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# Plus Maze Selection of Anxious and Non-Anxious Rats: A Behavioral and Neurochemical Evaluation

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**Abstract** The development of animal models of anxiety is of great importance to detect potential anxiolytic drugs and study the anxiety phenomenon. The selection of anxious and non anxious rats in animal may represent a good procedure to help study rats behavior when tested in animal models. The present study aimed to investigate the differences among rats selected in an elevated plus maze test. Rats were selected as anxious when they spent time measures 1 sd lower than the average in open arms of the maze and non anxious spent 1 sd higher than the average. The non anxious selected rats showed lower emotional reactivity when tested in open field test and social interaction test as well as decrease of 5HT/5HIAA ratio in frontal cortex when compared with anxious selected rats. The results point to differences among the selected rats what suggest the procedure should be considered in anxiety studies.

***Keywords:*** *anxiety, anxious rats, genetic selection, experimental behavioral tests, elevated plus maze, serotonin*

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## 1. Introduction

Anxiety disorders are the most common psychiatric disorders [1]. Thus the development of animal models of anxiety is of great importance to detect potential anxiolytic drugs [2]. The elevated plus maze (EPM) is a behavioral test that has been used to study anxiety since the early studies of Montgomery [3] and it was pharmacologically validated by Handley & Mithani [4]. The use of the EPM as an animal model of anxiety is based on the conflict that results from the natural tendency of the animals when they are faced with dangerous situations as the open arms spaces of the maze [5]. EPM is easy to use and has no require of aversive stimuli. That´s why is one of the most used experimental behavioral animal tests. However EPM is very sensitive to small exogenous changes that can alter animal behavior on the test. For instance, prior stress as light level, light dark cycle manipulation, a 2-h period isolation or a restraint period of 15 min before the EPM, is sufficient to reduce exploratory behavior in the EPM [2,6,7,8,9].

Serotonin, Glutamate and Gaba have been postulated as the main neurotransmitters in anxiety central modulation [1,10,11,12]. Serotonin modulates several central functions, such as food intake, energy expenditure, motor activity, mood and sleep [12]. Several studies have pointed the serotonergic system as one of the most important on anxiety modulation and we know a single exposure to diverse stressful situations can cause multiple neurochemical changes in serotonergic neurotransmitter system [13,14,15]. It was demonstrated that postnatal serotonin type 2 receptor blockade prevents the emergence of anxiety behavior and stress [14]. However, others results showed that decrease of 5-HT turnover can produce anxiolytic effect in rats submitted to other tests as EPM and open field [12].

EPM has been used to anxiolytic screening of drugssince benzodiazepinesincrease the percent of entries and the time spent in the open arms of the maze [2,4].However benzodiazepines shows no anxiolytic effects in a second exposure to a elevated plus maze, in a phenomena knows as "one trial tolerance" [15]. Serotonergic drugs show controversial results in EPM [13,17]. We could suggest as explanation for these variability of drugs effects the fact that rats tested in EPM are not anxious rats and then we couldn't observe anxiolytic effects.

Some studies have tried to select "anxious" and "non anxious" animals in experimental behavioral tests. Early studies in models as open field, avoidance model, elevated plus maze and also after a electrolytic lesion of the prelimbic area showed different pattern behavior of rats selected as "anxious" [18]. Results with EPM selection showed several differences between "anxious" and "non anxious" selected rats since corticoster one levels, pituitary reactivity to intravenously administered CRH, body temperature and locomotor activity following stress exposure [19]. It was also observed differences in benzodiazepines EPM effects. However the results are not concluded. Our study aimed to observe if selected anxious

rats in elevated plus maze showed the anxious behavior in two other experimental behavioral tests: open field and social Interaction test and if the selected rats showed differences in 5-HT system.

# 2. Methods

## 2.1. Subjects

Wistarrats 300-350 g weight from Bioterio Central da Universidade Federal de São Paulo were kept in light dark cicle of 12 h food and water *ad libitum*.

## 2.2. Apparatus and Procedure

### 2.2.1. Selection of Rats in Elevated Plus Maze

The elevated plus maze apparatus was made of wood and consisted of two opposed open arms (50 x 10 x 2cm) and two opposed closed arms (50 x 10 x 40cm), all facing a central platform (10 x 10cm), elevated 45cm from the floor. The subject was placed in a central box in the center of the maze, then the box was removed and his behavior was monitored for 5 min. It was measured the number of entries as well as the percent of time spent in the open and closed arms.

The rats were selected as "non anxious" when they spent 1 SD (standard deviation)higher the average of the percent of time spent in the open arms meanwhile the "anxious" rats spent 1 SD lower the average. From 350 Wistar rats 22 were selected as "non anxious" and 20 were selected as "anxious" animals.

### 2.2.2. Open Field

Seven days after the EPM selection the rats were tested in the open field test. Open field is based on a behavior analysis of the rodents exposed to a non familiar arena divided into peripheral and central quadrants during a determined period. Animals with lower emotional reactivity present lower motor activity represented by lower ambulation and frequency on the behavior of rearing and more time of immobility on the arena as compared to animals more reactive as well as a lower defecation score[7].

### 2.2.3. Social Interaction Test

The test was performed in an similar arena of the open field test, 7 days after the EPM selection. The rats were placed in pairs and were allowed to interact for 5 min of testing. It was measured the total time spent by the rats engaging in active social interaction (ie, sniffing, following, or grooming).

### 2.2.4. 5-HT Assays

Seven days after EPM exposure the animals were lightly anesthetized and sacrificed by decapitation. The brains were removed and hippocampus and frontal cortex were dissected out taking as reference Paxinos and Watson atlas [20]. The samples were homogenized in 0.2 M perchloric acid containing dihydroxy-benzylamine (DHBA), an internal standard to control for loss of tissue contents. It were centrifuged at 15,000 rpm for 20 min at 6ºC and stored at -70ºC for 7 days, and 50 µL was injected into the HPLC-EC system.

The HPLC system consisted of a Shimadzu LC-10 AD chromatograph, with a CBM-10A communication bus module, an on-line DGU-14A degassing unit, and an L-ECD-6A electrochemical detector with a glassy-carbon electrode, and an LC-10 AD pump. The system was equipped with a reverse-phase column (Hypurity Elite C18, 250 mm x 4.6 mm, 5 µm and 100-Å pore diameter particle size; Hypersil, Cheshire, UK), coupled with electrochemical detection. The mobile phase contained 150 mM chloroacetic acid, 120 mM NaOH, 0.67 mM EDTA, 0.86 mM sodium octylsulfate, 3.5% acetonitrile, and 2.6% tetrahydrofuran. It was adjusted to pH 3.0, filtered and pumped through the system at a flow rate of 1.2 mL/min. 5-HT and 5-HIAA were quantified by comparing the peak areas to standard curves. The 5-hydroxyindoleacetic acid (5-HIAA)/5-HT ratios was used as indicator of serotonergic activity [15,21].

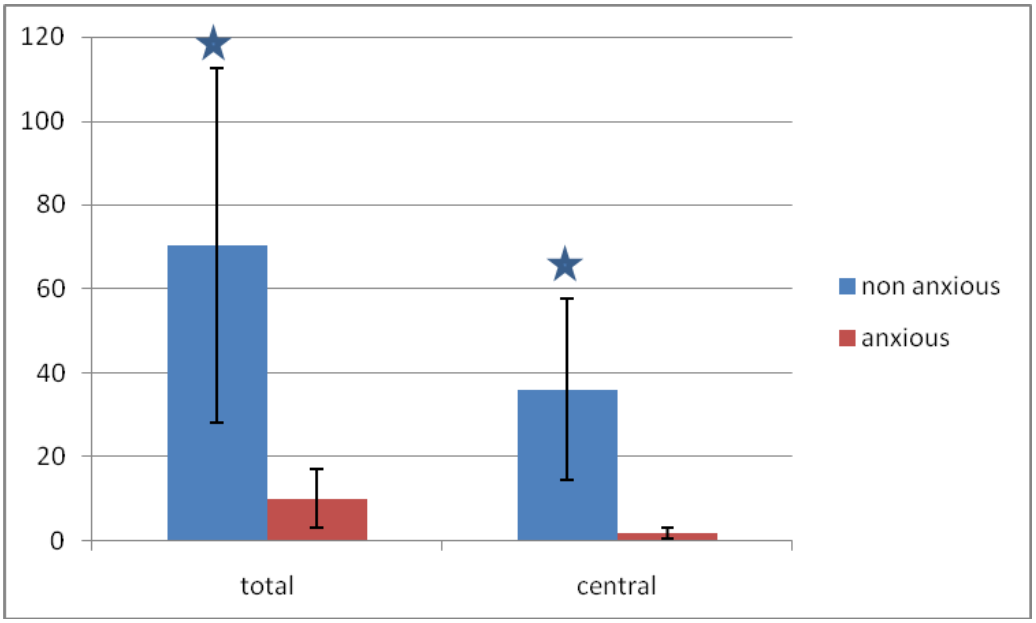


**Figure 1.** Total and central open field ambulation of non anxious and anxious selected rats in EPM. P<0,05

## 3. Results

Figure 1 shows the results for ambulation in the open field test. It was observed significant difference between non anxious and anxious rats when we compared total and central ambulation (70,8 ± 19,8 – 36,4 ± 20,1; 10,1± 18 – 1,8± 3,5). Figure 2 shows the results for time of freezing and rearing behavior. Non anxious rats showed significantly less time of freezing and more rearing behavior than anxious rats (22,7 s ± 12,7 - 40,9 s ±40,1; 27,3 ± 5,4 – 10,9 ± 9,4).No differences was observed in open field score of defecation (0,8± 1,2 – 1,9 ± 2,1).

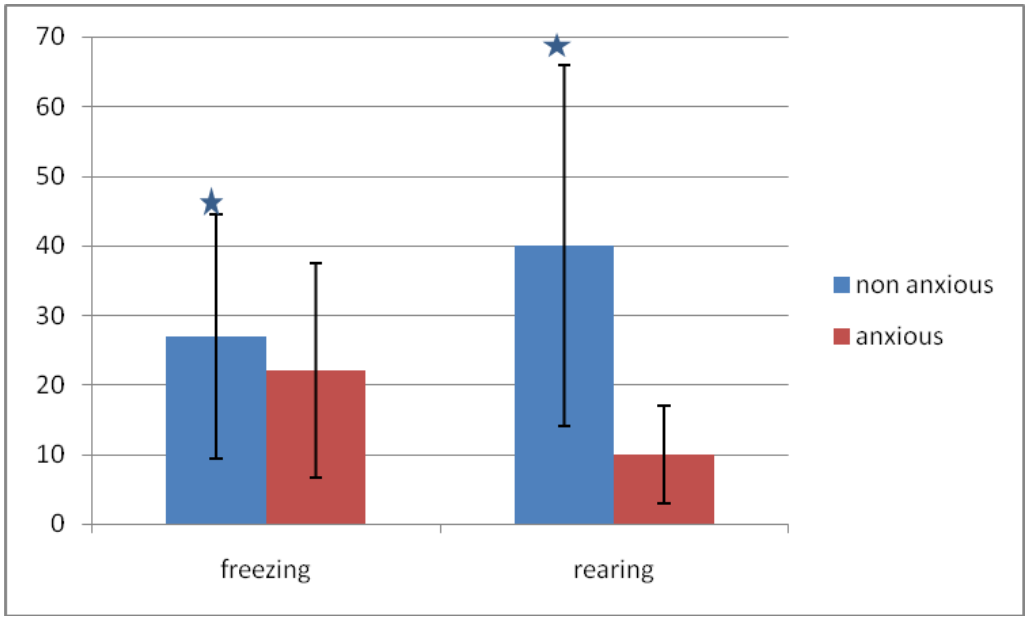


**Figure 2.** Time of freezing (seconds) and rearing behavior of non anxious and anxious selected rats in EPM submitted to open field teste. P<0,05

Non anxious rats showed significantly more time of active social interaction (90,7 sec ± 30) when compared with anxious rats (61,3 sec ± 22,2) in the social interaction test.

We can observe in Figure 3 the results for 5-HT and 5-HIAA levels in frontal cortex of non anxious (0,227 ng/mg ± 0,049; 0,186 ng/mg ± 0,058) and anxious rats (0.252 ng/mg ± 0,055; 0,146 ng/mg ± 0,056) and in hippocampus of non anxious (0,236 ng/mg ± 0,080; 0,346 ng/mg ± 0,213) and anxious rats (0.199 ng/mg ± 0,105; 0,251 ng/mg ± 0,083). There are no significant differences between the 5-HT measures in frontal cortex and hippocampus as well as 5-HIAA measures.

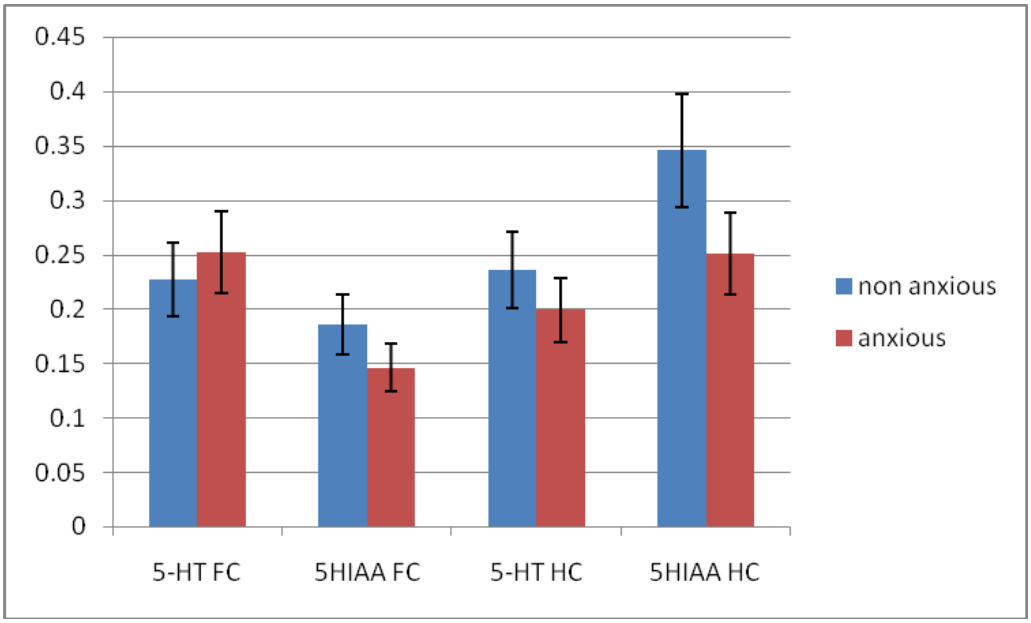


**Figure 3.** 5-HT and 5-HIAA levels ng/mg in frontal cortex (FC) and hippocampus (HC) of non anxious and anxious selected rats in EPM

In Figure 4 we can see the 5-HT/5HIAA ratio measures. There is no difference between measures of non anxious and anxious rats in hippocampus measures (0,994 ± 0,333 and 0,714 ± 0,253, respectively).However we observed a significant increase of 5-HT/5HIAA ratio in frontal cortex of non anxious (1,216 ± 0,308) rats when compared with anxious rats(1,996 ± 0,630) what could suggest lower 5-HT activity in frontal cortex of non anxious.

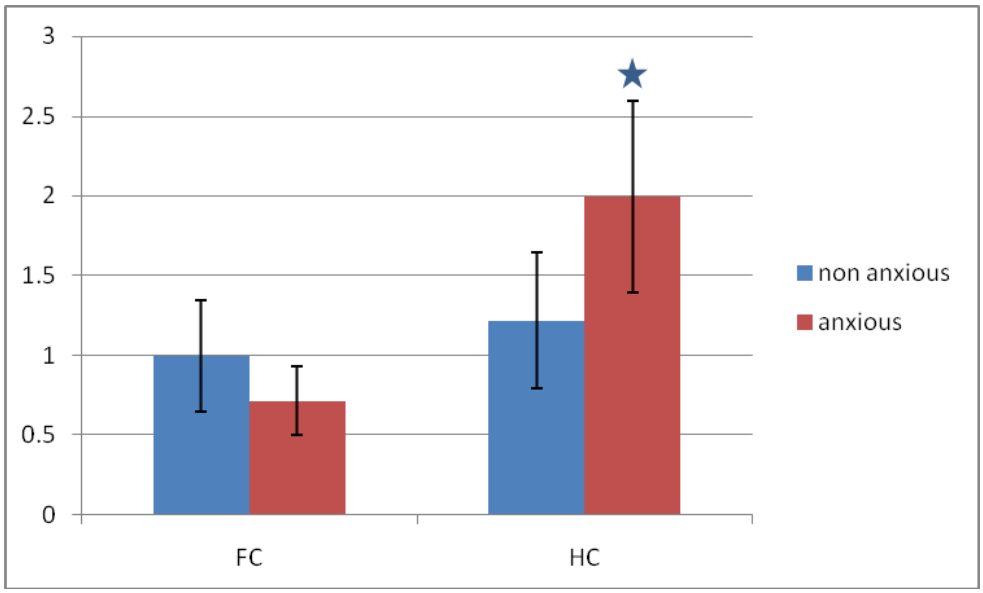


**Figure 4.** 5-HT/5-HIAA ratio in frontal cortex (FC) and hippocampus (HC) of non anxious and anxious selected rats in EPM. *P<0,05

## 4. Discussion

We could suggest the selection of anxious and non anxious rats behavioral tests to study anxiety is possible since emotionality of rats observed in tests as EPM and open field seems to be genetically determinated. Several genetic selections has been tried in animal tests and experiments of anxiety.

Selective breeding of strain rats based on ultrasonic vocalization (USV) rates in infancy shows that at third generation the selected “anxious” rats showed higher emotionality in EPM test [22]. Animals selectively bred for high levels of freezing in response to contextual cues were significantly more anxious than control rats both in social interaction test and EPM [23]. Roman low avoidance rats (RLA/Verh), genetically selected in two-way active avoidance, appear to be more anxious than their Roman high avoidance (RHA/Verh) counterparts, in the open field, elevated plus-maze, black/white box test, and in a new light/dark open field test [24]. Also inbred strains of Hatano high- (HAA) and low-avoidance (LAA) rats showed different behaviors when submitted an animal tests. HAA rats showed high anxiety-like behaviors compared with LAA rats in open field and elevated plus maze tests what suggests that HAA rats were predisposed to high anxiety compared with LAA rats [25].

Early studies demonstrated “anxious” selected rats in EPM selection showed higher corticosterone levels and pituitary reactivity to intravenously administered CRH than “non anxious” rats. It was also observed diazepamanxiolytic effect was less pronounced in “non anxious” rats as well as non anxious rats showed higherlocomotor activity following stress exposure [19]. Moreover, some studies showed that at the fourth generation bred rats selected as anxious in open field test showed anxious behavior in EPM and the black and white box [26-27].However, it was observed that rats selected as anxious in EPM did not differ from non anxious in alcohol consumption and, according to the tension reduction hypothesis, individuals with an elevated anxiety level may be more sensitive to the anxiolytic effects of alcohol and may, therefore, have a higher predisposition to consume alcohol [28].

It is noteworthy thatstudies withEPM selection arecontroversial since we know rats exposed in a second time to EPM develop a phobic state and shows no more response to anxiolytic drugs [15,29].

We observed that non anxious selected rats showed more rearing behavior and less freezing time in open field as well as higher central ambulation what is a pattern of less emotionality and less anxious behavior in open field. The results confirm previous studies that showed genetically anxious rats selected in open field test presented higher emotionality in EPM [26,27].

The social interaction test pointed to higher time of active interaction of pair ofselected “non anxious rats” when compared with “anxious” rats. There is no study for results with selected rats in EPM and tested in the social interaction test. However some previous results have showed that some drugs and procedures affect the same way the 2 models [23,29,30,31]. Rats selected as low sociability showed more anxious behavior when tested in open field and social interaction test [32]. The results suggest that anxious and non anxious selected rats would maintain the same behavioral pattern when submitted to other animal models what allow us to suggest selection of rats in EPM as a tool to study anxiety in experimental behavioral tests.

Several studies have proposed central 5-HT system plays an important role in the rats behavior in several experimental tests of anxiety [12,14,15,33]. Indeed, several 5-HT ligands has EPM effects. Agonists 5-HT2A and 5-HT2B show an anxiolytic-like response as well as 5-HT1B auto-receptor antagonist [13,17]. Fluoxetine, a specific 5-HT reuptakeinhibitor decrease open arms exploration [31]. Repeated administration of Nigella sp can decrease 5-HT turnover and shows anxiolytic response in EPM [16].

Experimental 5-HT manipulations has also showed EPM effects. It was observed that five weeks of food restriction, in rats, significantly decreased 5-HT turnover rates in the hippocampus and hypothalamus and produces anxiogenic-like behavior in EPM: more time in the closed

arms [34]. Carvalho et al, 2005, showed decrease in the content of 5-HT in several limbic structures (prefrontal cortex, amygdala, dorsal hippocampus, and nucleus accumbens) of rats soon after have been exposed to the open arms of EPM [15]. The decrease in 5-HT content in the limbic structures seems to be a long-lasting effect of the exposure to the EPM since it can be observed 24 h after the test [21,35].

Our results showed no differences between the two groups for the 5-HT and 5-HIAA measures in frontal cortex and hippocampus. However we could observe decrease of 5-HT/5HIAA ratio in frontal cortex what could point to a lower 5-HT activity of non anxious rats. These results point in the same way of Peerven et al, 2009, who observed a 5-HT turnover decrease after Nigella administration [16]. Despite the differences our results agreed with previous studies that suggest the 5-HT system plays an important role in determination of EPM rats behavior.

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