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Research report

# Dorsolateral periaqueductal gray stimulation prior to retrieval potentiates a contextual fear memory in rats

Cristiane R. Mochny[a], Grasielle C. Kincheski[a], Victor A. Molina[b], Antonio P. Carobrez[a,*]

[a] Departamento de Farmacologia, CCB, Universidade Federal de Santa Catarina, Campus Universitário Trindade, Florianópolis, SC 88040-900, Brazil
[b] IFEC-CONICET, Departamento de Farmacología, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, 5000, Argentina

## HIGHLIGHTS

- Dorsolateral periaqueductal gray stimulation as a negative emotional mnemonic trace.
- Negative emotional mnemonic trace potentiate contextual fear expression at recall.
- Enhancement of fear responses are long-lasting and evident after 6 days.
- Effect is contingent to the associated context.

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## ABSTRACT

The association of a neutral context with an aversive stimulus, such as foot-shock, result in a contextual fear memory. A growing number of evidence have revealed that prior exposure to diverse threatening situations facilitates the encoding of fear memory during acquisition and such reports support the widespread notion that emotionally arousal results in stronger and long-lasting memories. However, few studies have investigated if a threatening experience can affect the recall and the persistence of such fear memory trace. To test the hypothesis that an emotionally negative experience could modify the retrieval of a memory and potentiate the expression of a fear memory, the present study used the chemical stimulation (microinjection of NMDA) of the dorsolateral periaqueductal gray matter (dlPAG) of rats in order to induce an aversive emotional state. Such stimulation was performed one day after a weak fear training protocol, and the fear expression was analyzed in subsequent re-exposures to the conditioned context. The results showed that the negative emotional state induced by the dlPAG stimulation enhanced the fear memory trace when this trace was reactivated one day after this aversive experience. Additionally, the potentiation of the fear response was contingent to the associated context since no potentiation was evident when NMDA-stimulated animals were subsequently placed in a non-associated context. Finally, the model suggests that the enhancement of fear responses is long-lasting since NMDA-treated animals performed a robust fear response six days after memory retrieval.



## 1. Introduction

Excessive and uncontrollable fear is a main feature of several anxiety-related disorders. Therefore, it seems relevant to explore organismic or environmental challenges that could lead to inappropriate fear responses during the retrieval of an already established fear memory. A growing number of evidence has revealed that prior exposure to diverse threatening situations facilitates the encoding of fear memory during acquisition [1–4]. These reports, in fact, support the widespread notion that emotionally driven experiences results in stronger and long-lasting memories [1,4–6]. Despite the abundant evidence supporting the facilitating influence of stressful experiences on subsequent memory acquisition, it remains to be established how a threatening experience affect a fear memory trace when it is induced prior to the retrieval of a previously consolidated fear memory.

The periaqueductal gray matter (PAG) is an integrative area of the neuroaxis known to elicit various overt defensive responses. It is well established that this brain structure is not only a final common pathway for forebrain or diencephalic neural sites, but also plays a major role in the fear learning process [7–9]. Specifically its dorsal portion (dPAG) is long known to be involved in the modulation

* Corresponding author at: Departamento de Farmacologia, CCB, Campus Universitário Trindade, CCB, Bloco D, 3°. Andar, Laboratório de Neurobiologia Comportamental, Florianópolis, SC 88040-900, Brazil. Tel.: +55 48 3721 9491; fax: +55 48 3337 5479.
*E-mail addresses:* padua.carobrez@ufsc.br, apcarobrez@gmail.com (A.P. Carobrez).

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of aversive states (for a comprehensive review, see [10]) and in addition, the dPAG plays a relevant role in sustaining olfactory fear conditioning promoted by the chemical stimulation of the dorsal premammillary nucleus of the hypothalamus [9]. Moreover, recent results from our laboratory showed that the chemical stimulation of the dorsolateral column (dlPAG) can be used as an unconditioned stimulus (US) in an olfactory fear conditioning paradigm, pointing the PAG participation in the modulation of fear associations [11]. Additionally, an electrophysiological study suggested that PAG relays aversive information to the amygdala and instruct neural plasticity for conditioned associations [12].

It has been shown that during the encoding of a CS-US association, the training intensity is one of the factors to be considered for the formation and maintenance of a fear memory. Therefore, in the present study, we used the chemical stimulation of dlPAG as a negative emotional event followed by the recall of a fear memory induced by a weak fear conditioning protocol. We hypothesized that this event, performed one day after the encoding of the CS-US association, could affect the fear memory trace during the recall of the previous established fear memory, modifying the fear expression in response to the associated context in subsequent retrieval experiences.

## 2. Materials and methods

### 2.1. Subjects

122 Adult male Wistar rats, weighing 280–350 g obtained from the Department of Pharmacology of Federal University of Santa Catarina were used in this study. Animals were housed in polypropylene cages (50 cm × 30 cm × 15 cm) in groups of three or four, under a 12 h light/dark cycle (lights on at 7 am), in a temperature-controlled environment (23 ± 1 °C) with free access to food and water. The protocols used were approved by the Federal University of Santa Catarina, Animal Ethics Committee (23080.0055752/2006-64/UFSC) and the experiments were carried out in accordance with the Brazilian Society of Neuroscience and Behavior Guidelines for the Care and Use of Laboratory Animals.

### 2.2. Stereotaxic surgery

Rats were intraperitoneally anaesthetized with 1.5 ml/kg of a solution containing ketamine (10%; Cetamin®, Brazil) and xylazine (2%; Xilazin®, Brazil), associated with local anesthesia (3% lidocaine with norepinephrine 1:50,000, Dentsply, Brazil) and fixed in a stereotaxic frame (Stoelting Co., USA). A stainless steel guide cannula (13 mm length, 26G) was implanted unilaterally aimed at the caudal dlPAG according to Paxinos and Watson rat brain atlas [13] coordinates (midline = 1.9 mm; anteroposterior = 7.6 mm; dorsoventral = −2.0 mm from the skull surface at an angle of 22 °C) and fixed to the skull with a stainless steel screw and acrylic cement. A stylet introduced inside the guide cannula prevented obstruction. At the end of the surgery, subjects were injected intramuscularly with an antibiotic association of benzylpenicillin and streptomycin (1.0 ml/kg; Pentabiótico®, Brazil) to prevent possible infections. In addition, flunixin meglumine (2.5 mg/kg; Schering-Plough, Brazil), a drug with analgesic, antipyretic and anti-inflammatory properties, was administered subcutaneously. After seven days of recovery, rats were subjected to the experimental procedures.

### 2.3. Drugs and dlPAG infusion procedure

N-methyl-D-aspartate (NMDA; Sigma, St. Louis, MO) was dissolved in 0.1 M phosphate-buffered saline (PBS; pH 7.4) which alone served as vehicle control. The dose used was selected based on previous studies of our group, which showed that 100 pmol is a dose capable to stimulate the neurons of the dlPAG, triggering the expression of overt defensive behaviors [14] as well as olfactory fear conditioning acquisition [11]. The drug infusion (0.2 μl) of either vehicle or drug was performed at the rate of 0.6 μl/min using an infusion pump (Insight, Ribeirão Preto, SP, Brazil) with a thin dental needle (outer diameter = 0.3 mm), sized 16.2 mm, introduced through the guide cannula (13 mm), extending 3.2 mm below the cannula end, reaching the dlPAG. A polyethylene catheter (PE10; Clay Adams, USA) was interposed between the upper end of the dental needle and the microsyringe (5 μl; Hamilton), and an air bubble displaced inside the polyethylene was used to monitor the drug flow. Needle was removed 30 s after the end of drug infusion.

### 2.4. Apparatuses and behavioral measures

Three different chambers were used in the present study: a conditioning chamber, a propylene box and a glass box. During the experiment, the boxes were housed in a sound-attenuating room with illumination level of 80 lux. Before and after utilization, all three chambers were cleaned with a 10% ethanol solution (v/v) and dry towels.

The conditioning chamber (designated as context A, 23 cm × 20 cm × 26 cm) was constructed with stainless steel walls and a grid floor composed of 1 cm spaced stainless bars connected to a shock generator (Insight, Ribeirão Preto, SP, Brazil) that, when appropriate, delivered a 0.5 mA shock for 3 s. A propylene box (50 cm × 30 cm × 15 cm) was designated as the context B, where the animals were placed immediately after the microinjection at the dlPAG. A third context, a glass box (28 cm × 28 cm × 28 cm), was designated as context C, an environment completely different from the conditioning chamber and the context B.

Freezing duration was used as a fear response; this defensive behavior has been commonly used as a reliable index of fear and defined as the complete absence of body, head and vibrissae movement except those required for breathing [15]. The total time spent in freezing in each session was quantified in seconds and expressed as the percentage of total time.

### 2.5. Experimental procedures

All experiments were carried out during the diurnal phase, between 1:00 and 5:00 pm. Each experiment was recorded by a video camera while a monitor and a DVD-recording system were installed in an adjacent room.

#### 2.5.1. Weak contextual fear conditioning

In order to establish the experimental conditions for the emergence of a weak fear memory, animals were subjected to diverse training protocols, varying the number of footschocks applied. The training consisted in placing each rat in the chamber (context A) and allowing a 1 min acclimation period (pre-shock period); after this period, rats received one, three or five foot-shocks (0.5 mA, 3 s duration at an inter-shock interval 30 s; unconditioned stimuli). Animals remained in the chamber for an additional minute (post-shock period). Rats assigned to control group (0 foot-shock) were placed in conditioning chamber for 3 min without receiving any unconditioned stimuli. All groups remained a total of 3 min, except the group that received 5 shocks which remained 4 min in the conditioning chamber (context A). At the end of this period, rats were removed and subsequently placed in their home cages.

#### 2.5.2. dlPAG chemical stimulation with NMDA infusion

Rats were randomly assigned to receive either the infusion of NMDA 100 pmol or PBS (vehicle) into the dlPAG. Immediately after the drug infusion, subjects were placed in context B for 10 min, after this period animals were returned to their home cages.

#### 2.5.3. Test sessions

Depending on the experimental design, test sessions (test 1, 2 or 3) were performed either in context A or context C, 24 h, 48 h or 6 days after the dlPAG infusion. The test was performed by placing each rat in the selected context for 3 min, without receiving foot-shock, and the time spent in freezing was scored and expressed as percentage of total time (3 min).

### 2.6. Experimental design

#### 2.6.1. Experiment 1

This experiment was designed to establish a sub-threshold fear conditioning protocol that would enable a further increased fear response, when later exposed to the CS (context A). For this purpose, one, three or five foot-shocks were associated with a neutral context based on previous preliminary studies. Rats were submitted to the weak contextual fear conditioning (day 1) and the fear expression was evaluated in context A, on day 2 (test 1) and also on day 3 (test 2), when they were returned to the same context. Conditional emotional responses were assessed as previously described.

#### 2.6.2. Experiment 2

This experiment was designed to evaluate whether the chemical stimulation of the dlPAG, a midbrain structure known to signalize danger, affects the fear memory trace during recall and during a subsequent test session. On day 1, rats were subjected to the weak contextual fear conditioning protocol (context A) receiving 0, 1 or 3 foot-shocks; on day 2, rats were either infused intra-dlPAG with NMDA or vehicle (context B) and on day 3 rats were re-exposed to the context A for test 1. Six days later (day 9), an additional testing session was performed (test 2) in context A to measure fear conditioned responses and 24 h later, animals were again tested for their freezing response (test 3) in a novel context (context C).

#### 2.6.3. Experiment 3

The third experiment was designed to evaluate if the re-exposure to context A was a pre-requisite to observe the potentiated fear response in NMDA-treated rats that were previously conditioned. Therefore, in the test 1 (on day 3), rats were randomly assigned to two experimental groups depending on the context (re-exposure to context A or exposure to context C). This session last 3 min and the total time spent in freezing was scored. An additional test (test 2) was performed 24 h later by placing all rats (both groups) in context A, for 3 min and time spent in freezing scored.