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Aversive and Antiaversive Effects of Morphine in the Dorsal Periaqueductal Gray of Rats Submitted to the Elevated Plus-Maze Test

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MOTTA, V. AND M. L. BRANDÃO. *Aversive and antiaversive effects of morphine in the dorsal periaqueductal gray of rats submitted to the elevated plus-maze test.* PHARMACOL BIOCHEM BEHAV 44(1) 119–125, 1993.—The dorsal periaqueductal gray (DPAG) is a well-known region for processing defensive behavior in the brainstem. Rats implanted with cannulae in the DPAG were submitted to the elevated plus-maze test for 5 min. The effects of morphine following systemic (0.1–1.0 mg/kg) or DPAG administration (5–30 nmol) were compared with the benzodiazepine compound midazolam injected similarly (1–10 mg/kg, IP, and 10–80 nM, DPAG). Morphine and midazolam caused dose-dependent increases in the number of entries and time spent in the open arms. A systemic injection of naloxone in doses that block μ-opioid receptors reversed the effects of centrally administered morphine. Higher doses of morphine (70 nmol) induced a non-naloxone-reversible "fearful" hyperreactivity. It is suggested that low doses of morphine inhibit the neural substrate of aversion in the DPAG, probably through activation of μ-receptors, and that microinjections of higher doses of morphine cause proaversive actions not mediated by these opioid receptors.

DPAG Opioids Naloxone Escape Aversion Elevated plus-maze

IT has been established that a set of structures in the CNS comprised of the dorsal periaqueductal gray (DPAG), medial hypothalamus, and amygdala constitutes the main neural substrates for the integration of aversive states in the brain (1,13,24). These structures have been named, as a whole, as the brain aversive system (BAS) (12). Several lines of evidence have clearly implicated GABAergic, serotonergic, and amino acid-mediated mechanisms in the control of the neural substrates commanding defensive behavior in the BAS (4,6, 12,29). It has been suggested that 5-hydroxytryptamine$_2$ (5-HT$_2$) mechanisms exert a phasic modulatory role on this system because an increase in 5-HT activity in the DPAG attenuates the aversive consequences of DPAG stimulation and this effect can be blocked by 5-HT$_2$ antagonists such as ritanserin and ketanserin locally applied while 5-HT antagonists by themselves do not cause any apparent aversive responses (12,30). Support for a 5-HT$_2$ participation in the modulation of affective behavior in the DPAG has been given by recent electrophysiological studies showing that the majority of 5-HT receptors in this region belongs to the 5-HT$_2$ type (10). On the other hand, GABA is supposed to exert a tonic inhibitory control on the BAS because GABA agonists and benzodiazepine compounds (6) attenuate the aversive consequences of BAS electrical stimulation and GABA$_A$ antagonists, such as bicuculline and picrotoxin, reproduce them. These results together led to the suggestion that benzodiazepines might owe part of their well-known antiaversive effects to the increase of the efficiency of GABAergic mechanisms in these structures commanding defensive behavior.

Interest in the possible modulatory role played by opioid-mediated mechanisms in the BAS has grown lately. The effects of opioid agonists depend upon the type of receptors with which they interact. It has been shown that μ-agonists function as positive reinforcers and κ-agonists cause aversive states (2,5,22). Morphine presents more affinity for μ- than for κ-receptors (21). In this respect, the DPAG has significant levels of μ- and κ-receptors (20). As a matter of fact, it has been reported that microinjections of low doses of morphine into the DPAG attenuate in a dose-dependent manner the aversive consequences of electric stimulation at this site (7,8,16,17). High doses of morphine, however, when locally injected into the DPAG cause a behavioral activation together with jumps, which shows a great similarity to the reaction observed following electrical stimulation of or microinjections of GABA blockers into this structure (14,15). However, until now no study has been conducted to examine the involvement of opioid mechanisms using a test with a naturalistic approach such as the elevated plus-maze (EPM). It was therefore of interest

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to verify whether and how microinjections of morphine into the DPAG would affect the exploratory activity of rats submitted to this test.

METHOD

Animals

Two-hundred eighty male Wistar rats weighing 220–280 g were housed in groups of 6 with free access to food and water. They were kept in the experimental room for 48 h prior to the experiment on a 12 L : 12 D cycle (lights on at 7:00 a.m.) at 25–27°C.

Surgery

One-hundred fifty-four animals were anesthetized with sodium pentobarbital (45 mg/kg, IP) and fixed in a stereotaxic frame (David Köpf, Topanga, 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 (160 μm) was implanted in the midbrain, aimed at the DPAG. 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 such that the skull was leveled between bregma and lambda. The chemitrode was introduced vertically using the following coordinates, with lambda serving as the reference for each plane: posteroanterior, 0.3 mm; mediolateral, 0.5 mm; and dorsoventral, 4.5 mm (25). 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.

Apparatus

One week after surgery, rats were placed in an arena (circular, 60 cm in diameter and 50 cm high) with a floor divided into 12 sections. This arena was situated in an experimental compartment illuminated with a 40-W fluorescent lamp (350 lux at the arena floor level). Rats were allowed a 15-min period of habituation in the enclosure, after which the brain was stimulated electrically by means of a sine-wave stimulator (19). The stimulation current was monitored by measuring the voltage drop across a 1-K resistor with an oscilloscope (Labo, Brasil). Brain stimuli (AC, 60 Hz, 15 s) were presented at 1-min intervals with the current intensity increasing by steps of 5 μA for measurements of the aversive threshold. Escape threshold was operationally defined as the lowest intensity producing running (gallop) or jumping in two consecutive ascending series of electrical stimulation. Animals with an escape threshold above 200 μA (peak to peak) were discarded from the experiment.

Procedure

In the day following threshold determination, each rat was individually placed in a wooden arena (60 × 60 × 30 cm) for 5 min. Immediately afterward, the animal was transferred to an EPM similar to that previously described by Pellow et al. (26,27). Briefly, the maze was 50 cm from the floor and consisted of two open arms and two closed arms (with no roof) arranged such that like arms were opposite each other.

The rat was placed in the center of the EPM facing one of the closed arms. The number of entries into each type of arm was recorded for 5 min (all four paws defining an entry). The ratio of the number of entries and time spent in the open arms over the total number of entries and time spent in all arms × 100 were calculated. These ratios are considered to reflect the fear-induced inhibition to enter the open arms and can be related to the level of "anxiety" experienced by the test animal (26,27). Total exploratory activity was measured as the total number of arm entries. The measures were taken by an observer standing still in the same room for 5 min using standard electromechanical Grason-Stadler equipment. Rats that received IP injections did not receive any kind of surgery and were housed in groups of six for 2 days before the test.

Rats were randomly allocated to the following groups: a) vehicle control and IP midazolam (1.0, 3.0, 5.6, and 10 mg/kg); b) vehicle control and morphine IP (0.1, 0.3, 0.56, and 1.0 mg/kg); c) vehicle control and DPAG midazolam; d) vehicle control and DPAG morphine (5, 10, 30, and 70 nmol). Drugs and vehicles as controls were injected IP in a volume of 1 ml/kg. Intracerebral injections were made at the doses given above in a volume of 0.2 μl. Ten minutes after injections, rats were placed in a wooden arena for 5 min and immediately afterward in the EPM. Each rat received only one injection. Animals receiving 10 nmol morphine were also injected with either saline or naloxone (1 mg/kg, IP) 5 min after morphine administration. There were 10 rats per drug dose, selected at random from the various cages.

The intense behavioral activation together with jumps induced by 70 nmol morphine precluded the accomplishment of the EPM test. For studying the excitatory effects induced by this dose of morphine, animals were placed in the middle of the arena immediately after microinjections for recording locomotor activity. The following behavioral responses were recorded every minute for 1 h: number of crossings (i.e., number of floor sections traversed), number of rearings, jumps, and rotations. To examine the nature of the excitatory effect caused by morphine in an additional group of animals, naloxone (1 mg/kg, IP) was injected 5 min before DPAG morphine microinjection. Eight animals per group were used in these experiments.

Intracranial Injection Procedures

Animals were gently wrapped in a cloth, hand held, and a thin dental needle (o.d. 0.3 mm) introduced through the guide cannulae until its lower end was 1 mm below the guide cannulae, reaching the same depth as the electrode tips. The injection needle was linked to a 5-μl Hamilton syringe (Hamilton Co., Reno, NV) by means of a polyethylene tubing. A volume of 0.2 μl was injected during 20 s and the needle was held in place for an additional 10 s. The displacement of an air bubble inside the polyethylene (PE-10) catheter connecting the syringe needle to the intracerebral needle was used to monitor microinjection.

Drugs

Morphine sulphate (Roche Products Ltd., Brampton, Ontario, Canada), naloxone (Endo Laboratories, Garden City, NY), and midazolam (Roche) were each dissolved in physiological saline (0.9%) shortly before use. Physiological saline also served as vehicle control.

Histology

Upon completion of experiments, animals were deeply anesthetized with sodium pentobarbital and perfused intracardi-

ally with saline followed by formalin solution (10%). Three days later, brains were removed and 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 Paxinos and Watson (25). Only data from those animals with histologically correct cannulae placements were used for subsequent statistical analysis.

Statistical Analysis

Analysis of variance (ANOVA) was performed on the percentage of open arm entries and time spent in the open arms with drug treatment as the factor. ANOVA was also performed on the total number of arm entries. Whenever a drug increased or decreased both total arm entries and percentage of open arm entries, a correlation coefficient (product × moment correlation) was calculated to determine to what extent the alteration in open arm entries was independent of any effect on closed arm entries. Posthoc comparisons between means and controls were performed using Dunnett's *t*-test.

The hyperexcitability following 70-nmol morphine microinjections into the DPAG is expressed as the total number of crossings and jumps recorded in the arena. These data were not normally distributed so analysis was by the Mann–Whitney *u*-test.

RESULTS

The electrode tips were situated mostly in the dorsolateral aspects of the DPAG 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 DPAG electrical stimulation of rats

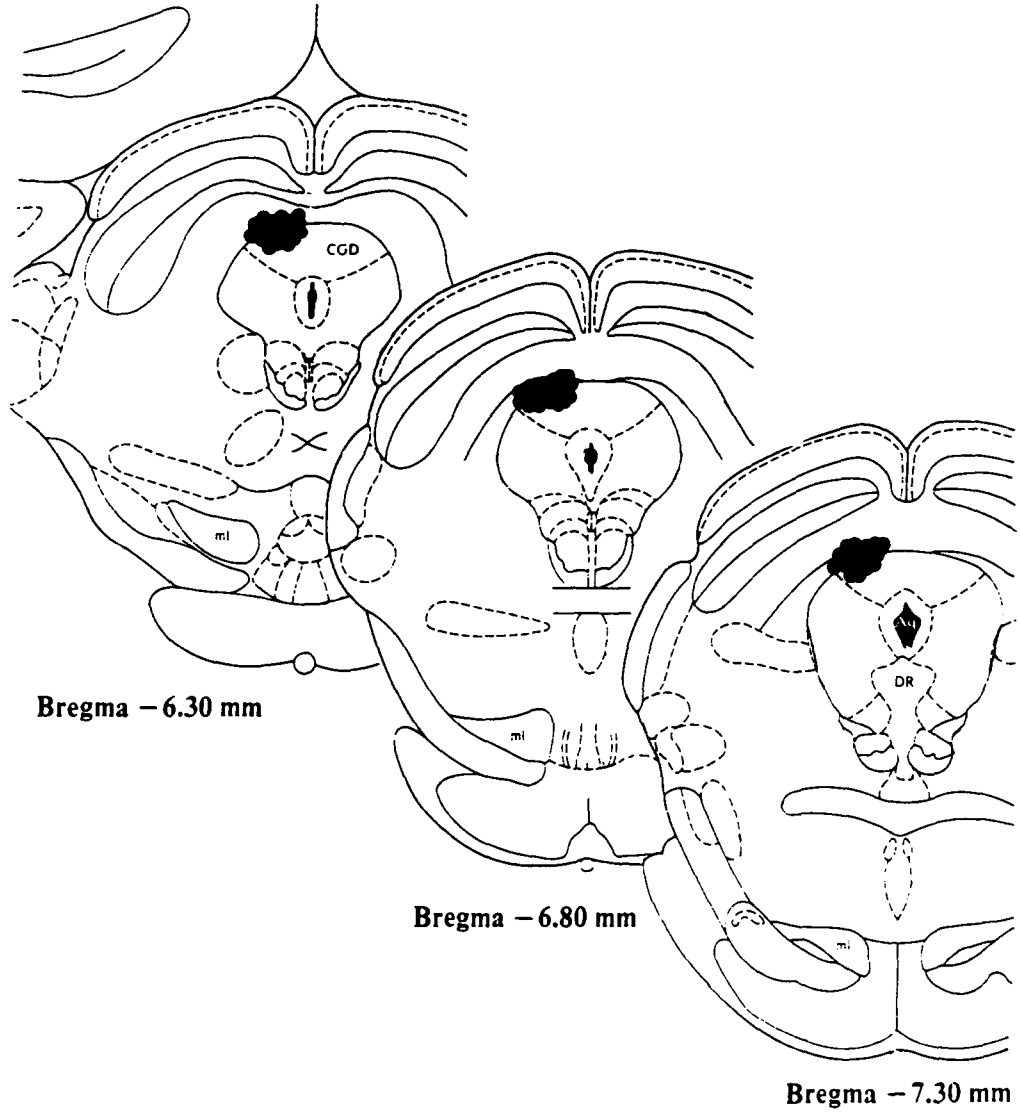

FIG. 1. Location of electrode sites on cross-sections from Paxinos and Watson's rat brain atlas (25). Figures represent the atlas coordinates in μm posterior to bregma. CGD, central gray; DR, dorsal raphe nucleus; ml, medial lemniscus; aq, cerebral aqueduct.

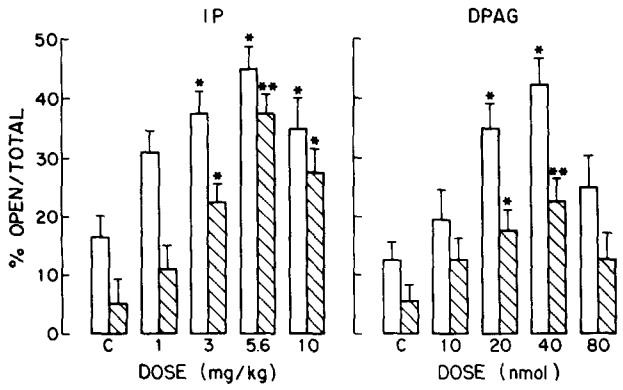

FIG. 2. Mean (±SEM) percentage of entries into and time spent in the open arms of the elevated plus-maze by rats tested with midazolam administered IP (left) or directly into the dorsal periacqueductal gray (DPAG) (right) 10 min before the test. Open columns represent number of entries and hatched columns indicate time spent in the arms. $n = 10$. *$p < 0.05$ and **$p < 0.01$, Dunnett's t-test.

tested in the arena caused a state of arousal followed by freezing behavior. Animals stopped their ongoing behavior as if they oriented themselves toward an environmental stimulus. With further increases in the intensity of electrical stimulation, freezing behavior gave way to a clear behavioral activation expressed by running and/or jumping that stopped as soon as the brain stimulation was switched off.

Figure 2 shows the effects of systemic (left) and DPAG (right) administration of midazolam on the number of entries into and time spent in the EPM. The effects of systemic administration of midazolam (1-10 mg/kg) on the overall exploratory activity of rats in the EPM are shown in Table 1. ANOVA revealed a nonsignificant effect on the total number of arm entries, $F(4, 45) = 1.89, p > 0.05$. As shown in Fig. 2, these doses of midazolam caused a significant effect on the percentage of entries into, $F(4, 45) = 3.12, p < 0.05$, and time spent, $F(4, 45) = 4.15, p < 0.01$, in the open arms. Posthoc analyses showed that 3, 5.6, and 10 mg/kg produced a significant increase in the percent of number of entries ($p < 0.05$ in all cases) and time spent ($p < 0.05$ for 3 and 10 mg/kg and $p < 0.01$ for 5.6 mg/kg) in the open arms. DPAG microinjection of rats with midazolam also caused a nonsignificant effect on the total arm entries, $F(4, 45) = 1.55, p > 0.05$ (see Table 1). In Fig. 2, it can be seen that this treatment caused a significant increase in the percentage of open arm entries, $F(4, 45) = 3.26, p < 0.05$, and percentage of time spent in the open arms, $F(4, 45) = 4.19, p < 0.01$. Posthoc analysis posthoc showed that these effects were mainly due to the doses of 20 ($p < 0.05$ for entries and time spent in the open arms) and 40 nmol ($p < 0.05$ for entries and $p < 0.01$ for time spent in the open arms).

Morphine (0.1-1.0 mg/kg) had a significant effect on the total number of arm entries, $F(4, 45) = 2.78, p < 0.05$. Posthoc analysis showed that at 0.3 mg/kg there was a significant increase in this measure ($p < 0.05$, see Table 1). As illustrated in Fig. 3 (left), systemic morphine did significantly alter the percentage of open arm entries, $F(4, 45) = 2.61, p < 0.05$, and time spent in the open arms, $F(4, 45) = 3.50, p < 0.05$. Posthoc analysis showed that this effect was only due to 0.3 mg/kg regarding the percentage of open arm entries ($p < 0.05$) and due to 0.1 and 0.3 mg/kg with respect to the percentage of time spent in the open arms (Fig. 3). Analysis of correlation showed that at the dose of 0.3 mg/kg the increase in open arm entries was interrelated to the effects in closed arm entries ($r = 0.86, p < 0.01$).

TABLE 1

MEAN (±SEM) TOTAL NUMBER OF ARM ENTRIES MADE BY RATS DURING A 5-MIN TEST IN THE ELEVATED PLUS-MAZE

Drug	IP (mg/kg)		DPAG (nmol)	
Midazolam	0	6.8 ± 1.34	0	8.1 ± 0.92
	1.0	9.8 ± 1.71	10	9.9 ± 1.39
	3.0	9.2 ± 1.62	20	9.1 ± 0.98
	5.6	10.2 ± 1.41	40	9.1 ± 0.73
	10.0	4.7 ± 1.00	80	6.3 ± 1.10
Morphine	0	7.9 ± 0.92	0	7.7 ± 0.58
	0.1	10.7 ± 1.44	5	8.2 ± 1.33
	0.3	12.3 ± 1.19*	10	10.6 ± 1.92
	0.56	9.1 ± 1.10	30	8.8 ± 0.29
	1.0	8.7 ± 0.73		
Morphine + naloxone	0		0	7.4 ± 1.15
	1.0		0	7.6 ± 0.80
	0		10	9.0 ± 0.62
	1.0		10	7.8 ± 1.20

Animals were given midazolam or morphine applied by IP route (left) or DPAG microinjections (right). Additional groups of animals received naloxone (1 mg/kg, IP) 5 min after saline or 10 nmol morphine microinjections into the DPAG.

*Different from controls ($p < 0.05$, Dunnett's t-test after ANOVA).

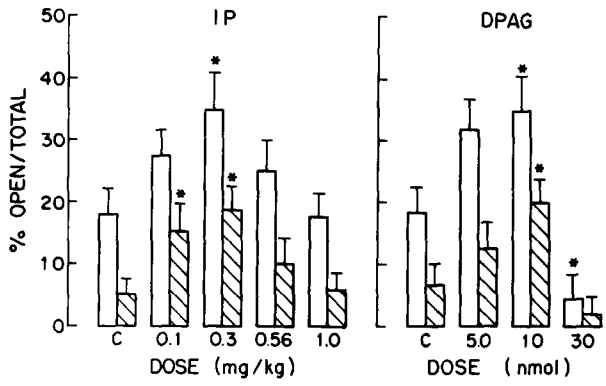

FIG. 3. Mean (±SEM) percentage of entries into and time spent in the open arms of the elevated plus-maze by rats tested with morphine administered IP (left) or directly into the dorsal periacqueductal gray (DPAG) (right) 10 min before the test. Open columns represent number of entries and hatched columns indicate time spent in the arms. $n = 10$. *$p < 0.05$, Dunnett's t-test.

DPAG microinjection of morphine (5-30 nmol) had no significant effect on the total number of arm entries, $F(3, 36) = 1.61$, $p > 0.05$, but showed significant effects on the percentage of open arm entries, $F(3, 36) = 4.18$, $p < 0.05$, and percentage of time spent in the open arms, $F(3, 36) = 3.96$, $p < 0.05$. Posthoc analysis revealed that these effects were due to the dose of 10 nmol morphine ($p < 0.05$, see Fig. 3, right). Thirty nanomoles of morphine caused an anxiogenic-like effect expressed by a reduction in the percentage of number of entries ($p < 0.05$).

As can be seen in Fig. 4, the antiaversive effects of morphine seem to be opioid mediated because prior administration of naloxone (1 mg/kg, IP) inhibited the antiaversive effect produced by 20 nmol morphine in the DPAG ($p > 0.05$ compared to control groups).

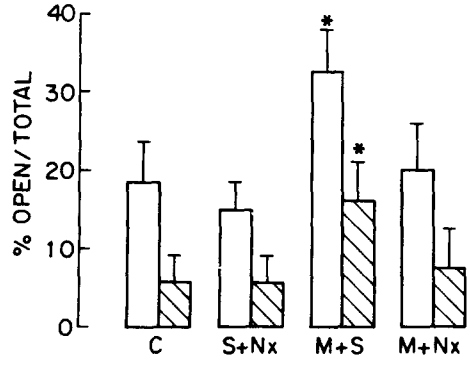

FIG. 4. Blockade by naloxone of the antiaversive action of morphine microinjected into the dorsal periacqueductal gray (DPAG). Data are represented as mean (±SEM) percentage of entries (empty columns) and time spent (hatched columns) in the open arms of the elevated plus-maze. Animals received a DPAG injection (0.2 μl, 30 s) followed by an IP administration 5 min later. The tests were conducted 5 min after the second injection. C, rats microinjected with saline into the DPAG and IP saline; S + Nx, animals injected with saline into the DPAG and IP naloxone; M + S, animals injected with 10 nmol morphine into the DPAG and IP saline; M + Nx, animals injected with 10 nmol morphine and IP naloxone. $n = 10$. *$p < 0.05$, Dunnett's t-test.

Microinjections of high doses of morphine (70 nmol) into the DPAG mimicked the effects observed following electrical stimulation of this structure. Microinjections of saline in the same sites produced no apparent change in the behavior of animals. Soon after microinjections of morphine, animals showed an intense hyperactivity lasting for about 45 min, characterized by intense running (Mann-Whitney $U = 0$, $p < 0.01$) and jumps (Mann-Whitney $U = 0$, $p < 0.01$). Few rearings and rotations could be observed. The behavioral activation induced by morphine was not significantly affected by pretreatment with 1 mg/kg naloxone ($p > 0.05$ for crossings and jumps in comparison with morphine group) (Fig. 5).

DISCUSSION

The present results show that gradual increase in the intensity of electrical stimulation of the DPAG of rats induce, in a progressive manner, characteristic aversive responses such as arousal, freezing, and escape behavior. A continuum of defense responses has also been described for rats that forage dangerous areas (11). Initially, they manifest a kind of stretched-approach response expressing their cautious behavior. If they encounter a predator, the next stage of defense is freezing. Next, they engage in vigorous escape attempts or jump attacks. Similar escape responses along with autonomic reactions resembling a defense reaction have also been observed following microinjections of GABA antagonists into the DPAG, suggesting that GABAergic mechanisms exert a tonic inhibitory control in neural substrates of aversion in this structure (6,9).

In the present work, we examined further the role played by opioid mechanisms on the neural substrate commanding aversive states in structures belonging to the BAS. Involvement of opioids in the modulation of aversive states has already been observed in structures of the brain aversive system such as the DPAG and medial hypothalamus (16,17). In conformity with these findings, microinjections of morphine into the DPAG also inhibit the mean blood pressure and heart rate rises induced by electrical stimulation of this structure (7,8). Accordingly, we observed here that DPAG microinjections of morphine attenuate in a dose-dependent manner the aversive consequences of exposure of rats to the open arms of the EPM. Although a diffusion of the drug to other structures

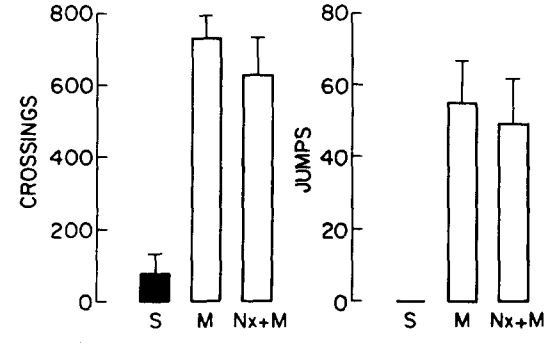

FIG. 5. Behavioral activation induced by morphine microinjections (70 nmol, 0.2 μl over 30 s) into the dorsal periacqueductal gray (DPAG). Hyperactivity is expressed as total crossings (left) and jumps (right) recorded during 45 min. S, saline; M, morphine; Nx + M, group injected with naloxone (1 mg/kg, IP) 5 min before morphine. $n = 8$.

cannot be discarded, earlier studies have indicated that when injected in the approximate volume used in these experiments the maximal effective spread of drugs is probably not more than 1.0 mm (3,18,23). This effect of morphine seems to be opioid in nature because it was antagonized by previous administration of naloxone. So, besides GABA mechanisms, opioid processes may also take part in the modulation of defensive behavior at brainstem structures.

In the present work, microinjections of high doses of morphine into the DPAG caused fearful hyperactivity. In this work, we presented evidence that high doses of morphine applied to this mesencephalic area cause proaversive effects that are not blocked by naloxone in doses reported to block μ-receptors (21,22,28). Based upon these data, we suggest that high doses of morphine act locally in the DPAG, causing aversive effects that are not mediated by μ-opioid receptors. Other workers have obtained results consistent with this suggestion (14,15). As bicuculline mimicks and GABA itself blocks this nonnaloxone-reversible action of morphine, Jacquet et al. (14) suggested that this morphine excitation was due in part to $GABA_A$ receptor blockade.

Another possibility that remains to be investigated is that morphine causes its proaversive effects in the DPAG by acting on other opioid receptors, for example, k-receptors. Increasing evidence suggests that opioid agonists can produce reinforcing or aversive effects depending upon the types of receptors with which they interact. Thus, μ- and δ-receptor agonists function as positive reinforcers and k-agonists produce aversive states as shown in several experimental paradigms (2,5,22,31). The DPAG of rats contains significant concentrations of μ- and k-receptors (20). These data, along with the well-known fact that morphine has much higher affinity for μ- than for k-receptors (21,28), could explain the differential effects caused by morphine depending upon the dose injected. Low doses, which are supposed to activate μ-receptors, produce antiaversive effects and proaversive effects may result from the activation of k-receptors by high doses of morphine. It still remains to use selective agonists and antagonists of the various opioid receptors to support the suggestions derived from this work.

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