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The benzodiazepine midazolam acts on the expression of the defensive behavior, but not on the processing of aversive information, produced by exposure to the elevated plus maze and electrical stimulations applied to the inferior colliculus of rats

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ABSTRACT

Electrical stimulation of midbrain tectum structures, particularly the dorsal periaqueductal gray (dPAG) and inferior colliculus (IC), produces defensive responses such as freezing and escape behavior. Freezing also results after termination of this stimulation (post-stimulation freezing; PSF). Whereas these responses are critically mediated by GABA in the dPAG, it is unclear how GABA-benzodiazepine mechanisms mediate the expression of fear (freezing and escape behaviors) and the processing of aversive information (PSF) produced by electrical stimulation of the IC. Since dorsal (ICd) and ventral regions (ICv) of the IC react differentially to aversive stimulation, we hypothesized that these regions might be sensitive to the action of benzodiazepine drugs when rats are submitted to animal models of anxiety: the elevated plus maze (EPM) and the IC electrical stimulation procedure. Midazolam (5, 10 or 20 nmol) was injected into the ICd or ICv of rats subjected to one of these tests. Intra-ICv, but not intra-ICd injections, of midazolam reduced the aversiveness of the IC electrical stimulation and decreased fear in the EPM, as assessed by its traditional and complementary measures. In contrast, the IC post-stimulation freezing remained unaltered with midazolam treatments. Thus, there is a clear pharmacological dissociation in the reactivity of dorsal and ventral regions of the IC to fear-provoking stimuli of the two animal models of anxiety used in this study. The present results support the proposal that benzodiazepine-mediated mechanisms are only involved in the output mechanisms of defensive behavior and not involved in the processing of ascending aversive information from the IC.

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1. Introduction

The inferior colliculus (IC) is a midbrain tectum structure, known for its pivotal role in acoustic processing. It is similar throughout a variety of species, but in the rat the IC is an ovoid bilateral structure, with the long diameter measuring 3.5 mm and the short diameter 2 mm (Faye-Lund and Osen, 1985). It is a region that has tonotopic and layered topography, morphologically prepared to encode and decipher auditory information in terms of spatial, spectral, and temporal properties (Winer and Schreiner, 2005).

Besides being a relay for auditory information (Aitkin and Phillips, 1984; Faingold et al., 1989), the IC is also capable of organizing responses to aversive stimuli. This notion has been refined over the last 30 years and today it is well accepted that the IC is part of the "Encephalic Aversion System (EAS)" that comprises other structures such as the dorsal periaqueductal gray (dPAG), the medial hypothalamus (MH), and the amygdala (AMYG). Neuroanatomical data establish reciprocal connections between the AMYG, MH and PAG (Bandler and Shipley, 1994; Brandao et al., 1999) but the way IC establishes neural

Abbreviations: BZD, Benzodiazepine; EAS, Encephalic Aversion System; EPM, Elevated plus maze; ICd, dorsal part of inferior colliculus; ICv, ventral part of inferior colliculus; MDZ, Midazolam.

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connectivity with the other structures of the EAS is still under investigation. It is known that electrical or chemical stimulation of the IC, as seen in other EAS areas, produces defensive responses, such as freezing and flight behavior (Pandossio and Brandao, 1999). It is reasonable to think that the IC would be able to elaborate some sort of immediate response after an initial (and rudimentary) processing of sounds that signaled some risk to survival (Casseday and Covey, 1996). However, it is known that this structure may also organize defensive behaviors that do not involve sounds. Indeed, rats submitted to the EPM without any acoustic stimulation present increased *Fos* activity in the IC, among other structures involved in the defense reaction (Silveira et al., 1993). Other *Fos* expression studies have also disclosed neural circuits underlying the freezing behavior elicited by stimulation of distinct regions of the IC (Borelli et al., 2006; Ferreira-Netto et al., 2007; Lamprea et al., 2002). NMDA injections aimed at the dorsal part of the IC (ICd) caused an increase of *Fos* expression in the medial geniculate nucleus, superior colliculus, dPAG and locus coeruleus, while injections of NMDA into the ventral region of IC (ICv) resulted in increased *Fos* expression in the prelimbic and cingulate cortices, basolateral and medial nuclei of AMYG, ventrolateral PAG, cuneiform nucleus and locus coeruleus (Ferreira-Netto et al., 2007). Bagri et al. (1991) found that electrical stimulations of either the dorsal part or ventral part of the IC both elicited wild running, being the ventral part more sensitive than the dorsal part. Indeed, the ICd has been recognized as the neural substrate of audiogenic seizures (Garcia-Cairasco and Sabbatini, 1991), while the ICv may play a key role in the sensorimotor gating of aversive information (Brandao et al., 1993).

The GABA system plays a tonic inhibitory role on the neural substrates of aversion in the inferior colliculus. It is estimated that 20%–40% of IC neurons are GABAergic (Caspary et al., 1990). GABA/benzodiazepine agonists attenuate the aversive outcome of electrical stimulation at this site (Melo et al., 1992; Pandossio and Brandao, 1999) as also shown previously in other related structures of the EAS (Audi and Graeff, 1987; Brandao et al., 1990; Graeff, 1981; Graeff et al., 1986, 1993; Schenberg et al., 1983). Instead of directly enhancing GABAergic neurotransmission with GABA agonists, the use of benzodiazepines, a class of drugs widely used in clinical practice, has proven to be very useful to modulate the GABA system during aversive states generated by an array of threatening conditions. Post-stimulation freezing (PSF) has been related to the processing of aversive information that is relayed to higher structures (Borelli et al., 2006; Brandao et al., 2008; Martinez et al., 2006; Vianna et al., 2001). A key question when we look at the anxiolytic-like actions of benzodiazepines is where, how and when they interfere with the emotional reactivity of the animal facing threatening situations. As for the IC, a recent study from this laboratory showed that there is a clear anatomical specificity regarding the dorsal and ventral regions of this structure as to the reactivity to aversive stimulation; only the ventral regions triggered fear responses to the local injections of the excitatory amino acid NMDA (Ferreira-Netto et al., 2007). From these findings we predicted that these IC regions might be sensitive to anxiolytic-like actions of benzodiazepine drugs when rats are submitted to animal models of anxiety: the elevated plus maze (EPM) and the IC electrical stimulation procedure. Besides, we were also interested in examining the effects of midazolam on the output mechanisms of defensive behavior (freezing and escape behaviors) and in the processing of ascending aversive information from the IC (post-stimulation freezing). A suitable candidate for this experimental purpose is midazolam, a short-acting, non-selective anxiolytic drug that is highly soluble in water, which makes it appropriate for intracerebral injections.

2. Materials and methods

2.1. Animals

Adult male Wistar rats ($n = 127$), weighing 275 ± 25 g, were housed in groups of four per cage, with free access to food and water. The cages were kept in a temperature-controlled room (20–25 °C) and 12-h light/dark cycle (lights on at 7:00 a.m.). All procedures followed the guidelines on the ethical use of animals by the Brazilian Society of Neuroscience and Behavior's (SBNeC) which follows the National Institutes of Health (NIH) guide for the care and use of laboratory animals. This work has also been approved by the Ethics Committee on Animal Use from the University of Sao Paulo (Protocol no. 11.1.308.53.9). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Guide cannulas and chemitrodes

Guide cannulas were made of 23G stainless steel needles (length = 13 mm; Becton–Dickinson, Franklin Lakes, NJ, USA), and were implanted in animals subjected to the EPM. Chemitrodes consisted of a guide cannula attached to a brain electrode. They were made of a stainless steel wire (0.27 mm, o.d.) (A-M Systems, Inc., USA) insulated throughout except at the cross section of the tip. The insulated wire was soldered to one pin of a miniature socket, parallel to a stainless steel guide cannula 1 mm shorter than the tip of wire. A non-insulated stainless steel wire was soldered to the other pin and to one of the anchor screws, thereby serving as the indifferent electrode. Chemitrodes were used in rats that received the drug at the same brain site used for electrical stimulation.

2.3. Stereotaxic surgery

The animals were anesthetized with ketamine/xylazine association (100/7.5 mg/kg respectively, i.p., Agener União, Embu-Guaçu, SP, Brazil) and fixed in a stereotaxic frame (David Kopf, Tujunga, CA). The upper incisor bar was set 3.3 mm below the interaural line to assure horizontality between bregma and lambda. Either a unilateral guide-cannula or chemitrode was implanted over the right IC. According to the atlas of Paxinos and Watson (2006) and with lambda serving as the reference point, the coordinates were a) for guide-cannulas: antero-posterior (AP) −0.9 mm, medio-lateral (ML) −1.7 mm and dorso-ventral (DV) −4.3 ± 0.5 mm (orthogonally) or b) for chemitrodes: AP +1.6 mm, ML -1.7 mm and DV either −4.3 or −5.3 mm (at a 15° angle). For all groups, the cannulas or chemitrodes were fixed to the skull with acrylic resin and two stainless steel anchor screws. Each guide cannula was sealed with a stainless steel wire to protect it from blockage. At the end of surgery, animals received an injection of a polyvalent veterinary antibiotic (Pentabiótico, 0.2 mL, i.m.; Fort Dodge, Campinas, SP, Brazil) and an injection of the anti-inflammatory and analgesic flunixin meglumine (Banamine®, 2.5 mg/kg, s.c.; Schering-Plough, Cotia, SP, Brazil). Afterward, rats were allowed 5 days to recover from the surgical procedure.

2.4. Drugs and microinjection procedure

Midazolam maleate (MDZ; Roche, Brazil) was used in doses of 5, 10 or 20 nmol. Control animals received saline (SAL, 0.9% NaCl) in the same volume and route of administration. The solutions were prepared shortly before use and injected at a rate of 0.2 μL/min by an infusion pump (Harvard Apparatus, Massachussets, USA). By using a volume of 0.2 μL, the drug diffusion was restricted to the target region (Myers, 1966). The doses of the drug and waiting times were selected based on previous studies (Borelli et al., 2006; Ferreira-Netto et al., 2007; Nobre and Brandao, 2004; Pandossio and Brandao, 1999). During the microinjection procedure, the animals remained free in a polypropylene box measuring 28 × 17 × 13 cm, lined with shredded paper. A dental needle (30 G, 14 mm long, 0.3 mm o.d.) was inserted into the cannula, passing it to 1 mm. This needle was connected to a polyethylene tube (PE- 10, Becton Dickinson, NJ, USA) and a 10 μL Hamilton syringe. The displacement of an air bubble inside the PE-10 tube was used to monitor the microinjection. After the end of the infusion, the needle was held in place for an extra minute to avoid reflux of the drug through the guide cannula.

2.5. Behavioral study

2.5.1. Elevated plus maze

A wood apparatus was used, consisting of two open arms (50 cm × 10 cm) crossed at right angles with two closed arms of the same size. The two closed arms were enclosed by walls 50 cm high, with the exception of the central part of the maze (10 cm × 10 cm) where the open and closed arms crossed. The entire apparatus was elevated 50 cm above the floor and it was placed in a room with minimal visual cues visible to the rat. To prevent the rats from falling, a rim of Plexiglas (0.5 cm high) surrounded the perimeter of the open arms. Luminosity at the level of EPM open arms was 30 lux. The experimental sessions were recorded by a video camera interfaced with a monitor and a VCR in an adjacent room. Fifteen minutes after the injections, each rat was gently placed in the central area of the EPM with its nose facing one of the closed arms and was allowed to freely explore the maze for 5 min. Conventional EPM measures (i.e. number of entries and time spent in each arm) were recorded (Pellow et al., 1985). For data analysis purpose, the percent time spent in open arms was used. It was calculated dividing the raw open time by the