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Involvement of the lateral habenula in the regulation of generalized anxiety- and panic-related defensive responses in rats

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ABSTRACT

Recently obtained evidence points to the involvement of the lateral habenular nuclei (LHb) in the mediation of coping defensive responses to threatening/stressful stimuli. Nevertheless, the role of this brain area in the regulation of defensive responses that have been associated with specific subtypes of anxiety disorders recognized in clinical settings is presently unknown. To address this question, we investigated the effects of either electrolytic lesions or chemical stimulation of the LHb on the defensive behaviors generated in rats by the elevated T-maze. This experimental model allows the measurement, in a same rat, of two defensive behaviors, inhibitory avoidance and escape, that have been related in terms of psychopathology to generalized anxiety and panic disorders, respectively. Bilateral electrolytic lesions of the LHb (1 mA, 10 s) impaired inhibitory avoidance acquisition and facilitated escape performance. On the other hand, chemical stimulation of the LHb by bilateral microinjection of kainic acid (30–60 pmol/0.2 μL) had the opposite effect, i.e., facilitated inhibitory avoidance and impaired escape. The present results indicate that the LHb exerts an opposed regulatory control on generalized anxiety- and panic-related defensive responses in rats.

Introduction

A wealth of evidence indicates that the habenular complex, an epithalamic structure that encompasses the medial (MHb) and the lateral (LHb) nuclei, represents a pivotal relay station between the basal ganglia and limbic forebrain structures (e.g. frontal cortex and septum) and regulatory serotonergic neurons located in the midbrain, mainly in the median (MRN) and dorsal (DRN) raphe nuclei (Aghajanian and Wang, 1977; Sutherland, 1982; Peyron et al., 1998; Lecourtier and Kelly, 2007). It has been shown that the LHb sends dense projections of excitatory nature to the DRN (Kalen et al., 1985, 1986; Lee et al., 2003) and that electrical stimulation of the former area can cause, depending on the parameters used, either excitation or inhibition (via action on post-synaptic GABAergic interneurons) of serotonergic cells in the DRN (Ferraro et al., 1996, 1997; Varga et al., 2003). For instance, increases in serotonin (5-HT) release are reported in the striatum, a DRN projection site (Vertes, 1991; Waselus et al., 2006), after 15 Hz stimulation of the LHb and this effect is counteracted by intra-DRN injections of the non-selective NMDA/GLY_B receptor antagonist kynurenic acid (Kalen et al., 1989).

By affecting the functioning of 5-HT neurons in the DRN, it is conceivable that the habenular complex is involved in the regulation of coping defensive responses to threatening/stressful stimuli and hence in fear, anxiety and affective states such as depression. For instance, Amat et al. (2001) have shown that the habenula plays an important role in the mediation of neurochemical and behavioral consequences to uncontrollable stress which has been related to depression. More specifically, these authors demonstrated that habenula lesions markedly attenuate the long-lasting rise in 5-HT levels observed in the DRN after exposure of rats to inescapable electric shocks. The same procedure also eliminated the deficit in escape performance that follows exposure to inescapable, but not escapable shocks. More recently, Yang et al. (2008) reported that lesions to the LHb of rats experimentally depressed by chronic exposure to mild stress have an antidepressive-like effect in the forced-swimming test, which is followed by enhanced 5-HT levels in the DRN. Altogether, this evidence indicates the involvement of the habenula-DRN pathway in the induction of learned helplessness/behavioral depression.

In the present study, we explored the role played by the habenula in the mediation of defensive responses that have been distinctively associated with anxiety and fear. More specifically, we evaluated whether electrolytic lesions or chemical stimulation of the LHb exerts a differential effect on inhibitory avoidance and escape behaviors generated by the elevated T-maze test of anxiety.

This animal model, derived from the elevated plus-maze (Pellow et al., 1985), allows the measurement, in a same rat, of the two

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behaviors mentioned above (Viana et al., 1994; Zangrossi and Graeff, 1997; Graeff et al., 1998; Pinheiro et al., 2007). The test is performed in a maze consisting of three elevated arms — one enclosed and two open. Inhibitory avoidance was measured by placing the rat at the end of the enclosed arm and recording the time to withdraw from this arm in three consecutive trials. One-way escape was measured by recording the time taken by animals to withdraw from one of the open arms in three consecutive trials. Based on a series of pharmacological studies (for a review see Graeff et al., 1998; Graeff and Zangrossi, 2002), inhibitory avoidance has been related to anxiety and one-way escape to fear. In terms of psychopathology, these responses have been associated with generalized anxiety and panic disorders, respectively (Blanchard et al., 2001, 2003; Gray and McNaughton, 2000; Deakin and Graeff, 1991; Graeff, 2002; McNaughton and Corr, 2004).

In order to control for non-specific lesion or drug effects on locomotion, animals' general exploratory activity was also evaluated in an open-field.

Methods

Animals

Male Wistar rats weighing 220–250 g were housed in pairs in Plexiglas-walled cages under a 12:12 dark/light cycle (lights on at 07:00 am) at 22±1 °C, and were given free access to food and water throughout the experiment. The experiments reported in this article were performed in compliance with the recommendations of the 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.

Apparatus

The elevated T-maze was made of wood and had three arms of equal dimensions (50×12 cm). One arm, enclosed by 40 cm high walls, was perpendicular to two opposed open arms. To avoid falls, the open arms were surrounded by a 1 cm high Plexiglas rim. The whole apparatus was elevated 50 cm above the floor.

The open field test was performed in a wooden square arena (60×60 cm), with 30 cm high walls. Luminosity at the level of the maze arms and arena center was 40 lx.

Surgery

Rats were anesthetized with 2,2,2-tribromoethanol (2.5%, 10 ml/Kg; Aldrich, USA) associated with local anesthesia (3% lydocaine with norepinephrine; Harvey, Brazil) and placed in a stereotaxic instrument (David-Kopf, USA).

Unipolar stainless steel electrodes, 0.12 mm in diameter, isolated with enamel except from the tip were bilaterally inserted into the LHb through holes drilled in the skull following the coordinates from the atlas of Paxinos and Watson (1997): AP: −3.6 mm from the bregma; LM: ±0.6 mm; DV: −4.8 mm. Bilateral electrolytic lesions of the LHb were performed by passage of a DC current of 1 mA for 10 s through the anode in the brain and a saline-soaked cotton swab on the tail as the cathode. Constant current was provided by a lesion-producing device (Ugo Basile, Italy). Sham operated rats were submitted to the same surgical procedure, but no electric current was applied through the electrode.

For the experiment involving the injection of sub-toxic doses of kainic acid, two 11 mm long guide cannulae, made of stainless steel (outer diameter 0.6 mm, inner diameter 0.4 mm), were bilaterally implanted 2 mm above the LHb. Burr holes were drilled in the skull following the coordinates: AP: −3.6 mm from the bregma; LM: ±0.6 mm; DV: −2.8 mm. The guide cannulae were fixed to the skull by means of acrylic resin and one stainless steel screw. At the end of the surgery, stylets with the same length as the guide cannulae were introduced inside them to prevent obstruction. Following this procedure, all animals were injected (1.0 ml/kg, i.m.) with an antibiotic association (Pentabiótico®; Fort Dodge, Brazil) to prevent possible infections. In addition, fluxinin meglumine (2.5 mg/kg; Schering-Plough, Brazil), a drug with analgesic, antipyretic and anti-inflammatory properties, was administered subcutaneously for post-surgery analgesia.

Behavioral tests

On the fourth and fifth days after the surgery for cannulae placement or lesion of the LHb, animals were gently handled by the experimenter for 5 min. On the sixth day, each animal was pre-exposed to one of the open arms of the elevated T-maze for 30 min. A wood barrier mounted on the border of the maze central area and the open arm's proximal end isolated this arm from the rest of the maze. It has been shown that a prior forced exposure to one of the open arms of the maze decreases the latencies to leave this arm on a later trial. This result has been attributed to the habituation of behavioral reactions to novelty (exploration, behavioral inhibition), which may interfere with one-way escape performance (Teixeira et al., 2000; Zanoveli et al., 2003).

On the seventh day, animals were tested in the elevated T-maze and open-field. In experiment 1, 15 sham-operated and 12 LHb-lesioned rats were tested. In experiment 2, animals were injected into the LHb with saline (n=10) or two different doses of kainic acid (30 and 60 pmoles; i.e. 0.0065 and 0.013 µg; n=10 for each group). Ten minutes later, they were exposed to the behavioral tests. The doses of kainic acid and the time interval between drug injection and testing were chosen based on previously published studies (Graeff et al., 1996; Viana et al., 1997; Pobbe and Zangrossi 2005; Dos Santos et al., in press).

For drug injections, a needle (0.3 mm outer diameter) was introduced through the guide cannula until its tip was 2 mm below the cannula end. A volume of 0.2 µl was injected over a period of 2 min (0.1 µl/min) using a 5 µl microsyringe (Hamilton 701-RN, USA) attached to a microinfusion pump (KD Scientific, USA). The displacement of an air bubble inside the polyethylene catheter connecting the syringe needle to the intracerebral needle was used to monitor the microinjection. The intracerebral needle was removed 1 min after the injection was finished.

The test in the elevated T-maze was initiated by measuring inhibitory avoidance. To this end, each animal was placed at the distal end of the enclosed arm of the elevated T-maze facing the intersection of the arms. The time taken by the rat to leave this arm with the four paws was recorded (baseline latency). The same measurement was repeated in two subsequent trials (avoidances 1 and 2) with 30 s intertrial intervals. Following avoidance training (30 s), each animal was placed at the end of the same open arm used in the pre-exposure session and the latency to leave this arm with the four paws was recorded in 3 consecutive trials (escapes 1–3), again with 30 s intertrial intervals. A cutoff time of 300 s was established for the avoidance and escape latencies. Immediately after being tested in the elevated T-maze, each animal was placed into the open-field for the evaluation of locomotor activity during a 5 min-period. The total distance traveled was analyzed by a video tracking system (Ethovision; Noldus, Holland). In experiment 2, we also evaluated the effect of intra-LHb injection (0.2 µl/2 min) of kainic acid (60 pmol) or saline (n=5 for each group) on the distance traveled in the open-field by experimentally naïve rats. This was done in order to investigate whether previous exposure to the elevated T-maze may have masked any putative motor effect of kainic acid in the open field. In this case, behavior in the open field was assessed 10 min after drug injection.

All behavioral tests were conducted from 1:00 to 6:00 PM.

Histology

After testing, animals were anesthetized with 25% urethane (10 ml/kg, i.p.; Sigma, USA) and those tested in experiment 2 (kainic acid study) were administered 0.1 µl of Evans Blue through the guide cannulae. Rats from both experiments were then perfused through the left ventricle of the heart with isotonic saline (0.9%), followed by 10% formalin. After removing the brain, and following a minimum period of 2 days immersed in a 10% formalin solution, 50 µm frozen sections were obtained in a cryostat. Sections were mounted on slides, stained with cresyl violet, and cover-slipped. The extent of lesions and the microinjection sites were localized in diagrams from the rat brain atlas by Paxinos and Watson (1997) using light microscopy.

Statistical analysis

Repeated measures analysis of variance was used to analyze both avoidance and escape data, with procedure (control or lesion) or drug treatment (saline or kainic acid) as the independent factors and trials as the repeated measure. When appropriate, post-hoc comparisons were performed by student *t* test or Duncan's test. Locomotor activity data were submitted to one-way ANOVA, followed by the student *t* test or Duncan post-hoc test.

Results

Fig. 1 depicts a schematic diagram of the smallest and largest lesions identified in the present study. Histological examination of the brain slices indicated that the lesioned areas were characterized by loss of neurons and extensive gliosis in the tissue. The anterior–posterior extension of the LHb lesions and injection sites were between −3.30 and −3.80 mm, caudal from bregma. Typically, lesions mainly encompassed the LHb. Nevertheless, in some cases (<30%), lesions also included some portions of the MHb, the mediodorsal thalamic nucleus and the ventromedial aspect of the dorsal hippocampus in at least one hemisphere. Fig. 1 also displays the sites of drug injections into the LHb of animals tested in the current study.

Experiment 1: bilateral electrolytic lesions of the LHb

As shown in Fig. 2 (upper panel), electrolytic lesions of the LHb impaired inhibitory avoidance acquisition [$F(1,25)=20.12$, $p<0.05$]. Two-way ANOVA also revealed a trial effect [$F(2,50)=33.96$, $p<0.05$] and a treatment by trial interaction [$F(2,50)=3.63$, $p<0.05$]. The student *t* test showed that damage to the LHb significantly decreased avoidance 1 and 2 latencies ($p<0.05$).

Fig. 2 (lower panel) shows that one-way escape was facilitated by LHb lesions [lesion effect — $F(1,25)=11.74$, $p<0.05$]. There was no trial

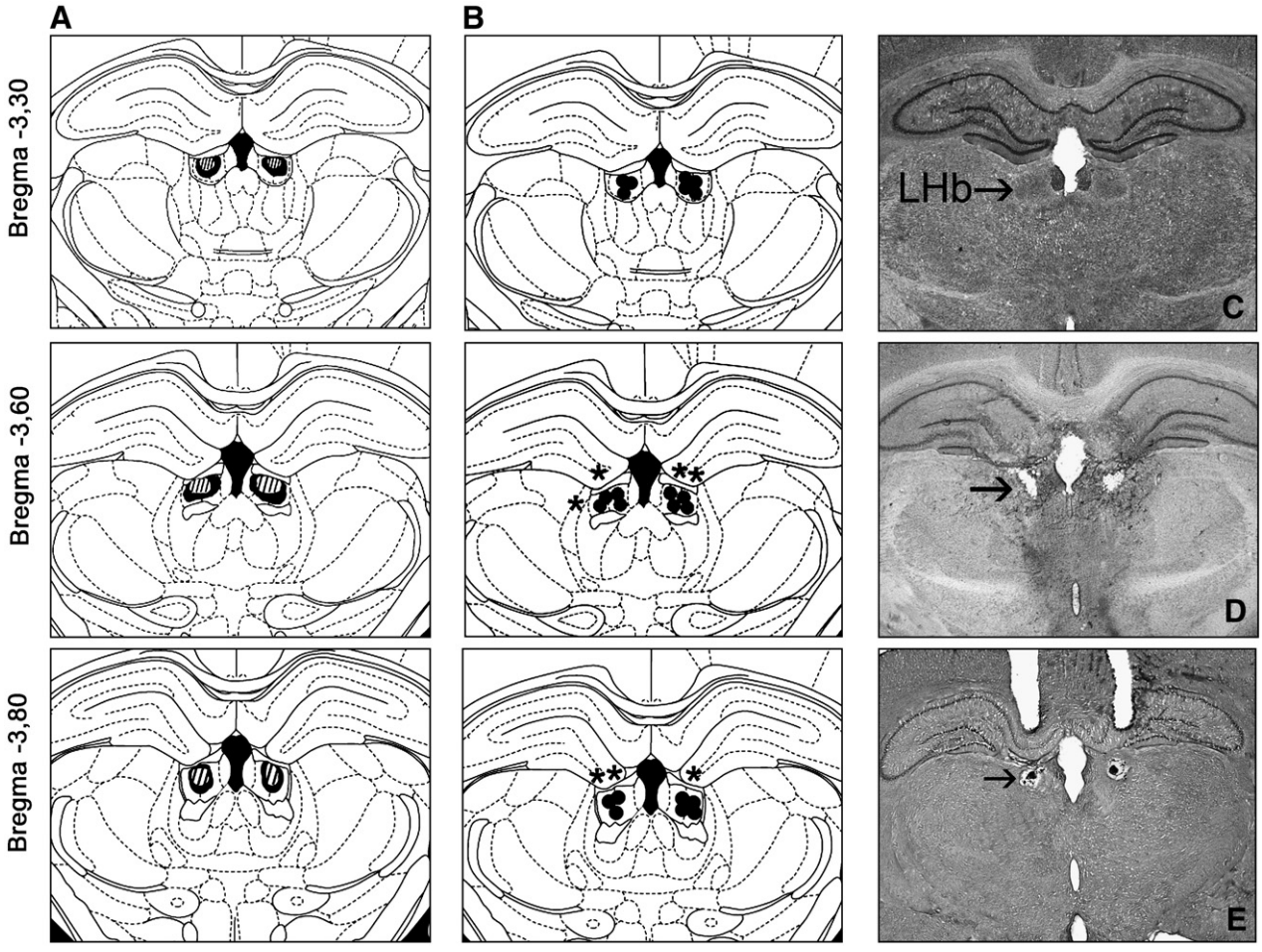

Fig. 1. Diagrammatic representation of coronal sections through the rat brain (Atlas of Paxinos and Watson, 1997) showing the smallest (*light dashed*) and largest (*dark shaded*) LHb electrolytic lesions (A) and the location of injection sites inside (*circles*) and outside (*asterisks*) the LHb (B). Representative photomicrographs showing typical sections through the habenula region of a sham-operated (C) and a LHb-lesioned rat (D). E: Photomicrograph showing typical injection sites in the LHb. The number of points shown in B is fewer than the total number of rats used because of several overlaps.

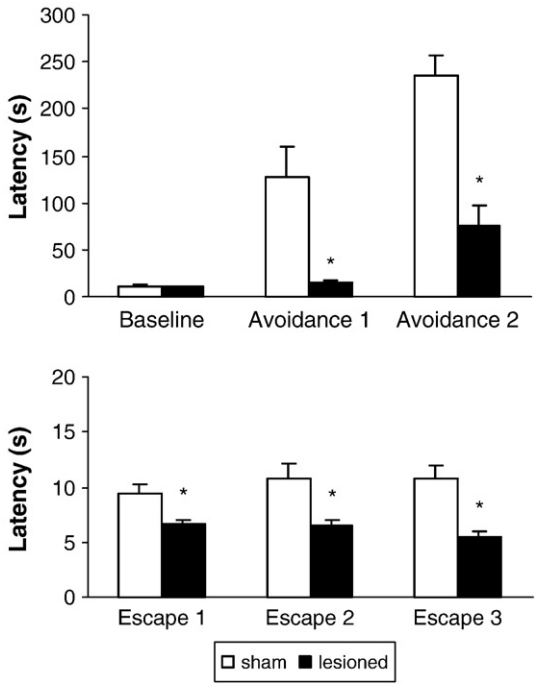

Fig. 2. Effects (mean ± SEM) of bilateral electrolytic lesions of the LHb on inhibitory avoidance (*upper panel*) and escape (*lower panel*) latencies measured in the elevated T-maze. $n = 12–15$ for each group; *$p < 0.05$ compared with the control group in the same trial.

effect [$F(2,50) = 0.63$, NS] nor a significant interaction between trial and lesion [$F(2,50) = 1.95$, NS]. The student t test revealed that LHb-lesioned rats had significantly ($p < 0.05$) shorter escape latencies across the three trials, when compared to control animals.

The total distance traveled in the open field was not significantly changed by LHb lesions (see Table 1).

Experiment 2: bilateral injection of kainic acid

As shown in Fig. 3 (upper panel), inhibitory avoidance acquisition was facilitated by bilateral administration of kainic acid into the LHb. Two-way ANOVA revealed a significant effect of treatment [$F(2,27) = 7.46$, $p < 0.05$], of trials [$F(2,54) = 129.31$, $p < 0.05$] and a significant treatment by trial interaction [$F(4,54) = 7.73$, $p < 0.05$]. The Duncan test showed that intra-LHb administration of the highest dose of kainic acid (60 pmoles) significantly increased avoidance 1 latency ($p < 0.05$).

Fig. 3 (lower panel) shows that one-way escape was inhibited by the injection of kainic acid into the LHb [treatment effect — $F(2,27) = 8.48$, $p < 0.05$]. There was no trial effect [$F(2,54) = 0.03$, NS] nor a significant interaction between trials and treatment [$F(4,54) = 0.32$, NS]. The Duncan post-hoc test showed that intra-LHb infusion of the two doses of kainic acid significantly increased escape latencies across trials ($p < 0.05$).

Locomotor activity measured in the open field was not significantly altered by the administration of kainic acid into the LHb either in naïve or T-maze-experienced rats (see Table 1).

Table 1
Effects (mean ± SEM) of LHb lesions or injection of kainic acid on the distance traveled in the open field test

Procedure	Distance traveled (m)
Experiment 1	
Control	25.00 ± 1.29
Lesioned	28.40 ± 1.54
Experiment 2	
T-maze experienced rats	
Saline	23.52 ± 1.37
Kainic acid (30 pmol)	24.78 ± 1.63
Kainic acid (60 pmol)	23.42 ± 2.35
Naïve rats	
Saline	27.28 ± 1.80
Kainic acid (60 pmol)	25.13 ± 0.77

Discussion

In this study we evaluated the role of the LHb in the regulation of two defensive tasks generated by the elevated T-maze, inhibitory avoidance and one-way escape. The results showed that electrolytic lesions of the LHb consistently affected the performance of these two behaviors. This procedure impaired inhibitory avoidance acquisition, indicating an anxiolytic-like effect, while at the same time, facilitated one-way escape, a panicogenic-like effect.

It is important to point out that lesion of the LHb did not significantly affect the distance traveled in the open-field, suggesting that motor alteration was not the main cause for the changes observed on inhibitory avoidance and one-way escape. This is also supported by the fact that inhibitory avoidance baseline latency, which is fundamentally dependent on locomotor ability, was not affected by this procedure.

In contrast with the results obtained in the lesion study, intra-LHb administration of sub-toxic doses of kainic acid facilitated inhibitory avoidance acquisition and impaired one-way escape, an anxiogenic- and a panicolytic-like effect, respectively. The micro-injection of this excitatory amino acid did not significantly interfere with animals' locomotion in the open-field either in groups of animals previously tested in the elevated T-maze or in naïve rats, which again excludes motor impairment as the critical factor accounting for the results obtained in the elevated T-maze.

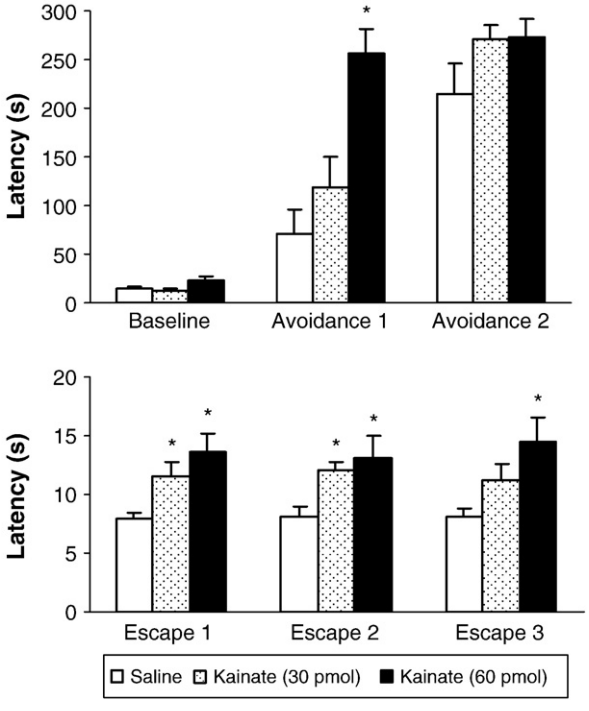

Fig. 3. Effects (mean ± SEM) of intra-LHb injections of kainic acid or saline on inhibitory avoidance (*upper panel*) and escape (*lower panel*) latencies measured in the elevated T-maze. $n = 10$ for each group; *$p < 0.05$ compared with the control group in the same trial.

Although prior studies have implicated the LHb in stress-related phenomena (Lee and Huang, 1988; Thornton and Bradbury, 1989; Heldt and Ressler, 2006), to our knowledge this is the first direct evidence of the involvement of this brain area in the modulation of defensive behaviors and hence in fear and anxiety.

Our data do not allow conclusive statements about the neural pathway(s) that was (were) affected by lesions or chemical stimulation of the LHb. In this respect, it is noteworthy to mention that this epithalamic structure, besides its dense connection with areas rich in serotonergic neurons, also sends fibers to dopaminergic cells located in the substantia nigra and ventral tegmental area (Herkenham and Nauta, 1979; Ji and Shepard, 2007) and to cholinergic neurons in the reticular formation (Araki et al., 1988; Nilsson et al., 1990). Although interference with any of these systems may ultimately alter rats' behavior, our results corroborate with findings showing that, in terms of responses to threatening/stressful stimuli, manipulations of the LHb fundamentally affect serotonergic neurotransmission at the level of the DRN (Speciale et al., 1980; Chastrette et al., 1991; Wirtshafter et al., 1994; Grahn et al., 2000). Moreover, as suggested by the study of Amat et al. (2001) regarding the effects of habenula lesions on the consequences of uncontrollable stress in rats, it seems that the habenula influence upon the DRN cells is of excitatory nature.

More specifically, in our study, the behavioral consequences of the intra-LHb injection of kainic acid were the same as those observed in the elevated T-maze after the pharmacological stimulation of the DRN with kainic acid, or of another compound that non-specifically excites 5-HT cells, the inverse agonist of benzodiazepine receptors FG-7142 (Sena et al., 2003, Pobbe and Zangrossi, 2005). The same is true when the outcome of our lesion experiment is compared to studies employing intra-DRN injections of drugs that inhibit the activity of serotonergic cells, such as the 5-HT_{1A} receptor agonist 8-OH-DPAT or the $GABA_A$ receptor agonist muscimol (Sena et al., 2003). Interestingly, the pharmacological manipulation of another 5-HT rich area, the MRN, which is also innervated by the LHb (Pasquier et al., 1976), does not follow the pattern of results presently shown. In this case, neither intra-MRN injection of 8-OH-DPAT/muscimol nor kainic acid/FG-7142 interferes with escape performance in the elevated T-maze (Dos Santos et al., in press).

If our hypothesis that the behavioral effects reported here are mediated through the pathway connecting the LHb to the DRN is correct, the nature of the neurotransmitter involved in this process remains to be explored. Based on the results of a series of electrophysiological and neurochemical studies (Kalen et al., 1986; Conley et al., 2002; Liu et al., 2002), glutamate and substance P (SP) pose as the main candidates. Accordingly, Kalen et al. (1989) showed that intra-DRN injections of the non-selective NMDA/GLY_B receptor antagonist kynurenic acid blocked the increases in 5-HT release in the striatum induced by the LHb electrical stimulation. In parallel, another line of evidence shows that whereas the levels of SP in the DRN are decreased after damage to the habenula (Neckers et al., 1979), local injection of this neurokinin in the DRN increases extracellular levels of 5-HT in a terminal area, the ventral hippocampus (Gradin et al., 1992). As indicated by a study of Liu et al. (2002), SP has a local excitatory influence upon DRN 5-HT neurons by an indirect mechanism which seems to involve the excitation of a local population of glutamatergic inputs to these neurons. We are currently evaluating the role played by NMDA-receptors in the DRN on the behavioral effects induced by chemical stimulation of the LHb in rats exposed to the elevated T-maze.

In conclusion, the present results indicate that the LHb exerts an opposed regulatory role on defensive responses that have been associated with anxiety and fear. A failure in this regulatory mechanism may be of importance in the pathophysiology of generalized anxiety and panic disorders.

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