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Blockade of μ- and activation of κ-opioid receptors in the dorsal periaqueductal gray matter produce defensive behavior in rats tested in the elevated plus-maze

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Abstract

We studied the effects of morphine injected into the dorsal periaqueductal gray using conventional and novel ethological measures of the behavior of rats submitted to the elevated plus-maze test. Morphine (20 and 40 nmol) applied into the dorsal periaqueductal gray produced dose dependent aversive effects with reduced entries and time spent in the open arms. Freezing behavior was the most prominent novel ethological measure produced by microinjections of these doses of morphine. These pro-aversive effects were not inhibited by previous dorsal periaqueductal gray microinjection of [D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2](CTOP) (1 nmol), a selective peptide antagonist for μ-opioid receptors. On one hand, microinjection of CTOP produced a dose dependent increase in scanning and stretched attended postures, by its own. On the other hand, the aversive effects of morphine into the dorsal periaqueductal gray microinjections were significantly reduced by systemic administration of nor-binaltorphimine, an opioid receptor antagonist with a tardive and selective action at κ-opioid receptors. These findings suggest that mechanisms mediated by μ-opioid receptors in the dorsal periaqueductal gray may be involved in the control of risk assessment behavior. On the other hand, the pro-aversive effects produced by microinjections of morphine into the dorsal periaqueductal gray are probably mediated by κ-opioid receptors.

Keywords: Dorsal periaqueductal gray; μ-Opioid receptor; κ-Opioid receptor; CTOP; Nor-binaltorphimine; Aversion; Elevated plus-maze

1. Introduction

It has been reported that systemic injections of morphine may produce euphoric or dysphoric effects (Reisine and Pasternak, 1996). Indeed, opioid ligands may mediate reward or aversive processes depending on the structures where they are acting and on the type of receptors with which they interact (Mucha and Herz, 1985; Bechara and Van der Kooy, 1987; Bals-Kubik et al., 1989). Given the importance of this matter, many laboratories have spent a lot of effort to characterize where and how these functions could be carried out in the brain.

It has been reported that microinjections of low doses of morphine into the dorsal periaqueductal gray matter attenuates in a dose dependent manner the aversive consequences of electrical stimulation of this site (Jenck et al., 1983, 1986; Brandão et al., 1985, 1990). High doses of morphine, however, when locally injected into the dorsal periaqueductal gray cause a behavioral activation together with jumps which shows a great similarity with the reaction observed following aversive electrical stimulation of or microinjections of γ-aminobutyric acid (GABA) receptor antagonists into this structure (Brandão et al., 1982; Jacquet et al., 1987; Jacquet and Squires, 1988; Brandão et al., 1994, 1999). Similarly, low doses of morphine in the dorsal periaqueductal gray caused anti-aversive effects while high doses produced pro-aversive effects in rats tested in an open field and in the elevated plus-maze (Motta and Brandão, 1993; Anseloni et al., 1999). Other studies have reported that μ agonists produce place preference and κ agonists cause place aversion, through centrally mediated mechanisms (Bals-Kubik et al., 1989;

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Mucha and Herz, 1985; Bechara and Van der Kooy, 1987). In our laboratory we have shown that the aversive effects of dorsal periaqueductal gray microinjections of high doses of morphine in rats tested in the elevated plus-maze was inhibited by nor-binaltorphimine, in doses reported to block κ-opioid receptors (Portoghese et al., 1987), *administered i.p. 30 min before the test*. As dorsal periaqueductal gray has significant amounts of κ receptors (Mansour et al., 1987, 1988, 1995) it has been hypothesized that these aversive effects may result from activation of κ receptors in the dorsal periaqueductal gray (Motta et al., 1995). One problem with this interpretation is that the antagonist nor-binaltorphimine may act as a non-selective opioid receptor antagonist *within the first hours after its systemic administration* and has a tardive long-lasting selective antagonist action at κ receptors *later on* (Endoh et al., 1992). To approach this issue, the effects of morphine in the elevated plus-maze were evaluated 24 h after the pretreatment with nor-binaltorphimine. We also determined whether or not [D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2] (CTOP), a potent and highly selective antagonist for μ-opioid receptors (Hawkins et al., 1989), affects the aversive effects caused by high doses of morphine in the dorsal periaqueductal gray. These treatments were evaluated on the ethological behavioral categories currently used in this laboratory for measuring the exploratory behavior of rats submitted to the elevated plus-maze test.

2. Material and methods

2.1. Animals

Male Wistar rats weighing 230–280 g were housed in groups of two with free access to food and water. They were kept in the experimental room for 48 h prior to the experiment on a 12 h light/dark cycle (lights on at 7 a.m.) at 22 ± 1°C.

2.2. Surgery

Forty animals were anaesthetized with sodium pentobarbital (45 mg/kg, i.p.) and fixed in a stereotaxic frame (David Kopf, USA). The upper incisor bar was set at 3.3 mm below the interaural line such that the skull was leveled between bregma and lambda. A guide cannula made of a hypodermic needle (o.d. 0.6 mm, i.d. 0.4 mm) was implanted in the dorsal periaqueductal gray matter. The guide-cannula was introduced in the dorsal periaqueductal gray, with an angle of 12°, using the following coordinates with the lambda serving as the reference for each plane: postero-anterior 0.3 mm; medio-lateral 0.5 mm; and dorso-ventral 5.2 mm. The guide-cannula was fixed to the skull with acrylic resin and three stainless steel screws. At the end of the surgery, each guide-cannula was sealed with a stainless steel wire to protect it from congestion.

2.3. Behavioral procedure

One week following the surgery, each rat was individually placed in an elevated plus-maze similar to that previously described (Anseloni et al., 1995; Anseloni and Brandão, 1997). The maze was made in wood with two open arms (50 × 10 cm) and two enclosed arms of the same size with 50 cm high walls. The level of illumination was 100 lx on the floor level of the walled arms of the mazes. The maze was configured such that arms of the same type were opposite to each other, and the whole maze was raised 50 cm from the floor. The walls of the closed arms of the maze were made of transparent Plexiglas.

All testing was conducted during the midportion of the light phase. The rats were placed individually in the center of the maze facing a closed arm and allowed 5 min of free exploration. The behavior of the animals was recorded by a video camera positioned to the side of the maze, allowing for the discrimination of all behaviors, with the signal

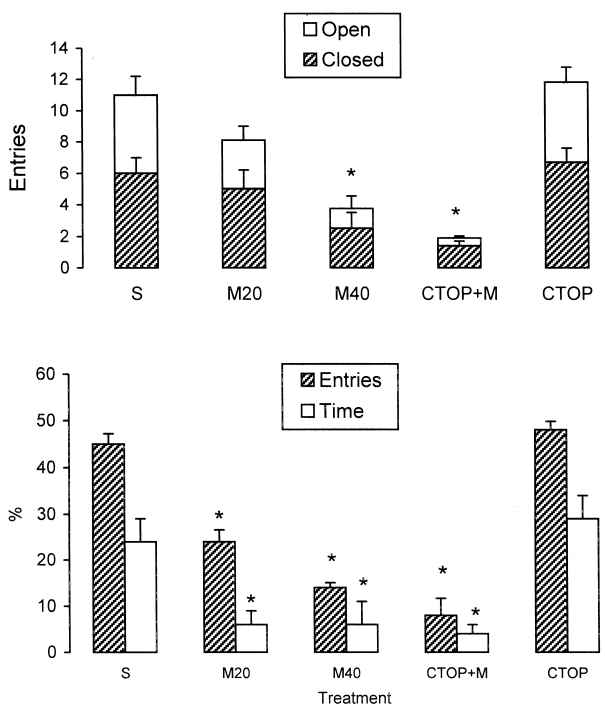

Fig. 1. Effects of morphine (20 and 40 nmol) and CTOP (1 nmol) microinjections into the dorsal periaqueductal gray on exploratory behavior of rats (mean ± S.E.M.) in the elevated plus-maze. Each animal was injected twice (0.2 μl); saline or CTOP (1 nmol) followed by morphine or saline. The interval between the first and second injections was of 5 min. Top: number of entries into both types of arms. Bottom: % of entries and time spent in the open arms in relation to totals. *, Different from entries and time spent in that type of arm in the control group (Tukey test, $P < 0.05$). S, saline; M20, 20 nmol morphine; M40, 40 nmol morphine; CTOP (1 nmol). $N = 8$ for each group.

relayed to a monitor in another room via a closed circuit TV-camera. The maze was thoroughly cleaned after each test using damp and dry cloths. All rats were tested just once.

Videotapes were subsequently scored by an observer using an ethological analysis software (Observer) developed by Noldus (Netherlands). Using separate location and behavior keys, this software allows the real-time scoring of videotapes of any behavior by direct keyboard entry to a PC. Behaviors scored from videotape included standard (open and closed arm entries) and ethological measures. This software only records the next behavioral category after a "stop" key is pressed, this way allowing for the recording of duration and frequency of prolonged behaviors as freezing, rearing, stretched attend postures etc.

The performance of each animal in the maze was analyzed taking into account the standard measures recorded in each section of the maze (closed and open arms, central platform) comprising the frequency of open and closed arm entries (arm entry defined as all four paws into an arm), total arm entries and the amount of time spent by the animals in each section of the maze. These data were used to additionally calculate % open arm entries, % time in open arms, % time in central platform.

The ethological items recorded were end-arm exploration, rearing, peeping out, stretched attend posture, flat back approach, scanning, freezing and head-dipping. These categories were defined after work in rats (Blanchard et al., 1991; Anseloni and Brandão, 1997) and in mice (Rodgers and Cole, 1994; Rodgers and Johnson, 1995). Rearing: the partial or total rising onto the hind limbs. Scanning: scrutinizing in any direction, including sniffing (olfactory exploration of maze floor and walls). Head-dipping: exploratory movement of head/shoulders over sides of the maze and down towards the floor. Freezing: arrest of movement over 6 s accompanied, at least, by two of the following autonomic responses: arching back, piloerection, defecation, micturition, exophthalmus or ear retraction. End-arm exploration: number of times the rat reached the end of an open arm. Peeping out: stretch the head/shoulders from the closed arms to the central platform. Stretched attend posture: when the animal stretches to its full length and turns back to the anterior position. Flat back-approach: locomotion when the animal stretches to its full length and cautiously moves forward.

2.4. Intracranial microinjections

The animals were gently wrapped in a cloth, hand-held and a thin dental needle (o.d. 0.3 mm) was introduced through the guide-cannula until its lower end was 1 mm below the guide-cannula. The injection needle was linked to a 5 μl Hamilton syringe 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 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 the microinjection.

2.5. Drugs

Morphine sulphate (Verano, Argentina) and CTOP (RBI, USA) and nor-binaltorphimine (RBI) were each dissolved

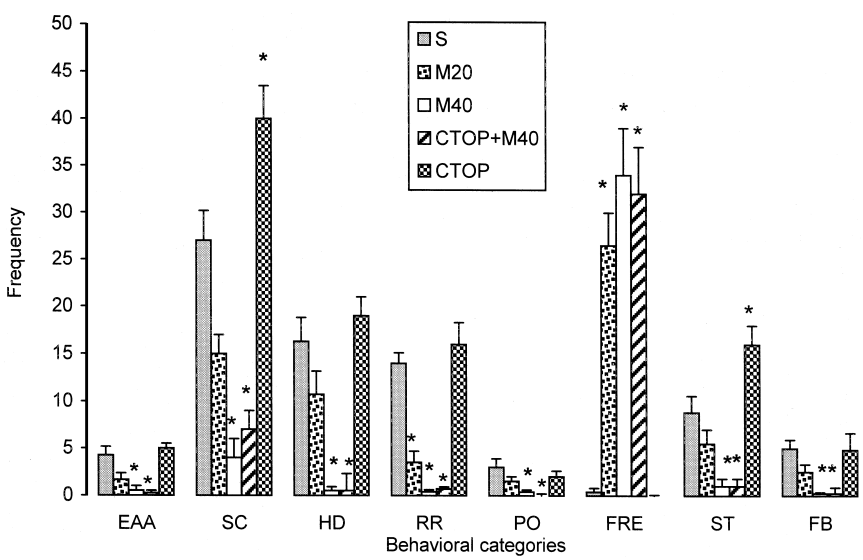

Fig. 2. Effects of injections of morphine (M) and CTOP on the frequency (mean ± S.E.M.) of the ethological measures recorded in both arms of the elevated plus-maze. Each animal was injected twice (0.2 μl); saline or CTOP (1nmol) followed by morphine or saline. The interval between the first and second injections was of 5 min. *, Different from the respective control group (Tukey test, $P < 0.05$). S, saline stands for injected controls and M for morphine. EAA, end-arm activity; SC, scanning; HD, head dipping; RR, rearing; PO, peeping out; FRE, freezing; ST, stretched attend postures; FB, flat back approach. $N = 8$ in each group.

in physiological saline (0.9%) shortly before use. Physiological saline served as vehicle control either injected into the dorsal periaqueductal gray (0.2 μl) or systemically (i.p., 1 ml/kg).

The rats were allocated to one of the following treatment groups: Part I (CTOP in combination with morphine; $N = 8$ for each treatment): (a) saline + saline, (b) saline + morphine 20 nmol (15.2 μg/0.2 μl), (c) saline + morphine 40 nmol (30.4 μg/0.2 μl), (d) CTOP 0.5 nmol (0.53 μg/0.2 μl) + saline, (e) CTOP 1 nmol (1.06 μg/0.2 μl) + morphine 40 nmol, and (f) CTOP 1 nmol (1.06 μg/0.2 μl) + saline. The animals were injected twice. They received either saline or CTOP intracerebral microinjection (0.2 μl) followed 5 min later by another microinjection of saline or morphine (20 or 40 nmol), and tested in the plus-maze 15 min after the second injection. Part II (nor-binaltorphimine in combination with morphine): (a) Sal + Sal; (b) nor-binaltorphimine (2 mg/kg) + saline, $n = 8$; (c) saline + morphine 40 nmol, $n = 8$; (d) nor-binaltorphimine + morphine 40 nmol, $n = 8$. The animals received nor-binaltorphimine (2 mg/kg, i.p.) or saline (1 ml/kg, i.p.) followed 24 h later by dorsal periaqueductal gray microinjections of morphine (40 nmol) or saline, and tested in the elevated plus maze 15 min later, as described above. The doses of the drugs and the time for testing used here were chosen on the basis of previous studies showing that they do not produce any apparent behavioral changes in the animals (Motta and Brandão, 1993; Motta et al., 1995; Anseloni et al., 1999, Sante et al., 2000). We also tested the dose of 5.0 nmol of CTOP in nine animals. However, six animals showed pre convulsive signs, such as excessive grooming, bobbing, wet dog shakes, masticatory movements, asymmetrical postures with head turnings and the three remaining rats also presented jerks and short running fits.

2.6. Statistical analysis

Data are reported as means ± S.E.M. Comparisons of the exploratory activity of rats submitted to the plus-maze were performed by one-way analyses of variance (ANOVA). Tukey post-hoc comparisons were carried out whenever significant overall *F*-values were obtained.

2.7. Histology

Upon completion of the experiments, the animals were deeply anesthetized with sodium pentobarbital and perfused intracardially with saline followed by formalin solution (10%). Three days later the brains were removed and frozen. Serial 50-μm brain sections were cut using a microtome and stained with neutral red in order to locate the positions of the microinjection sites (Paxinos and Watson, 1997). Data from rats with injection cannula tips located at sites outside the dorsal periaqueductal gray were not included in the present study.

3. Results

The points of drug injections in the midbrain tectum were located in the dorsal aspects of the periaqueductal gray, as described elsewhere (Anseloni et al., 1999).

ANOVA performed on the data obtained in the Experiment I revealed significant effects of drug injections into the dorsal periaqueductal gray upon the number of entries on the open arms ($F(5,42) = 7.32$; $P < 0.001$), closed arms ($F(5,42) = 6.43$; $P \leq 0.001$) and both arms ($F(5,42) = 9.28$; $P < 0.001$) of the maze. Tukey's test showed that all treatments that included morphine 20 or 40 nmol were different from controls ($P < 0.05$) regardless whether pre-injected with saline or CTOP 1 nmol (Fig. 1A). The same happened with the % open/total entries ($F(5,42) = 11.03$; $P < 0.001$) and % time open/total spent in the maze ($F(5,42) = 9.65$; $P < 0.001$) (Fig. 1B). CTOP, by its own, did not produce effects on these conventional measures different from the saline control group.

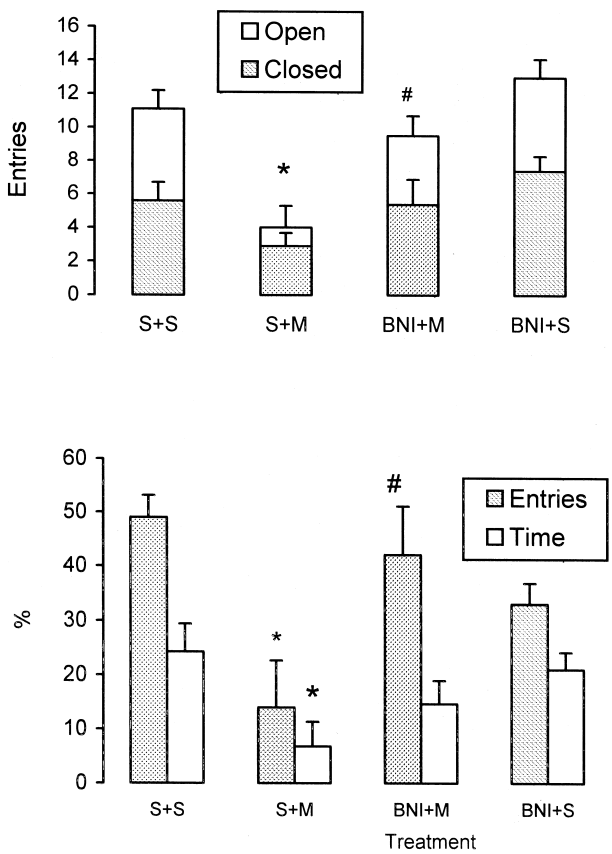

Fig. 3. Effects of nor-binaltorphimine (2 mg/kg, i.p.) on the effects of morphine (40 nmol) microinjections into the dorsal periaqueductal gray on exploratory behavior of rats (mean ± S.E.M.) in the elevated plus-maze. Each animal was injected twice; saline or NBI followed by dorsal periaqueductal gray morphine or saline (0.2 μl). The interval between the first and second injections was of 24 h. Top: number of entries into both types of arms. Bottom: % of entries and time spent in the open arms in relation to totals. *, Different from entries and time spent in that type of arm in the control group (Tukey test, $P < 0.05$). S, saline; M, 40 nmol morphine; BNI = nor-binaltorphimine. $N = 8$ for each group.

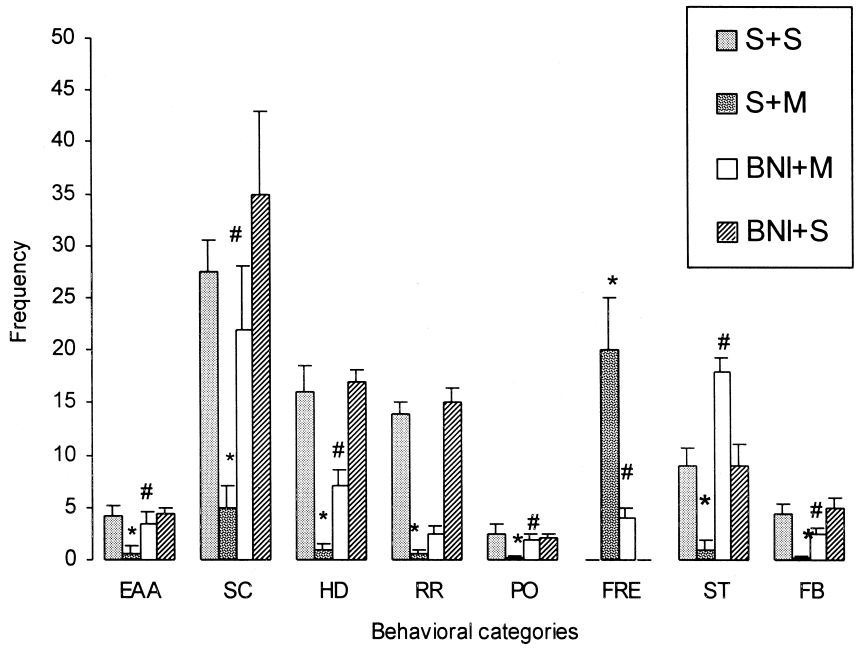

Fig. 4. Effects of nor-binaltorphimine (2 mg/kg, i.p.) on the aversive effects of microinjections of morphine into the dorsal periaqueductal gray on the frequency (mean ± S.E.M.) of the ethological measures recorded in both arms of the elevated plus-maze. Each animal was injected twice; saline or nor-binaltorphimine followed by morphine (40 nmol/0.2 μl) or saline (0.2 μl). The interval between the first and second injections was of 24 h. S, saline stands for injected controls, M for morphine and BNI for nor-binaltorphimine. EAA, end-arm activity; SC, scanning; HD, head dipping; RR, rearing; PO, peeping out; FRE, freezing; ST, stretched attend postures; FB, flat back approach. *, Different from the respective control group (S); #, different from the S + M group ($P < 0.05$, Tukey test). $N = 8$ in each group.

Fig. 2 illustrates the effects of morphine and CTOP injections into the dorsal periaqueductal gray on the ethological measures. ANOVA detected significant effects on stretched attend postures ($F(5,42) = 21.91$; $P < 0.001$), flat back approach ($F(5,42) = 10.56$; $P < 0.001$), peeping-out ($F(5,42) = 6.30$; $P < 0.001$), rearing ($F(5,42) = 29.20$; $P < 0.001$), scanning ($F(5,42) = 26.12$; $P < 0.001$), end-arm activity ($F(5,42) = 9.42$; $P < 0.001$), head dipping ($F(5,42) = 22.73$; $P < 0.001$) and freezing ($F(5,42) = 26.07$; $P < 0.001$). Tukey post-hoc analysis ($P < 0.05$) showed that morphine produced a dose dependent increase on freezing and a compensatory decrease in all remaining measures. Previous injections of CTOP (1 nmol) did not influence these effects of 40 nmol of morphine. On the other hand, this antagonist had detectable effect per se; stretched attend postures and scanning were significantly increased by CTOP 1 nmol while 0.5 nmol only increased stretched attend postures in relation to the saline controls.

ANOVA performed on the data obtained in Experiment II (nor-binaltorphimine against morphine) revealed significant effects of drug injections into the dorsal periaqueductal gray upon the number of entries on the open arms ($F(3,28) = 3.48$; $P = 0.03$), closed arms ($F(3,28) = 2.61$; $P = 0.07$) and both arms ($F(3,28) = 3.90$; $P = 0.02$) of the maze. Tukey's test showed that the reduction in these measures was due to the treatment with 40 nmol of morphine and that nor-binaltorphimine significantly inhibited these effects ($P < 0.05$) (Fig. 3A). The same occurred with the % open/total entries ($F(3,28) = 4.80$; $P = 0.008$) and % time open/total spent in the maze ($F(3,28) = 3.05$; $P = 0.04$) (Fig. 3B). The effects of nor-binaltorphimine on its own on these conventional measures were not different from the saline control group.

Fig. 4 illustrates the effects of morphine and nor-binaltorphimine injections into the dorsal periaqueductal gray on the ethological measures. ANOVA detected significant effects on stretched attend postures ($F(3,28) = 20.30$; $P < 0.001$), flat back approach ($F(3,28) = 10.70$; $P < 0.001$), peeping-out ($F(3,28) = 3.24$; $P = 0.04$), rearing ($F(3,28) = 60.13$; $P < 0.001$), scanning ($F(3,28) = 26.12$; $P < 0.001$), end-arm activity ($F(3,28) = 4.82$; $P = 0.008$), head dipping ($F(3,28) = 28.24$; $P < 0.001$) and freezing ($F(3,28) = 48.26$; $P < 0.001$). Tukey post-hoc analysis ($P < 0.05$) showed that morphine produced a dose dependent increase on freezing and a compensatory decrease in all remaining measures. Previous injections of nor-binaltorphimine reversed these effects of 40 nmol of morphine ($P < 0.05$). On the other hand, this antagonist did not have any effect per se.

4. Discussion

Several lines of evidence have implicated GABAergic, serotonergic and amino acid mediated mechanisms among others in the control of the neural substrates mediating

anxiety in the brain aversion system (Brandão et al., 1982, 1986, 1994, 1999; Bandler and Carrive, 1988; Graeff, 1990). Opioid mechanisms have also been implicated in the integration of defensive behavior in the midbrain tectum. In conformation with this proposal, it has been reported that microinjections of low doses of morphine into the dorsal periaqueductal gray attenuated, in a dose dependent manner, the aversive consequences of the exposure of rats to the open arms of the elevated plus-maze and also inhibited the mean blood pressure and heart rate rises induced by electrical stimulation of this structure (Jenck et al., 1983, 1986; Brandão et al., 1985, 1990; Cardoso et al., 1992; Brandão, 1993; Motta and Brandão, 1993; Anseloni et al., 1999). These effects of morphine seem to be opioid in nature since they were antagonized by naltrexone. The anti-aversive effects of low doses of morphine are supposed to be the result of the activation of μ opioid receptors as they are antagonized by naltrexone, which has preferential affinity for μ-opioid receptors and because they mimic the anti-aversive effects of DAMGO (D-Ala-*N*-Me-Phe-Gly-ol enkephalin), a highly selective agonist of these receptors (Motta et al., 1995, Anseloni et al., 1999). In this sense, the present findings related to the effects of CTOP per se may bring some light in the understanding of the role of opioid mechanisms in the defense reaction. Although this μ-opioid receptor antagonist did not significantly change the number of entries and the time spent in both closed and open arms by rats exposed to the plus-maze it caused a specific increase in stretched attend postures and scanning, ethological measures related to risk assessment behavior (Rodgers and Cole, 1994; Rodgers and Johnson, 1995; Brandão et al., 1997). One possibility for explaining these results is that endogenous opioid ligands may regulate defense-related behaviors associated to risk evaluation generated at the dorsal periaqueductal gray level, through μ-opioid receptors.

High doses of morphine (20–40 nmol) caused clear pro-aversive effects with a significant reduction in the behavioral categories inversely related to anxiety as number of entries and time spent in the open arms, head-dippings and end-arm activity as well as produced a dose-dependent increase in freezing. These effects cannot be attributed to a motor deficit as previous work of this laboratory has shown that still higher doses of morphine (70 nmol) caused a behavioral activation with jumps in an arena (Motta and Brandão, 1993; Motta et al., 1995). In this study the pro-aversive effects of microinjections of morphine in rats exposed to the elevated plus-maze were significantly blocked 24 h after systemic injection of norbinaltorphimine. This latter drug has a tardive and long-lasting blocking effect at κ-opioid receptors and acts as a non-selective opioid receptor antagonist within the first hour of injection (Endoh et al., 1992). In support for an involvement of κ-opioid receptors in the elaboration of aversive states in the dorsal periaqueductal gray, we have recently shown that the microinjection of U-50488-H, a 1,2-aminoamide with specific agonistic actions at κ receptors, directly into the dorsal periaqueductal gray mimicked these pro-aversive of morphine in rats submitted to the plus-maze test. Based on these findings we suggest that high doses of morphine act locally in the dorsal periaqueductal gray causing an aversive state, which is consequence of the activation of κ-opioid receptors.

The aversive effects of morphine as the result of an action on μ-opioid receptors of the dorsal periaqueductal gray could also be considered in view of the significant distribution of these receptors at this midbrain level. Because of this, we also examined the effects of CTOP — a selective μ-opioid receptor antagonist — on the exploratory behavior of rats submitted to the elevated plus-maze. This drug by its own produced dose-dependent aversive effects. The pretreatment with CTOP (1 nmol) did not change the aversive states produced by microinjection of high doses of morphine in the midbrain tectum. The results obtained in the present experiments seem to dismiss the possibility of an aversive action of morphine at μ-opioid receptors in the dorsal periaqueductal gray.

Based on the data obtained in this work, it seems reasonable to speculate that aversive states may result if environmental or internal cues signaling danger or threatening situations disrupt the balance μ- or κ-receptor-mediated mechanisms toward κ-receptor activity at the dorsal periaqueductal gray level. A combined action on μ and κ receptors could underlie the well-known naloxone-precipitated aversive reactions in rats made physically dependent by morphine infusion in the periaqueductal gray (Bozarth and Wise, 1984; Wise, 1992). This aversive reaction could be the result of opiate antagonism of μ receptors by naloxone and morphine action on κ receptors, to which naloxone shows lower affinity than for μ receptors (Tempel et al., 1985; Bigelow and Preston, 1995). The potential involvement of delta-opioid receptors was not assessed in the present study because few delta receptors appear to be present in the periaqueductal gray matter (Mansour et al., 1987, 1995).

In summary, the present data bring new evidence for the brain neurochemical mechanisms involved in the behavioral effects of morphine. These findings suggest that mechanisms mediated by μ-opioid receptors may be involved in the control of risk assessment-related responses generated in the dorsal periaqueductal gray during aversive situations. On the other hand, the aversive effects produced by microinjections of high doses of morphine in the dorsal periaqueductal gray are mediated by other mechanisms, probably through κ-opioid receptors.

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