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Research Report

Fos-like immunoreactivity in the brain associated with freezing or escape induced by inhibition of either glutamic acid decarboxylase or $GABA_A$ receptors in the dorsal periaqueductal gray

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Abstract

GABAergic neurons exert tonic control over the neural substrates of aversion in the dorsal periaqueductal gray (dPAG). It has been shown that electrical stimulation of this region at freezing or escape thresholds activates different neural circuits in the brain. Since electrical stimulation activates cell bodies and fibers of passage, it is necessary to use chemical stimulation that activates only post-synaptic receptors. To investigate this issue further, reduction of GABA transmission was performed with local injections of either the GABA-A receptor antagonist bicuculline or the glutamic acid decarboxylase (GAD) inhibitor semicarbazide into the dorsolateral periaqueductal gray (dlPAG). Local infusions of semicarbazide (5.0 μg/0.2 μl) or bicuculline (40 ng/0.2 μl) into this region caused freezing and escape, respectively. The results obtained showed that freezing behavior induced by semicarbazide was associated with an increase in Fos expression in the laterodorsal nucleus of the thalamus (LD) and ventrolateral periaqueductal gray (vlPAG), while bicuculline-induced escape was related to widespread increase in Fos labeling, notably in the columns of the periaqueductal gray, hypothalamus nuclei, the central amygdaloid nucleus (Ce), the LD, the cuneiform nucleus (CnF) and the *locus coeruleus* (LC). Thus, the present data support the notion that freezing and escape behaviors induced by GABA blockade in the dlPAG are neurally segregated: freezing activates only structures that are mainly involved in sensory processing, and bicuculline-induced escape activates structures involved in both sensory processing and motor output of defensive behavior. Therefore, the freezing elicited by activation of dlPAG appears to be related to the acquisition of aversive information, whereas most brain structures involved in the defense reaction are recruited during escape.
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Abbreviations: AHC, anterior hypothalamus; BIC, bicuculline; BLA, basolateral amygdaloid nucleus; c, caudal; Ce, central amygdaloid nucleus; Cg, cingulate cortex; CIC, central inferior colliculus; CnF, cuneiform nucleus; Contra, contralateral; dPAG, dorsal periaqueductal gray; dlPAG, dorsolateral periaqueductal gray; DMH, dorsomedial hypothalamus; dmPAG, dorsomedial periaqueductal gray; DRN, dorsal raphe nucleus; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; i, intermediate; Ipsi, ipsilateral; LC, *locus coeruleus*; LD, laterodorsal nucleus of the thalamus; LEnt, entorhinal cortex; LH, lateral hypothalamus; lPAG, lateral periaqueductal gray; MnR, median raphe nucleus; PAG, periaqueductal gray; PaV, paraventricular hypothalamic nucleus; PMD, dorsal premammillary nucleus; PrL, prelimbic cortex; r, rostral; SC, superior colliculus; SCB, semicarbazide; vlPAG, ventrolateral periaqueductal gray; VMHdm, ventromedial hypothalamic nucleus, dorsomedial part

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1. Introduction

It has been established that the longitudinal columns of the periaqueductal gray (PAG) coordinate distinct patterns of behavioral and physiological reactions that are critical for survival [6,20]. Although escape behavior has been the main focus of attention, interest in freezing behavior induced by electrical or chemical stimulation of the dorsolateral PAG (dlPAG) has been growing lately. Indeed, electrical or chemical stimulation of this brain region leads to defensive behavior characterized by alertness, freezing, escape behavior, analgesia and autonomic reactions [7,10,23,38,61]. Thus, there is general agreement on that dlPAG is not only part of a fight/flight system but that several other defensive reactions may be integrated at this level [15,61]. Lately, defensive immobility elicited by chemical stimulation of the dlPAG has gained considerable attention since freezing behavior elicited by this procedure has been reported to resemble human panic attacks [9,36,60,64]. In this context, it has been shown that animals assume different strategies depending on the kind of threatening situation they are faced with [3,8,14]. Thus, while freezing behavior is the usual response when the animal perceives the danger, escape is the natural reaction when the predator is close [8]. This line of research has evolved to disclose the structures and the neural mechanisms that might participate in the acquisition of aversive information and expression of the different types of defensive behaviors.

Studies using electrical stimulation of the midbrain tectum have shown that defensive strategies as distinct as avoidance, freezing and escape are likely to be organized in different networks [8,40,45,63]. However, electrical stimulation has the disadvantage of exciting not only perikarya but also axons and fibers of passage. To circumvent this problem, the use of chemicals that stimulate only postsynaptic receptors is preferable in studies on the organization of neural circuits that are responsible for the defense reaction. From these studies, it has been shown that defensive behavior elicited by stimulation of the dlPAG is a complex process mediated by a number of neurotransmitters and neuromodulators. Among them, it has long been demonstrated that GABAergic mechanisms exert a tonic inhibitory control on the neural substrates of aversion in the dlPAG [10,13,14,23,31]. This study goes one step further with the measurement of Fos distribution in serial sections of the brain following chemical stimulation of the dlPAG. We take advantage of the fact that microinjections of the inhibitor of the glutamic acid decarboxylase (GAD) semicarbazide, or the GABA-A receptor antagonist bicuculline, into the dlPAG cause freezing or escape behavior, respectively [11,44]. The freezing behavior is the result of a gradual reduction of the GABA levels in the region injected, which is established *pari passu* with the enzyme inhibition, so that the blockade is not abrupt as it occurs with the GABAergic receptor antagonism [1,11,52]. Saline-injected animals were exposed to the same procedure in order to control for the novelty of the experimental setting as well as for the locomotor activity. At first, we chose to concentrate on those nuclei known for having monosynaptic connections with PAG; that is, cingulate (Cg) and prelimbic (PrL) areas [2,34,39], central amygdaloid nucleus (CeA) [48,49], ventromedial (dorsomedial part, VMHdm) and premammillary (PMD) nuclei of the hypothalamus [16,18,56], cuneiform nucleus (CnF) [29,47], inferior colliculus (IC) [40], the *locus coeruleus* (LC) [32] and the dorsomedial (dmPAG), dorsolateral (dlPAG), lateral (lPAG) and ventrolateral (vlPAG) subdivisions of the periaqueductal gray [6,20,51,63]. In view of the results obtained, other structures were also included in the course of the study.

2. Materials and methods

2.1. Animals

Naive male Wistar rats weighing 230–250 g were used. Animals were kept under controlled temperature (22 ± 2 °C) and a 12-h light:12-h dark cycle (lights on at 07:00 h). They were housed in two per cage and had free access to food and water throughout the experiment. The experiments were conducted between 9:00 and 14:00 p.m. The experiments were performed in compliance with the recommendations of SBNeC (Brazilian Society of Neuroscience and Behavior), which are based on the US National Institutes of Health *Guide for Care and Use of Laboratory Animals*.

2.2. Surgery

The animals were anesthetized with tribromoethanol (250 mg/kg, ip) and fixed in a stereotaxic frame (David Kopf, U.S.A.). A brain cannula was implanted in the midbrain, aimed at the dlPAG. The stainless steel guide-cannula (13 mm length, o.d. 0.6 mm, i.d. 0.4 mm) was introduced at an angle of 16° using the following coordinates, with the lambda serving as the reference for each plane: antero-posterior, 0 mm; medio-lateral, 1.9 mm; and dorso-ventral, 4.1 mm [46]. The upper incisor bar was set at 2.5 mm below the interaural line so that the skull was horizontal between bregma and lambda. The cannula was fixed to the skull by means of acrylic resin and two stainless steel screws. At the end of the surgery, each guide-cannula was sealed with a stainless steel wire to prevent obstruction.

2.3. Microinjections

The animals were put in a plastic box so that a thin dental needle (o.d. 0.3 mm) could be introduced through the guide-cannula until its lower end was 1 mm below the tip of the cannula. The injection needle was linked to a 5 μl Hamilton syringe by means of polyethylene tubing connected to a microinfusion apparatus (Harvard, USA). A constant volume of 0.2 μl was injected during 30 s. The displacement

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