Journal of Neuroscience Methods 366 (2022) 109429

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

A new method to study learning and memory using spontaneous locomotor activity in an open-field arena

Jaise Silva Ferreira [a], Joaquim Barbosa Leite Junior [a], João Marcos de Mello Bastos [a], Richard Ian Samuels [b], Robert J. Carey [c], Marinete Pinheiro Carrera [a,*]

[a] Behavioral Pharmacology Group, Laboratory of Animal Morphology and Pathology, State University of North Fluminense Darcy Ribeiro, Avenida Alberto Lamego, 2000, Campos dos Goytacazes 28013-602, RJ, Brazil
[b] Department of Entomology and Plant Pathology, State University of North Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brazil
[c] Department of Psychiatry SUNY Upstate Medical University, 800 Irving Avenue, Syracuse, NY 13210, USA

ARTICLE INFO

Keywords:
Novel environment
Latent habituation measurement
Exploratory behavior
Central zone
Morphine
Post-trial conditioning

ABSTRACT

Background: Reduced locomotion with repeated exposure to a novel environment is often used as a measure of the basic adaptive learning process of habituation. While this is a well-established and reliable measure of habituation, it is not useful for the investigation of neurobiological changes before and after habituation because of the uncontrolled differential activity levels in a novel versus habituated environment.
In this study we report a behavioral method that uses spontaneous locomotion to measure habituation, in which the total spontaneous locomotion in an initially novel environment does not change with repeated testing but, the ratio of central to peripheral activity does change and is indicative of habituation. The test sessions are brief (5 min) and the locomotion is measured in 2 separate zones. The peripheral zone comprises 8/9 of the test arena and the central zone 1/9 of the arena.
Results/Comparison with existing methods: In contrast to methods that use between-session reductions in locomotion to assess habituation, this method employs brief test sessions in which overall activity between sessions does not change, but the distribution of locomotion in the periphery versus the central zone of the arena does change. The brevity of the test session also enables us to utilize post-trial drug treatment protocols to impact memory consolidation.
Conclusions: The progressive change in the central/peripheral activity ratio with repeated testing can be determined independently of total activity and provides a habituation acquisition function that permits the measurement of neurobiological changes without the complication of effects related to changes in locomotor activity per se. The present report also presents evidence that this method can be used with post-trial drug treatment protocols to study the learning and memory effects of the post-trial treatments without the use of explicit rewards and punishments.

1. Introduction

A decrease in spontaneous motor activity with repeated tests in a novel environment has been widely cited as an example of the basic learning process of habituation. In fact, following exposure to an initially novel environment, the reduction in activity in subsequent exposures to the same environment has been employed as a spontaneous learning and memory model, in that it circumvents the complications of experimenter application of positive/aversive stimuli (Sadile et al., 1978; Tomaz et al., 1990; Rosat et al., 1992; Izquierdo et al., 1993; Lukaszewska, 1993; Pellicano et al., 1993; Cerbone and Sadile, 1994; Carey et al., 1998). While these studies have shown that certain drug treatments given prior to testing in a novel environment can prevent the reduction in activity on a subsequent test session indicative of an absence of habituation and suggestive of an impairment of memory for the novel environment, there are other effects of the drug treatment

Abbreviations: HAB, Habituation; MOR, Morphine; N/MOR, Novel/Morphine; VEH, Vehicle; N/VEH, Novel/Vehicle; HAB/VEH, Habituation/Vehicle; HAB/MOR, Habituation/Morphine; TD, Total Distance traveled; CZD, Central Zone Distance.

* Corresponding author.
E-mail address: marinete@uenf.br (M.P. Carrera).

https://doi.org/10.1016/j.jneumeth.2021.109429
Received 17 August 2021; Received in revised form 25 October 2021; Accepted 24 November 2021
Available online 28 November 2021

unrelated to memory processes that could account for the effect (Järbe et al., 1981; Taukulis, 1996; Carey et al., 2002). It is now established that administering treatments post-test after a learning experience is the appropriate methodology to investigate memory, in that the memory tests are conducted in the non-drug state at least 24 h after the drug treatment. An extensive post-test consolidation literature (McGaugh and Roozendaal, 2009; Gasbarri and Tomaz, 2012) has demonstrated that drug treatments given shortly after a new learning experience or in the early acquisition stages can significantly modify retention. If the same treatments are administered after the acquisition process is completed, then these treatments have no effect on learning or memory. In preclinical studies, using mice or rats' psychostimulant drugs such as amphetamine given post-test can enhance the acquisition of a learned behavior, whereas opioid drugs such as morphine impair acquisition. While the reduction in spontaneous behavioral activity with repeated testing is the hallmark of habituation to a novel environment, a shortcoming of this method is that it is less useful for post-test treatment effects on memory, in that the memory may have been consolidated during within session habituation that occurs during the typical 20–30 min testing session. For this reason, we have employed brief 5 min exposures to a novel environment so that an immediate post-test treatment can occur during consolidation/reconsolidation of the memory for the test environment (Santos et al., 2015, 2017, 2018; Oliveira et al., 2019). Using this strategy, we have previously shown that drugs given immediately post-test after a 5 min novel environment test session can have major effects upon the subsequent behavioral response to the same environment (Santos et al., 2015, 2017, 2018; Oliveira et al., 2019). The same post-test treatments administered immediately post session have no effect however, if the animals had received several prior 5 min exposures to the previously novel test environment (Santos et al., 2018; Ferreira et al., 2020). These finding are indicative of habituation to the test environment even though session activity levels did not change over the course of the repeated 5 min exposures to the initially novel open-field environment. While the repeated 5 min tests would have presumptive face validity for habituation even in the absence of a reduction in activity, it would be desirable to be able to link a behavioral change to this presumptive acquired familiarity with the test environment. A potential behavioral marker independent of total activity is the location of activity within an open-field arena following repeated exposures. Rats have a strong initial activity preference for the periphery of an open-field arena (Schwarting et al., 1991) but it was shown (Dai et al., 1995) that following several brief exposures to a novel open-field an animals' proclivity to enter the central zone increases. We hypothesized that while overall activity does not change in brief 5 min tests, that there could be an increase in the percent of spontaneous total activity that occurs within the central zone of the open-field arena as the animal develops habituation to the arena walls. In this way, it might be possible to detect a habituation learning effect in the absence of a change in the overall activity in the open-field arena. In the first experiment, we report that with repeated 5 min tests, a modest but highly reliable increase in the contribution of central activity to total activity emerges and reaches asymptote following 5–6 sessions in the absence of any change in total activity. This gradual progressive shift in the location of activity within the open-field arena is suggestive of a habituation acquisition function. We assessed this acquisition of habituation possibility in a second experiment in which we administered morphine post-test following placement in a novel open-field arena. In that post-test morphine impairs acquisition of new learning (Izquierdo, 1979; Castellano et al., 1994; Costanzi et al., 2004), this experiment had the objective of assessing whether post-test morphine would prevent the acquisition of habituation using the relative increase in central zone activity as the behavioral indicator of habituation. Additionally, to validate that the post-test morphine effects on changes in central zone activity were associated with the consolidation of the memory for the novel environment, the post-test morphine treatments were administered after the asymptotic increase in the proportion of total activity contributed by central zone activity had been reached.

2. Methods

2.1. Animals

Male Wistar albino rats provided by the State University of North Fluminense Darcy Ribeiro, initially weighing 200–300 g, were housed in individual plastic cages (25 × 18 × 17 cm) until the end of the experiment. Individual housing was used in the experiment to avoid the potential group stressor effects of returning an animal to group housing while under the influence of the potent psychoactive drug morphine (10 mg/kg), that was administered post-test in some phases of the experiment. Food and water were provided ad libitum. The vivarium was maintained at a constant temperature (22 ± 2 ºC), and a 12 h/12 h light/dark cycle (lights on at 07:00 h and off at 19:00 h). All experiments occurred between 8:00 and 12:00 h. For 7 days prior to all experimental procedures, each animal was weighed and handled daily for 5 min. All groups were equated for body weight, so body weight was in effect the selection criterion for group assignment. The handling process included being placed in a transport cage and taken to the injection administration bench located in an anteroom adjacent to the experimental testing room. The animals were then given VEH injections. In this way the animals were familiarized with the handling and injection aspects of the testing protocol prior to the start of experimentation. All the experiments were performed in compliance with the Brazilian Council for Animal Experimentation (CONCEA) regulations, which are based on the US National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Drugs

Morphine sulfate (Cristalia, São Paulo, Brazil) was used from 10 mg ampoules (1 ml) and was injected subcutaneously in the nape of the neck at a dose of 10 mg/kg. A 0.9% saline solution was used as vehicle. All doses were administered in a volume of 1.0 ml/kg body weight. Drug solutions were freshly prepared before each experiment.

2.3. Apparatus and behavioral measurements

The behavioral measurements were conducted in a black open field chamber (60 × 60 × 45 cm). A closed-circuit camera (IKEGAMI, model ICD-49) mounted 60 cm above the arena was used to record behavioral data. Locomotion, measured as distance traveled (m), was automatically analyzed using EthoVision (Noldus, the Netherlands) that tracked the center of the body mass. The complete test procedure was conducted automatically without the presence of the experimenter in the test room. All behavioral testing was conducted under dim red light to avoid the possible aversive quality of white light (Nasello et al., 1998) enhancing the contrast between the white subject and the dark background of the test chamber and to reduce the animal's shadow. The parameters of the test chamber and the central zone were selected so that the rat could easily penetrate the central zone without having to move very far from the chamber wall (20 cm). The rationale for this arrangement was to create an environment in which the animal would have a low but reliable probability of entering the central zone. The central zone (20 × 20 cm) comprised 1/9 of the floor area and was monitored independently from the rest of the floor area and could only be distinguished by the computer analyzer. A fan in the experimental room provided masking noise. The rat was placed in the center of the open-field facing away from the experimenter and then the fan was turned on and then was turned off upon removal of the animal from the experimental arena.

In order to provide for the opportunity to review and re-input data, the test sessions were recorded using a digital video recorder connected to the camera. This apparatus arrangement has been used in a number of previous experimental reports and the details of the experimental setup

including a diagram of the open-field environment have been previously reported in detail (Dai and Carey, 1994; Carey and Gui, 1997).

2.4. Experimental procedure

2.4.1. Experiment 1: Habituation induction

The first experiment was undertaken to assess the utility of using activity in the central zone of a novel open-field as a behavioral change associated with repeated 5 min exposures to the open-field. Seven rats were used as subjects and immediately prior to the 5 min placement in the open-field arena, each animal was administered a saline injection (VEH). Also, after removal from the open-field arena each animal was again injected with saline. The subjects were given ten 5 min test sessions, one per day, on successive days. The injection protocol was used in conjunction with the five min tests in order to follow the injection regimen to be used in the subsequent experimentation. In this and in each subsequent experimental protocol, animals were injected pre-trial with VEH and post-trial with either VEH or MOR depending upon group assignment by an experimenter blind to the drug treatment and then placed back into the transport cage and returned to the vivarium. The order of testing vehicle and morphine groups was always counterbalanced. The complete injection process required 15–20 s. The pre-test injections were made in order to have the possibility of administering a drug pre-test at some future time without introducing an injection procedure variable.

2.4.2. Experiment 2: Novel environment morphine phase

In this experiment 14 animals were used and subdivided into two groups (n= 7) the (N/VEH) and (N/MOR) groups. Each group received their first post-trial injections after their first exposure to the open-field arena when it was completely novel. Both groups received 5 daily 5 min test sessions and immediately post-test after each session, were injected with MOR (10 mg/kg) N/MOR group or VEH N/VEH group.

2.4.3. Experiment 3: Habituated environment morphine phase

In this experiment 14 animals were used and subdivided into 2 groups (n = 7). Both groups underwent the 15 daily 5 min test sessions. The first 10 sessions followed the same habituation protocol as in experiment 1. On test sessions 11-15 the MOR phase was initiated. In this morphine treatment phase, one group (HAB/VEH) received VEH injections immediately post-test and the second group (HAB/MOR) received morphine (MOR) 10 mg/kg immediately post-test sessions 11 to 15. In order to control for differences in handling and injections between the HAB groups and N groups, the N groups in experiment 2 were taken to the experimental test room and given VEH injections on 10 successive test days to approximate the additional handling and injections received by the HAB groups in experiment 3.

2.5. Data analysis

For the results of experiment 1 as well as for the habituation phase of experiment 2, a one-way analysis of variance (ANOVA) with repeated measues was used to determine the effect of the days on the total distance traveled (TD), central zone distance (CZD) traveled and CZD/TD percentages. The CZD/TD percentages were the central zone distance divided by total distance. For the post-trial morphine effects in the novel (N) environment and habituation (HAB) phases of experiment 2, a repeated two-factor analysis of variance (ANOVA) was used to determine the treatment, days, and interaction effects. When a significant effect of treatment group versus induction day interaction was obtained, data were further evaluated by independent *t*-tests using $p < 0.05$ as the criterion for statistical significance.

3. Results

3.1. Experiment 1: Habituation induction

Fig. 1 presents the results of experiment 1. For the total distance (TD) traveled in the open-field arena (Fig. 1A), a one-way ANOVA showed that there was no effect of days of treatment [$F(9, 60) = 0.20$; $p > 0.05$].

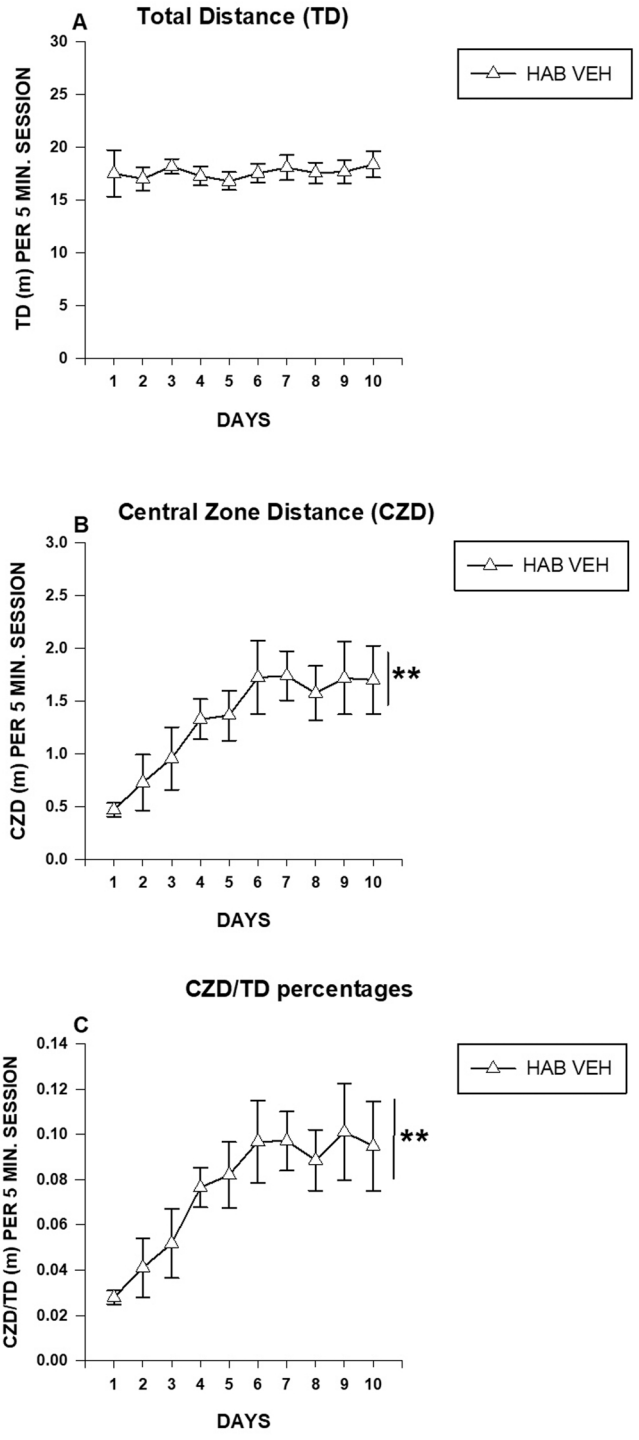

Fig. 1. Means and S. E. M.s for total distance (A), central zone distance (B) and CZD/TD percentages (C) during ten successive daily 5 min tests in the open-field arena in experiment 1. * * denotes a significant difference between the first and the last day of the habituation sessions (One-way ANOVA followed by the Tukey test).

For the central zone distance (CZD) traveled in the open-field arena, there was a progressive increase, reaching a plateau after 5–6 sessions (Fig. 1B). A one-way ANOVA showed that there was an effect of days of treatment [$F(9, 60) = 3.0$; $p < 0.01$. And similarly, for the CZD/TD percentages (Fig. 1C), a one-way ANOVA showed there was an increase in the CZD/TD percentages across habituation days [$F(9, 60) = 3.10$; $p < 0.01$]. Thus, over the 10 test sessions the TD did not increase but CZD increased and the percentage of TD contributed by CZD progressively increased, reaching asymptote in 5–6 sessions, suggestive of a habituation acquisition function.

3.2. Experiment 2: Novel environment morphine phase

Fig. 2 presents the total distance (TD), central zone distance (CZD) and central zone distance divided by total distance CZD/TD during the morphine phase for the N-MOR and N-VEH groups. In that the morphine treatments were administered post-trial, the first post-trial morphine treatment test day was a VEH test day for both groups, as the first test occurred prior to the first post-trial morphine treatment. For TD (Fig. 2A), a repeated two-way ANOVA showed that there was an interaction group x day [$F(4, 48) = 8.60$; $p < 0.01$], an effect of groups [$F(1, 12) = 13.22$; $p < 0.01$] and an effect of days of treatment [$F(4, 48) = 4.32$; $p < 0.01$]. An independent *t*-test to further analyze the interaction group X days, showed that on day 1 [$t(12) = 0.10$; $p > 0.05$] there was no difference between the groups. On day 2 [$t(12) = 2.82$; $p < 0.01$], the N-MOR group had higher locomotion than the N-VEH group as well as on day 3 [$t(12) = 3.13$; $p < 0.01$], on day 4 [$t(12) = 3.70$; $p < 0.01$] and day 5 [$t(12) = 5.07$; $p < 0.01$]. For central zone distance (CZD) (Fig. 2B), a repeated two-way ANOVA showed that there was only an effect of days of treatment [$F(4, 48) = 13.73$; $p < 0.01$]. There was no effect of groups [$F(1, 12) = 2.50$; $p > 0.05$] and no interaction group x day [$F(4, 48) = 2.20$; $p > 0.05$]. Analyzing across the days of induction, a one-way ANOVA [$F(4, 65) = 4.60$; $p < 0.01$] followed by Tukey test showed that there was an increase of locomotion in the central zone across the days of induction phase, i. e., the last days had higher locomotion in the central zone than during the first days ($p > 0.05$). For the CZD/TD percentages (Fig. 2C), a repeated two-way ANOVA showed that there was interaction group X day [$F(4, 48) = 5.22$; $p < 0.01$], an effect of groups [$F(1, 12) = 6.63$; $p < 0.05$] and an effect of days of treatment [$F(4, 48) = 9.20$; $p < 0.01$]. An independent *t*-test to further analyze the interaction group X days showed that on day 1 [$t(12) = 0.22$; $p > 0.05$], day 2 [$t(12) = 1.31$; $p > 0.05$] and day 3 [$t(12) = 1.72$; $p > 0.05$], there were no differences between the groups. On day 4 [$t(12) = 3.30$; $p < 0.01$] and day 5 [$t(12) = 3.80$; $p < 0.01$], the N-VEH group had a higher CZD/TD score than the N-MOR group. N-VEH group [$F(4, 30) = 3.74$; $p < 0.01$] had higher CZD/TD percentages on the last day than on the first day of the induction phase, but for the N-MOR group, the day 1 and day 5 scores were not different ($p > 0.05$).

3.3. Experiment 3: Habituated environment morphine phase

The first 10 sessions were essentially a replication of experiment 1 and the results were virtually the same. Fig. 3 summarizes day 1 to 15 habituation and morphine phases on day 1 and day 10 showed the effects of the 10 habituation test sessions on TD, CZD and CZD/TD and days 11 and 15 show the effects of post-trial MOR versus VEH. As can be seen in Figs. 3A and 3D, TD did not change over the 15 test sessions. In Figs. 3B and 3E, CZD increased substantially from session 1 and remained unchanged from sessions 10 to 15, indicating that the MOR treatment did not affect this activity and the result was similar for CZD/TD as shown in Figs. 3C and 3F. A two-way ANOVA comparing groups across days 1,10,11 and 15 for TD showed no effect of groups [$F(2, 62) = 0.04$; $p > 0.05$], no effect of days [$F(2, 62) = 0.50$; $p > 0.05$] and no interaction groups X days [$F(2, 62) = 0.11$; $p > 0.05$]. For central zone distance (CZD), the results showed that there was only an effect of days

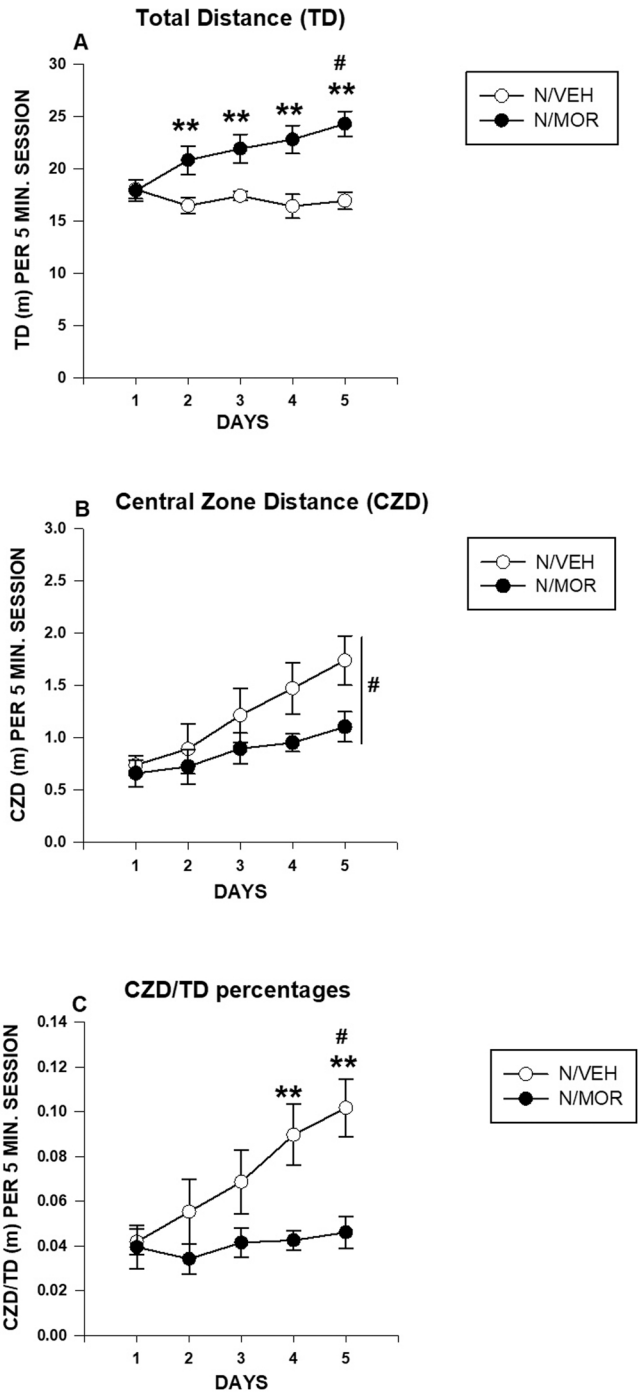

Fig. 2. Means and S. E. M.s for total distance (A), central zone distance (B) and CZD/TD percentages (C) during experiment 2 novel environment morphine phase. * * denotes difference between N/VEH and N/MOR groups. # denotes a significant difference between the first and last day (repeated ANOVA followed by independent *t*-test or by the Tukey test).

[$F(2, 62) = 18.70$; $p < 0.01$]. There was no effect of groups [$F(2, 62) = 0.07$; $p > 0.05$] and no interaction groups X days [$F(2, 62) = 0.10$; $p > 0.05$]. The Tukey test showed that day 1 had lower central zone distance locomotion than the other days ($p < 0.05$) that did not differ from each other ($p > 0.05$) For the CZD/TD percentages, the results showed that there was only an effect of days [$F(2, 62) = 19.62$; $p < 0.01$]. There was no effect of groups [$F(2, 62) = 0.0$; $p > 0.05$] and no interaction groups X days [$F(2, 62) = 0.22$; $p > 0.05$]. The Tukey test showed that day 1 had lower CZD/TD percentages than the other days

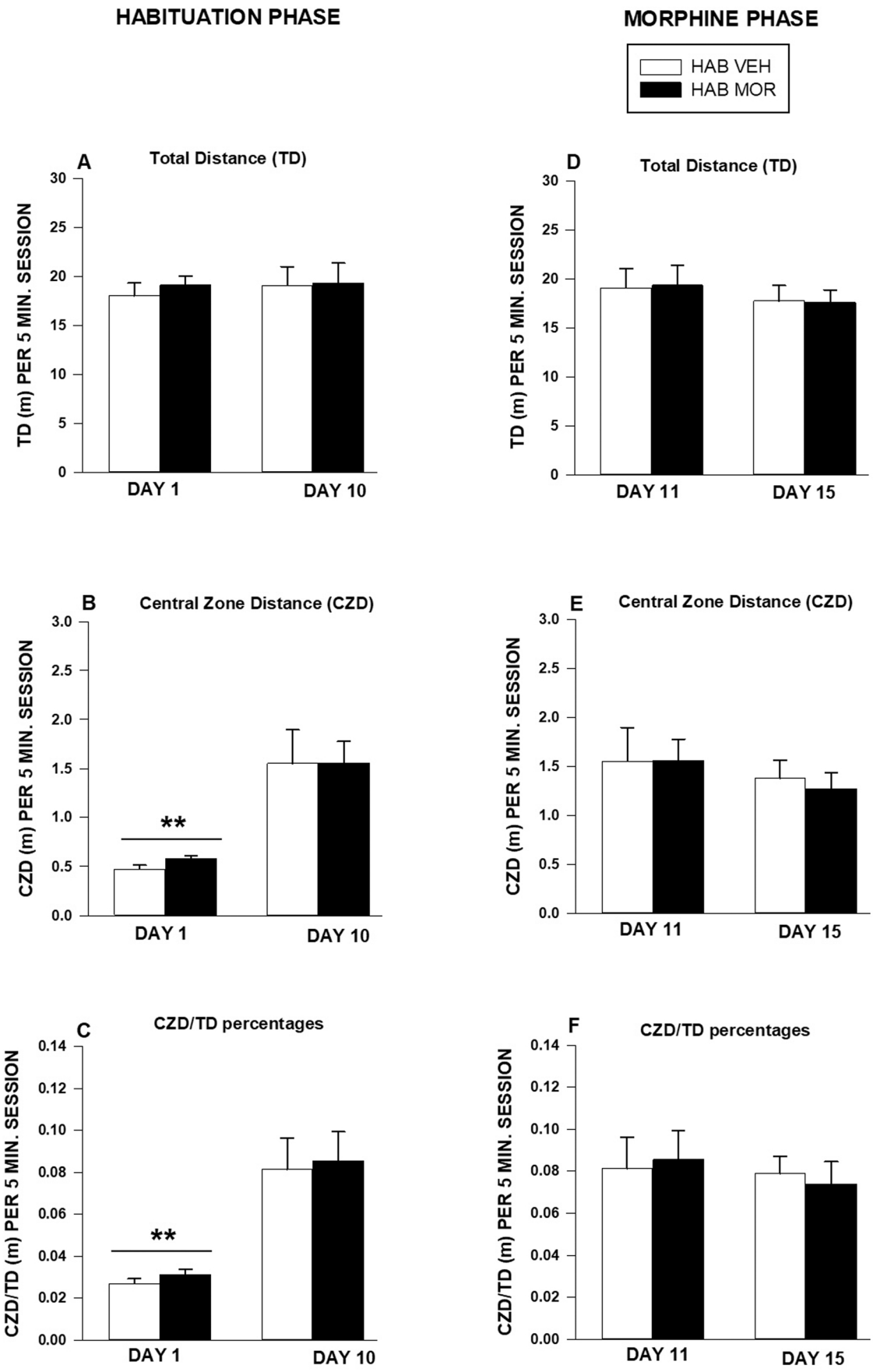

Fig. 3. Means and S. E. M.s of locomotor activity during the habituation phase (A, B and C) and morphine phase (D, E and F) of experiment 3: Habituated environment morphine phase. ** denotes lower locomotion than the other days (two-way ANOVA followed by the Tukey test).

($p < 0.05$) and that days 10, 11 and 15 did not differ from each other ($p > 0.05$).

4. Discussion

In the initial experiment, the effect of ten daily 5 min tests on total distance scores and the distance within the central zone of an initially novel open-field arena showed for these non-drug animals that total distance remained stable and unchanged over the course to the 10 test sessions. In contrast, the central zone distance scores and the percent of total distance contributed by activity in the central zone underwent a substantial change. This change in central zone activity resembled a conventional acquisition function with a progressive increase over the initial 5–6 sessions to reach an apparent asymptotic performance level.

In the subsequent morphine post-trial experiments, total activity and central zone activity were measured as dependent variables. In the morphine novel environment experiment, the post-trial morphine treatments were initiated after the first 5 min exposure to the novel environment. Whereas total activity levels in the N/VEH group did not change over the course of the 5 test sessions, the fraction of total distance contributed by central zone distance scores did markedly change over the course of the 5 sessions. In the N/VEH group, the overall pattern of increases in central zone distance scores with repeated test sessions both in terms of absolute scores as well as percent of total activity essentially replicated the progressive increases observed with repeated test sessions in the VEH group in the first experiment. The first test session for the N/MOR group was also a non-drug test in that morphine was administered post-test and the central zone/total activity scores for the N/VEH and N/MOR were equivalent. The subsequent tests for the N/MOR group reflected the effects of the prior day MOR treatment. Total activity and central zone activity increased in the N/MOR group over the course of these 4 test sessions and were higher than the N/VEH group. While total activity increased, it did not prevent central zone activity in that central zone activity increased with repeated testing rather, central zone activity scores as a percent of total distance did not increase but remained at the same low day 1 non-drug level over the course test sessions 2–5. Thus, in terms of central zone activity scores as a percent of total distance, this did not change with repeated testing in the N/MOR group, indicative of an absence of habituation and consistent with the effects of post-test morphine on the impairment of acquisition of new behavior.

In the habituated environment MOR experiment, the same post-trial morphine protocol was used but was initiated after the VEH and MOR groups had first completed ten 5 min VEH sessions. As in the first experiment, total activity did not change over the first ten 5 min VEH sessions prior to the post-test morphine treatments, but the percent of total activity occurring in the central zone increased. These two habituated (HAB) groups were then given another 5 daily 5 min test sessions followed immediately post-session by vehicle or 10 mg/kg MOR injections. Over the course of the 5 treatment sessions, the total distance and central zone distance fraction of total distance scores for the VEH/HAB and MOR/HAB groups did not differ from each other and remained essentially unchanged from their immediately prior HAB scores regardless of post-trial treatment. Thus, the ten 5 min HAB sessions eliminated the post-trial MOR increase in total distance and also did not reverse the CZ distance fraction of total distance scores.

The finding that the MOR treatment in the HAB/MOR group had no effect on behavior is certainly consistent with the idea that the ten 5 min test sessions did induce habituation and is consistent with the absence of an amnestic post-test treatment effect on behavior after acquisition of new learning has been completed. From this perspective then, the 5 min test sessions initiated in a novel environment can be seen as habituation acquisition test sessions that are analogous to conventional learning acquisition protocols, but without explicit rewards/reinforcements (Izquierdo, 1980; Platel and Porsolt, 1982; Bardo and Bevins, 2000).

Habituation to a novel environment is typically manifested as a diminution in behavioral activity but with the brief test sessions used in the present protocol, it is manifested in a change in the location of the behavioral activity. It has been a long-known feature of open-field behavior that rats have a strong preference for wall contact and the importance of vibrissa for this thigmotactic scanning has been extensively studied (Steiner et al., 1988; Schwarting et al., 1991). One way this initial wall preference behavior has been characterized is that it represents a fear of open spaces, such that the animal maintains contact with the walls of the open field. As the animal becomes more familiar with the open field environment it has been suggested that the increase in activity in the central zone open area of the test environment reflects a decrease in anxiety Liebsch et al., 1998). Inconsistent with applying an anxiety analysis to the findings obtained in the present study is the report by Nasello and co-workers (1998) which showed that when open-field tests are conducted under red light conditions that exploratory behavior is elicited and not behavior indicative of anxiety. Furthermore, it has been demonstrated in conditioned place preference studies (Bardo et al., 1995; Bardo and Bevins, 2000), that novel environments are preferred to familiar habituated environments, indicative of positive reinforcement effects of a novel environment. Indeed, it has been extensively demonstrated (Berlyne, 1966) that novel stimuli have a positive reward effect. Regardless of which interpretation is given to the relative increase in activity that occurs in the central zone, this change occurs reliably with repeated tests in a novel environment in the absence of a change in overall activity. Indeed, it has been pointed out by Sadile and co-workers (1978) that habituation involves cognitive and emotional processes.

When the central zone results are considered in terms of a basic learning acquisition process, then the failure of the post-trial N/MOR treatment group to increase central zone distance, as a fraction of total distance, can be characterized as an impairment in the acquisition of new learning and be seen as consistent with an extensive literature (Izquierdo, 1979; Castellano et al., 1994; Costanzi et al., 2004), wherein MOR post-trial impairs learning. The present findings with the N/MOR and HAB/MOR groups also appear consistent with memory consolidation processes (de Mello Bastos et al., 2020) in which memory consolidation is vulnerable to modification in the initial stages of acquisition but not after the learning process is completed. In the N/MOR group the MOR treatment would occur during the consolidation of the memory for the novel environment whereas for the HAB/MOR group the consolidation process would have been completed so the post-trial MOR would not affect consolidation, thus would be without effect.

Certainly, a unique feature of using the present post-trial drug treatment method to investigate effects on acquisition of a new behavior is that the effects of the post-trial treatment can be observed in terms of direct effects on behavior instead of only indirect effects such as on the acquisition of a complex behavior such as maze learning. The present results show that the increase in total activity by the post-trial morphine treatment essentially mirrors the direct effects of increased activity induced by morphine administered in a conventional pre-test morphine protocol (Vanderschuren et al., 1999; Powell and Holtzman, 2001; Li et al., 2010; Liu et al., 2014). This similarity in pre- and post-trial morphine effects on locomotor activity suggests that the morphine drug response is incorporated into the memory consolidation of the environmental experience and the morphine drug response is expressed in behavior during the subsequent non-drug test one day later. When morphine post-trial effects that impair acquisition of new behaviors are considered from this perspective, then the acquisition impairment by morphine can be seen as the disruptive effect of a morphine drug response becoming incorporated into the consolidation process. In the subsequent acquisition test, the cues present in the behavioral test environment could then elicit the morphine drug response. Seemingly, the impairment of the acquisition of new behaviors by post-trial morphine would be as if the morphine were administered pre-test and testing occurred during the morphine drug state. While this remains speculative, it does have heuristic value in suggesting new ways to

experimentally investigate and compare pre- and post-trial drug treatments on the acquisition of new behaviors.

In conclusion, open-field arenas have been and remain widely used for assessing basic spontaneous behaviors. The present method makes a straightforward adaptation of the open-field, using readily available image analysis systems to quantify movements in the periphery and the central sections of the open-field. The present findings point to the utility of incorporating the measurement of central zone activity in brief test sessions in a novel environment as a quantitative measure habituation without a change in total arena activity. By considering the repeated tests in the novel environment as a learning process, post-trial treatments can be administered such as morphine in the present study to investigate drug effects on memory consolidation of the novel environment experience without administering positive and/or aversive stimuli. Furthermore, if neurobiological measurements are made following exposure to a novel environment versus measurements after repeated tests in the same environment, the effects cannot be attributed to a change in activity levels per se. This is typically the case when longer test intervals are used and there is a decrease in activity with repeated testing. In that a brief exposure to a novel environment increases dopamine activity (Pedrazza et al., 2007; Menezes et al., 2015), it now becomes feasible to determine the effects of repeated treatments on the dopamine response without the complication of differential levels of locomotor activity. As has been pointed out by Sadile and co-workers (1978), habituation involves cognitive and emotional changes, so that the capability to assess the neurotransmitter and other neurochemical changes that occur during habituation without the complication of concurrent locomotor activity changes, will enable a better opportunity to investigate these important issues in this fundamental adaptive behavioral process.

CRediT authorship contribution statement

Jaise Silva Ferreira: Methodology, Data curation, Investigation, Formal analysis.; **Joaquim Barbosa Leite Junior:** Data curation, Investigation, Resources. **João Marcos De Mello Bastos:** Data curation, Investigation, Resources. **Richard Ian Samuels:** Resources, Funding acquisition. Writing – review & editing. **Robert J Carey:** Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Marinete Pinheiro Carrera:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors have no competing interests to declare.

Acknowledgments

This research was supported by the Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF) and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (no E-26/200.895/2017). J.B.L.J. and J.M.M.B. are recipients of fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). R.I.S. is a Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) research fellow.

References

Bardo, M.T., Bevins, R.A., 2000. Conditioned place preference: what does it add to our preclinical understanding of drug reward? Psychopharmacology 153, 31–43. https://doi:10.1007/s002130000569.

Bardo, M.T., Rowlett, J.K., Harris, M.J., 1995. Conditioned place preference using opiate and stimulant drugs: a meta-analysis. Neurosci. Biobehav Rev. 19, 39–51. https://doi:10.1016/0149-7634(94)00021-r.

Berlyne, D.E., 1966. Curiosity and exploration. Science 153, 25–33 (https://doi:10.1126/science.153.3731.25).

Carey, R.J., DePalma, G., Damianopoulos, E., 2002. 8-OHDPAT effects upon cocaine unconditioned and conditioned behaviors: a role for drug stimulus effects. Pharmacol Biochem Behav 72, 171–178. https://doi.org/10.1016/s0091-3057(01)00750-x.

Carey, R., Gui, J., 1997. A simple and reliable method for the positive identification of pavlovian conditioned cocaine effects in open-field behavior. J. Neurosci. Methods 73, 1–8. https://doi:10.1016/s0165-0270(96)02203-0.

Carey, R.J., Dai, H., Gui, J., 1998. Effects of dizocilpine (MK-801) on motor activity and memory. Psychopharmacology 137, 241–246. https://doi:10.1007/s002130050616.

Castellano, C., Cestari, V., Cabib, S., Puglisi-Allegra, S., 1994. The effects of morphine on memory consolidation in mice involve both D1 and D2 dopamine receptors. Behav. Neural Biol. 61, 156–161. https://doi:10.1016/s0163-1047(05)80069-x.

Cerbone, A., Sadile, A.G., 1994. Behavioral habituation to spatial novelty: interference and noninterference studies. Neurosci. Biobehav Rev. 18, 497–518. https://doi:10.1016/0149-7634(94)90004-3.

Costanzi, M., Battaglia, M., Rossi-Arnaud, C., Cestari, V., Castellano, C., 2004. Effects of anandamide and morphine combinations on memory consolidation in cd1 mice: involvement of dopaminergic mechanisms. Neurobiol. Learn Mem. 81, 144–149. https://doi:10.1016/j.nlm.2003.09.003.

Dai, H., Carey, R.J., 1994. The NMDA antagonist MK-801 can impair attention to exteroceptive stimuli. Behav. Brain Res 62, 149–156. https://doi:10.1016/0166-4328(94)90022-1.

Dai, H., Krost, M., Carey, R.J., 1995. A new methodological approach to the study of habituation: the use of positive and negative behavioral indices of habituation. J. Neurosci. Methods 62, 169–174. https://doi:10.1016/0165-0270(95)00073-9.

Ferreira, J.S., Bastos, de Mello, Leite Junior, J.M., Samuels, J.B., Carey, R.I., Carrera, R. J., M.P, 2020. Morphine administered post-trial induces potent morphine conditioned effects if the context is novel but not if the context is familiar. Pharm. Biochem Behav. 196, 172978. https://doi:10.1016/j.pbb.2020.172978.

Gasbarri, A., Tomaz, C., 2012. Memory and motivational/emotional processes. Front Behav. Neurosci. 6, 71. https://doi:10.3389/fnbeh.2012.00071.

Izquierdo, I., 1979. Effect of naloxone and morphine on various forms of memory in the rat: possible role of engogenous opiate mechanisms in memory consolidation. Psychopharmacology 66, 199–203. https://doi:10.1007/BF00427631.

Izquierdo, I., 1980. Effect of beta-endorphin and naloxone on acquisition, memory, and retrieval of shuttle avoidance and habituation learning in rats. Psychopharmacology 69, 111–115. https://doi:10.1007/BF00426531.

Izquierdo, I., da Silva, R.C., Bueno e Silva, M., Quillfeldt, J.A., Medina, J.H., 1993. Memory expression of habituation and of inhibitory avoidance is blocked by CNQX infused into the entorhinal cortex. Behav. Neural Biol. 60, 5–8. https://doi:10.1016/0163-1047(93)90651-w.

Järbe, T.U., Swedberg, M.D., Mechoulam, R., 1981. A repeated test procedure to assess onset and duration of the cue properties of (-) delta 9-THC, (-) delta 8-THC-DMH and (+) delta 8-THC. Psychopharmacology 75, 152–157. https://doi:10.1007/BF00432178.

Li, X., Li, J.X., Zhu, X., Cui, R., Jiao, J., 2010. Effects of physostigmine on the conditioned hyperactivity and locomotor sensitization to morphine in rats. Behav. Brain Res. 206, 223–228. https://doi:10.1016/j.bbr.2009.09.016.

Liebsch, G., Montkowski, A., Holsboer, F., Landgraf, R., 1998. Behavioural profiles of two Wistar rat lines selectively bred for high or low anxiety-related behaviour. Behav. Brain Res. 94, 301–310. https://doi:10.1016/s0166-4328(97)00198-8.

Liu, X.S., Hou, Y., Yan, T.L., Guo, Y.Y., Han, W., Guan, F.L., Chen, T., Li, T., 2014. Dopamine D3 receptor-regulated NR2B subunits of N-methyl-d-aspartate receptors in the nucleus accumbens involves in morphine-induced locomotor activity. CNS Neurosci. Ther. 20, 823–829. https://doi.org/10.1111/cns.12276.

Lukaszewska, I., 1993. Scopolamine impairs object exploration but not habituation in rats. Acta Neurobiol. Exp. 53, 243–250. ⟨https://www.ane.pl/pdf/5326.pdf⟩.

McGaugh, J.L., Roozendaal, B., 2009. Drug enhancement of memory consolidation: historical perspective and neurobiological implications. Psychopharmacology 202, 3–14. https://doi:10.1007/s00213-008-1285-6.

de Mello Bastos, J.M., Leite, J.B.J., Samuels, R.I., Carey, R.J., Carrera, M.P., 2020. Post-trial low dose apomorphine prevents the development of morphine sensitization. Behav. Brain Res. 380, 112398. https://doi: 10.1016/j.bbr.2019.112398.

Menezes, J., Alves, N., Borges, S., Roehrs, R., de Carvalho Myskiw, J., Furini, C.R., Izquierdo, I., Mello-Carpes, P.B., 2015. Facilitation of fear extinction by novelty depends on dopamine acting on D1-subtype dopamine receptors in hippocampus. Proc. Natl. Acad. Sci. USA 112, E1652–E1658. https://doi:10.1073/pnas.1502295112.

Nasello, A.G., Machado, C., Bastos, J.F., Felicio, L.F., 1998. Sudden darkness induces a high activity-low anxiety state in male and female rats. Physiol. Behav. 63, 451–454. https://doi:10.1016/s0031-9384(97)00462-9.

Oliveira, L.R., Santos, B.G., de Mello Bastos, J.M., Samuels, R.I., Carey, R.J., Carrera P., M., 2019. Morphine administered post-trial can induce potent conditioned morphine effects. Pharm. Biochem Behav. 179, 134–141. https://doi:10.1016/j.pbb.2019.02.014.

Pedrazza, E.L., Riboldi, G.P., Pereira, G.S., Izquierdo, I., Bonan, C.D., 2007. Habituation to an open field alters ecto-nucleotidase activities in rat hippocampal synaptosomes. Neurosci. Lett. 413, 21–24. https://doi:10.1016/j.neulet.2006.11.023.

Pellicano, M.P., Siciliano, F., Sadile, A.G., 1993. NMDA receptors modulate long-term habituation to spatial novelty: dose- and genotype-dependent differential effects of posttrial MK-801 and CPP in rats. Physiol. Behav. 54, 563–568. https://doi:10.1016/0031-9384(93)90250-j.

Platel, A., Porsolt, R.D., 1982. Habituation of exploratory activity in mice: a screening test for memory enhancing drugs. Psychopharmacology 78, 346–352. https://doi:10.1007/BF00433739.

Powell, K.R., Holtzman, S.G., 2001. Parametric evaluation of the development of sensitization to the effects of morphine on locomotor activity. Drug Alcohol Depend. 62, 83–90. https://doi:10.1016/s0376-8716(00)00167-8.

Rosat, R., Da-Silva, R.C., Zanatta, M.S., Medina, J.H., Izquierdo, I., 1992. Memory consolidation of a habituation task: role of N-methyl-D-aspartate, cholinergic muscarinic and GABA-A receptors in different brain regions. Braz. J. Med Biol. Res. 25, 267–273.

Sadile, A., De Luca, B., Cioffi, L., 1978. Long-term habituation to a novel environment amnesic and hypermesic effects of various post-exposure treatments. Abh. Akad. Wiss. DDR 5, 203–218.

Santos, B.G., Carey, R.J., Carrera, M.P., 2015. Post-trial induction of conditioned apomorphine stimulant and inhibitory response effects: evidence for potent trace conditioning of drug effects. Pharm. Biochem Behav. 129, 79–86. https://doi:10.1016/j.pbb.2014.12.003.

Santos, B.G., Carey, R.J., Carrera, M.P., 2017. The acquisition, extinction and spontaneous recovery of Pavlovian drug conditioning induced by post-trial dopaminergic stimulation/inhibition. Pharm. Biochem Behav. 156, 24–29. https://doi:10.1016/j.pbb.2017.04.002.

Santos, B.G., Carey, R.J., Carrera, M.P., 2018. Repeated pre-trial and post-trial low and high dose apomorphine treatments induce comparable inhibitory/excitatory sensitization and conditioned drug effects. Pharm. Biochem Behav. 175, 108–115. https://doi:10.1016/j.pbb.2018.09.011.

Schwarting, R.K., Steiner, H., Huston, J.P., 1991. Asymmetries in thigmotactic scanning: evidence for a role of dopaminergic mechanisms. Psychopharmacology 103, 19–27. https://doi:10.1007/BF02244068.

Steiner, H., Bonatz, A.E., Huston, J.P., Schwarting, R., 1988. Lateralized wall-facing versus turning as measures of behavioral asymmetries and recovery of function after injection of 6-hydroxydopamine into the substantia nigra. Exp. Neurol. 99, 556–566. https://doi:10.1016/0014-4886(88)90172-0.

Taukulis, H.K., 1996. Pavlovian conditioning to a diazepam cue with yohimbine as the unconditional stimulus. Neurobiol. Learn Mem. 65, 223–232. https://doi:10.1006/nlme.1996.0027.

Tomaz, C., Aguiar, M.S., Nogueira, P.J., 1990. Facilitation of memory by peripheral administration of substance P and naloxone using avoidance and habituation learning tasks. Neurosci. Biobehav Rev. 14, 447–453. https://doi:10.1016/s0149-7634(05)80067-3.

Vanderschuren, L.J., Schoffelmeer, A.N., Mulder, A.H., De Vries, T.J., 1999. Dopaminergic mechanisms mediating the long-term expression of locomotor sensitization following pre-exposure to morphine or amphetamine. Psychopharmacology 143, 244–253. https://doi:10.1007/s002130050943.