Categories of Wistar Rats Based on Anxiety Traits: A Study Using Factor and Cluster Method

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Keywords

Anxiety traits · Wistar rats · Elevated plus maze · Open field test · Cluster analysis · Factor analysis

Abstract

Background: Outcomes of neurobehavioral studies are invariably affected by the variations in the anxiety traits of animals in the same species. Identifying those traits and categorizing them accordingly would improve the reproducibility of results, and reduce the variation in the results from different laboratories. **Purpose:** The present study was done to identify the possible groups among the normal population of outbred adult male Wistar rats. **Methods:** Anxiety traits were measured in elevated plus maze (EPM) test and open field test (OFT). The various anxiety responses from these tests were subjected to exploratory factor and hierarchical cluster analyses. Different clusters thus derived were compared with each other. **Results and Conclusion:** In ex-

ploratory factorial analysis, 2 components, that is, anxiety and activity were derived from the EPM and OFT parameters. Cluster analysis of EPM parameters classified the rats into 3 groups “high anxiety and low activity”, “medium anxiety and high activity”, and “low anxiety and medium activity”. Whereas, cluster analysis on OFT parameters identified one more group namely “low anxiety and high activity”. The rats which came under the clusters formed from the EPM and OFT parameters were not identical. Moreover, EPM and OFT may be measuring different aspects of anxiety.

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Introduction

Rodent models are the indispensable parts of neuroscience research providing significant insights in various physiological, behavioral, and pharmacological studies. However, one of the most common dilemmas associated

with these models is the non-reproducibility of data from various laboratories especially in neurobehavioral studies. These inconsistencies are attributed to the individual genetic variations, testing environment, protocols, and the rearing environment of the rodents [1, 2]. Standardizing the testing and rearing facilities is found to be effective in reducing many of the variations observed in the results [2], but may not be enough to make the data replicable [3]. Considering this, it would be important to examine the variations (or traits) among the rodents of the same strain.

Anxiety is defined as “a psychological, physiological, and behavioral state induced in animals and humans by a threat to well-being or survival, either actual or potential” [4]. Anxiety may be the “state” which is due to some external stimuli or “trait” which is innate. According to Gottesman and Gould, animal models based on endophenotypes that represent evolutionarily selected and quantifiable traits may lend themselves better in investigation of psychiatric phenomena than models based on face-valid diagnostic phenotypes [5].

In rats, anxiety-like behavior is assessed by measuring their response to a novel and potentially threatening environment using batteries of tests [6, 7]. Among them, elevated plus maze (EPM) test is the most common and simple method to assess anxiety in rodents [8, 9]. It measures the innate behavior while exploring a new area, and at the same time takes into account their fear of moving into an open area with face, construct, and predictive validity in terms of anxiety measurement [10]. The rats are classified on the basis of high and low anxiety-related behavior (HAB and LAB) in the EPM test [11]. Similarly open field test (OFT) is ideal for an initial screening of behavior in rats, wherein the anxiety determines the response to an unfamiliar environment [12]. Moreover, most of the studies on behavior are performed in small sample size of 5–8 rats/group. Hence, it may be important to categorize each strain taking large sample size for various neurobehavioral studies. Therefore, the present study was aimed to examine the anxiety groups among the normal population of outbred adult male Wistar rats using exploratory factor and hierarchical cluster analyses on the basis of parameters measured in OFT and EPM test.

Materials and Methods

Animals

A total of 104 outbred adult male Wistar rats (250–300 g) housed in polystyrene cages, kept in controlled temperature ($26 \pm 1^\circ\text{C}$) and light-dark schedule of 12 h (lights on at 06:00 h), with

food and water provided *ad libitum* were used in the study. All the procedures employed in this study were approved by the Institutional Animal Ethics Committee of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala.

Study Design

The non-habituated Wistar rats were tested for anxiety in the EPM test ($n = 104$) and OFT ($n = 88$ out of 104) on different days at 14:00 h using ANY-maze video-tracking system (version 4.82) from Stoelting Co. (USA). The acrylic EPM had 2 open arms (50×10 cm) and 2 closed arms ($50 \times 10 \times 50$ cm), arranged in plus shape kept 45 cm above the floor. The test was conducted for 5 min after placing the rat in the center facing the opposite open arm. The parameters measured includes entries to open and closed arm, time spent in the open and the closed arm, total distance traveled, average speed, and the total time mobile on the maze. The index of open arm avoidance ($100 - [\text{percentage time spent in open arm} + \text{percentage entries in to open arm}]/2$) was also measured. Ethologically derived parameters which include head dipping, rearing, and grooming were noted.

The acrylic OFT maze chamber ($100 \times 100 \times 40$ cm) consisted of 3 concentric zones, namely outer, middle, and inner zone. The test was conducted for 5 min after placing the rat in the center of the maze. Parameters measured included entries and time spent in the inner and outer zones, average speed, time mobile on the maze, total distance traveled, and ethologically derived parameters like rearing and grooming. OFT was always done 1 or 2 days after EPM test in order to ensure that the pre-exposure to another test does not influence the findings [2].

Statistical Analysis of Data

All analysis was done in SPSS software (version 16.0). Parameters measured from OFT and EPM test were subjected to 2-fold exploratory factor analysis to identify the related variables. The parameters were categorized into different components based on their correlation. After identifying the components, the parameters were subjected to hierarchical cluster analysis to categorize the animals into groups based on their anxiety behavior.

Factor Analysis

In the factor analysis, principal component was used as the extraction method and since the distribution was identified as orthogonal, varimax with Kaiser normalization was used as the rotation method. Principal components analysis with permutation was used [13]. The number of components was fixed on the basis of raw data Eigenvalue (greater than 1) and was further confirmed using parallel analysis Eigen value Monte Carlo test [14] and scree plot.

Cluster Analysis

The parameters of the anxiety tests which were validated in the factorial analysis were subjected to agglomerative hierarchical cluster analysis to determine the number of clusters. Squared Euclidean distance was used in the proximities matrix [15, 16]. Weighted average linkage was used as the clustering method since the size of the clusters was not expected to be similar [15, 16]. The difference between the number of cases and the data after point of cluster termination (step of “elbow”) was taken as the valid cluster

Fig. 1. Scree plot showing the principle component analysis for EPM and OFT parameters. Scree plot showing the comparison of raw data Eigen value obtained through principle component analysis with random data Eigen value at the 50th and 95th percentile obtained through the confirmatory parallel analysis. (a) Scree plot

for EPM test and (b) scree plot for OFT. From the plot, the number of components revealed is equal to 3 since the raw data Eigen value of 3 roots are greater than 1 as well as the 50th and 95th percentile random data Eigen value.

solution. Hierarchical cluster analysis was redone by specifying the cluster solution to categorize the entire data into groups based on the defined clusters.

Non-Parametric Statistics

After clustering into various subgroups, Kruskal-Wallis test was used to compare the anxiety parameters among the groups and Mann-Whitney test was used to identify the pair-wise differences between the groups.

Results

Components and Subgroups Based on EPM Test

The exploratory factor analysis revealed 3 components (78% of total variance) on the basis of EPM parameters (Fig. 1). The first component defined the activity of rats on the basis of distance travelled, average speed, and mobility on maze; and the second component defined the anxiety trait of rats on the basis of entries and time spent in open and closed arm, index to open arm avoidance, and head dipping behavior. The third component comprising of grooming and rearing, did not satisfy the reliability test. Henceforth, the third component was not taken for further analysis.

In the hierarchical cluster analysis, 3 cluster solutions were chosen for the EPM test parameters (Fig. 2). Table 1 summarizes the values of various anxiety parameters for the total sample and for the 3 groups (Groups I, II, and III). Among the 3 groups all the parameters were showing

significant difference (Kruskal-Wallis test: $p < 0.001$) except for grooming and rearing.

Rats in Group I (41.35%) showed “high anxiety and low activity”, Group II (19.23%) rats displayed a “medium anxiety and high activity”, and Group III (39.42%) rats had a “low anxiety and medium activity” pattern of response.

Components and Subgroups Based on OFT

The exploratory factor analysis revealed 3 components (73% of total variance) for OFT parameters (Fig. 1). First component defined the activity of rats on the basis of total distance traveled, average speed, total time mobile, and rearing behavior. Second component defined the anxiety trait of the rats on the basis of entries and time spent in outer and inner zone. Third component which comprised grooming did not satisfy the reliability test and was not taken for further analysis.

In the hierarchical cluster analysis, 4 cluster solutions were obtained for OFT parameters (Fig. 3). Table 2 summarizes the values of various anxiety parameters for the total sample and for the 4 groups (Groups I, II, III, and IV). Among the 4 groups, all the parameters showed significant difference (Kruskal-Wallis test: $p < 0.001$) except for the grooming.

Rats in Group I (9.09%) displayed a “high anxiety and low activity”, Group II rats (4.55%) showed a “low anxiety and medium activity”, Group III rats (11.36%) had a “low

Fig. 2. Scree plot showing the agglomeration schedule for EPM test parameters. Scree plot representing the agglomeration schedule output for EPM test parameters. Arrow shows the point of termination of clustering process (elbow) or the point after which there is a large increase in the coefficient. From the plot, the number of clusters revealed is equal to 3 ($104 - 101 = 3$).

Table 1. Various anxiety parameters measured from EPM test before and after clustering

EPM parameters	Unclustered total sample	Clustered		
		Group I	Group II	Group III
Total distance traveled, m	11.63±07.7	06.94±02.9	24.35±07.4***	10.38±03.8***, ###
Average speed, m/s	00.04±00.0	00.02±00.0	00.08±00.0***	00.03±00.0**, ###
Time mobile, %	42.91±25.7	25.30±10.3	87.20±16.2***	39.84±12.2***, ###
Head dipping	11.87±07.4	06.60±03.7	11.35±05.5**	17.50±06.9***, ###
Time in open arm, %	15.98±14.6	03.45±03.3	17.34±07.3***	28.15±13.6***, ###
Time in closed arm, %	61.31±23.0	82.17±11.0	64.78±10.8***	38.32±12.8***, ###
Entries in to open arm, %	18.84±10.2	10.80±07.7	24.83±08.1***	24.23±07.7***
Entries in to closed arm, %	33.91±09.1	40.68±06.6	33.86±05.0***	27.00±07.4***, ###
Index of open arm avoidance	82.59±11.4	92.88±04.7	78.92±05.8***	73.81±09.6***, #

Values are represented as mean ± SD.

* Indicates significant difference from Group I and # indicates significant difference from Group II. $n = 43$ for Group I, 20 for Group II, and 41 for Group III. Pair-wise comparison was performed using Mann-Whitney test. *, # $p \leq 0.05$, ** $p \leq 0.01$, ***, ### $p \leq 0.001$. Group I includes rats showing “high anxiety and low activity”, Group II includes rats displaying “medium anxiety and high activity”, and Group III rats displayed “low anxiety and medium activity”.

anxiety and high activity” while the pattern of response in Group IV which had majority of the total number of rats (75%) was “medium anxiety and high activity”.

Similarity of EPM Test and OFT Subgroups

Table 3 shows the percentage of animals which overlap under various subgroups of OFT and EPM test. All the rats which represented Group II in OFT came under Group I of EPM test. Rats categorized in Group II of EPM test were equally divided into Group III and IV subgroups of OFT. However, rats representing Group III of EPM test which spent maximum time in the open arm were distributed into subgroups I, III, and IV of OFT.

Discussion

This study examined the anxiety traits of the normal population of adult male Wistar rats. Activity and anxiety, the 2 components of anxiety traits, identified by factorial analysis from OFT and EPM test parameters in Wistar rats were similar to those reported in other rodents [17, 18]. In the present study, time spent and entries into the open and closed arm belonged to the anxiety component, and mobility and distance traveled on the maze represented the best measure to define activity component.

Cluster analysis identified 3 groups of anxiety traits from EPM test parameters, whereas OFT parameters classified them into 4 groups. It is noted that Castro et

Fig. 3. Scree plot showing the agglomeration schedule for OFT parameters. Scree plot representing the agglomeration schedule output for OFT parameters. Arrow shows the point of termination of clustering process (elbow) or the point after which there is a large increase in the coefficient. From the plot, the number of clusters revealed is equal to 4 ($88-84 = 4$).

Table 2. Various anxiety parameters measured from OFT before and after clustering

OFT parameters	Unclustered total sample	Clustered			
		Group I	Group II	Group III	Group IV
Total distance traveled, m	23.31±07.6	11.09±04.2	18.09±05.6	26.06±07.7***	24.69±06.5***
Average speed, m/s	00.08±00.0	00.04±00.0	00.06±00.0	00.09±00.0***	00.08±00.0***
Time mobile, %	65.94±15.5	31.73±10.2	66.18±08.7***	75.05±08.4***	68.70±11.4***
Rearing	13.15±07.6	05.88±04.0	05.50±05.1	08.80±07.5	15.15±07.1*, #, \$
Time in outer zone, %	79.52±14.9	96.58±02.7	42.13±12.5***	59.48±10.4***, ##	82.76±08.6***, ###, \$\$\$
Time in inner zone, %	06.07±06.5	01.20±01.4	18.38±05.4***	16.11±11.2***	04.39±02.8###, \$\$\$
Entries in to outer zone, %	48.54±03.4	38.90±06.6	31.22±08.4	24.47±04.5***	34.12±04.7\$\$\$
Entries in to inner zone, %	18.13±06.8	14.73±06.3	19.33±07.5	29.10±09.0***, #	16.81±04.7\$\$\$

Values are represented as mean ± SD.

* Indicates significant difference from Group I, # indicates significant difference from Group II and \$ indicates significant difference from Group III. $n = 4$ for Group I, 8 for Group II, 10 for Group III, and 66 for Group IV. Pair-wise comparison was performed using Mann-Whitney test. *, #, \$ $p \leq 0.05$, ## $p \leq 0.01$, ***, ###, \$\$\$ $p \leq 0.001$. Group I includes rats displaying “high anxiety and low activity”, Group II includes rats showing “low anxiety and medium activity”, Group III includes rats displaying “low anxiety and high activity”, and Group IV includes rats showing “medium anxiety and high activity”.

Table 3. Percentage of animals which overlap under various subgroups of EPM and OFT test

	OFT subgroups			
	Group I	Group II	Group III	Group IV
EPM test subgroups				
Group I	87.5	100.0	70.0	65.7
Group II	0.0	0.0	20.0	20.9
Group III	12.5	0.0	10.0	13.4

Values are represented in %. In EPM test, Group I includes rats showing “high anxiety and low activity”, Group II includes rats displaying “medium anxiety and high activity”, and Group III rats displayed “low anxiety and medium activity”. In OFT, Group I includes rats displaying “high anxiety and low activity”, Group II includes rats showing “low anxiety and medium activity”, Group III includes rats displaying “low anxiety and high activity”, and Group IV includes rats showing “medium anxiety and high activity”.

al. [19] classified Sprague Dawley rats into 4 groups based on the mean score and interaction studies on EPM and OFT parameters, namely low anxiety and low exploration, low anxiety and high exploration, high anxiety and low exploration, and high anxiety and high exploration. In the present study, even though a “high anxiety low activity” group was observed, a “low anxiety and high activity” group, which spent more than 50% of test time in open arm of EPM [20], was not observed. However, few animals within Groups II and III did show a “low anxiety and high activity” pattern of response. Nonetheless, they could not form a separate group in the cluster analysis. On the other hand, based on their performance in OFT, rats belonging to Group III (11.36%) showed a “low anxiety and high activity” pattern of response. Surprisingly, 70% of rats belonging to this group represented Group I in EPM test. This probably suggests that the anxiety and the activity components, which both EPM and OFT measure, do not overlap. Both these tests measure entirely different aspects of anxiety as reported previously [21]. So, multiple tests may be required to assess the actual anxiety trait of an animal. Moreover, in the present study, the anxiety and activity components of EPM test and OFT were found to be less correlated. When anxiety was low, activity was more or less at a medium level and when activity was high, anxiety was at a medium level. In BL6 and DBA mouse model also, no correlation was reported between the anxiety and activity components [22].

A medium level of anxiety, normally observed in rodents, based on the free-exploratory paradigm [23] and EPM test [24], is consistent with the finding of the present study. In the present study, rats displaying medium level of anxiety spent 10–50% of the test time in the open arm of EPM which may be considered as normal anxiety trait in rats according to previous report [20].

Anxiety trait of an animal is found to influence their response toward stress, drugs, environmental enrich-

ment, diseases, etc. Vulnerability toward certain conditions like stress is not only species specific, but also varies in different animals. Some animals show high vulnerability whereas some are relatively resistant to stress [25, 26]. If this factor is not taken into consideration, it may lead to unreliable and non-reproducible results. Identifying the anxiety trait of an animal is extremely important in neurobehavioral research. The rats showing “high anxiety low activity” and “low anxiety and low exploration” profiles are useful models to study anxiety disorders, stress, and associated depression [11, 19].

In conclusion, the present study examined the behavior of adult male Wistar rats in EPM test and OFT and categorized them into various groups using cluster analysis based on the anxiety and activity component extracted from the principle component analysis. Cluster analysis on the EPM parameters in the present study did not find a low anxiety and high activity group similar to the LAB group. The study suggests that a single test may not be enough to conclude about the anxiety trait of an animal model as rats subdivided into different groups on the basis of EPM test and OFT did not have overlapping features. The tests may be improvised to identify additional components (exploration, defecation score) to accurately define the anxiety trait of an animal model.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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References

- 1 Chesler EJ, Wilson SG, Lariviere WR, et al: Identification and ranking of genetic and laboratory environment factors influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. *Neurosci Biobehav Rev* 2002;26:907–923.
- 2 Izidio G, Lopes D, Spricigo L Jr, Ramos A: Common variations in the pretest environment influence genotypic comparisons in models of anxiety. *Genes Brain Behav* 2005;4: 412–419.
- 3 Crabbe JC, Wahlsten D, Dudek BC: Genetics of mouse behavior: interactions with laboratory environment. *Science* 1999;284:1670–1672.
- 4 Steimer T: The biology of fear-and anxiety-related behaviors. *Dialogues Clin Neurosci* 2002;4:231–249.
- 5 Gottesman II, Gould TD: The endophenotype concept in psychiatry: etymology and strategic intentions. *Am J Psychiatry* 2003;160:636–645.
- 6 Litvin Y, Pentkowski NS, Pobbe RL, et al: Unconditioned models of fear and anxiety. *Handbook of Behavioral Neurosci* 2008;17:81–99.
- 7 Sahgal A: *Behavioural Neuroscience. A Practical Approach*. Oxford, UK, Oxford University Press, 1993.

- 8 Pellow S, Chopin P, File SE, Briley M: Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 1985;14:149–167.
- 9 Rao RM, Sadananda M: Influence of state and/or trait anxieties of Wistar rats in an anxiety paradigm. *Ann Neurosci* 2016;23:44–50.
- 10 Walf AA, Frye CA: The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc* 2007;2:322–328.
- 11 Landgraf R, Wigger A: Born to be anxious: neuroendocrine and genetic correlates of trait anxiety in HAB rats. *Stress* 2003;6:111–119.
- 12 Prut L, Belzung C: The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. *Eur J Pharmacol* 2003;463:3–33.
- 13 Tabachnick BG, Fidell LS: *Using Multivariate Statistics* (ed 5). Boston, MA, Allyn & Bacon, 2007.
- 14 O'Connor BP: SPSS and SAS programs for determining the number of components using parallel analysis and Velicer's MAP test. *Behav Res Methods Instrum Comput* 2000;32:396–402.
- 15 Sosulina L, Meis S, Seifert G, et al: Classification of projection neurons and interneurons in the rat lateral amygdala based upon cluster analysis. *Mol Cell Neurosci* 2006;33:57–67.
- 16 Yim O, Ramdeen KT: Hierarchical cluster analysis: comparison of three linkage measures and application to psychological data. *Tutor Quant Methods Psychol* 2015;11:8–21.
- 17 File SE: The biological basis of anxiety; in Meltzer HY, Nerrozzi D (eds): *Current Practices and Future Developments in the Pharmacotherapy of Mental Disorders*. Amsterdam, Elsevier, 1991.
- 18 Trullas R, Skolnick P: Differences in fear motivated behaviors among inbred mouse strains. *Psychopharmacology (Berl)* 1993;111:323–331.
- 19 Castro JE, Diessler S, Varea E, et al: Personality traits in rats predict vulnerability and resilience to developing stress-induced depression-like behaviors, HPA axis hyper-reactivity and brain changes in pERK1/2 activity. *Psychoneuroendocrinology* 2012;37:1209–1223.
- 20 Muigg P, Scheiber S, Salchner P, et al: Differential stress-induced neuronal activation patterns in mouse lines selectively bred for high, normal or low anxiety. *PLoS One* 2009;4:e5346.
- 21 Cruz AdM, Frei F, Graeff F: Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacol Biochem Behav* 1994;49:171–176.
- 22 Ohl F, Roedel A, Binder E, Holsboer F: Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice. *Eur J Neurosci* 2003;17:128–136.
- 23 Goes TC, Antunes FD, Teixeira-Silva F: Environmental enrichment for adult rats: effects on trait and state anxiety. *Neurosci Lett* 2015;584:93–96.
- 24 Correia D, Ribeiro AF, Godard AL, Boerngen-Lacerda R: Trait anxiety and ethanol: anxiolysis in high-anxiety mice and no relation to intake behavior in an addiction model. *Prog Neuropsychopharmacol Biol Psychiatry* 2009;33:880–888.
- 25 Southwick SM, Vythilingam M, Charney DS: The psychobiology of depression and resilience to stress: implications for prevention and treatment. *Annu Rev Clin Psychol* 2005;1:255–291.
- 26 Feder A, Nestler EJ, Charney DS: Psychobiology and molecular genetics of resilience. *Nature Rev Neurosci* 2009;10:446–457.