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Conspecific Release Is Influenced by Anxiety-Like Behavior of Female Rats in a Prosocial Task

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Abstract

The concept of prosocial behavior encompasses actions that promote the well-being of others, often motivated by emotional connections and empathy within a group. This behavior is also observed in non-human species, including rodents, frequently studied in neuroscience research. A previous study proposed the releasing of a trapped conspecific as a task to study prosocial behavior in laboratory rats. Nevertheless, the empathetic/prosocial motivation to liberate the trapped rat is controversial. In this study, we used a similar protocol to evaluate behavioral factors that may influence the performance of the conspecific release task (CRT). Adult female Wistar rats were submitted to the CRT conducted in an open field arena. An acrylic restrain box, in which a conspecific was trapped, was placed in the center of the arena. In Experiment 1, the rats were evaluated in six CRT trials, and the occurrence of the liberating behavior (opening trapping box) varied among subjects. We established a criterion for categorizing the opening behavior: the free rats that performed the CRT in 2 sessions or more were categorized into "opener" and the rats that performed the behavior one time, or never performed the CRT were categorized as "non-openers". After separating the groups, behavioral parameters that possibly influenced CRT performance were compared between openers and non-openers. At the end of experiment 1, anxiety-like behavior was observed in animals belonging to the non-opener group, as expressed by diminished exploration of the center of the arena. In experiment 2 we applied pharmacological manipulation to modulate the anxiety-like behavior of the free rat, evaluating the effects of these variations on the liberating behavior. The rats that were categorized as non-openers were treated with the classical anxiolytic diazepam and the free rats categorized as openers received fluoxetine, that is anxiogenic when given acutely. Treatments were applied before the day of the test to evaluate performance in CRT. Non-openers treated with diazepam freed their

cage mates, whereas openers treated with fluoxetine did not. The data suggests that anxiety-like levels influence CRT behavior.

Keywords

Distress Recognition, Empathy, Social Behavior, Anxiety, Diazepam and Fluoxetine

1. Introduction

Prosocial behavior occurs when an action can promote the well-being of another individual. It is often motivated by the affinity between individuals within a group [1]. This behavior is also motivated by the desire for interaction through emotional actions, often mediated by putting oneself in the place of the other and internalizing distress to alleviate suffering [2].

This socially motivated behavior is common in humans, who can help individuals, whether family or strangers [3]. Notably, similar prosocial behaviors are found in other species like non-human primates and rodents, where familial or social bonds and emotional contagion drive altruistic actions [2] [4]-[6].

Empathy, a complex and multifaceted concept, is classically defined as an emotional state in which an individual shares the feelings of another [2]. However, applying this concept to non-human animals is a challenge. In this regard, studies have suggested that non-human animals can experience feelings and emotions such as joy, sadness, and empathy [7]. Likewise, emotional contagion (a form of empathy) is also observed among mammals and extends to species living in societies, such as ants, which exhibit prosocial behavior of rescuing conspecifics in dangerous situations [8]. While studies in rodents often reveal facets of emphatic phenomena such as emotional contagion, the precise role of empathy in motivating prosocial behavior remains poorly understood [9] [10].

Pioneering studies, similarly those by Ben-Ami Bartal *et al.* [10], provide insights into the empathetic capacities of rodents, demonstrating their propensity for prosocial behavior in response to the distress of conspecifics. The prosocial behavior proposed in the study was the spontaneous liberation of a trapped conspecific by a free rat [10]. Subsequent research efforts have highlighted the persistence and learning associated with this behavior, exploring the complex interplay between empathy and prosociality [11] [12]. However, the interpretation of this task as empathy-motivated behavior has been questioned by some researchers [13] [14].

Studies have explored various factors that might motivate the release of a conspecific in the task proposed by Bartal and colleagues. For instance, Silberberg *et al.* [15] demonstrated that the free rat sought to release its conspecific motivated by the desire for social contact. Conversely, Sato *et al.* [11] showed that the free rat released the trapped conspecific to alleviate the trapped rat's stress. Silva *et al.* [16]

found that social interaction between the trapped and free rats was not a significant motivator; rather, task learning might have facilitated the door opening and release of the trapped animal. More recently, studies by Breton *et al.* [17] have demonstrated that helping behavior can be influenced by the age of the free rat, indicating that adolescent rats do not exhibit social biases between familiar and unfamiliar rats.

While other factors may influence empathetic behavior, studies have shown that anxiogenic situations, such as maternal separation [18], can interfere with emotional contagion behaviors. Similarly, anxiolytic contexts promote empathy-driven behaviors [19] [20]. Accordingly, animals administered oxytocin, a substance capable of reducing social anxiety, have demonstrated improved performance in the conspecific release task [21]. In this regard, a study by Kitano *et al.* [22] revealed that oxytocin knockout animals performed worse compared to wild-type animals, corroborating the possible role of oxytocin in reducing social anxiety and facilitating the completion of the conspecific release task.

In parallel, the behavior of releasing a trapped conspecific seems to vary among the different studies and even within the same study. A recent review revealed a 70% success rate in opening the trap, including in Bartal's experiments [23]. In another work, Wu *et al.* [24] reported that 66.6% (8 out of 12) of rats successfully opened the trapping box.

Our study aims to evaluate the free rat behavior and the influence of different behavioral patterns in the performance of the conspecific releasing task (CRT). Particularly, we evaluated anxiety-like behavior of the free animals and examined the effects of anxiolytic and anxiogenic substances on the releasing performance.

2. Materials and Methods

2.1. Subjects

One hundred and twenty-six female Wistar rats, aged 10 - 12 weeks, were housed under a 12/12 h light-dark cycle at a temperature of 21˚C (±3˚C), with unrestricted access to food and water. Rats were allocated in pairs two weeks prior to the experiments to familiarize themselves with their partners and the new cage environment. One rat in each pair was randomly and permanently assigned to the role of "Free rat," while the partner was assigned to the role of "Trapped rat." We used a simple randomization procedure, with equal probability of assignment between subjects. All procedures were carried out according to the Brazilian rules for the use of animals in scientific research (Law n˚ 11.794) and approved by the local ethics committee (CEUA/UNIFESP No. 6485310820/2020).

2.2. Apparatus

The CRT was performed in a circular wooden open field (**Figure 1(A)** – 96 cm × 32 cm). In the center of the arena, we placed an acrylic restrain box (25 × 7.5 × 7.5 cm). The box had small ventilation holes and a door that could only be opened outwards (**Figure 1(B)**). The behavior of the animals was recorded by a webcam

placed over the apparatus and analyzed by a video-tracking software (Anymaze, Stoelting Co. USA). Only the free rat was tracked. To generate contrast with the bottom of the arena, a black mark was placed on the tail of the free rat.

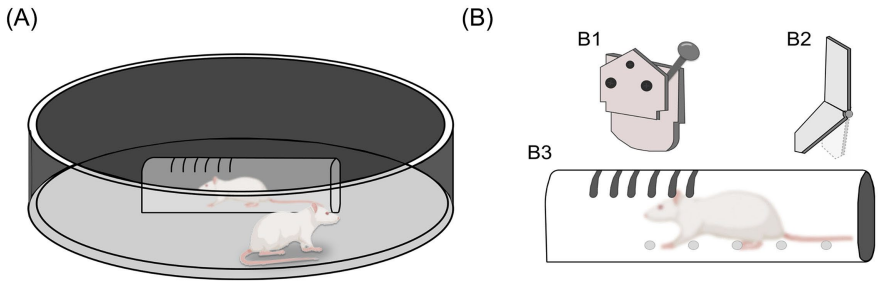

Figure 1. Schematic representation of the apparatus: (A) open field apparatus with restraining box and animals during the test; (B) Restraining box; First door (B1); second door (B2); and restrain box (B3).

The arena was virtually divided into three zones for analysis: outer, intermediate, and inner zones. The outer zone (OZ) was defined as the peripheral region of the arena (the closest to the border), the intermediate zone (IZ) was defined as the area closer to the restraining box excluding the region of the restraining box itself, and the inner zone (iZ) was defined as the area closer to the restraining box including the restraining box itself. The regions were defined as the Outer Zone (OZ), Intermediate Zone (IZ) and Restraining Box (RB), as described in Figure 2. Additionally, the occupation of the Inner Zone (time spent in intermediate zone plus time spent on the restraining box) was also considered in the analysis.

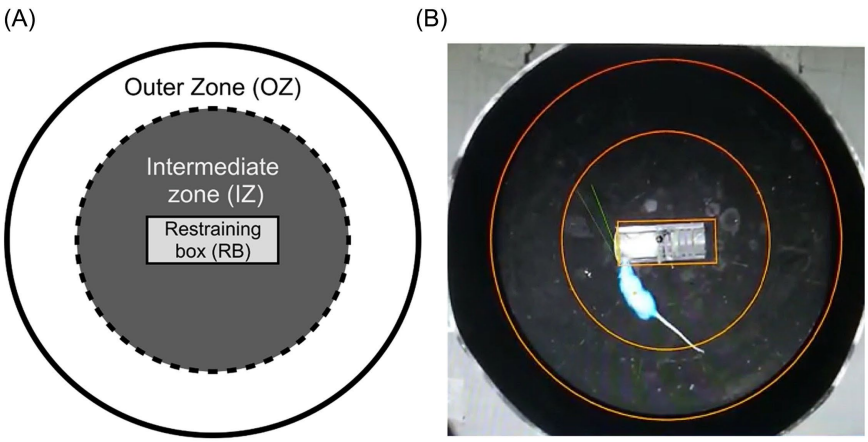

Figure 2. Schematic representation of the apparatus: (A) Open field apparatus with markings OZ—Outer Zone; IZ—Intermediate Zone; and RB—Restraining Box. (B) Example of screen image from the video-tracking software with virtual delimitations of the zones.

2.3. Substances

In Experiment 2 we used the classical anxiolytic diazepam (Roche), at a dose of 1 mg/kg, in a volume of 0.2 mL per 100-gram, 30 min before the test, as described previously [25]. As an anxiogenic agent, we used fluoxetine (Sigma Chemical Co.,

United States), at a dose of 2 mg/kg, in a volume of 0.1 mL per 100-gram, 60 minutes before the test as described by Zienowicz [26]. This previous study has shown anxiogenic action of this fluoxetine dosage, as well as pilot studies in our laboratory. Saline was administered before the categorizing sessions, at a volume of 0.1 mL per 100-gram, 30 min before the test. We use saline to dilute all substances, and all injections were intraperitoneal.

2.4. General Procedures

The rationale of the task is the behavior of opening the door of the restraining box and releasing a conspecific animal by the free rat in the arena. The protocol used the methodology previously described by Ben-Ami Bartal *et al.* [10] and Silva *et al.* [16] with minor modifications. Initially, rats were randomly chosen and marked to be the free rats (n = 9 - 10). For each experimental test, the animals' cages were brought to the test room, where they remained in their cages at least 30 minutes before the trial. Afterwards, the marked rats were individually placed in the apparatus to freely explore the arena and the restraining box without the door. After the habituation period (four daily habituation trials), the experimental CRT protocol was initiated. The trapped rat (unmarked cage mate) was placed in the restraining box at the center of the apparatus (**Figure 1(A)**). Next, the free rat was introduced into the arena. If the free rat opened the door and released its conspecific, a second door without a lever was placed to prevent entry and exploration of the restraining box by both the animals. If the free rat did not open the door after 20 minutes, the experimenter released the confined conspecific rat, allowing interaction for 10 minutes.

The behavioral parameters registered were: 1) Latency (in seconds) to open the restraint door; 2) Relative opening frequency of the restraint door by the free rat in trials. The relative frequency was calculated by dividing the number of door openings by the number of animals; 3) Time in the different open field zones; 4) the rate of time spent in social interaction after release. The social interaction rate was calculated as the time spent in social interaction (s) divided by the remaining session time after release (s). Social interaction was recorded when the animals approached each other, with at least one directing or contacting its vibrissae toward the partner. Behaviors such as sniffing, following, and walking over or under the partner were also categorized as social interaction.

2.5. General Procedures

Experimental designs are schematized in **Figure 3**.

2.5.1. Experiment 1

This experiment was conducted in six 30-minute trials (six days, a trial per day). The animals randomly chosen to be trapped were first placed in the restriction box and then the free rat was introduced in the arena. Forty-four pairs of rats participated in this experiment. A criterion was established for the separation of ani-

mals into two groups. If the free rats performed the door opening in two or more trials, they were classified as "openers" (OG; n = 20). Conversely, if they performed the door opening in just one trial or did not perform the behavior in any trial, they were classified as "non-openers" (NOG; n = 24). The assignment of rats to these groups was conducted by an experimenter blind to the experimental conditions and was performed only after the conclusion of the CRT session.

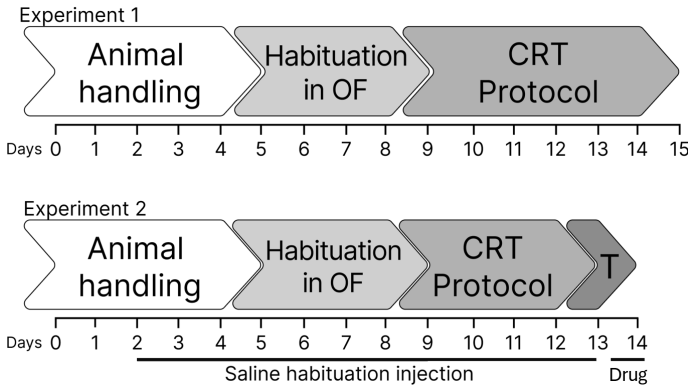

Figure 3. General procedures. Timeline of experiment 1 and experiment 2.

2.5.2. Experiment 2

As described below, the main difference between NOG and OG, besides the releasing behavior in experiment 1, was related to anxiety-like behavior. Nineteen pairs of rats participated in this experiment. Therefore, we choose to manipulate the basal anxious state of the free animals with anxiolytic and anxiogenic drugs and evaluate the effects of these changes in the opening behavior. Rats were randomly marked and designated as "free rats" and were submitted to five-minute sessions of human handling for two consecutive days. Starting from the third session of human handling, the rats received an intraperitoneal injection of saline solution (0.1 ml per 100-gram) at the end of the manipulation period (5 days in total). After the handling procedures, the free rats were exposed to the open field apparatus for four habituation sessions as described above. After the habituation period, the CRT protocol was initiated. In all sessions (habituation and trial sessions), the rats received an intraperitoneal injection of saline (0.9%) 30 minutes before the trials. At the end of the four trials, the free rats were classified and separated into two groups: "openers (OG)" and "non-openers (NOG)", according to the criteria established in experiment 1. After group separation, an additional CRT trial (fifth trial), designated as the test day, was conducted. In this additional trial, the non-opener-free rats received an intraperitoneal injection of diazepam (1 mg/kg) 30 minutes before the start of the trial and the opener-free rats received an intraperitoneal injection of fluoxetine (2 mg/kg) 60 minutes before the start of the trial. Only four trials and one test were conducted to prevent overexposure of the animal to the open field apparatus, while still allowing for the differentiation between groups after four exposures. Analysis of this experiment were conducted by an experimenter blind to treatment and open/non-opener classification.

2.6. Statistical Analysis

All data were checked for normality using the Kolmogorov-Smirnov test. Latency to opening, frequency of social interaction, and zone occupation were analyzed by repeated measures ANOVA followed by pairwise comparisons tests with Bonferroni adjust. Repeated measures ANOVA with Greenhouse-Geisser or Huynh-Feldt corrections were applied when necessary. Latency in each behavioral session was analyzed by Student's test and paired Student's t-tests when applicable. The opening rates were analyzed by Fisher's exact test. The results are presented as the mean ± standard error of the mean (±SEM), and effect sizes are presented as partial eta-square (η_p^2) and Cohen's D (d). A level of statistical significance of $p < 0.05$ was adopted in all analyses.

3. Results

3.1. Experiment 1

The results of opening the restriction box were used to categorize the animals into the groups OG and NOG.

After categorization, the OG comprised 20 rats, while the NOG comprised 24 rats, corresponding to 43% and 57%, respectively. For the latency to open the restraining box, repeated measures ANOVA revealed a significant main effect of session [$F(5, 41) = 2.440$, $p = 0.036$; $\eta_p^2 = 0.055$], and a significant interaction between group and session [$F(5, 41) = 4.809$, $p = 0.001$; $\eta_p^2 = 0.103$] (**Figure 4(A)**). Rats in the NOG had a significantly longer latency to open doors compared to the group of opener rats (**Figure 4(A)**). Specifically, the pairwise analysis with Bonferroni adjustment demonstrated a significant difference between groups on trial 2 ($p = 0.001$); trial 3 ($p < 0.001$); trial 4 ($p < 0.001$); trial 5 ($p < 0.001$); and trial 6 ($p < 0.001$). As expected, the Fisher exact test revealed increased opening rates of OG compared to NOG in all trials ($p = 0.05$), except for trial 1 (**Figure 3(B)**).

For the time spent in the inner zone, repeated measures ANOVA demonstrated a significant main effect of group [$F(1, 41) = 12.527$, $p = 0.001$; $\eta_p^2 = 0.230$]. The pairwise comparison with Bonferroni adjusts demonstrated OG spent more time in the inner zone than NOG (**Figure 5(A)**). For the intermediate zone, repeated measures ANOVA did not demonstrate a significant effect of the group or time, but the pairwise comparison with Bonferroni adjust analysis demonstrated OG spent more time in the intermediate zone than NOG (**Figure 5(B)**).

Considering the time spent in the outer zone of the apparatus, the repeated measures analysis demonstrated a significant main effect of group [$F(1, 41) = 11.775$, $p = 0.001$; $\eta_p^2 = 0.219$]. The pairwise comparison with Bonferroni adjusts demonstrated that OG spent more time in the intermediate zone than NOG (**Figure 5(C)**). For the time rate in the restraining box, repeated measures ANOVA did not demonstrate a significant main effect of the group in the occupancy time by free-ranging rats during CRT trials (**Figure 5(D)**).

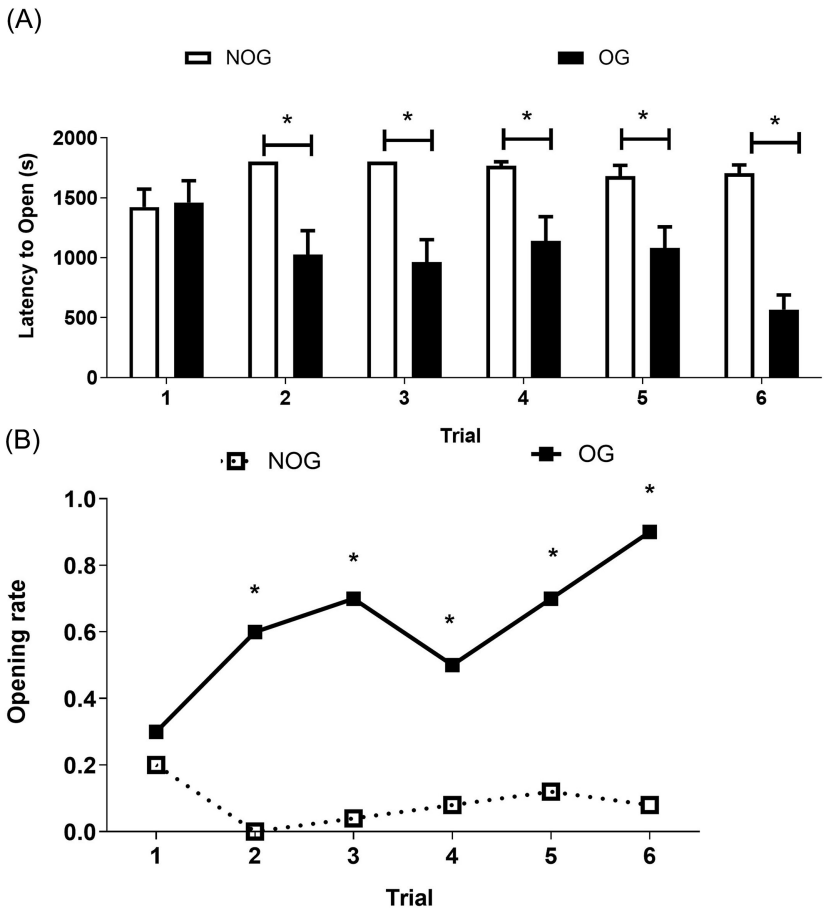

Figure 4. (A) Latency to open the restraining box in each trial. Data show means + SEM. (B) Opening rate for Opener group (OG; n = 20) and non-opener group (NOG; n = 24) in each trial. The opening rate was calculated by dividing the number of door openings by the number of animals. *p < 0.05 comparing openers and non-openers (Repeated measures ANOVA for latency and Fisher's exact test for opening rate).

Lastly, a repeated measures ANOVA showed no significant difference between the OG and NOG in the social interaction rate (Figure 6).

3.2. Experiment 2

After categorization, the OG consisted of ten female rats, while the NOG comprised nine female rats. This corresponds to 53% and 47%, respectively. For the latency to open the restraining box, repeated measures ANOVA demonstrate a significant main effect of group [F (1, 17) = 14.190, p = 0.002; η_p^2 = 0.422], and a significant interaction between group and session [F (4, 17) = 3.395, p= 0.014; η_p^2 = 0.166]. A pairwise comparison with Bonferroni adjust demonstrated that OG had decreased opening latency compared to NOG in the third (p = 0.005) and fourth (p = 0.001) training trials (Figure 7(A)). For the opening rates, the Fisher exact test revealed a difference between the OG and NOG in all training trials (p < 0.05). Conversely, on the day of the test, the OG group demonstrated a decreased opening frequency compared to the NOG group (Figure 7(C)).

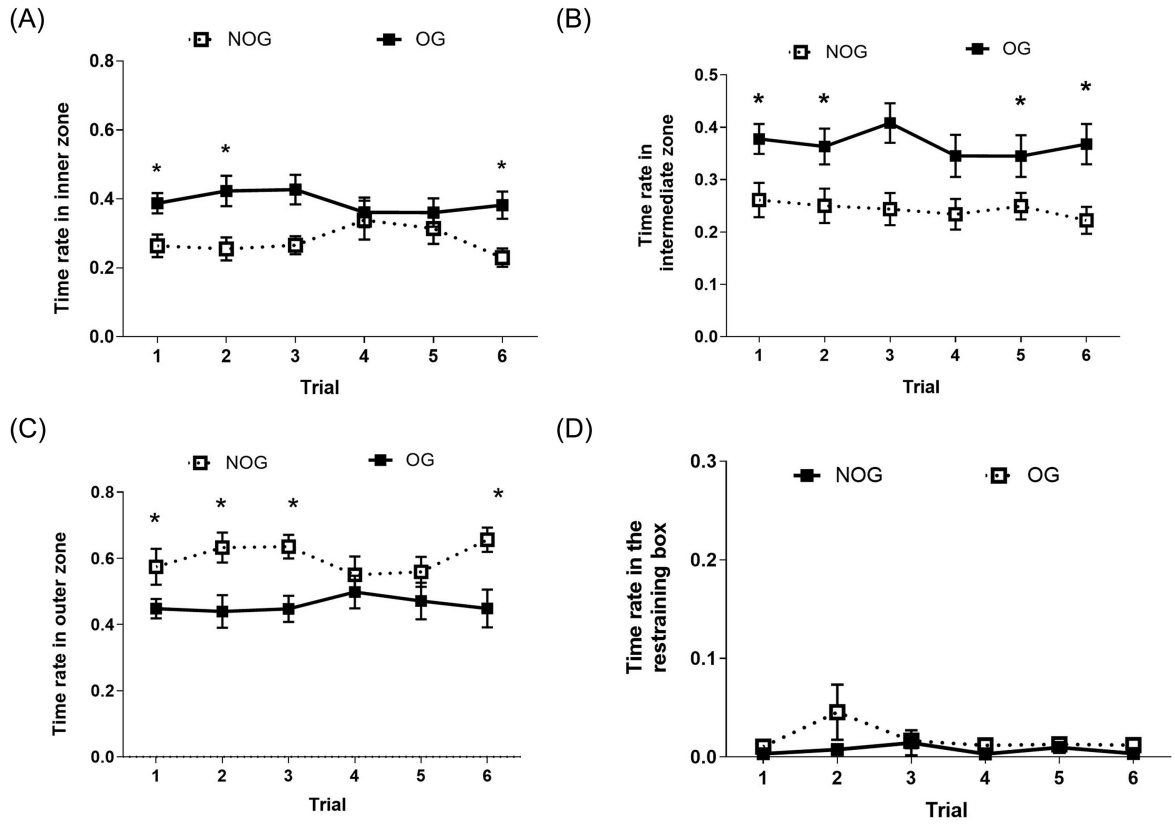

Figure 5. Time rate in different zones of the apparatus. (A) Time rate t in the inner zone. (B) Time rate in the intermediate zone. (C) Time rate in the outer zone. (D) Time rate in the restraining box. Data show means ± SEM. *p < 0.05 (repeated-measure ANOVA followed by Bonferroni adjust analysis, n = 20 - 24).

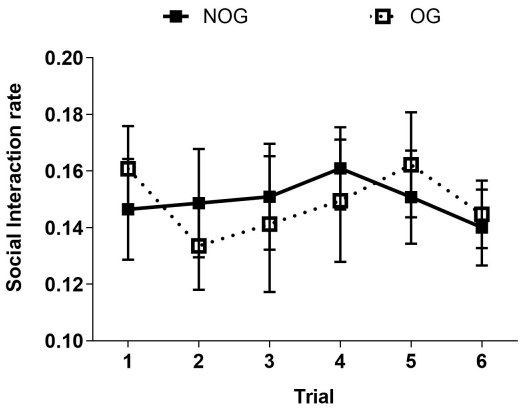

Figure 6. Social interaction rate after releasing in each trial. Data show means ± SEM. (Repeated measures ANOVA, n = 20 - 24).

On the test day, the free rats from the OG received fluoxetine, while the NOG received diazepam, to verify the influence of anxiety level in the prosocial behavior (**Figure 7(B)**). Unlike the training trials, Student's t-test did not demonstrate a significant difference when comparing the Opener and Non-opener groups on the test day. Additionally, the paired samples t-test demonstrated a significant difference when comparing session 4 with the test day for openers. The opener rats,

which received fluoxetine on the test day, showed a longer latency to open on the test day, t (9) = −2.443, p = 0.037; d = −0.77. Similarly, the paired-samples t-test revealed a significant difference when comparing session 4 and the test day of non-openers. The non-opener rats, which received diazepam on the test day, showed a shorter latency to open on the test day, t (8) = 3.148, p = 0.014; d = 1.05 (Figure 7(B)).

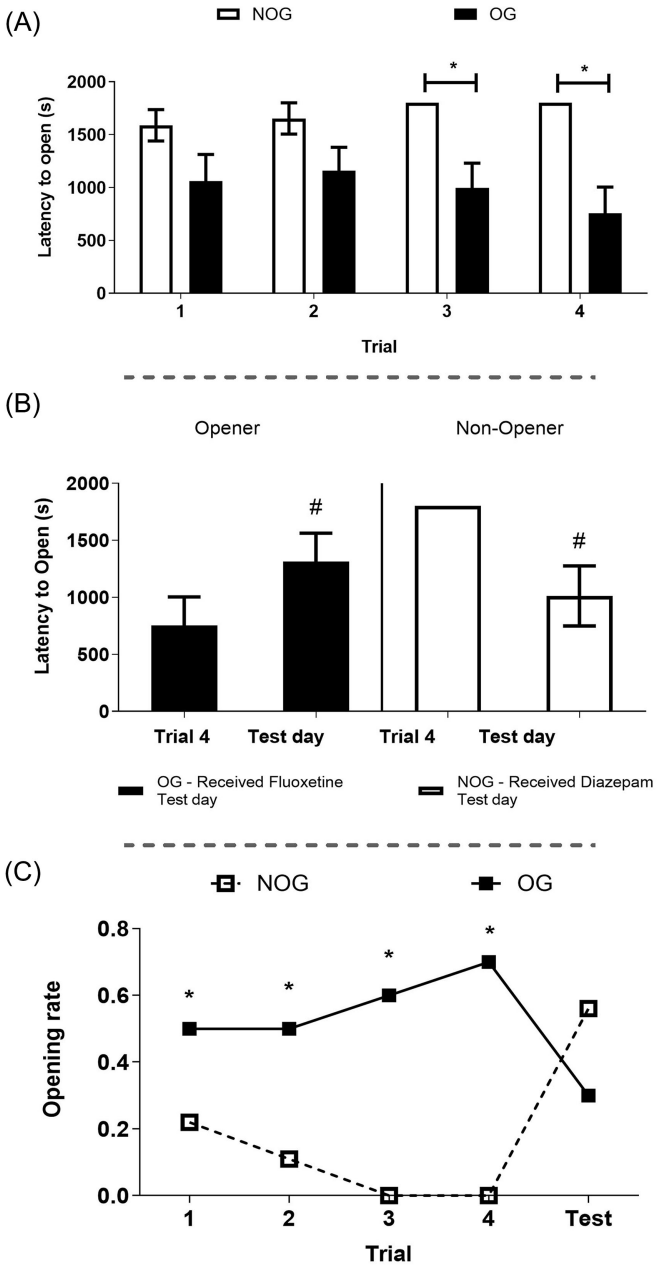

Figure 7. (A) Latency to open the restraining box in trials 1 - 4. *p < 0.05 comparing OG (n = 10) vs NOG (n = 9) group (ANOVA with repeated measures). (B) Latency to open the restrain box in trial 4 and test day. #p < 0.05 compared to trial 4 in each group (paired-samples Student's t-test). (C) Opening rate in all trials. *p < 0.05 comparing OG vs NOG group (Fisher's exact test).

Regarding the time spent in each zone, repeated measures ANOVA with a Huynh-Feldt correction was conducted to evaluate the occupancy of the inner zone by the free rats across different sessions. The analysis revealed a significant interaction between group and session [F (2.232, 17) = 3.164, p = 0.046; η_p^2 = 0.157], with a mean square of 0.115 (ε = 0.581, Figure 8(A)). Similarly, a repeated measures ANOVA with a Greenhouse-Geisser correction was conducted to assess the occupancy of the intermediate zone by the free rats across sessions. The analysis revealed a significant interaction effect between group and session [F (2.181, 17) = 4.374, p = 0.017; η_p^2 = 0.205], with a mean square of 0.161 (ε = 0.545). Pairwise comparisons with Bonferroni adjust demonstrated that OG spent more time in the intermediate zone than NOG when comparing the groups in the fourth training trial (p = 0.039, Figure 8(B)). Considering the time spent in the outer zone (Figure 8(C)) and the restraining box (Figure 8(D)) of the apparatus, repeated measures ANOVA did not demonstrate significant main effects. Lastly, repeated measures ANOVA showed no significant difference between the OG and NOG in the social interaction rate (Figure 9).

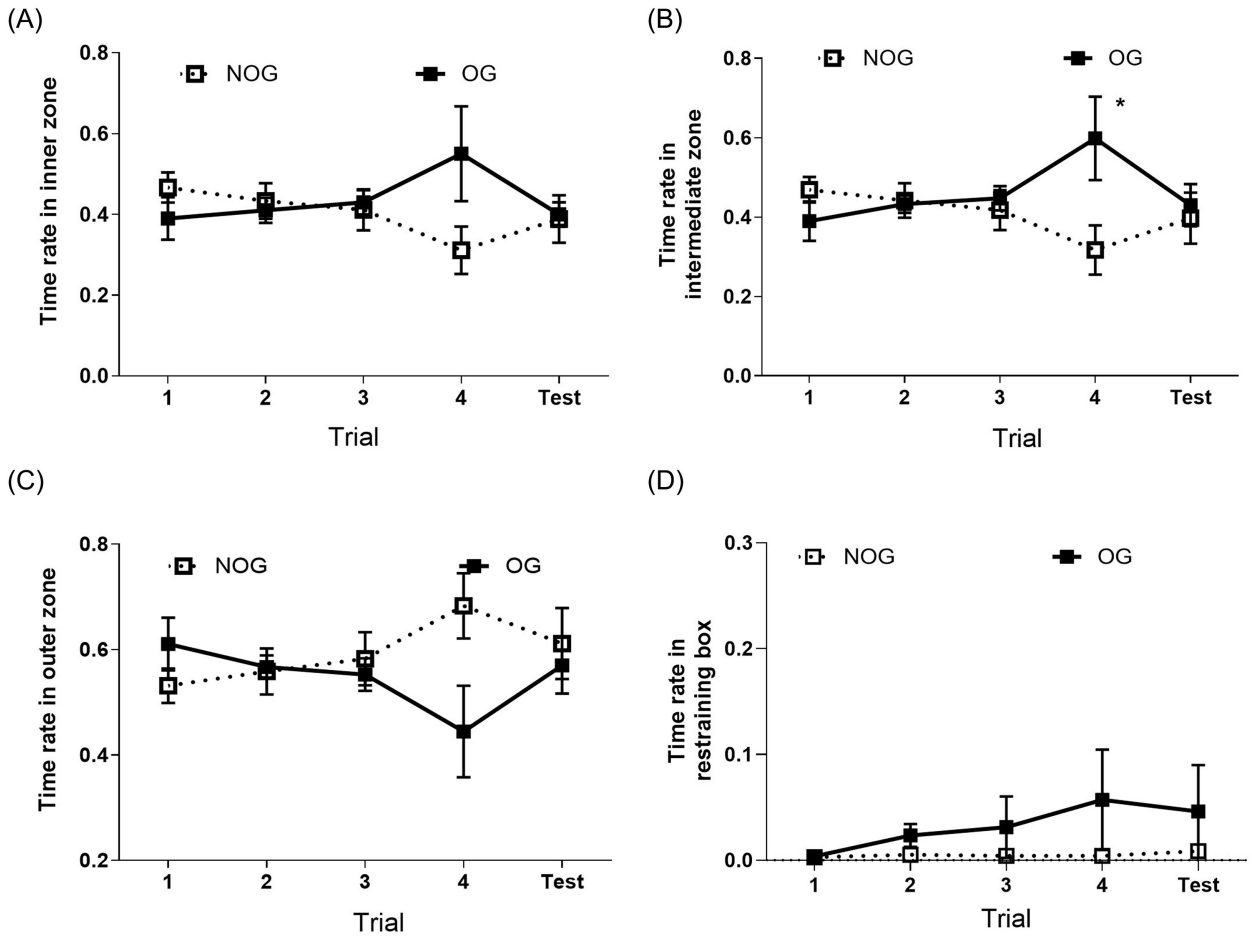

Figure 8. Time rate in different zones of the apparatus. (A) Time rate in the inner zone. (B) Time rate in the intermediate zone. (C) Time rate in the outer zone. (D) Time rate in the restraining box. Data show means ± SEM. *p < 0.05 comparing OG and NOG (Repeated measures ANOVA, n = 9 - 10).

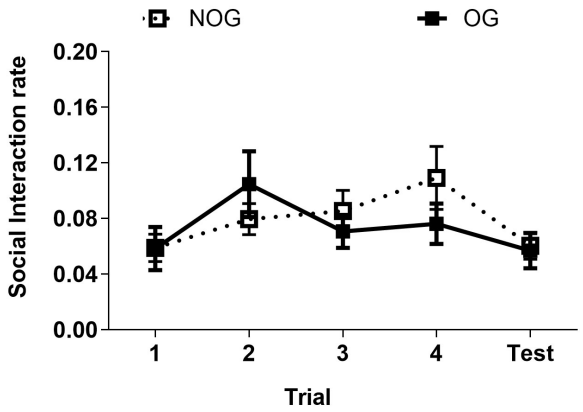

Figure 9. Social contact rate during each trial. Data show means ± SEM (Repeated measures ANOVA, n = 9 - 10).

4. Discussion

The experimental protocol of CRT, first described by Ben-Ami Bartal *et al.* [10] and the adapted version of the protocol by Silva *et al.* [16], reveals an interesting aspect of rats' behavior. When placed in an open arena with their cage mate confined in a restraining box at the center, the free rat often demonstrates helping behavior by aiding in the liberation of its cage mate within a few days of training. However, our data demonstrates low adherence to CRT among the tested rats. The data suggest a clear division between rats that exhibit helping behavior (openers—OG) and those that do not (non-openers—NOG).

Comparing the findings of this study with the literature and previous findings of our group, we can observe that the rats diverge between the OG and NOG, with a higher proportion of non-openers than openers. This divergence contrasts with the first study [10], as well as from the findings of our group [16], which reported similar findings using comparable experimental protocols. Recently, Ben-Ami Bartal *et al.* [27] published data showing that door-opening behavior may not occur when animals are exposed to an individual outside their lineage or social group or in the presence of reinforcements such as chocolate. This contrasts with Silva *et al.* [16], which demonstrated a reduction in the rate of opening the restraining box when it was empty but did not show a behavior of non-opening between groups of the same strain, as seen in our findings.

An important point to consider is the difference in the rat strain used in the first study, that employed Sprague-Dawley rats [10], and our group results [16], including the present study, that used Wistar rats. This strain differences main explain the lower CRT adherence in the Wistar rats, than the Sprague-Dawley rats. Additionally, another study has shown a release rate of 76% for female rats and 51.7% for male rats using the Sprague-Dawley lineage in the conspecific liberation task [20]. Even when following a protocol similar to that used by Silva *et al.* [16] there was a difference in CRT adherence. This could be attributed to the change in laboratory location—previously conducted in Natal-RN and currently in São Paulo-SP (both in Brazil). Moreover, the dimensions of the open-field arena (62.5

cm × 31.5 cm) used by Silva *et al.* [16] differ from those used in this protocol (96 cm × 32 cm). Finally, in both experiments conducted under this protocol, 43% and 53% of female free rats, performed the CRT in experiments 1 and 2, respectively. This percentage is quite significant for the opener group, allowing for comparison with the non-opener group and enabling the investigation of factors that influence the reduction in CRT performance.

Some of important points have to be considered, like 1) differences in rat strains, Sprague-Dawley rats [10] and Wistar rats, performed in the present study and previously [16]; 2) sexual difference in release ratio (76% for female and 51.7% for male) in Sprague-Dawley rats [20]; and 3) country region of the experimental performance [between the same research group, in the northeast [16] and the southeast (performed in the present work)], wherein the background and environment of the animals can influence in the reproducibility of studies [28] [29]; (4) minor apparatus modifications, whereupon the size dimensions of the open field can modify the behavior, as seen in the previous group study [16] and the present study.

Nevertheless, in both experiments conducted in the present study, 43% and 47% of female free rats, performed the CRT in experiments 1 and 2, respectively. This percentage is quite significant for the opener group, allowing for comparison with the non-opener group and enabling the investigation of factors that influence the reduction in CRT performance.

However, the latency for opening the restraining box in the presence of the confined rat reveals a significant difference between OG and NOG. Non-openers consistently presented prolonged latency periods across all sessions, whereas openers show a reduction in latency over time. These findings are consistent with the literature [10] [16] and collectively indicate that task learning occurred.

It is important to mention that, even with the possibility of social contact, non-opener rats maintain extended latency periods, contrasting with findings by Silberberg *et al.* [15] that suggested reduced latency when social interaction was offered. Furthermore, Silva *et al.* [16] showed that efficiency in performing opening behavior was not correlated with amount of social interaction after release, corroborating our results, wherein it was not seen a significant difference in social contact between OG and NOG.

The hypothesis of other factors influencing the low performance of CRT was evaluated. Subsequently, we analyzed the time spent in the different areas of the apparatus. In this respect, the evaluation of anxiety-like behavior in rodents can be performed in various apparatuses. One of them is the open field, in which the evaluation of the time spent in different zones, such as external (the closest zone to the wall) and internal (the center of the apparatus) is used to estimate anxiety-like behavior [30]. In addition, some protocols use an intermediate zone to scrutinize these analyses. In this context, it is consensus that the animals that spend more time in the external zone (in our case, Outer Zone) show increased anxiety-like behavior [20] [31]-[34], and this behavior can be modified by anxiolytic and

anxiogenic manipulations [30] [32].

The data on the occupancy of the zones during CRT sessions show that NOG animals explore more the outer zone, and less the internal and intermediate zones, potentially indicative of anxious-like behavior. Thus, we raised the hypothesis that increased anxiety could hinder CRT execution, that aligns with findings by Fontes-Dutra *et al.* [35] and Tuncak *et al.* [36] that demonstrated impairment in the development of CRT related to anxious-like behavior in an animal model of autism. Ben-Ami Bartal *et al.* [5] further demonstrated the impact of stress on CRT performance, wherein the high or low corticosterone levels hindering box-opening behavior. These data suggest that under high-stress levels, free rats remain in the periphery of the apparatus for extended periods and do not perform CRT, or under low-stress levels, rats also do not perform CRT. Accordingly, Mason [23] showed that high levels of anxiety impair helping behavior in rats, and a moderate level of anxiety favors the execution of this behavior. Our data of exploration of the inner and intermediate zone reveal a significant difference between the OG and NOG, in line with the findings of Ben-Ami Bartal *et al.* [27], who demonstrate that opener rats spend more time near the restraining box compared to non-opener rats. We can observe that this difference occurs in sessions 2, 3, and 6 of CRT, in which opener rats spend more time in the zones near the containment box where their cage mate is confined. Interestingly, there is some correspondence with the higher rate of opening the restraining box in sessions 3 and 6 of CRT.

In Experiment 2, we aimed to investigate the interference of anxious-like behavior in the opening behavior of free rats during CRT. During the regular CRT sessions, the behavior of free-ranging rats regarding the opening of the containment box was similar to that observed in Experiment 1. Thus, we also categorized the animals into two distinct groups: "opener" and "non-opener." As expected, this behavior showed statistically significant differences between the groups during the CRT sessions. Indeed, the latency to open the restraint box observed in Experiment 2 indicated that the animals in the OG performed the CRT more rapidly throughout the training sessions. In contrast, the animals in the NOG did not develop the CRT.

In this case, we subjected the rats to an additional session, a test session, to observe the behavior of free-ranging rats under the pharmacological effects of anxiogenic (fluoxetine at a dose of 2 mg/kg) and anxiolytic (diazepam at a dose of 1 mg/kg) substances. The results showed that during the test session there was a reversal in the latency to open the containment box between the OG and NOG, indicating that typical anxious-like behavior can influence the development of CRT [37]. There was also a reversal in the opening rate in the test session, indicating that diazepam at a dose of 1 mg/kg reduced anxiety levels in rats in the NOG, enabling them to perform the CRT, releasing the conspecific. This finding contrasts with Ben-Ami Bartal *et al.* [5] findings, which showed a reduction in containment box opening latency when administering high doses of benzodiazepines (midazolam 2 mg/kg). Nevertheless, in our study, we administered diaze-

pam acutely 30 minutes before exposure to the apparatus and observed the increase opening behavior by the rats in the NOG. Moreover, analyzing the training session 4 and the test session, rats in the NOG demonstrated better CRT performance in the test session compared to session 4 of training, indicating that low dose of diazepam, administered acutely, favored CRT development. Diazepam is a classic anxiolytic used in animal models to assess anxiety-like behavior and task performance. Its efficacy is commonly evaluated in paradigms such as the open field test, plus-maze or elevated plus-maze. Thus, diazepam's anxiolytic properties probably facilitated the performance of tasks by decreasing anxious behavior [16] [32] [38]-[40].

The anxiogenic effects of acute treatment with fluoxetine can be observed in various animal models of anxiety, such as the social interaction test [41]-[43] and exploration of the open arms in a plus-maze [40] [44]. Specifically, the anxiogenic effect of acute fluoxetine was observed by Griebel *et al.* [45], when administered 30 minutes prior to exposure to the elevated plus-maze, resulting in reduced exploration of the open arms. Furthermore, in an air puff test, fluoxetine increased the escape behavior, and promoted a 55% reduction in social interaction time, reinforcing the anxiogenic effect of fluoxetine when administered acutely [43].

In the present study, we observed a reduction in containment box opening by rats in the OG when treated with acute fluoxetine at a dose of 2 mg/kg 1 hour before exposure to the apparatus. This observation indicates that fluoxetine promoted an anxiogenic-like effect in OG rats reducing the containment box opening [46]-[48]. These findings support the hypothesis of a decrease in the performance of the CRT due to increased anxiety. This behavior change could be due to decreased motivation to social interaction on the test day, although our data from social interaction does not indicate the opening behavior is motivated by desire of social contact. Alternatively, a reduced general exploration of the apparatus could be related to this effect. Nevertheless, if this was the case, there would be a reduction of exploration in all apparatus, including in the external zone, which was not the case.

5. Conclusion

Our data suggests that the performance of the conspecific release task, a behavior possibly motivated by empathy, requires a low level of anxious behavior in the free animal. In addition, our results suggest that non-opener rats may present deficits in recognizing signs of distress from their cage mate, or lack of empathic motivation, due to increased anxiety levels.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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