Influence of State and/or Trait Anxieties of Wistar Rats in an Anxiety Paradigm

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Elevated plus maze · Restraint · Stress · Population · Behavior

Abstract
Systematic individual differences between male Wistar rats can be detected in paradigms such as the elevated plus maze (EPM), which is a widely used behavioral paradigm that measures fear-motivated avoidance behavior. It has been extensively used to assess anxiety profiles with face, construct and predictive validities. During a typical EPM test, animals actively avoid the open arms in favour of the closed arms. We investigated whether individuals carry inherent trait anxiety profiles and whether perturbations of different intensities influence anxiety measures. Inherent anxiety levels and coping strategies following stress have become critical determinants in pre-disposition to other neuropsychiatric disorders and affect biomedical interventions in individuals. One group of rats was screened on EPM and in the activity box. Another set of rats were randomly divided into groups and subjected to perturbations of acute and sub-chronic isolation or restraint and tested in the EPM. Based on open-arm time in the EPM, low or high anxiety profiles were identified with significant differences in all measures. Perturbations of different intensities induced differential anxiety measures as expressed in the EPM. Anxiety levels were significantly reduced in sub-chronic restrained subjects, while isolation did not show marked difference. Anxiety profiles become evident from broad sample sizes and could constitute a critical limiting factor in personalized treatments. Stress-induced anxiety disorders could implicate comorbidity to other neuropsychiatric disorders in individuals. Coping strategies come to the fore in repeated sub-chronic perturbations indicating adaptive responses to the stressor, while acute perturbation enhances expression of anxiety behaviors.

Introduction
The elevated plus maze (EPM) is the most widely used behavioral paradigm that measures fear-motivated avoidance behavior in rodents. It is based on the conflict between the animal’s innate behavioral urge to explore novel spaces and its fear of open, brightly lit spaces; in other words, it asssays approach and avoidance. This test has been validated using behavioral, physiological and pharmacological approaches. Anxiolytic drugs increase the time spent on the open arms; hence, rats that avoid open spaces (arms) to a greater extent are said to be more anxious. The principal construct here is that movements are cautious in neophobia, while neophilia is characterized...
by ample locomotion and inspection or exploration. EPM thus is an unconditioned test for anxiety that demonstrates face, construct and predictive validities of the anxiety model. However, it can be used only once as it demonstrates an one-trial tolerance effect or test decay on second exposure [1].

Work with selectively bred and outbred Wistar rats [2, 3] based on the percentage of open-arm time in the EPM has shown that male adult Wistar rats demonstrate either low or high anxiety-like behavior. This ‘trait’ has been reported to remain stable under re-test conditions (in non-EPM paradigms) and is correlated with other behaviors where anxiety plays a role, such as in object burying and avoidance learning [4, 5]. This behavioral trait of high or low anxiety is also manifested in the emission of ultrasonic vocalizations and in striatal serotonin and cytokine levels [6].

Any stimulus that interferes with brain homeostasis acts as a stressor and evokes conservation or withdrawal as a behavioral response. Re-establishment of homeostasis is achieved by evoking adaptive responses leading to a coping strategy [7]. Coping mechanisms have an adaptive value in a stressful situation [8] and could induce resilience to stress. The elicited responses may differ depending on the type of stress, sex, age and hormonal state of the individual. The limbic hypothalamic-pituitary-adrenal (HPA) axis is the primary circuit that is involved in initiation, regulation and termination of a stress response, with the brain areas involved in stress control and response being similar [9]. Earlier studies have shown behavioral changes after exposure to inescapable stress such as restraint [10].

For a better understanding of state and trait anxieties, the EPM was used with certain perturbations that could influence expression of anxiety-like behaviors. Our aim was to ascertain whether rats identical in strain, sex and age differ systematically in anxiety-like behavior. The second aim was to test whether anxiety traits are also manifested in other behavioral paradigms, such as a novel activity box. Finally, we used acute and sub-chronic pre-test perturbations to observe how ‘state’ or induced anxiety impacts anxiety behaviors.

**Methods**

**Subjects**

Young adult male outbred Wistar rats (Sri Venkateshwara Enterprises, Bangalore), weighing 137.84 ± 2.42 g (n = 65) at the beginning of the experiment were used. They were housed in groups of 4 in polypropylene cages under standard laboratory conditions with food and water ad libitum. The housing room was maintained on a 12-hour light/dark cycle. Ambient temperature was between 25 and 27°C. All animals were handled on 3 consecutive days before the experiments began. All experiments were conducted in the light cycle (9:00–17:00 h) in accordance with the ethical regulations for animal experimentation laid down by CPCSEA and cleared by the Institutional Animal Ethics Committee (SAC/IAEC/105/2011) of the host institution. All measures were taken to minimize pain and discomfort, other than that critical to the experiment.

**Elevated Plus-Maze**

The EPM apparatus was made of acrylic (black) and consisted of 2 open arms (50 × 10 cm) and 2 closed arms with no roof (50 × 10 × 40 cm) at right angles to each other and an open square (10 × 10 cm) in the center. The maze was elevated 50 cm above the floor. The animals were placed into the center, facing the same open arm each time. The maze was cleaned thoroughly with 1% acetic acid before the next animal was introduced. If an animal fell from the maze, it was immediately placed back in the position from which it had fallen. The EPM recording was of 5 min duration.

**Activity Box**

The activity box consists of an open cube of dimensions 40 × 40 × 40 cm, placed at an elevation of 50 cm above the ground. The activity box was cleaned thoroughly with 1% acetic acid before the next animal was introduced. The recording was of 5 min duration.

**Overt Behavior Recording and Analysis**

Behavior was recorded using a Panasonic CCD camera fed to a Piccolo frame grabber card and analyzed using a tracking and video recording software, Ethovision® XT version 8.0 (Noldus, Netherlands). Zones were marked and a template was created to digitize physical distances. The following zones were defined: for activity box – center versus periphery; for EPM – open arms, closed arms and center. An entry was defined as the 4 paws of the subject being inside the template zone. Rearings were scored by a person blind to the experimental conditions. All other measures listed below were software generated, such as duration, frequency, latency, distance moved in the activity box and EPM.

**Perturbations**

Another set of rats were then randomly divided into 5 groups: (1) group-held controls; (2) 2 h of isolation in bare new cage (acute); (3) 2 h of physical restraint in restrainer (acute); (4) 2 h of isolation for 5 consecutive days (sub-chronic) and (5) 2 h of physical restraint for 5 consecutive days (sub-chronic). Restraining was done using 20 × 6.3 cm transparent cylindrical tube procured from Orchid Scientifics, Nashik, India. One end of it was enclosed with the provision for breathing. The other end was clamped using a cylindrical disc. The bottom had a slit allowing the animal to urinate and excrete freely. After the perturbations, the animals were placed in the EPM, recorded and analyzed as described above.

**Statistical Analyses**

All results are expressed as mean ± SEM. The t test or one-way analysis of variance (ANOVA) with Tukey’s post hoc tests was performed. The level of significance was defined as p ≤ 0.05.
Results

Subjects demonstrated great variation in open arm times. A scatter plot of open-arm times across all animals showed individual differences from no time spent in the open arm to close to one-third of the entire testing period spent on the open arm. Based on the time spent in the open arms, animals could be classified into 2 groups using a median split: (1) high anxious (HA) or low open-arm time and (2) low anxious (LA) or high open-arm time.

LA animals showed an increased open-arm time compared to the HA group (fig. 1a) which was highly significant ($t_{28} = 5.74$, $p = 0.001$). The converse was true of closed-arm time ($p < 0.001$). Latency to first entry of an open arm was 3 times more in HA animals (26.46 ± 6.43 s) than in the LA group (8.72 ± 2.66 s), which was significant ($p < 0.05$). HA animals spent 85% of the entire testing period in the closed arm when compared to the LA animals that spent 70%, which was highly significant ($p < 0.001$). Open-arm entries of LA animals were twice as much as those of HA animals, which was very significant ($p < 0.01$). Time spent in the center of the EPM of HA animals was significantly ($p < 0.05$) lower than in LA animals. Number of entries into the closed arm was comparable between HA and LA rats. The mean ± SEM values of all parameters are given in table 1.

When open-arm time and open-arm entries are taken in relation to total number of entries (both arms) and total time spent on the EPM, an anxiety index or measure ranging from 0 to 1 is obtained (fig. 1b). Here, anxiety measures range from 0.6 to 1.0, with HA and LA rats showing significant differences in anxiety levels ($t = 4.043$; d.f. = 28; $p = 0.0004$).

The novel activity box paradigm returned no significant differences in ambulation or exploratory behavior between HA and LA animals. Specifically, measures (distance moved, center entries, rearing frequency) yielded no significant differences between HA and LA rats. Time spent in the center was marginally increased in LA animals, with a corresponding marginal decrease in periphery time, while the reverse was observed for HA animals. These data however did not reach significance ($p = 0.09$). The mean ± SEM values of all measures are given in table 2.

Table 1. EPM measures – HA vs. LA

| Variable   | Zone           | HA              | LA              | Statistic, p value |
|------------|----------------|-----------------|-----------------|-------------------|
| Duration, s| Open arm       | 11.68±2.15      | 44.68±5.33***   | $t_{28} = 5.74$, $p = 0.001$ |
|            | Closed arm     | 255.44±6.71     | 211.21±8.38***  | $t_{28} = 4.12$, $p = 0.001$ |
|            | Centre         | 41.62±6.72      | 69.34±8.77*     | $t_{28} = 2.50$, $p = 0.018$ |
| Frequency  | Open arm       | 3.6±0.63        | 6.33±0.57**     | $t_{28} = 3.20$, $p = 0.003$ |
|            | Closed arm     | 10.13±1.4       | 11.8±1.05       | $t_{28} = 0.95$, $p = 0.349$ |
| Latency, s | Open arm       | 26.46±6.43      | 8.72±2.66*      | $t_{28} = 2.54$, $p = 0.016$ |

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. 

Fig. 1. a Scatter plot of open arm time on the EPM. Open-arm times of all animals were plotted serially. The dividing line indicates the median split used to classify the rats into 2 groups based on open-arm time into HA and LA groups. b Anxiety index of LA group was significantly lower than HA group. Anxiety index was calculated as: $1 - (\text{open-arm time/total time}) + (\text{open-arm entries/total entries})/2$. The read-out of the anxiety index correlates with the open-arm times. The whiskers of the box plot represent minimum to maximum. *** $p < 0.001$. 

Table 1. EPM measures – HA vs. LA
Physical restraint for 2 h (acute) induced a decrease in open-arm time (1.68 ± 0.68 s) when compared to controls (6.25 ± 2.22 s) and time-matched isolates (6.71 ± 2.96 s). Latency to enter the open arm was significantly increased for acute restrained animals thus demonstrating a first open-arm entry after 177.8 ± 42.49 s, which is after half of the test time when compared to controls (14.71 ± 7.27 s; p < 0.001) and the time-matched isolates (30.36 ± 17.1 s; p < 0.01). Acute physical restraint of 2 h induced a significant decrease in ambulation with differences emerging with the controls (p < 0.01) and with the time-matched isolates (p < 0.05). This was expressed also in the significantly reduced closed-arm entries when compared to controls (p < 0.01), though it was not different from time-matched isolates.

In the sub-chronic group that underwent 2 h of physical restraining on 5 consecutive days, open-arm time was increased. A one-way ANOVA returned highly significant differences across groups (F_{4,30} = 6.14, p = 0.001; fig. 2). When compared to time-matched isolates, it was significantly (p < 0.05) increased. Ambulation was increased in the sub-chronic restrained group when compared to time-matched isolates, but did not reach significance (table 3).

All the perturbations, whether acute or sub-chronic restraint and time-matched isolations induced a reduction in ambulation, which also translated into a significant reduction (F_{4,30} = 4.85, p = 0.002) in closed-arm entries. When compared to controls, closed-arm entries were very significantly reduced (p < 0.01) in acute re-
straint, while they were significantly reduced in sub-chronic restraint and both the isolate groups (p < 0.05). Latency to enter the open arm was also significantly different (F_{4,30} = 6.36, p = 0.001), with post hoc tests returning a highly significant increase in the acute restrained group only when compared to controls (p < 0.001) and when compared to their time-matched isolates (p < 0.01).

When comparing acute with sub-chronic restraint, the sub-chronic restraint group demonstrated an increased open-arm time that was highly significant (p < 0.001). Latency to enter open arm was also significantly reduced (p < 0.05) when compared to the acute restrained group. No other parameters were affected as a result of repeated restraint.

The anxiety index parameter was significantly increased (p < 0.05) in acute restraint when compared to repeated restraint. The mean ± SEM and all other parameters are given in table 3.

### Discussion

In recent years, research conducted at behavioral, physiological, biomedical applicational and intervention- al levels are all taking the individual into account as innumerable studies [11] have demonstrated individual differences across species including humans. Individual differences in behavior are determined both by genetic variances and by events in the surroundings that influence an individual’s anxiety levels. Individual differences seen in stress physiology and behavioral profiles could determine how each individual copes with stress, a critical factor in biomedicine [8]. Research in this direction will expose building blocks of affect-related personalities and the differences in them, opening up plausible strategies towards personalized treatments, by revealing substrates for vulnerability. The differences in these substrates appear to cause inter-individual differences, giving way to variability in susceptibility to various psychiatric disorders. Neurobiological foundations to these affective disorders may help in determining if a particular drug or treatment will be useful for a given individual or not. As invasive analyses are not possible with human subjects, animal studies of affective neuroscience are on the rise [12].

Decreased open-arm time in the EPM is often correlated with freezing behaviors or immobility that index anxiety levels. All measures are based on the principle of developing an avoidance approach conflict and indicate whether the animal follows its innate urge to explore new spaces, that is, the open arms or its fear of elevated, open spaces. It is to be expected that individuals in a population vary, but that it can also be obtained in a single randomized trial, as we have shown here, which has implications in humans, as individual differences could predict predisposition to affective disorders [13]. These individual differences are apparent when faced with environmental challenges or stressful situations like a novel environment [11]. Consistent individual differences have been seen even at the physiological level [14]. Preliminary studies indicate that HA-LA rats [15] also demonstrate subtle differences in memory retention of spatial information when tested in the radial arm maze [16].

### Table 3. Comparative measures of anxiety behaviors following pre-test perturbations: control; acute – 2 h isolation or restraint and sub-chronic isolation and restraint. n = 7 in each group

| Variable            | Control | Acute isolation (2 h) | Acute restraint (2 h) | Sub-chronic isolation (2 h × 5) | Sub-chronic restraint (2 h × 5) |
|---------------------|---------|-----------------------|-----------------------|----------------------------------|-------------------------------|
| OA time, s          | 6.25±2.22 | 6.71±2.96               | 1.68±0.68             | 9.90±3.64                        | 22.49±4.23**                 |
| CA time, s          | 267.8±7.03 | 263.1±8.67              | 280.2±5.2             | 262.6±10.51                      | 254.2±8.5                     |
| Centre time, s      | 25.78±5.14 | 40.96±8.7                | 20.02±5.2             | 30.28±7.82                       | 23.80±5.01                     |
| OA latency, s       | 14.71±7.27 | 30.36±17.1               | 177.8±42.49***        | 51.98±26.67                      | 45.48±21.37**                |
| OA entries, n       | 2±0.68     | 1.25±0.45                | 1.12±0.58             | 2.7±0.77                         | 3.33±0.79                     |
| CA entries, n       | 15±1.03    | 8.45±1.47*               | 6.37±1.17**           | 8.55±1.57*                       | 8.44±2.02*                    |
| Dist moved, cm      | 1.568±91.46 | 1.458±138.5              | 946.3±123.1**        | 976.2±148.9**                    | 1.325±115.1                   |
| Anxiety index       | 0.91±0.02  | 0.89±0.03                | 0.94±0.02             | 0.89±0.02                        | 0.81±0.03*                   |

* Indicates significant differences between all perturbed groups vs. control. # Denotes significant differences in acute condition between isolates and restrained animals and in sub-chronic condition between isolates and restrained animals. € Indicates significant differences in sub-chronic restraint when compared to acute restraint. **, #, € p < 0.05; ***, #, €€€ p < 0.01; ****, #, €€€€ p < 0.001.
That pre-test manipulations induce different anxiety profiles when tested in adolescents and adults have been shown in SD rats [17]. Studies show that individually housed rats were hyper-reactive in novel environments and showed slower habituation at all time points tested [18, 19] though the isolates tested here were only socially isolated for the duration of the experiment. Different periods of social isolation also have short- and long-term influences on behavior. However, all these isolation periods were for longer durations than the brief period of 2 h used here.

Restraint stress increases anxiety-like behavior. Activity box measures were not taken here as various other studies have shown that restraint stress reduces locomotor activity [20]. Adolescent rats demonstrated increased anxiety in the light-dark box following restraint when compared to adults [21]. The differences in behavior induced by acute and sub-chronic restraint correlated with corticosterone levels (unpublished data), while other studies show concomitant plasma ACTH levels [22], with increase from acute restraint and a decrease after the third day of restraint. The latter study showed a decrease in open arm entries and time following acute restraint (15 min, 30 min, 1 h), but also at a sub-chronic level of 3 days, which is at variance from what was observed in this study. Possibly, 3 days was not sufficient to increase activity on the open arms. Moreover, the restraint period used in the latter study was for 1 h only.

The behavioral differences in the acute and sub-chronic groups could also be due to differences between escapable and inescapable stress [7]. Acute restraint, which is inescapable and by itself anxiogenic, leads to increased anxiety in the EPM [23]. However, during 5 consecutive exposures, the situation already involves a cognitive appraisal and, therefore, does not induce the state anxiety observed in the first exposure [24]. Serotonin levels are also increased after 1 h of restraint, more so in the hypothalamus, indicating the activation of the HPA axis again [25]. The difference in state anxiety in acute and sub-chronic exposures could be explored further by the use of anxiolytics.

Here, previous, repeated exposure to the same stressor evoked a different response with repeated sub-chronic restraint having no effect on anxiety behaviors. Whether it is because of repeated handling in the days preceding the test remains to be seen, however other studies indicate that this is not the case [1]. Prior exposure to a novel environment is said to increase motor activity in the EPM and could have translated into the increased entries into the open arm observed here [1]. Moreover, aversiveness of the test condition and prior exposure are important factors that may influence the expression of anxiety.

Genetic pre-disposition in combination with environmental risk factors contribute toward susceptibility to affective disorders. Thus, measures of individual anxiety levels can facilitate early detection of a psychiatric disorder [12]. Persistence and co-segregation of a trait validates the presence of a phenotype and are among the criteria used in a selection strategy based model of inter-individual differences. Persistence of the endophenotype, even under environmental changes, further substantiates the presence of that trait.

Behavioral differences do not affect survival. If the variation is neutral, random or non-adaptive, those differences are maintained without having major effects. However, neophobic individuals were found to have shorter lifespan compared to neophilics [26]. The present study assessing differences in behavioral responses due to inherent anxiety levels or ‘trait’ and induced anxiety through acute and sub-chronic exposures, that is ‘state’ anxiety, suggests that inter-individual differences could possibly be present even at structural and functional level, reflected in personalities and coping styles owing to endophenotypes and interactions with adverse events. This could help assess vulnerability to psychiatric disorders and help evolve perspectives and interventions in psychopharmacology.

Author Contributions

R.M.R. carried out the experiment and did the analysis and graphs. R.M.R. and M.S. wrote the paper.

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