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Antinociception Elicited by Aversive Stimulation of the Inferior Colliculus

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CASTILHO, V. M., V. AVANZI AND M. L. BRANDÃO. *Antinociception elicited by aversive stimulation of the inferior colliculus.* PHARMACOL BIOCHEM BEHAV **62**(3) 425–431, 1999.—We have shown that the inferior colliculus is involved in the integration of defensive reactions. Electrical and chemical stimulation of this structure elicits fear and escape behavior, expressed respectively by immobility (freezing) and wild running, followed by jumps. In this study, we analyzed whether the defensive behavior integrated at this level of the midbrain tectum is also followed by antinociception and its chemical mediation. In addition, we further addressed whether or not the aversive states and the stress-induced analgesia share the same neural substrates in the inferior colliculus. To this end, animals chronically implanted with a chemitrode, an electrode glued to a guide cannula, in the inferior colliculus were injected with naltrexone, methysergide, ketanserin, and midazolam. The animals were submitted to gradual increases in the electrical stimulation of the inferior colliculus, which allowed the measurement of the thresholds for aversive responses—vigilance, freezing, and escape. Following the induction of the aversive behavioral responses the animals were submitted to the tail-flick test. The results obtained show that midazolam was the only treatment that changed the aversive thresholds. On the other hand, while naltrexone and midazolam did not affect the fear-induced analgesia, it was inhibited by microinjections of the serotonergic blockers, methysergide and ketanserin. These results emphasize previous data demonstrating the nonopioid nature of the unconditioned analgesia to brain-aversive stimulation. Because methysergide is a nonspecific antagonist of 5-HT receptors, and ketanserin acts with a high degree of specificity at 5-HT_2/5-HT_{1C} receptors, the present results suggest that activation of 5-HT_2/5-HT_{1C} receptors may be implicated in the antinociception induced by stimulation of the inferior colliculus. Moreover, the present data also indicate that aversive reactions and analgesia from inferior colliculus stimulation can be pharmacologically dissociated. © 1999 Elsevier Science Inc.

Stress-induced analgesia 5-HT receptors Inferior colliculus Naltrexone Midazolam

MANY investigations have reported that stimulation of some midbrain tectum structures, such as the dorsal periaqueductal grey matter (DPAG), deep layers of the superior colliculus, and inferior colliculus, elicit—in a progressive manner—aversive responses characterized by arousal, freezing, and escape behavior (5,7,16,18,21,39,48). It has also been shown that defensive reactions produced by electrical stimulation of these structures is generally accompanied by antinociception (11, 12,14,15,44,47). In general, it is believed that the analgesia and autonomic manifestations observed in these situations occur to give support to the expression of fear-related behaviors (6,11,12,25). Electrical stimulation of midbrain, areas such as the periaqueductal gray, elicits these antinociceptive processes through activation of pathways that inhibit sensitive neurons in the spinal cord (1,2,28,48,51). This antinociceptive system can also be activated by a variety of external or environmental nociceptive stimuli exerting an important function on the control of defensive and affective behaviors (13–15,47,49).

The nonopioid nature of the mechanisms involved in the integration of the aversive states and analgesia have also been demonstrated (11,12,36,41,49). Studies on these neural and hormonal mechanisms provide evidence for the involvement of corticotrophins, corticosterone, and serotonin (5-HT) in some kinds of antinociception (2,22,23,31,32,46,50). Recently, we presented evidence that the analgesia associated with the fear induced by electrical and chemical stimulation of the DPAG is of a nonopiod nature, and that serotonergic mechanisms could be involved in these processes (11,12). In the present work, we examine the occurrence of fear-induced analgesia elicited by electrical stimulation of the inferior colliculus at three aversive thresholds—vigilance, freezing, and escape—and the chemical nature of this antinociception. Taking into account that the aversive reactions and antinociception

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can be dissociated by pretreatment with benzodiazepines in the DPAG and amygdaloid complex, and that the neural substrate subserving the defense reaction and nociception in these structures are independent (6,8,25,33,40), we also examined whether or not they share the same neural substrates at the level of the inferior colliculus. To this end, we studied the effects of microinjections into the inferior colliculus of midazolam (a benzodiazepine compound), naltrexone (an opioid antagonist), methysergide (a nonselective 5-HT antagonist), and ketanserin (a selective 5-HT_2 antagonist) on the behavioral responses and antinociception elicited by electrical stimulation of this brainstem structure.

METHOD

Subjects

Male Wistar rats weighing 210–240 g from the animal house of the Campus of Ribeirão Preto at the University of São Paulo were used. These animals were housed in individual Plexiglas-walled cages and given free access to food and water throughout the experiment.

Surgery

The animals were anesthetized with sodium pentobarbital (45 mg/kg, IP) and fixed in a stereotaxic frame (David Kopf, Tujunga, CA). A chemitrode made of a stainless steel guide cannula (o.d. 0.6 mm, i.d. 0.4 mm) glued to a brain electrode was implanted in the midbrain, aimed at the inferior colliculus. The electrode was made of stainless steel wire, 160 μm in diameter, insulated except at the cross-section of the tip reaching 1 mm below the lower end of the cannula. The upper incisor bar was set at 3.3 mm below the interaural line so that the skull was horizontal between bregma and lambda. The chemitrode was introduced vertically using the following coordinates with the lambda serving as the reference for each plane: anteroposterior, −0.9 mm; mediolateral, 1.2 mm; and dorsoventral, 4.5 mm (42). The chemitrode was fixed to the skull by means of acrylic resin and three stainless steel screws. The electrode wire was connected to a male pin, parallel to the outer end of the cannula. Together they could be plugged into an amphenol socket at the end of a flexible electrical cable and used for brain stimulation. At the end of the surgery each guide cannula was sealed with a stainless steel wire to protect it from congestion.

Nociceptive Testing

One week after surgery, all rats were tested for antinociception behavior, i.e., a reduction of the responsiveness to noxious stimuli, using the tail-flick test. Each animal was placed in an acrylic tube and its tail laid across a nichrome wire coil that was then heated by the passage of an electric current. The current raised the temperature of the coil at the rate of 9° C/s. A small adjustment of the current was made if necessary at the beginning of the experiment to obtain three consecutive baseline tail-flick latencies (TFL), between 2.5 and 3.5 s. If at any time the animal failed to flick its tail within 6 s, the tail was removed from the coil to prevent damage to the skin. Each TFL was normalized by an index of analgesia (IA) using the formula:

$$IA = \frac{(TFL_{test}) - (\overline{TFL}_{control})}{6 - (\overline{TFL}_{control})}$$

Three baselines of tail-flick latencies were taken at 3-min intervals. TFL were also taken following aversive reactions induced by electrical stimulation of the inferior colliculus at the vigilance, freezing, and escape thresholds.

Measurement of Aversive Thresholds

Immediately after three control tail-flick latencies were taken the rats were placed in an arena (circular enclosure, 60 cm in diameter and 50 cm high) with the floor divided in 12 sections. This arena was situated in an experimental compartment illuminated with a 40-W fluorescent lamp (350 lx at the arena floor level). The rats were allowed a 10-min period of habituation in the enclosure at the beginning of each experimental procedure. Afterwards, three tail-flick tests were applied to assess the animal reactiveness to noxious stimuli. Immediately thereafter the brain was electrically stimulated by means of a sinewave stimulator. The stimulation current was monitored by measuring the voltage drop across a 1-K resistor (AC, 60 Hz, 15 s). It was presented at 1-min intervals with the current intensity increasing by steps of 5 μA (rms) for measurement of the aversive thresholds. Each aversive threshold was operationally defined as the lowest intensity producing the given behavior in two consecutive series of electrical stimulation of the inferior colliculus. "Alert" was defined as the arrest of any ongoing behavior. The freezing threshold was defined by the occurrence of immobility accompanied by at least two of the following autonomic reactions: urination, defecation, piloerection, or exophthalmus. The escape threshold

TABLE 1

PROCEDURE: DATA COLLECTION FOR ANIMALS SUBMITTED TO INFERIOR COLLICULUS MICROINJECTIONS AND TESTED FOR DETERMINATIONS OF AVERSIVE THRESHOLDS—ALERT, FREEZING AND ESCAPE— AND TAIL-FLICK LATENCIES (TFL)

Groups	Habituation		Inferior Colliculus Stimulation		Treatment	Doses (nmol)	Inferior Colliculus Stimulation	
Control				TFL	saline	—		TFL
1	10 min,	TFL	aversive	test after	naltrexone	13	aversive	test after
2	each day of	Baseline	thresholds	each	methyserg	5	thresholds	each
3	testing			aversive	ketanserine	15		aversive
4				threshold	midazolam	40		threshold
				↑			↑	
				15 min			15 min	

Refer to the Methods section for a complete description.

was defined by the current intensity produced running (gallop) or jumping. Animals with an escape threshold above 200 μA (peak-to-peak) were discarded from the experiment.

Procedure

Each animal was habituated to the experimental procedure for 10 min. The procedure consisted of the measurement of three TFL baseline latencies followed by the determination of the aversive thresholds (alert, freezing, and escape), being each one followed immediately by the subsequent tail-flick test. The effects of microinjections into inferior colliculus were evaluated 15 min later. Independent groups of animals were used for each group (control, naltrexone, methysergide, ketanserin, and midazolam). For each one of these groups, the animals were submitted to the electrical stimulation procedure and to the tail-flick test again 15 min after drug or saline administration into the inferior colliculus (Table 1).

Injection Procedures

The animals were gently wrapped in a cloth, hand held, and a thin dental needle (o.d., 0.3 mm) was introduced though the guide cannula until its lower end was 1 mm below the guide cannula, reaching the same depth as the electrode tip. The injection needle was linked to a 2-μl Hamilton syringe by means of a polyethylene tubing. A volume of 0.2 μl was injected for 20 s, and the displacement of an air bubble inside the polyethylene (PE-10) catheter connecting the syringe needle to the intracerebral needle was used to monitor the microinjection. The effects of drug microinjections were then evaluated on the analgesia induced by electrical stimulation of the mesencephalic tectum.

Drugs

Methysergide bimaleate (RBI), naltrexone hidrochloride (Sigma), ketanserin tartrate (RBI), and midazolam maleate (Roche) were each dissolved in physiological saline (0.9%) shortly before use. Physiological saline also served as vehicle control. The doses used were: 5 nmol (2.5 μg) of methysergide, 15 nmol (7.5 μg) of ketanserin, 13 nmol (5.0 μg) of naltrexone, and 40 nmol (16 μg) of midazolam.

Histology

Upon completion of the experiments, the animals were deeply anaesthetized with sodium pentobarbital and perfused intracardially with saline followed by formalin solution (10%). The brains were removed and 3 days later were frozen. Serial 50 μm brain sections were cut using a microtome and stained with neutral red to localize the positions of the electrode tips according to the Paxinos and Watson atlas (41). Data from rats with electrode tips located outside the inferior colliculus were not included in the statistical analysis.

Analysis of Results

The tail-flick latencies are expressed as graphs of averaged Iindex of analgesia (IA) values against the type of aversive responses (vigilance, freezing, or escape). Aversive thresholds and IA are reported as means ± SEM before and after drug microinjections. Data obtained for each treatment were subjected to a two-way analysis of variance (condition × thresholds). Factor condition refers to pre- and postinjections of drugs or its vehicle in the inferior colliculus. The second factor refers to the alert, freezing, and escape thresholds. A factor analysis was also performed considering condition as the first factor and treatment as the second factor for each aversive threshold (alert, freezing, and escape). Factors found to be significant were tested with Newman–Keuls comparisons. The number of animals per group was 13 for methysergide, 12 for ketanserin, 12 for naltrexone, and 8 for midazolam and 12 for saline (control).

RESULTS

Electrical stimulation of the sites studied in this work induced escape reactions that were always followed by antinociception. The electrode tips were situated mostly in the central nucleus of the inferior colliculus as illustrated in Fig. 1. Because both the lower end of the injection needle and the electrode tip reached 1 mm below the guide cannula of the chemitrode, brain injections were made close to these electrodes.

Gradual increases in the inferior colliculus electrical stimulation of rats tested in the arena caused a state of alertness followed by freezing behavior. The animals stopped their ongoing behavior as if they oriented themselves towards the stimulus. With further increases in the intensity of the electrical stimulation, freezing behavior gave way to a clear behavioral activation expressed by running and/or jumping.

The data obtained for each aversive threshold determined by electrical stimulation of the inferior colliculus were submitted to a two-way ANOVA. For the saline group this analysis revealed that the differences between vigilance (58.12 ± 7.19 μA), freezing (73.50 ± 10.33 μA), and escape thresholds (106.25 ± 10.24 μA) were highly significant, $F(2, 53) = 14.42$, $p < 0.001$. This treatment did not significantly affect any aversive thesholds, $F(1, 53) = 1.08$, $p > 0.05$, and no interaction between condition and thresholds could be obtained, $F(2, 53) = 0.14$, $p > 0.05$. These findings confirm previous studies from this laboratory showing significant differences in the intensity of electric current applied to the midbrain tectum in the pro-

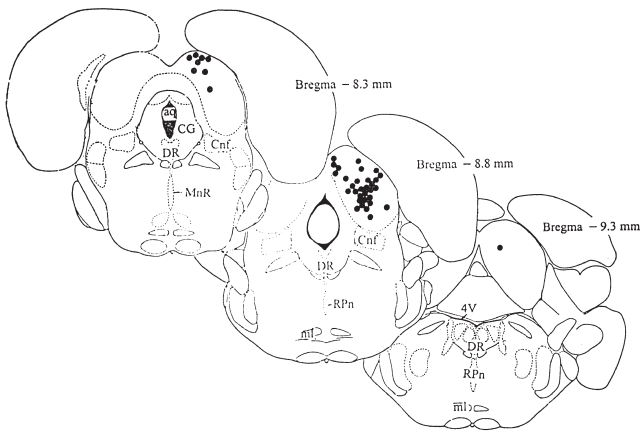

FIG. 1. Location of central microinjections sites of saline, midazolam, opioid, and serotoninergic antagonists in cross-section diagrams of the mesencephalon, according to the Paxinos and Watson atlas. The number of of points is less than the total of animals studied because of several overlappings. CG = central gray; Aq = aqueduct; Cnf = cuneiform nucleus; DR = dorsal raphe nucleus; ml = medial lemniscus; MnR = median raphe nucleus; 4V = 4th ventricle; RPn = raphe pontis nucleus.

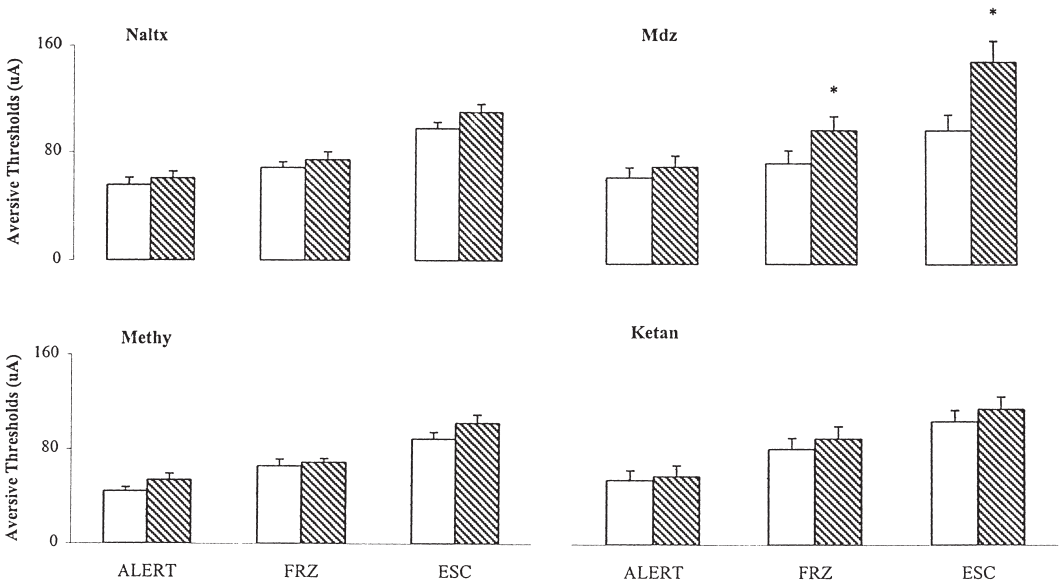

FIG. 2. Effects of inferior colliculus microinjections of naltrexone (Naltx, 13 nmol/0.2 μl), methysergide (Methy, 5 nmol/ 0.2 μl), ketanserin (Ketan, 15 nmol/0.2 μl), and midazolam on the aversive responses to the electrical stimulation of the inferior colliculus, at the alert, freezing (FRZ), and escape thresholds (ESC). Columns represent the means of the aversive thresholds and bars the SEM. Open column represent control sessions without drug pretreatment, and hatched columns those measurements in presence of the treatment applied 15 min before inferior colliculus electrical stimulation. $*p < 0.05$.

duction of alert, freezing, and escape responses (11,1 2). Naltrexone, $F(1, 54) = 3.22$, $p > 0.05$, methysergide, $F(1, 59) = 3.49$, $p > 0.05$, and ketanserine, $F(1, 55) = 0.78$, $p > 0.05$, microinjections did not change the aversive thresholds determined through the procedure of electrical stimulation of the inferior colliculus. The opposite profile of effects could be observed with midazolam. This drug produced a significant increase in the aversive thresholds, $F(1, 33) = 6.83$, $p < 0.05$. Post hoc

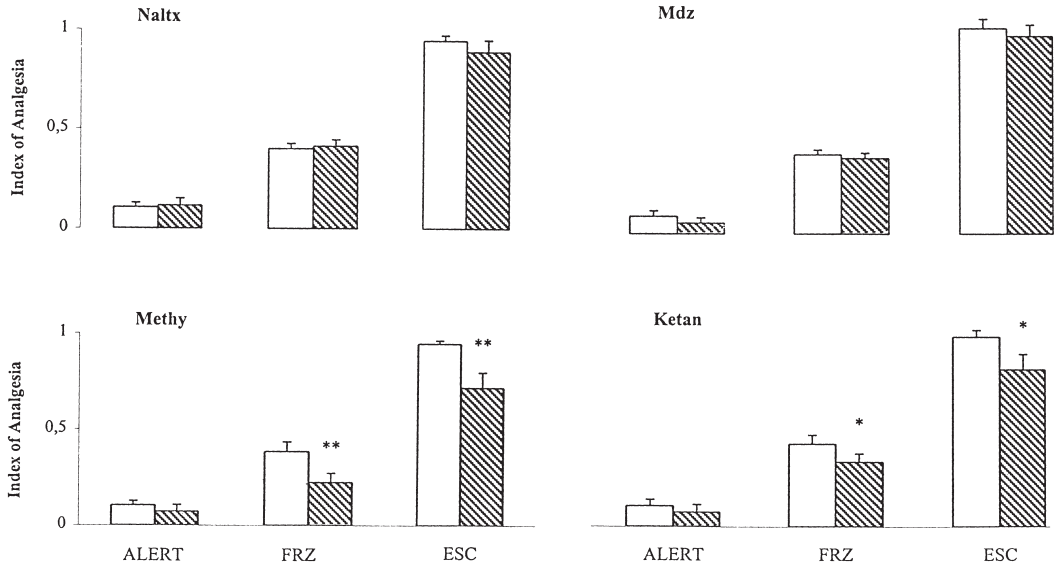

FIG. 3. Effects of inferior colliculus microinjections of naltrexone (Naltx, 13 nmol/0.2 μl), methysergide (Methy, 5 nmol/0.2 μl), ketanserin (Ketan, 15 nmol/0.2 μl), and midazolam (Mdz, 40 nmol/0.2 μl) on the index of analgesia measured following aversive responses to the electrical stimulation of the inferior colliculus, at the alert, freezing (FRZ), and escape thresholds (ESC). Columns represent the means of the index of analgesia and bars the SEM. Open columns represent control sessions without drug pretreatment, and hatched columns represent antinociception induced by inferior colliculus stimulation in presence of the treatment applied 15 min before inferior colliculus electrical stimulation. $*p < 0.05$, $**p < 0.01$.

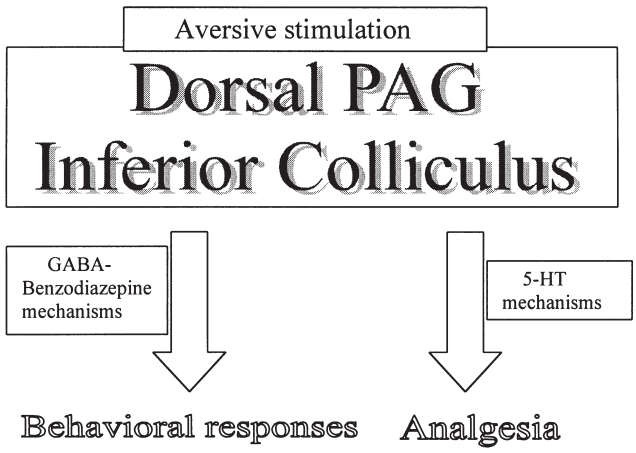

FIG. 4. Diagram showing the pharmacological dissociation between the behavioral responses and antinociception of the defense reaction elicited by stimulation of the midbrain tectum.

analysis revealed that these effects were due to changes on freezing and escape thresholds ($p < 0.05$). There was no significant interaction between treatment and thresholds, $F(2, 33) = 1.38$, $p > 0.05$. A factor analysis with repeated measures considering treatment as the second factor for each aversive threshold produced qualitatively similar results. Also, in this case the only significant interaction was saline × midazolam at freezing, $F(1, 16) = 7.48$, $p < 0.05$, and escape, $F(1, 18) = 9.78$, $p < 0.01$, thresholds. These effects are illustrated in Fig. 2.

Analgesia was invariably detected following aversive reactions induced by electrical stimulation of the inferior colliculus. Treatment with saline did not significantly affect this analgesia, $F(1, 53) = 0.19$, $p > 0.05$. Pretreatment with local microinjections of naltrexone in the inferior colliculus, 15 min before the electrical stimulation procedure, caused no significant effect on the analgesia measured at the aversive thresholds, $F(1, 54) = 0.10$, $p > 0.05$. The lack of effect of this opioid antagonist could not be attributed to the dose used (13 nmol), because this dose has been shown to be effective in other experimental situations (17). In contrast, pretreatment with local microinjections with methysergide in the inferior colliculus caused a significant inhibition, $F(1, 59) = 11.56$, $p < 0.05$, of the analgesia elicited by electrical stimulation. Post hoc comparisons showed that these effects were due to the effects of the drug on the analgesia produced by stimulation at the freezing and escape thresholds ($p < 0.05$). Significant effects were also obtained with central microinjections of ketanserine in the midbrain tectum, with the dose of 15 nmol, $F(1, 55) = 6.58$, $p < 0.05$. Post hoc analysis also revealed that these effects occurred at the same levels of aversive stimulations ($p < 0.05$, at freezing and escape thresholds). There was no interaction between treatments and aversive thresholds when the pretreatment was naltrexone, $F(2, 54) = 0.55$, $p > 0.05$, methysergide, $F(2, 59) = 1.75$, $p > 0.05$, or ketanserine, $F(2, 55) = 0.92$, $p > 0.05$. No significant effect could be seen in the tail-flick test, $F(1, 33) = 0.67$, $p > 0.05$, following microinjections of midazolam into the inferior colliculus. A factor analysis with repeated measures considering treatment (saline × drug) as the second factor for each aversive threshold produced qualitatively similar results. The serotonergic blockers were the only drugs that interacted significantly with saline, $F(1, 23) = 6.91$, $p < 0.05$ (for methysergide), and $F(1, 22) = 5.53$, $p < 0.05$ (for ketanserin). All drug effects on tail-flick latencies are illustrated in Fig. 3.

DISCUSSION

The present results show that gradual increases in the electrical stimulation of the inferior colliculus of rats induce, progressively, characteristic aversive responses such as arousal, freezing, and escape behavior. This pattern of responses conform with defense reactions usually seen in rats confronted with predators (3,13). Electrical stimulation of the inferior colliculus shares many of the aversive properties of the stimulation of structures belonging to the aversive brain system such as DPAG, deep layers of superior colliculus, medial hypothalamus, and amygdala (4,18,21,39). It is possible that the inferior colliculus is also involved in the neural circuit responsible for aversive reactions. Indeed, reciprocal anatomical connections have been demonstrated between the inferior colliculus and superior colliculus and DPAG (10,20,24,29,38), which is consistent with the demonstration of functional connections between the inferior colliculus and these structures (27).

Of all drugs tested in this study, microinjections of midazolam into the inferior colliculus was the only treatment that increased the aversive thresholds determined by the brain electrical stimulation procedure. These findings support the notion that GABA–benzodiazepine mechanisms exert an inhibitory control on the neural substrates of aversion in the midbrain tectum (4,8). The lack of effects observed with microinjections of opioid and serotonergic antagonists in the inferior colliculus on the aversive responses also supports previous suggestions that these neurochemical mechanisms exert a phasic regulation on the neural substrates of aversion in the midbrain tectum. Indeed, opioid and 5-HT receptors agonists cause clear antiaversive effects when microinjected into the inferior colliculus (7–9,30,31).

Electrical stimulation of the inferior colliculus at each aversive threshold produced analgesia. There was no effect of previous experience with aversive thresholds on the nociceptive test, as no interaction occurred between thresholds and conditions. Antinociception usually follows the defensive reactions elicited by stimulation of the neural substrates of fear in the dorsal mesencephalic structures. Stimulation of some midbrain sites has frequently been reported to be aversive in animals [for a review, see (8,19)] and humans (35), as well as to be strongly antinociceptive (41,43). However, analgesia and the behavioral manifestations of the defense reaction may have different neurochemical substrates. The opioid receptor antagonist, naltrexone, reduces fear-induced analgesia, but does not reduce the occurrence of freezing responses when injected into ventral PAG (13). On the other hand, it has been reported that the analgesia that follows unconditioned responses to aversive stimulation of the dorsal periaqueductal gray is of nonopioid nature (11,12). In accordance with these latter findings, our results show that naltrexone was unable to cause significant changes in the analgesia that followed the aversive responses induced by electrical stimulation of the inferior colliculus.

In the same way, serotoninergic mechanisms may be independently triggered during the aversive states and the analgesia induced by stimulation of the inferior colliculus. The present results show that methysergide, a nonspecific serotoninergic antagonist, applied directly to the inferior colliculus, blocks the antinociception induced by electrical stimulation of this

structure. These data are in agreement with studies showing an involvement of serotonergic mechanisms in the DPAG stimulation-produced analgesia (11,12,36). In support of this, an immunohistochemical study has described appreciable amounts of serotonergic fibers in the inferior colliculus (45). In support of these findings, our results indicated an involvement of 5-HT_2/5-HT_{1C} receptor subtypes in fear-induced analgesia elicited by electrical stimulation of the inferior colliculus. The specific 5-HT_2 receptor antagonist, ketanserin, applied directly into this region blocked the antinociception elicited by its aversive stimulation. This contrasts with the lack of effects of the 5-HT antagonists on the aversive thresholds. In fact, in view of previous evidence (30,31), we might even expect an enhancement of the aversive reactions by the 5-HT antagonists used in this study. Thus, an opposite role may be played by 5-HT mechanisms in the behavioral reaction and antinociception induced by aversive states, and these components of the defense reaction can be independently integrated at the level of the inferior colliculus. Many studies have reported that serotonin has clear antiaversive effects and a facilitatory action on the analgesia induced by stress, acting through the activation of 5-$HT_{2/1C}$ receptors in the DPAG (19,36,37).

The independence between antinociception and behavioral defense mechanisms have been postulated by several authors (11,12,25,26,33,44). This dissociation at the level of the dorsal periaqueductal gray matter is mainly supported by experiments showing that benzodiazepines inhibit the defensive reactions without affecting the antinociception induced by aversive stimulation of this region (25,26,33). In this study, we have demonstrated that the aversive effects of inferior colliculus stimulation was clearly inhibited by midazolam, whereas the antinociception elicited by this stimulation was not. As illustrated in the diagram depicted in Fig. 4, these findings provide additional support for the independence of the neural substrates of analgesia and fear-related behaviors integrated at the level of the inferior colliculus.

The data obtained here suggest that serotonergic mechanisms are involved in the modulation of the stress-related analgesia induced by brain stem electrical stimulation. An involvement of opioid mechanisms of the inferior colliculus in the modulation of this response could not be detected with the technique used in the present study. The possibility of such modulation cannot be discarded, as microinjections of opioid antagonists into the inferior colliculus may not influence opioid release in other brain regions due to electrical stimulation of fibers of passage or output neurons.

Evidence obtained in this laboratory show a combined opioid and serotonergic modulation of the behavioral responses to aversive stimulation of the inferior colliculus (9). Similar findings have also been observed at the level of the dorsal periaqueductal gray; that is, the behavioral responses to aversive stimulation of this region are under control of opioid (34) and serotonergic mechanisms (37), whereas the antinociception produced by this stimulation is mediated only by 5-HT_2 receptors (11,12). Thus, it is not unlikely that opioid and serotonergic processes may regulate different facets of the defense reaction induced by brain stem electrical stimulation. Whereas the behavioral aversive reaction seems to be a multimediated process, the analgesia related to fear induced by midbrain tectum electrical stimulation is specifically mediated by serotonergic mechanisms. The pharmacological dissociation of the behavior and antinociception elicited by inferior colliculus electrical stimulation is further substantiated by the differential effects of midazolam on both processes.

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