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Research report

# Testing conditions in shock-based contextual fear conditioning influence both the behavioral responses and the activation of circuits potentially involved in contextual avoidance

Juliette Viellard[a], Marcus Vinicius C. Baldo[b], Newton Sabino Canteras[a,*]

[a] Dept. Anatomy, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-000, Brazil
[b] Dept. Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP 05508-000, Brazil

## HIGHLIGHTS

- Animals confined to the conditioning chamber or with free access to the conditioning chamber were tested using shock-based fear conditioning.
- Different testing conditions yielded different contextual responses and engaged different circuits related to aversive responses.
- Animals with free access to the conditioning chamber displayed risk assessment responses and engage a distinct hypothalamic – PAG circuit.

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## ABSTRACT

Previous studies from our group have shown that risk assessment behaviors are the primary contextual fear responses to predatory and social threats, whereas freezing is the main contextual fear response to physically harmful events. To test contextual fear responses to a predator or aggressive conspecific threat, we developed a model that involves placing the animal in an apparatus where it can avoid the threat-associated environment. Conversely, in studies that use shock-based fear conditioning, the animals are usually confined inside the conditioning chamber during the contextual fear test. In the present study, we tested shock-based contextual fear responses using two different behavioral testing conditions: confining the animal in the conditioning chamber or placing the animal in an apparatus with free access to the conditioning compartment. Our results showed that during the contextual fear test, the animals confined to the shock chamber exhibited significantly more freezing. In contrast, the animals that could avoid the conditioning compartment displayed almost no freezing and exhibited risk assessment responses (i.e., crouch-sniff and stretch postures) and burying behavior. In addition, the animals that were able to avoid the shock chamber had increased Fos expression in the juxtadorsomedial lateral hypothalamic area, the dorsomedial part of the dorsal premammillary nucleus and the lateral and dorsomedial parts of the periaqueductal gray, which are elements of a septo/hippocampal–hypothalamic–brainstem circuit that is putatively involved in mediating contextual avoidance. Overall, the present findings show that testing conditions significantly influence both behavioral responses and the activation of circuits involved in contextual avoidance.



## 1. Introduction

Animals form contextual fear memories to life-threatening events (such as an encounter with a predator), to social defeat (such as interactions with aggressive conspecifics) and to harmful events that may endanger their physical integrity [see 1,2]. Previous studies have shown that risk assessment behaviors, such as the crouch-sniff and stretch postures, are the primary contextual fear responses to predatory and social threats [2,3], whereas freezing behavior is the main contextual fear response to physically harmful events, such as electric footshock [4,5]. Moreover, contextual fear responses to painful stimuli, predators and aggressive conspecifics have been shown to be processed by independent neural circuits involving the hippocampus, amygdala and downstream hypothalamic and brainstem circuits [see 1].

Notably, studies from our group have tested contextual responses to predators and social threats using a behavioral apparatus that differs from the traditional fear conditioning chamber used

* Corresponding author.
*E-mail address:* newton@icb.usp.br (N.S. Canteras).

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to assess contextual fear responses to painful stimuli. Thus, during the test for contextual fear after exposure to a predator threat or social defeat, animals were placed outside the threat-associated chamber and could either enter or avoid this compartment [see 2,3]. Conversely, in most studies using shock-based fear conditioning, the animals are usually confined inside the conditioning chamber during the contextual fear test and do not have the option to escape or avoid the conditioning chamber [4,5].

As mentioned above, studies have shown that different threats elicit different contextual fear responses and activate distinct neural circuits; therefore, in this study, we wanted to determine whether these differences are due, at least in part, to the different behavioral testing conditions used for shock-based fear conditioning and other forms of threats (i.e., predatory exposure and social defeat). We tested shock-conditioned animals for contextual fear responses using an apparatus similar to what we have used for predator exposure and social defeat [2,3]. Specifically, following footshock conditioning on the test day, the animals were placed in a home cage linked to a corridor with access to the shock-associated chamber. These animals thus could either enter or avoid the conditioning compartment. After the contextual fear test, we examined the pattern of Fos expression in specific regions of the prefrontal cortex, amygdala, hypothalamus and brainstem. These results were compared with the behavior and Fos expression of conditioned animals that were confined to the shock chamber during the contextual fear test. Overall, the present findings show that testing conditions significantly influence both the behavioral responses and the activation of circuits involved in contextual avoidance.

## 2. Materials and methods

### *2.1. Animals*

Adult male Wistar rats (n = 21), weighing approximately 250 g, were obtained from local breeding facilities. The animals were kept in the animal facility under controlled temperature (23 °C) and illumination (12 h cycle) conditions and had free access to water and standard laboratory chow.

### *2.2. Ethics*

The experiments were carried out in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, 1996), and all experimental procedures were approved by the Committee on the Care and Use of Laboratory Animals of the Institute of Biomedical Sciences, University of São Paulo, Brazil (protocol No. 085/2012). In addition, all attempts were made to minimize the number of animals used and their suffering.

### *2.3. Experimental groups*

The following experimental groups were tested for contextual fear responses:

- A control group tested outside of the conditioning chamber (**CG-Out**, n = 5): Rats were habituated to the apparatus for 5 d. On the fear conditioning day, the animals were placed in the shock chamber (grid compartment) for 8 min without receiving footshock. On the test day, the animals were placed outside the conditioning chamber and allowed to freely explore the experimental apparatus;
- The fear conditioned placed outside of the conditioning chamber group (**FC-Out**, n = 6): Rats were habituated to the apparatus for 5 d. On the fear conditioning day, the animals were placed in the shock chamber (grid compartment) and received footshocks. On the test day, the animals were placed outside the conditioning chamber and allowed to freely explore the experimental apparatus;
- A control group tested inside of the conditioning chamber (**CG-In**, n = 5): Rats were habituated to the apparatus for 5 d. On the fear conditioning day, the animals were placed in the shock chamber (grid compartment) for 8 min without receiving footshock. On the test day, the animals were confined inside the conditioning chamber;
- The fear conditioned confined inside the conditioning chamber group (**FC-In**, n = 5): Rats were habituated to the apparatus for 5 d. On the fear conditioning day, the animals were placed in the shock chamber (grid compartment) and received footshocks. On the test day, the animals were confined inside the conditioning chamber.

### *2.4. Experimental apparatus and procedure*

The experimental apparatus was made of clear Plexiglas and consisted of a 25 × 25 × 25 cm home cage connected by a hallway (12.5 cm wide, 80 cm long and 25 cm high) to another 25 × 25 × 25 cm chamber (the grid compartment). The floor of the grid compartment was made of stainless steel rods (5 mm diameter, spaced 1.2 cm apart) that were linked to a shock delivery apparatus (Insight Instruments, Ribeirão Preto, SP, Brazil). Between the grid compartment and the hallway, there was a sliding door (12.5 cm wide and 26 cm high) that was closed when the animal was intended to be confined to the grid compartment. All experiments were carried on under 50 W red light illumination. The rats were habituated for 5 d at the beginning of the dark phase and were individually placed in the home cage and allowed to explore the rest of the apparatus for 10 min. On the conditioning day, the rats entered the grid compartment, and the investigator shut the sliding door. After three min, the rat received seven footshocks (unconditioned stimulus (US), 0.9 mA, 1 s duration with 30 s intervals) [see 6]. The rats were left undisturbed for an additional 2 min before they were removed from the apparatus. The control group remained in the grid compartment for 8 min on the conditioning day but did not receive the US. On the test day, the rats in the CG-Out and FC-Out groups were placed in the home box and allowed to freely explore the apparatus (including the grid compartment) for 6 min. The rats in the CG-In and FC-In groups were confined to the grid compartment for 6 min. During the test day, the animals were recorded using a horizontally mounted video camera. The rats were sacrificed using sodium pentobarbital (40 mg/kg, i.p., Cristália, Campinas, SP, Brazil) 90 min after the behavioral testing, and the brains were processed for histology and Fos immunohistochemistry.

### *2.5. Behavior analysis*

The animals' behavior was scored by a trained observer using ethological analysis software (The Observer, version 5.0, Noldus Information Technology, Wageningen, Netherlands). The analysis included spatiotemporal (the time spent in the home cage, hallway or grid compartment) and behavioral measurements, which were measured in terms of duration (total duration per session) and included the following: freezing (cessation of all movement, excluding that associated with breathing); risk assessment responses, including crouch-sniff posture (animal immobile with the back arched but actively sniffing and scanning the environment) and stretch posture (including both the stretch-attend posture, during which the body is stretched forward and the animal is motionless, and the stretch-approach posture, which consists of movement directed toward the grid compartment with the animal's body in a stretched position); burying behavior (pushing the