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Research report

Acquisition and expression of fear memories are distinctly modulated along the dorsolateral periaqueductal gray axis of rats exposed to predator odor

Rimenez R. Souza, Antonio P. Carobrez*

Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, Brazil

HIGHLIGHTS

- Cat odor induces avoidance and increases risk assessment in rats, as well as contextual fear conditioning.
- NMDA receptors within the dlPAG mediate both innate and contextual fear to predator odor.
- Acquisition, but not consolidation, of predator fear is mediated by rostral dlPAG NMDA receptors.
- Expression of predator-induced contextual fear is mediated by caudal dlPAG NMDA receptors.
- Innate and contextual fear to predator odor is differently mediated by rostro and caudal dlPAG.

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ABSTRACT

The dorsolateral region of the midbrain periaqueductal gray (dlPAG) modulates both innate and conditioned fear responses. However, the contribution of the rostrocaudal portions of the dlPAG to defense reactions and aversive memories remains unclear. Here, we sought to investigate the effects of N-methyl-D-aspartate (NMDA) receptor blockade within rostral or caudal dlPAG of rats exposed to innate and learned fear to cat odor. For this, adult male Wistar rats were microinjected with the NMDA antagonist D-2-amino-5-phosphono-pentanoate (AP5; 3 or 6 nmol/0.2 μl) into the rostral or caudal dlPAG before and after the exposure to the cat odor or to the context paired with the predator odor. The results demonstrated that cat odor exposure induced unconditioned defensive behaviors as well as contextual fear. AP5 microinjected in the rostral dlPAG reduced the defensive responses to cat odor and impaired the acquisition, but not consolidation of contextual fear. On the other hand, AP5 infused within the caudal dlPAG promoted long-lasting reduction of contextual fear expression. Altogether, our data suggest that NMDA receptors mediate a functional dichotomy in the rostrocaudal axis of dlPAG regulating unconditioned and conditioned defensive reactions to predatory cues.

1. Introduction

Predator odor exposure is a biologically relevant stimulus that has been used to investigate the neurobiology of fear in laboratory rodents. Both rats or mice exposed to cat odor exhibit intense defensive reactions such as avoidance and risk assessment behaviors, freezing and reduction of exploratory activity [1,2], increased activity of the hypothalamus-pituitary-adrenal axis and sympathetic nervous system [3,4], as well as long-lasting contextual fear, anxiety-like behaviors and hyperarousal [2,5,6]. Due to these robust fear-induced responses, exposure of laboratory rodents to cat odor has been proposed as an animal model of posttraumatic stress disorder (PTSD) [7,8]. In addition, it has been demonstrated that aversive olfactory cues are strong precipitators of distressing episodes in PTSD patients [9], reinforcing the usefulness of such approach to unfolding the neurobiology of traumatic memories.

Both human and rodent studies have shown that the midbrain periaqueductal gray (PAG) mediates innate and conditioned fear responses [10–16]. The PAG is organized in longitudinal columns surrounding the mesencephalic aqueduct [17], and its dorsolateral column (dlPAG) is described as an essential substrate for integration and execution of defensive reactions towards environmental threats [12,13,18,19]. Although a variety of neurotransmitters and neuromodulators are present in the dlPAG, the excitatory amino acid glutamate is described as essential for the dlPAG-mediated

* Corresponding author. Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil.
E-mail address: padua.carobrez@ufsc.br (A.P. Carobrez).

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defense reaction [20–24]. Hence, the activation of *N*-Methyl-D-aspartate (NMDA) glutamate receptors in the dlPAG induces not only defensive behaviors [20,21], but is described to promote synaptic plasticity and facilitate memory formation [25,26]. Also, dlPAG stimulation by local administration of NMDA works as an unconditioned stimulus instructing forebrain structures for the acquisition of olfactory fear conditioning [11,27]. Furthermore the integrity of glutamatergic transmission within dlPAG is necessary for the acquisition of fear conditioning after stimulation of the dorsal premammillary nucleus [28], a key region for anti-predatory defensive responses [4,19].

Although most studies have addressed the caudal portion of dlPAG as a site for execution of defensive reactions towards contextual threats [11,13,24,29–32], the pattern of neuronal activity during live predator or predator odor exposure engages mostly the rostral portion of dlPAG [2,12,18]. Neuroanatomical studies have shown longitudinal differences in neuronal connectivity between dlPAG and fear circuits [12,19,23,33,34], as well as heterogeneous expression of neurotransmitter systems [23,24,35–37], suggesting that rostral and caudal dlPAG may have distinct contributions in the modulation of different types of defensive responses.

Thus, the present study was aimed to investigate if the NMDA receptors in the rostral and caudal portions of dlPAG axis mediate the expression of innate fear, as well as the acquisition and retrieval of predator-induced contextual fear. For this, we performed intra-rostral or caudal dlPAG infusions of the NMDA antagonist AP5, either before the exposure to the cat odor or before the exposure to contextual fear induced by previous exposure to cat odor. Intra-dlPAG infusion of AP5 was also performed immediately after the exposure to evaluate possible effects on consolidation of contextual fear.

2. Materials and methods

2.1. Animals and housing

Male *Wistar* rats weighing 300–420 g and at 15–16 weeks of age at the time of testing were used. The animals were housed in groups of 3–4 per cage (50 × 30 × 15 cm) in standard environmental conditions (23 ± 1 °C on a 12 h/12 h dark/light cycle, lights on 7:00 a.m.), with food and water available *ad libitum*. Experiments were performed during the middle phase of the light cycle. All animal procedures were approved by the Institutional Ethics Committee (23080.006118/2004-36/UFSC/BRAZIL) and were in accordance with NIH animal care guidelines [38].

2.2. Drugs

The NMDA antagonist AP5 (±2-amino 5-phosphopentanoic acid, 3 or 6 nmol/0,2 μl, RBI, USA), was solubilized in phosphate buffered saline (PBS, pH = 7.4), which alone served as vehicle control. The solutions were prepared in 500 μl eppendorfs immediately before use and protected from light with aluminum foil. The doses were chosen based on previous studies from our laboratory [28,39].

2.3. Stereotaxic surgery and dlPAG infusion procedure

Rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (90 mg/kg, Dopalen®, Vetbrands, Brazil) and xylazine (10 mg/kg, Dopaser®, Calier, Spain) solution and securely placed in a stereotaxic frame. A 0.1 ml of local anesthetic with vasoconstrictor (Xylestesin®, Cristália, Brazil) was subcutaneously injected under the scalp as a pre-surgical care, and after five minutes a longitudinal incision was made. The bone was exposed and asepsis was made. A stainless steel guide cannula (diameter = 0.7 mm; length = 13 mm) was then implanted with the tip aimed at the rostral or caudal dlPAG (coordinates from bregma: AP = −5.8 and −7.6 mm for rostral and caudal dlPAG, respectively; ML = +1.9 mm; DV = −2.0 mm from surface at an angle of 22°), according to the coordinates of rat brain atlas [40]. The cannulas were anchored to the skull with two stainless steel screws and acrylic cement. After surgery, rats received acute treatment with pentabiotic (Pentabiótico Veterinário, Fort Dodge, Brazil) and the anti-inflammatory/analgesic flunixin meglumine (Banamine, Schering-Plough, Brazil) for post-operative care. Rats were allowed to recover for a week prior to behavioral experiments. During this period, rats were monitored for thermoregulation and signs of pain or discomfort.

The intracerebral injection was carried out by inserting a 16.2 or 16.4 mm long (rostral and caudal dlPAG, respectively) stainless steel needle through the guide cannula while the rat was gently restrained. The needle was previously connected to a 5 μl Hamilton microsyringe by polyethylene tubing (PE10, Clay Adams, USA). An infusion pump (B12000, Insight Ltda., Ribeirão Preto, Brazil) allowed for microinjections over a 20-s time period (0.6 μl/min rate). The forward movement of a small air bubble inside the polyethylene tubing confirmed drug flow. The needle was kept in the cannula for an additional 30-s period after drug infusion to prevent backflow of the solution.

2.4. Experimental apparatus and general procedure

Testing sessions were conducted in a black acrylic rectangular arena (60 × 26 × 36 cm), except for the front wall, which had a transparent side allowing video recording. The apparatus comprised two different areas: a covered enclosed "hide area" (20 × 26 × 36 cm) and an uncovered "open area" (40 × 26 × 36 cm), as that previously described by Souza et al. [41]. Compartments were separated by a small door (6 × 6 cm) that allowed free transit of the rat between the two areas. On the opposite wall of the open area, a clean cloth (45 × 30 cm) folded was attached for familiarization and context sessions. A similar folded cloth with cat odor was used in the conditioning session. The cat odor was obtained by leaving the cloth in the bed of a domestic cat for 3 days and an additional 10 min rubbing at the back of the cat 30 min before testing sessions. The cat odor cloth was kept in a sealed plastic bag prior to the experiments and was always handled with surgical gloves. Each cloth was exposed to a maximum of ten rats per day. Each experimental session lasted no more than 180 min, and no significant reduction of fear was observed in the control rats (vehicle). To prevent possible bias by a reduction in the intensity of the olfactory cue, at least one control rat was exposed to cat odor in the beginning and at the end of each experimental session.

At the beginning of the each session, each rat was placed into the apparatus near the small door and facing the cloth. Sessions were named: familiarization (day 1), cat odor (day 2; cat odor as the unconditioned stimulus – US), context (day 3; context in the absence of cat odor), and context 2 (day 4; as in day 3). Each session lasted 10 min. Between subjects' test and after each session, the apparatus was cleaned with ethanol solution 10% or 70% (v/v), respectively. The experiments were carried out in a room with low illumination (4 lux at the center of the apparatus). A video camera was placed 50 cm in front of the apparatus and connected to a recording system located in an adjacent room, enabling a trained observer to score the following parameters: *approach time (%)* – the percentage of time that animals stayed within a 7 cm perimeter (approach line) near the cloth; *hide time (%)* – the percentage of time spent in the hidden area; and *head-out (s)* – the time performing stretched attend postures from the enclosed compartment towards the open area. All scores were made by an observer blind to the subject's treatment.