shed light on neurochemical changes induced by FST. Previous studies have also reported variable results regarding the effect of stress on the levels of monoamines in rat hippocampus, including increases^[20–22], decreases^[23,24] and no changes^[23,25,26]. These inconsistencies can be attributed to the species, age and sex of animals used, as well as the type and the intensity of stress. For example, in the hippocampus, the FST does not significantly alter the 5-HT levels in rats^[25] whereas it induces a significant decline in mice^[26]. Similarly, in hippocampus of rats, the chronic mild stress significantly reduces the 5-HT levels^[23] whereas chronic restraint stress elicits a significant increase^[20]. In addition, it is well-documented that stress increases circulating adrenalin levels^[10]. Here, neither acute nor sub-chronic FST stress significantly altered adrenalin levels compared with unstressed rats. This suggests that the animals were not under stress, thereby supporting the lack of FST stress-induced declines in monoamine levels found in this study. Furthermore, forced immobilization stress has been reported to cause an increase in circulating adrenalin levels by stimulating the sympatho-adrenal medullary system^[27], which becomes significant after 30 min, indicating the impor-

tance of duration of stress^[28]. Likewise, here the absence of changes in adrenalin levels after FST stress may be due to the inadequacy of the commonly used 4–6 min FST stress to stimulate the adrenal medulla and significantly alter adrenalin levels.

Stress has been shown to cause neurodegeneration, most likely via elevated cortisol levels^[11,12]. Olfactory bulbectomy, which serves as a model of depression, has been reported to cause neurodegeneration^[29], whereas to our knowledge the present study was the first to assess the effects of FST on neurodegeneration. Rotenone treatment is a well-established method to induce neurodegeneration. In this study, fluoro-jade C staining revealed that the brains of rotenone-treated animals (a model of Parkinson's disease^[30], a positive control) had injured neurons, however, 14 d of FST stress (chronic) did not cause neuronal damage (Fig. 1). This is consistent with a report^[31] that the immobility time in FST has no correlation with the level of the stress hormone cortisol.

In conclusion, animals exhibiting long immobility time (depressed) in the FST showed neither a decline in hippocampal monoamines nor elevated adrenalin levels. Neurodegeneration was not observed either. It therefore can be inferred that the physical stress indicator (immobility time) has no detectable correlation with the internal markers of depression/stress (monoamines, adrenalin and neurodegeneration). Thus, it is questionable whether the immobility time in FST really indicates that an animal is under stress.

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强迫性游泳压力对大鼠单胺水平和神经退行性变化没有影响

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摘要: 目的 本文旨在研究强迫性游泳试验中的不动时间(压力的行为性指示)与海马中单胺水平(抑郁指标)、血浆中肾上腺素水平(循环系统中的压力指标)以及神经退行性变化(fluoro-jade C染色法检测)的关系。**方法** 给予雄性Sprague-Dawley大鼠急性、亚慢性(7天)或慢性(14天)强迫游泳的压力,并在强迫游泳试验中记录大鼠的不动时间。试验结束后,用高效液相色谱电化学检测法测定大鼠海马中去甲肾上腺素、5-羟色胺和多巴胺以及血浆中肾上腺素的水平,并对大鼠脑片进行fluoro-jade C染色。**结果** 急性、亚慢性和慢性压力的大鼠均显示出较长的不动时间,分别为(214 ± 5) s, (220 ± 4) s和 (231 ± 7) s,表明这些大鼠都处在压力状态下。然而,与非压力状态下的对照组大鼠相比,急性和亚慢性压力下的大鼠海马中单胺类分子的水平并没有显著降低,并且血浆中肾上腺素的水平也没有显著升高。此外,慢性压力大鼠的脑片呈现出fluoro-jade C染色阴性。**结论** 强迫性游泳试验中的不动时间与抑郁和压力并不相关,提示此法可能不能准确地指示压力水平。

关键词: 强迫性游泳; 不动时间; 去甲肾上腺素; 5-羟色胺; 多巴胺; 肾上腺素; 神经退行性变化