Differential effects of chronic predictable and unpredictable stress on neurobehavioral and biochemical responses in rats

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The present study was designed to investigate the effects of exposure to chronic predictable and unpredictable stress on neurobehavioral and biochemical responses in rats. Male Wistar rats (200-250g) were exposed to either chronic predictable stress (CPS) i.e. immobilization for 1 hour/day for 14 days or chronic unpredictable stress (CUS) i.e. daily a different, random, novel stressor sequence (immobilization stress for 1h, footshock, cold stress, overnight food and water deprivation and social isolation) for 14 days. Behavioral responses were assessed by the elevated plus maze (EPM) test and biochemical parameters, viz. malondialdehyde (MDA, a marker of oxidative stress) and stable NO metabolites (NOx, marker of nitrosative stress), were measured in brain homogenates of the rats. Exposure to chronic CPS resulted in adaptation to neurobehavioral suppression in the EPM (as observed after acute Restraint stress), which was not seen after CUS. These behavioral changes after CPS and CUS were closely paralleled by alterations in the levels of brain MDA and NOx. These results suggest that CPS and CUS results in differential modulation of the neurobehavioral profile and oxidative/nitrosative stress markers in the brain.

Keywords: Chronic predictable stress; Chronic unpredictable stress; Elevated Plus Maze; Malondialdehyde; Nitric Oxide


Introduction

Stress is an integral part of our life and pathophysiological conditions related to stress are subject of extensive research in recent years. Exposure to stress activates the Hypothalamo-Pituitary-Adrenal (HPA) axis and result in increased release of Corticotrophin Releasing Hormone (CRH), adrenocorticotropic (ACTH) and glucocorticoids from the hypothalamus, anterior pituitary and adrenal cortex respectively [1]. Glucocorticoids prepare the organism for coping with stress induced changes in energy and metabolism by affecting the expression of various proteins by regulating genes through nuclear receptors. Chronic stress is reported to be involved in the etiopathogenesis of a variety of disease states viz. hypertension, coronary heart disease, gastric ulcers, diabetes, immunosuppression, mental depression and memory loss. To survive through any external or internal challenges/stress, all living organisms maintain an extremely complicated equilibrium known as ‘homeostasis’. This harmony is constantly perturbed by the stressors and an organism copes with such situations by adapting to such aversive stimuli. Effects of stress on an organism depend upon various factors viz. type, intensity, and the duration of a particular stressor or physiological factors like strain/gender of the subjects. Stressors, particularly of chronic nature, are known to influence the physiological milieu and disturb the homeostasis resulting in either disease states or development of adaptive mechanisms [2]. Psychological stress includes social conflicts, competition for resources, etc. which accompanies anxiety and fear [3].
Immobilization/restraint stress can produce both physical and psychological stress together and can induce visible changes in behavior as well as internal biochemical changes in an organism \(^6\). Different responses to acute and chronic stress have been documented on various systems viz. neuroendocrine, visceral and immune systems. It is widely accepted that acute stress tends to enhance immune functioning, whereas chronic stress results in suppressed immune response \(^5\). Similarly effects of restraint stress in male and female genders have been reported to be different \(^6\). Further, predictable or unpredictable stress exposure can differentially affect various responses but these mechanisms are not well defined. Thus, stress may represent a modulator of various responses, whose outcome depends on a multitude of factors. Many studies have reported the effects of various stressors but still there is a paucity of studies which can indicate/establish the differences in response to chronic predictable stress or unpredictable stress. The present study was thus designed to investigate effects of chronic predictable and unpredictable stress on neurobehavioral and biochemical markers in rats.

**Material and Methods**

**Experimental Animals**

Inbred male Wistar rats weighing 200–250 g were used for the study and maintained under standard laboratory conditions of temperature (22±2 °C) and a 12 h light: 12 h dark cycle. Six rats were used in each group and they had free access to food and water throughout the experiments. The study protocol was approved by the Institutional Animal Ethical Committee (IAEC) and guidelines prepared by Indian National Science Academy (INSA), New Delhi, on care and use of animals in scientific research were followed.

**Grouping of Animals**

Animals were randomly divided into following groups, Group 1 served as Control which was not exposed to Restraint Stress (RS), Group 2 was given single exposure to RS for 1h. and served as acute RS group, Group 3 was exposed to Chronic Predictable Stress (CPS) i.e. same stressor was given daily and Group 4 was exposed to Chronic Unpredictable Stress (CUS) i.e. exposure to different stressors as per protocol mentioned below.

**Stress protocols**

1) Chronic predictable stress (CPS) - Rats were immobilized/restrained for 1 hour/day at room temperature in specific Plexiglas restrainers (INCO, Ambala) \(^2, 7\), for 14 days i.e. multiple sessions of same stress were employed in case of CPS. Restraint stress is a widely used and a validated animal model of inducing emotional stress as it ensures minimum movement including that of tail and involves no pain. This technique induces all the components of stress response viz. activation of the HPA axis thus resulting in elevation of blood pressure and heart rate \(^8, 9\).

2) Chronic unpredictable stress (CUS) - Rats were subjected to one stressor daily that followed a sequence for 14 days \(^10, 11\). Five different stressors employed were: (i) immobilization stress for 1 hour; (ii) foot shock (in foot shock chamber 150 V, 2mA, for 3 min); (iii) cold stress (exposure in cold chamber at 4-6°Celsius for 1 hour); (iv) food and water deprivation overnight; (v) social isolation overnight. No single stressor was applied consecutively for 2 days and during 14 days of exposure, each stressor was applied two or three times.

Both CPS and CUS groups were exposed to immobilization stress for 1 hour on first day of exposure. After completion of the stress protocol the neurobehavioral parameters of animals were assessed in the Elevated Plus Maze (EPM).

**Preparation of brain homogenates**

After neurobehavioral assessment in the EPM the animals were sacrificed and their brains were dissected out, washed in ice cold isotonic saline and weighed. The brain was then minced, and a homogenate (10% w/v) was prepared in chilled phosphate buffer. The homogenate was used for estimating levels of malondialdehyde (MDA) and NOx.

**Neurobehavioral Studies: EPM test**

The elevated plus maze (EPM) test is a widely used and validated method for testing anxiety like behavior. This test involves exposure of animals to a conflict situation between exploring open versus enclosed arms of the maze \(^12\).

The EPM (Caterpillar Instruments) consisted of two open opposite arms and two enclosed arms with 40 cm high walls of the same measurement (40×10cm). The arms are connected in the center to give the maze a plus sign (+) like look. The entire maze was placed in a quiet dimly lit room, elevated 50 cm above ground. The individual rats were placed in the center of the elevated plus maze such that they face the closed arms. Then they were observed for five minutes by using Any Maze software and video tracking system, latest version 4.2 (Stoelting, U.S.A) for number of entries into the open and closed arms and time spent in them. One entry into an arm of the maze was defined by software when 75% of body of the animal crosses the middle square of the maze and enters into closed/open arm. Subsequently,
Table 1. Effects of chronic predictable stress (CPS) and chronic unpredictable stress (CUS) on the elevated plus maze (EPM) test parameters and brain MDA and NOx levels in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>EPM test parameters</th>
<th>Biochemical Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OAE (%)</td>
<td>OAT (%)</td>
</tr>
<tr>
<td>Control</td>
<td>30.2 ± 4.2</td>
<td>12.0 ± 1.5</td>
</tr>
<tr>
<td>CPS</td>
<td>27.3 ± 4.0</td>
<td>10.4 ± 1.8</td>
</tr>
<tr>
<td>CUS</td>
<td>16.2 ± 2.3a</td>
<td>7.8 ± 1.0a</td>
</tr>
</tbody>
</table>

All values are expressed as Mean ± S.E.M; n = 6 rats per group; a, p < 0.05 compared to control (no stress) group; MDA: Malondialdehyde; NOx: Nitric oxide (NO) metabolites

the percentage of open arm entries and the percent time spent in open arms with respect to total number of entries (in open arms plus closed arms) and total time spent in both open and closed arms (during 5 min exposure to EPM), respectively, were calculated, i.e. The percentage of time spent in open arms was calculated as follows:

\[
\text{% Open arm time} = \frac{\text{Open arm time}}{\text{Open arm time + Closed arm time}} \times 100
\]

The animals (rodents) usually have a tendency to remain in the closed arms in EPM, hence whenever there is an increase in the percent of open arms entries and/or open arm time, it indicates an anxiolytic state. This EPM test is a standard, time tested and well established technique of evaluating the anxiolytic or anxiogenic effects of stimuli or drugs [12, 13].

Biochemical Assays

Malondialdehyde (MDA) estimation

Malondialdehyde (MDA) levels, a marker of lipid peroxidation, was determined in the aliquots of brain homogenates of rats following method of Okhawa et al [14]. 0.2 ml of brain homogenate was added to the reaction mixture which consisted of 0.2 ml of 8.1% sodium lauryl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA). Distilled water was added to make up the volume of mixture to 4 ml and heated at 95 °C for 60 min. The test-tubes containing mixture were cooled and 5 ml of n-butanol and pyridine (15:1, v/v) and 1 ml of distilled water were added followed by centrifugation. The organic layer was separated out and its absorbance was read at 532 nm using a UV-visible spectrophotometer (UV 5740 SS, ECIL). Protein concentration in the samples were measured as per the method of Lowry et al. [15] and purified bovine serum albumin was used as standard. MDA content was expressed as nmol/mg protein.

Brain Nitric Oxide (NOx) estimation

NO is a highly labile and reactive molecule with a very short half-life. It gets quickly converted to the more stable nitrates and nitrites (NOx), which are good markers for NO levels. Brain NOx levels were determined by the method of Tracey et al [16] in which all nitrates present in sample were coupled with NADPH and FAD and converted into nitrites using aspergillus nitrate reductase. Brain samples were homogenized in 5 ml distilled water and centrifuged at 10,000 g for 15 min at 4 °C. 50 μl test sample/supernatant was added to the assay mixture containing 10 μl of 0.86 mM/l NADPH, 10 μl of 0.11 mM/l FAD, 10 μl nitrate reductase (2 U/ml) and 20 μl of 310 mM/l Potassium phosphate buffer. Samples were incubated at 37 °C for 1 h in the dark, followed by addition of 5 μl of 1 M/l Zinc sulphate which precipitated the proteins. After centrifugation, 50 μl of supernatant from each microtube was transferred into individual wells of 96 well microplate followed by addition of 100μl Griess reagent which resulted in color development. Absorbance was recorded after 10 min in 96 well assay plates at 540 nm in a UV-visible spectrophotometer (UV 5740 SS, ECIL). Standard curve was plotted using known concentration of Sodium nitrate and converted to NOx content by using a nitrate standard curve and data was expressed as NOx/mg protein.

Statistical analysis

The results were expressed as Mean ± SEM (n= 6 per group). The behavioral and biochemical values were analyzed by one-way analysis of variance (ANOVA) followed by Student’s t test. A p value of less than 0.05 was considered as statistically significant. Data analysis was performed using the software Graph Pad Prism for Windows Version 5.02; Graph Pad Software, Inc. USA.

Results

The EPM test is a robust experimental model for evaluating anxiogenic/anxiolytic responses and our present results showed that exposure to CPS and CUS elicited markedly different neurobehavioral responses in rats. Whereas CUS induced significant reductions in both OAE and OAT, as compared to control values (p < 0.05, in each case), such magnitude of changes were not observed in the
CPS group of rats (p > 0.05). In fact, the changes in % OAE of the CUS group were much greater than those seen in the CPS group (Table 1).

Acute RS induced suppressions in both EPM parameters and the values for % OAE and OAT were 12.7 ± 1.5 and 5.8 ± 1.9 respectively, as compared to 30.2 ± 4.2 (% OAE) and 12.0 ± 1.5 (% OAT) of the controls. Exposure to CPS and CUS induced differential nature of responses in the EPM parameters. Whereas CUS induced significant reductions in both OAE and OAT (p<0.05, in both cases) as compared to control (no stress) group, no significant differences were observed after CPS exposure (p>0.05). These results are summarized in Table 1.

Biochemical assay of the brain homogenates revealed that, in CPS exposed rats, MDA levels were elevated and NOx levels were lowered in comparison to controls (p<0.05, in each case). However, no such changes were seen in brain MDA or NOx levels in the CPS group of animals. Further, as observed in neurobehavioral studies, comparison of these biochemical markers between CUS and CPS showed that elevations of MDA levels and reduction in NOx levels were greater in magnitude in CUS as compared to CPS group of rats. These neurobehavioral and biochemical data are summarized in Figure 1.

Discussion

Stress activates the HPA and sympatho-adreno-medullary axes and elevates levels of corticosterone and catecholamines which are part of the humoral adaptive response to the stressor [1, 17]. However, when the similar type of stressor is applied it could result in decrease, increase or no change in these neuroendocrine markers [18-20]. The present study confirms earlier reports from our laboratory that in EPM test, acute RS produced neurobehavioral suppression as was evident from reduced % OAE and % OAT, suggestive of an anxiogenic response [2, 6, 21]. Acute RS served as the comparator for both CPS and CUS as both groups were exposed to restraint stress (RS) for 1 hr on first day of chronic stress schedule. The results of the chronic predictable stress (CPS) group were different from those of the acute group. Exposure to CPS reversed neurobehavioral suppression i.e. greater number of OAE and OAT as compared to that after single RS were observed in EPM, thus suggesting adaptation had occurred after such repeated stressful stimuli. Interestingly, in contrast to the CPS, exposure to CUS did not show adaptation to the anxiogenic response in the EPM test and % OAE and % OAT were significantly less than that observed in control and CPS groups. From these results it can be suggested that there are differences between CPS and CUS responses, and the unpredictable nature of the latter probably prevents the development of adaptation and coping strategies seen after CPS. Our results are supported by another study in which cocaine (a psychostimulant) treated animals reacted differently to predictable and unpredictable stress [9].

Earlier studies have shown that NO mimetics reversed stress-induced anxiogenesis while elevating brain NOx levels thus indicating that NO may act as a crucial regulator of stress responses [2, 21-24]. In agreement with these findings, our present study showed that brain NOx levels (stable metabolites of NO) decreased after acute RS. Similar to that observed in behavioral studies, CPS resulted in reversals of brain NOx levels towards normalcy - the magnitude of changes being lesser than seen after acute RS, indicating adaptation to stress. However, such adaptive changes were not seen after CUS.

As reported in earlier studies, acute RS resulted in increased brain MDA levels, a marker of oxidative stress, as compared with non-stressed rats. However, increased brain MDA levels in CUS were seen as compared to CPS thus showing increased lipid peroxidation and more oxidative damage during exposure to the latter stress paradigm.

Our findings are in general agreement with an earlier study of de Boer et al. [25], where it was reported that following chronic predictable noise stress the corticosterone levels came to a stable level as compared to chronic unpredictable noise stress in rats. However our results differ from those of Fabiola et al. [26] who reported more pronounced impairments after repeated restraint stress as compared to that seen after variable stress. Taken together, our results show that the impact of chronic predictable stress may differ from that of chronic unpredictable stress and rats
exposed to CPS, but not CUS, readily adapt to the stressor. Further, complex interactions between reactive oxygen and reactive nitrogen species may contribute to such effects.

Conflicting Interests

The authors do not have any conflicting interests in this study.

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