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Research report

Functional mapping of the periaqueductal gray matter involved in organizing tonic immobility behavior in guinea pigs

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ABSTRACT

Tonic immobility behavior (TI) is an innate response characterized by profound motor inhibition that is exhibited by prey when physical contact with a predator is prolonged and the situation inescapable. The periaqueductal gray matter (PAG) is intimately associated with the somatic and autonomic components of defensive reactions. This study investigated whether the TI response was able to recruit specific functional columns of the PAG by examining c-fos immunolocalization in guinea pigs. In the TI group, the innate response was invoked in animals through inversion and physical contention for at least 15 min. In the control group, the animals were physically manipulated only. Our results demonstrate that the defensive behavior of TI is capable of promoting the expression of Fos protein in different areas of the PAG, with higher levels of staining in the ventrolateral (vl) and lateral (l) columns. In addition, our results demonstrate increased Fos immunoreactivity (FOS-IR) in the dorsal raphe nucleus, the Edinger–Westphal nucleus, the cuneiform nucleus and the superior colliculus. In contrast, there were no significant alterations in the number of FOS-IR cells in the inferior colliculus or the oculomotor nucleus. Analysis of the results suggests that neuronal activation after the TI response differs by functional column of the PAG.

1. Introduction

Tonic immobility (TI) is an innate response characterized by a reversible state of profound physical inactivity and relative lack of responsiveness to the environment that is induced by situations that generate intense fear. The objective of the behavior is to protect the animal from attacks by predators [31,70]. This response can be triggered in a wide range of vertebrate and invertebrate species and is also known as animal hypnosis or feigning death [62,70]. In the laboratory, TI can be induced by postural inversion and manual restriction of movements, maneuvers that emphasize the tactile and proprioceptive sensations that are important for the induction of this behavior.

Several studies have been conducted to determine the central nervous system regions that control this response. Use of the transection technique has defined the levels of the nervous system that participate in this modulation in toads and rabbits. These experiments show that the TI response may be related to the activation of a pool of interneurons in the reticular formation of the brainstem that influence descending neurons that, in turn, inhibit the motor neurons of the spinal cord [38]. Experiments have also shown that the integrity of midbrain regions is essential for the expression of this defensive behavior [17,38], though other brain regions may also modulate the TI response. In fact, previous studies have suggested that the neural substrates involved in mediating the TI and other defensive behaviors, aversive reactions and emotional states [32] fundamentally include the periaqueductal gray matter (PAG) [54], the parabrachial region [53], hypothalamus [58], and the amygdala [45,46]. Additionally, the deep layers of the superior colliculus (SC) and the inferior colliculus play an important role in the integration of defensive behaviors [20,22] through a pathway to the dorsal raphe nucleus and to the locus coeruleus [30]. In this context, the PAG integrates a mesencephalic locomotor region that receives projections from various forebrain areas and projects to spinal motor neurons [68]. Thus, the PAG may serve as an interface between the limbic forebrain regions and spinal cord in the expression of fear behavior [41].

According to Bandler and Carrive [3], the "defense reaction" is often referred to as the pattern of behavioral and cardiovascular change characteristic of an animal's reaction to threatening or stressful stimuli. It is well established that the PAG controls a wide range of functions, including the modulation of pain [4,5,29], analgesia [64], autonomic responses [48], vocalization [36] and different patterns of behavior, such as threat display, fight, flight

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and immobility responses [3,38,54,57]. Each of these patterns of behavior can be elicited in specific parts of the PAG by electrical or chemical stimulation [3,67,74]. More specifically, strong or moderate immobility, characterized by a period of profound inactivity, is observed after stimulating the ventrolateral PAG of the cat, while a strong flight response is observed after stimulating the dorsomedial PAG [74].

In the last decade, efforts have been made to evaluate the neural substrates activated during emotional and motivational defensive responses. Several laboratories have specialized in the study of behavioral responses induced by methods based on the predator versus prey paradigm [15,23,33] and in invasive techniques to promote fear-induced behavior [14,21]. These experimental approaches often produce a state of increased vigilance and anxiety, characterized by defensive attention and immobility, avoidance responses and risk assessment [8,25,33]. Certain experimental approaches use c-fos immunolocalization to identify the neural substrate activated in threatening situations, e.g., when small rodents are exposed to a live predator [15,23]. In these studies, rats exposed to a cat exhibited increased Fos-like immunoreactivity in specific neurons of the medial hypothalamus and PAG. In contrast, studies involving acute footshock induced Fos expression in the supraoptic nucleus, the central, basolateral and lateral amygdaloid nuclei and the nucleus of the solitary tract [13,26,61]. It is possible that different neural circuitry is mobilized during exposure to predators and aversive stimuli.

Based on the these previous findings, the present study was designed to investigate whether the TI response is able to recruit specific functional columns of the PAG, as well adjacent areas such as the Edinger–Westphal nucleus (EW), the dorsal raphe nucleus (DRN), the superior colliculus (SC), the inferior colliculus (IC), the cuneiform nucleus (CnF) and the oculomotor nucleus (NIII), by means of c-fos immunolocalization in guinea pigs.

2. Materials and methods

2.1. Animals

Adult male guinea pigs (*Cavia porcellus*, University of São Paulo, Campus of Ribeirão Preto, Brazil) weighing 450–500 g ($n = 27$) were kept in Plexiglas wall cages (56 cm × 17 cm × 39 cm, five guinea pigs per cage) in a room maintained at 24 ± 1 °C, on a 12 h light–dark cycle, with free access to water and food throughout the experimental period. The experiments were conducted during the light phase of the cycle. The experimental protocol was analyzed and approved by the Committee for Animal Care and Use of the University of São Paulo, Campus of Ribeirão Preto (no. 05.1.84.53.7).

2.2. Experimental procedure

Guinea pigs were habituated (24 h) to the experimental room before being divided into two experimental groups. In the control group ($n = 13$), the guinea pigs were manipulated for a few minutes and then kept in a room under the same conditions as the experimental groups. In the TI group ($n = 14$), each guinea pig was exposed to five TI induction maneuvers, and the duration of the episodes were recorded. TI induction was conducted as described previously [42]. Briefly, TI induction was attempted by holding the guinea pig around the thorax with the hands, quickly inverting it, and pressing it down into a shaped plywood trough (25 cm long × 15 cm high). In this study, the average duration of TI was 15 min.

2.3. Fos protein immunohistochemistry

Guinea pigs were deeply anesthetized 2 h after the onset of the experimental procedure with an intramuscular injection of ketamine (150 mg/kg) plus xylazine (14 mg/kg) and then transcardially perfused with 200 mL of phosphate buffered saline (PBS, 0.01 M, pH 7.4), followed by 200 mL of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) at 4 °C. The brains were rapidly removed and fixed in the above solution for 2 h (at 4 °C), then cryoprotected overnight in a 30% sucrose/phosphate buffer, after which the brains were frozen in isopentane at −40 °C. Subsequently, 40 μm coronal sections were cut by cryostat and processed for Fos immunocytochemistry. Briefly, tissue sections were successively washed and incubated overnight with primary Fos antibody (SC 52, Santa Cruz Biotechnology, USA) at a concentration of 1:2000 in PBST (0.1 M PBS with 0.2% Triton-X and 0.1% bovine serum albumin, BSA). The sections were then processed using the avidin-biotin-immunoperoxidase method (Vectastain ABC Kit, Vector Laboratories, USA) and FOS-IR was revealed by the addition of the chromogen 3,3′-diaminobenzidine (DAB, 0.02%) and 1% hydrogen peroxide. The polyclonal anti-c-Fos antibody was omitted in negative controls. The sections were rinsed in PBS, mounted on gelatin-pretreated slides, dehydrated through serial ethanol solutions, xylene-cleared and cover slipped for microscopic observations. In all experiments, tissue from the control and experimental guinea pigs were processed together. The brainstem regions analyzed in this study were as follows: three distinct rostrocaudal levels of the PAG, the EW, the dorsal raphe nucleus (DRN), the SC, the inferior colliculus (IC), the CnF and the oculomotor nucleus (NIII).

2.4. Data quantification and analysis

The anatomical localization of Fos immunoreactivity (FOS-IR, brown staining) was aided by comparing representative sections stained with cresyl violet to a stereotaxic atlas for guinea pigs [65]. To describe the distribution pattern of Fos-labeled cells in the guinea pig PAG, we employed the designations proposed in the Paxinos and Watson Atlas [60], which are, from the rostral to the caudal levels, the dorsomedial (dm), dorsolateral (dl), lateral (l) and ventrolateral (vl) columns.

For a cell to be counted as expressing FOS-IR, the nucleus of the neuron had to be of an appropriate size (cell neuron diameter ranging from approximately 8 to 15 μm) and shape (oval or round) and had to be distinct from the background at 10× magnification. For quantitative analysis of Fos-labeled cells, three consecutive sections of the brainstem region of interest were taken from each guinea pig. In each section, the number of Fos-positive neurons was counted unilaterally in the study area by one observer blinded to treatment, using a light microscope with a 10× objective. The absence or presence of labeled neurons was registered using an image analysis system (Image J). This number was standardized to a tissue area of 0.2 mm^2 and the average of these counts was calculated for each guinea pig.

2.5. Statistical analysis

The results for each brain area are reported as the mean ± SEM for each group and were analyzed by means of a two-way analysis of variance (ANOVA), with area and groups (control and TI) as the factor for each distinct rostrocaudal levels of the PAG. For each area, this analysis was followed by a one-way ANOVA with groups as the between-group factor (control and TI). Newman–Keuls post hoc tests were done when appropriate. The significance level was set at $P = 0.05$.

3. Results

Sample micrographs of the PAG regions with the resulting immunoreactions are presented in Fig. 1. Analysis of the results determined that TI behavior promoted an increase in FOS-IR in all PAG columns and in the EW, DRN, SC and CnF. In contrast, the experimental induction of TI response did not alter FOS-IR in the IC and NIII. Relatively sparse FOS-IR cells were observed throughout the PAG and adjacent areas in controls (Fig. 2).

At the rostral level of the brainstem, two-way ANOVA revealed that a significant difference occurred between area ($F_{5,147} = 24.363$, $P < 0.001$) and group ($F_{1,147} = 177.664$, $P < 0.001$), and there was an interaction between area and group ($F_{15,160} = 28.076$, $P < 0.01$). The Newman–Keuls post hoc test revealed a significant difference between the number of FOS-IR cells in the TI group compared to the control group ($P < 0.05$) in all PAG columns and within the CnF and EW. In contrast, within the NIII, no difference occurred between the control and TI groups (Fig. 2).

Two-way ANOVA applied at the intermediary level revealed a significant difference between area ($F_{6,123} = 20.894$, $P < 0.001$) and group ($F_{1,123} = 150.760$, $P < 0.001$), and there was an interaction between area and group ($F_{6,123} = 5.163$, $P < 0.001$). The Newman–Keuls post hoc test showed a significant difference between the number of FOS-IR cells in the TI and control groups in all PAG columns ($P < 0.05$, Fig. 2), the SC and the DRN. In contrast, no difference in FOS-IR was observed in the IC between the control group and the TI group (Fig. 2).

At the caudal level of the brainstem, the two-way ANOVA showed that there was a significant difference between area ($F_{3,51} = 14.052$, $P < 0.001$) and group ($F_{1,51} = 86.138$, $P < 0.001$), and there was an interaction between area and group ($F_{3,51} = 3.259$, $P < 0.05$). The Newman–Keuls post hoc test ($P < 0.05$) revealed that the number of FOS-IR cells in all PAG columns and within the DRN was different in the TI group compared to the control group (Fig. 2).