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GABAergic and glutamatergic modulation of exploratory behavior in the dorsomedial hypothalamus

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Abstract

Systemic injection of glutamate NMDA receptor antagonists or drugs that facilitate $GABA_A$-mediated neurotransmission produces anxiolytic effects. The dorsomedial hypothalamic (DMH) region is proposed to be a possible site of action of these drugs. The objective of the present study was to investigate if facilitation of $GABA_A$-mediated neurotransmission or blockade of NMDA receptors in the DMH would produce anxiolytic effects in the elevated plus-maze (EPM). Seven days after surgery, male Wistar rats with unilateral cannulas in the DMH were submitted to the behavioral studies. Results showed that midazolam, a benzodiazepine anxiolytic (30–60 nmol/0.3 μl), produced a dose-dependent increase in open arm exploration without changing the number of enclosed arm entries, indicating an anxiolytic effect. This effect was antagonized by previous treatment with flumazenil, a benzodiazepine receptor antagonist (60 nmol/0.3 μl). Flumazenil alone had an anxiogenic effect, decreasing exploration of the open arms of the EPM. 2-Amino-7-phosphonoheptanoic acid (AP7), an NMDA receptor antagonist (0.2–2 nmol/0.3 μl), did not modify open arm exploration but decreased general exploratory activity. These results indicate that benzodiazepine receptors located in the DMH could modulate anxiety. Interference with NMDA receptor-mediated neurotransmission in this region, however, seems to change general exploratory activity rather that anxiety.

Keywords: Aversion; Benzodiazepine; Elevated plus-maze; Flumazenil; Anxiogenic; Endogenous ligands; Glutamate; NMDA

1. Introduction

The discovery of benzodiazepine compounds more than 30 years ago produced a major change in the clinical treatment of anxiety disorders (Gray and McNaughton, 2000). It is now recognized that these drugs act on specific binding sites located in the $GABA_A$ receptor complex, exerting an allosteric facilitation of GABAergic neurotransmission (Mehta and Ticku, 1999; Zorumski and Isenberg, 1991).

Benzodiazepine receptors are widely distributed throughout the central nervous system (Doble and Martin, 1992). Studies using intracerebral injections have tried to elucidate the brain regions more likely involved in the effects of these drugs. Using this approach, anxiolytic effects have been found, after microinjection, in the amygdaloid complex and the dorsal periaqueductal gray matter (DPAG; Hodges et al., 1987; Russo et al., 1993). These regions are proposed to be part of a brain aversive system that would also include the medial hypothalamus (Graeff, 1990).

Several pieces of evidence support the involvement of the dorsomedial hypothalamus (DMH) in the organization of defensive reactions. For example, rats exposed to anxiety models show increased levels of catecholamines in this region (Sajdyk et al., 1997; Shekhar et al., 1994), blockade of GABA receptors in the DMH increases corticosterone and ACTH plasma levels (Shekhar and Keim, 1996), lesion of the DMH causes anxiolytic effects (Inglefield et al., 1994) and exposure to anxiety tests induces c-Fos expression in this region (Aspley and Marsden, 1997).

Interference with GABA-mediated neurotransmission in the medial hypothalamus is suggested to modulate anxiety. Injection of GABA agonists into the DMH decreases the aversive consequences of direct electrical or chemical stimulation (Milani and Graeff, 1987; Silveira and Graeff, 1992). These drugs also induce anxiolytic effects in animal models such as the elevated plus-maze (EPM) and the social interaction tests (Shekhar, 1993; Shekhar and Katner, 1995).

Another important neurotransmitter related to anxiety is glutamate. Anxiolytic effects of glutamate receptor antago-

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nists have been found after systemic (Bennett and Amrick, 1986; Stephens et al., 1986) or intra-DPAG administration (Guimarães et al., 1991; Matheus and Guimarães, 1997; Matheus et al., 1994) in several animal models. The DMH could also be involved in these effects. Glutamate receptors are present in this region (Meeker et al., 1994) and local kainic acid injection produces defensive reactions (Silveira and Graeff, 1992).

Few studies, however, have directly investigated a possible involvement of the DMH on the anxiolytic effects of benzodiazepine or glutamate antagonists. An exception was the study by Milani and Graeff (1987), where midazolam antagonized the flight reaction induced by DMH electrical stimulation. The role of benzodiazepine receptors in the DMH, however, has not yet been investigated under conditions that do not involve the use of direct stimulation of this structure.

Therefore, the objective of the present study was to investigate the effects of a benzodiazepine receptor agonist or a glutamate NMDA receptor antagonist microinjected into the DMH of rats submitted to the EPM, an ethological model of anxiety.

2. Materials and methods

2.1. Subjects

Male Wistar rats weighting 200–250 g at the beginning of the experiment were housed in pairs with free access to food and water in a temperature-controlled room (23 ± 1°C) and a 12-h light, 12-h dark cycle (lights on at 6:00 a.m.). Procedures were conducted in conformity with the Brazilian Society of Neuroscience and Behavior guidelines for care and use of laboratory animals, which are in compliance with international laws and policies. All efforts were made to minimize animal suffering.

2.2. Drugs

Midazolam maleate (30–60 nmol; Roche, Brazil) and 2-amino-7-phosphonoheptanoic acid (AP7; 0.02–2 nmol; Ciba Geigy) were dissolved in sterile isotonic saline. These doses were in the range where positive anxiolytic effects had been previously found in the dorsolateral periaqueductal gray (Guimarães et al., 1991; Russo et al., 1993). Flumazenil (60 nmol; Roche) was dissolved in a saline–Tween-80 2% solution. The dose was also based in a previous work (Russo et al., 1993).

2.3. Apparatus

The wood plus-shaped maze consisted of two opposite open arms (50 × 10 cm), crossed at a right angle by two arms of the same dimensions enclosed by 40-cm-high walls with no roof. The maze was located 50 cm above the floor and a 1-cm-high edge made of Plexiglas surrounded the open arms to avoid falls. Rodents naturally avoid the open arms of the EPM, probably because they cannot engage in thigmotaxic behavior (Treit et al., 1993). Anxiolytic compounds typically increase the exploration of these arms without changing the number of enclosed arm entries (File, 1992).

Since AP-7 decreased enclosed arm entries (see below), we decided to use an open circular arena (72 cm in diameter with 45-cm-high Plexiglas walls) to measure the drug effect on the distance moved by the animals.

The experiment was carried out in a sound-attenuated, temperature-controlled (23 ± 1°C) room. The environment was illuminated by two 40-W fluorescent lights placed 1.3 m away from the EPM. The observer sat in the same room 1 m away from the maze. Exploratory activity in the open arena was videotaped and later analyzed with the help of Ethovion (version 1.9; Noldus, the Netherlands) software.

2.4. Surgery

Rats were anesthetized with 2.5% 2,2,2-tribromoethanol (10 ml/kg ip) and fixed in a stereotaxic frame. A stainless steel guide cannula (0.7 mm OD) aimed unilaterally at the DMH (coordinates — A: −3.0 mm from bregma, L: 0.6 mm, D: 7.2 mm) was introduced. The cannula was attached to the bones with stainless steel screws and acrylic cement. A stylet filled up the guide cannulas to prevent obstruction.

2.5. Procedure

Seven days after the surgery, the animals were randomly assigned to one of the treatment groups. In the first experiment, the animals ($n = 5$ per group) received isotonic saline or midazolam 30 or 60 nmol. In the second experiment, the rats ($n = 7–9$ per group) received a first microinjection of vehicle (saline–Tween-80 2% solution) or flumazenil (60 nmol) followed, 5 min later, by a second microinjection of saline or midazolam (60 nmol). In the third experiment, the animals received saline ($n = 19$) or AP7 (0.02, 0.2 or 2 nmol, $n = 7$ per group). Ten minutes after the last microinjection, they were placed into the center of the EPM facing an enclosed arm. The number of entries and time spent on open and enclosed arms were recorded for 5 min.

In the experiment using the open arena, the animals ($n = 5$ per group) received, 10 min before the test, a microinjection of saline or AP7 (2 nmol). The distance moved in the arena was recorded for 5 min.

Intracerebral injections were performed with a thin dental needle (0.3 mm OD) introduced through the guide cannula until its tip was 1.5 mm below the cannula end. A volume of 0.3 μl was injected, in 30 s, using a Microsyringe infusion pump (Kd Scientific, USA). The movement of an air bubble inside the PE-10 polyethylene tubing connecting the microsyringe with the dental needle confirmed drug flow.

After each trial, the maze or the open arena was cleaned with an alcohol solution. In all experiments, saline- and drug-treated groups were run in parallel.

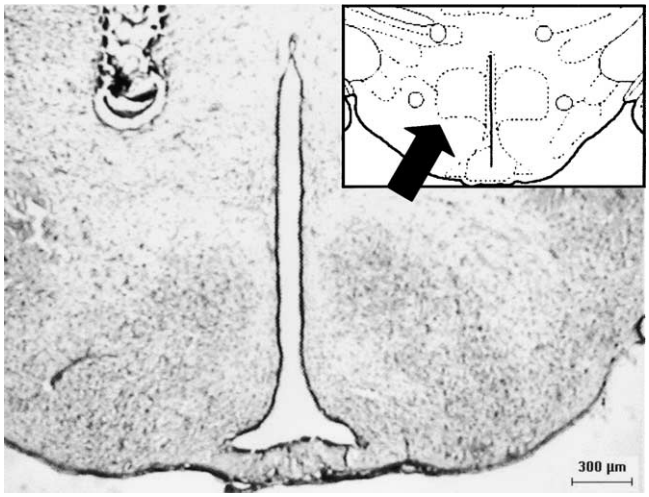

Fig. 1. Photomicrograph of the injection site in the DMH. The arrow in the right panel indicates the localization of the DMH in a figure based on the atlas of Paxinos and Watson (1986).

2.6. Histology

After behavioral tests, rats were killed under deep urethane anesthesia and their brains perfused through the left ventricle of the heart with isotonic saline followed by 10% formalin solution. After that, a dental needle was inserted through the guide cannula and a 0.2-µl microinjection of Evans blue was performed. The brains were removed and, after a minimum time period of 3 days immersed in a 10% formalin solution, frozen sections of 50 µm were obtained in a cryostat (Cryocut 1800). Injection sites were localized in diagrams from the rat brain atlas of Paxinos and Watson (1986). In order to test the anatomical specificity of the drug effect, animals that received midazolam microinjections outside the DMH were joined together in an additional group (OUT). The number of animals in the other experiments with injection sites outside the DMH was small (28%). Consequently, they were discarded from analysis.

2.7. Statistical analysis

In experiments involving the EPM, the percentages of open arm entries (100 × open/total entries) and of time spent in the open arms (100 × open/open + enclosed) were calculated for each animal. These data, and the number of enclosed arm entries, were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan test for multiple comparisons. In case of significant effect in the number of enclosed arm entries, an analysis of covariance (ANCOVA) was performed in the percentage of time spent in open arms using the number of enclosed arm entries as covariate. Data from the open arena were analyzed by a repeated measure MANOVA, with factors being treatment and time. In case of significant interaction between these two factors, the groups were compared by *t* test.

3. Results

A representative injection site can be seen in Fig. 1. Midazolam microinjection into the DMH produced a dose-dependent increase in the percentage of time spent in the open arms ($F_{3,21} = 5.26$, $P < .01$; Duncan, $P < .05$) and tended to do the same with the percentage of entries onto these same arms ($F_{3,21} = 2.95$, $P = .056$; Duncan, $P < .05$). The drug did not change the number of enclosed arm entries ($F_{3,21} = 1.91$, NS; Fig. 2). Animals that received midazolam outside the DMH were not different from controls (Duncan, $P > .05$). The increase in the percentage of entries and time spent in the open arms induced by midazolam (60 nmol) was antagonized by a previous treatment with flumazenil ($F_{3,28} = 7.37$, $P < .05$ and $F_{3,28} = 7.23$, $P < .05$, respectively; Fig. 3). The group that received the flumazenil–saline treatment showed a significant decrease in these two parameters, as compared to control (vehicle–saline; Duncan, $P < .05$). No effect was found in the number of enclosed arm entries ($F_{3,28} = 0.54$, NS; Fig. 3).

The dose of 2 nmol of AP7 produced a significant decrease in the percentage of entries ($F_{3,39} = 6.7$, $P < .001$; Duncan, $P < .05$) and time spent ($F_{3,39} = 2.96$, $P < .05$; Duncan, $P < .05$; Fig. 4) in the open arms. The same dose

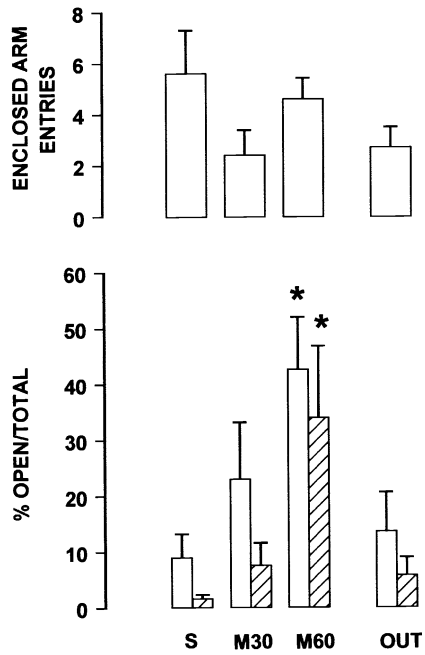

Fig. 2. Effect of midazolam, 30 (M30) or 60 (M60) nmol, microinjected into the DMH of rats ($n = 5$ per group) tested in the EPM. Animals ($n = 10$) that received the drug outside the DMH were analyzed together in an OUT group. Columns represent the means, and vertical bars the S.E.M. In the upper panel, open columns refer to the number of entries made into enclosed arms. In the lower panel, the open columns represent the percentage of entries onto open arms while the hatched columns refer to the percentage of time spent in open arms. Asterisks signal significant differences from saline-treated (S) group detected by the Duncan test ($P < .05$).

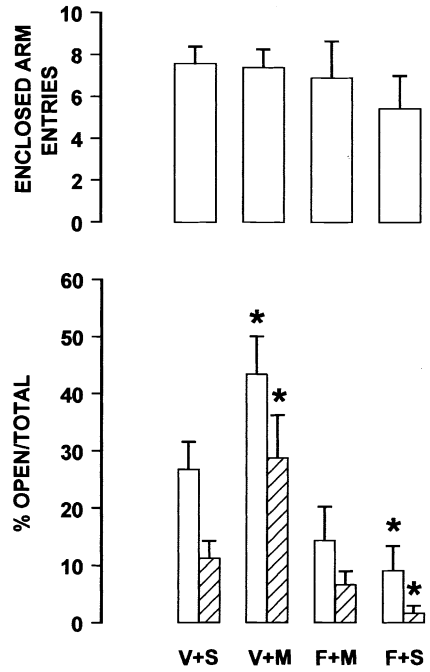

Fig. 3. Effect of midazolam and flumazenil microinjected into the DMH of rats tested in the EPM. Animals ($n=7-9$ per group) received a first microinjection of vehicle (V) or flumazenil (F, 60 nmol) followed, 5 min later, by a second microinjection into the same site of saline (S) or midazolam (M, 60 nmol). Asterisks signal significant differences from vehicle + saline (V + S) group detected by the Duncan test ($P<.05$). Further specifications as in Fig. 2.

also induced a significant decrease in the number of enclosed arm entries ($F_{3,39}=5.59$, $P<.05$; Duncan, $P<.05$). The significant effect on time spent in open arms disappeared when an ANCOVA was performed using the number of enclosed arm entries as covariate ($F_{3,38}=1.56$, NS).

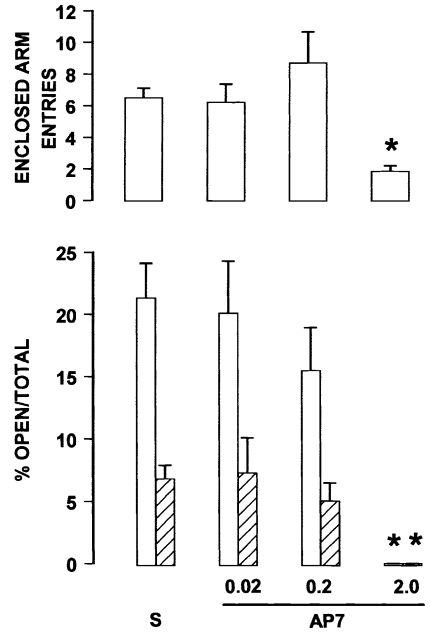

Fig. 4. Effect of AP7 (0.02, 0.2 and 2 nmol, $n=7$ per group) microinjected into the DMH of rats tested in the EPM. Asterisks signal significant differences from saline group ($n=19$) detected by the Duncan test ($P<.05$). Further specifications as in Fig. 2.

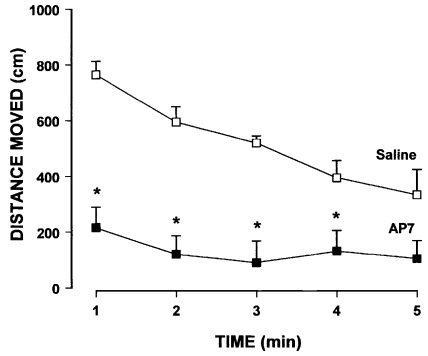

Fig. 5. Effect of AP7 (2 nmol) or saline (0.3 μl) microinjected into the DMH of rats ($n=5$ per group) tested in an open circular arena. Points represent the mean (+S.E.M.) distance moved (in cm) at each minute. Asterisks signal significant difference from saline group detected by t test ($P<.05$).

Data from the open arena showed a significant Time ($F_{4,32}=11.15$, $P<.001$), Treatment ($F_{1,8}=32.33$, $P<.001$) and Treatment × Time ($F_{4,32}=4.86$, $P<.01$; Fig. 5) effects. The drug caused a significant decrease in distance moved inside the arena from the first to the fourth minute (t test, $P<.05$).

4. Discussion

Midazolam, a benzodiazepine full agonist, increased open arm exploration without changing the number of enclosed arm entries when injected into the DMH. This effect is typical of an anxiolytic compound (File, 1992). No drug effect was found when the injections were made outside this region.

The anxiolytic effect of midazolam was antagonized by previous local treatment with flumazenil, a benzodiazepine receptor antagonist (Richards et al., 1986). Together, the results suggest that activation of benzodiazepine receptors located in the DMH produces anxiolytic effects. These results agree with those obtained by Milani and Graeff (1987), using direct electrical stimulation of this nucleus. They are also coherent with studies showing anxiolytic effects of $GABA_A$ receptor agonists injected into the DMH (Shekhar, 1993; Shekhar and Katner, 1995).

The DMH is proposed to be part of a brain system, which includes the amygdala and the DPAG, related to active defense reactions to threatening stimuli (Graeff, 1990). Several neurotransmitters, including GABA, glutamate and serotonin, are involved in the modulation of these structures (Graeff, 1990). Previous studies showed that benzodiazepine agonists injected into the amygdala or the DPAG produced anxiolytic effects in ethologically based models of anxiety such as the EPM (Russo et al., 1993; Zangrossi and Graeff, 1994). We now extend these results

by showing that similar effects could also be obtained in the DMH.

In addition to a direct inhibition of this defense system, however, other mechanisms could also be mediating the anxiolytic effects found in the present study. The DMH may participate in the neural control of theta frequency in the hippocampus. Disruption of this frequency is another mechanism proposed to explain the anxiolytic effects of benzodiazepine drugs (Gray and McNaughton, 2000).

Flumazenil produced an anxiogenic effect by itself in the DMH. Although there is a report of anxiolytic effect of flumazenil in healthy volunteers submitted to a clinical model of anxiety (Kapczinski et al., 1994), many studies using systemic drug injection showed anxiogenic effects (File et al., 1982; Lee and Rodgers, 1991; Nutt et al., 1990). In experiments similar to ours, flumazenil failed to alter anxiety when injected into the DPAG (Russo et al., 1993), but was anxiogenic when injected into the amygdala (Cunha et al., 1993).

The anxiogenic effect of flumazenil found in the present work suggests the presence of endogenous benzodiazepine receptor ligands (Medina et al., 1989) in the DMH. Further investigations, using a systematic approach to study the effects of different doses of flumazenil in areas related to anxiety, could help to elucidate these possibilities.

The NMDA receptor antagonist produced a decrease in open arm exploration of the maze, suggesting an anxiogenic effect. There was, however, a significant decrease in the number of enclosed arm entries. This parameter has been proposed to reflect changes in general exploratory activity in the maze (File, 1992). Therefore, this apparent 'anxiogenic' effect probably reflected a general reduction of maze exploration. Confirming this possibility, the same dose of AP7 that decreased open and enclosed arm entries also decreased the distance moved in the open arena. Moreover, the significant effect on time spent in open arms disappeared when an ANCOVA was performed using the number of enclosed arm entries as covariate (File, 1992).

Several pieces of evidence point to a participation of glutamate-mediated neurotransmission in the DMH in the modulation of defense reactions. All major glutamate receptor subtypes are present in the hypothalamus, with higher concentration in the ventromedial and dorsomedial regions (Meeker et al., 1994). Kainic acid injection into the DMH produces defensive reactions (Silveira and Graeff, 1992), whereas blockade of glutamate receptors attenuates stress-induced cardiovascular changes (DiMicco et al., 1996). Selective agonist of group II metabotropic glutamate receptor, which inhibits glutamate release, prevents lactate-induced behavioral and physiological changes in animals with chronic inhibition of GABA synthesis (Shekhar and Keim, 2000). Moreover, AP7 and other glutamate antagonists show anxiolytic effects in the DPAG, an area closely related to the DMH (Guimarães et al., 1991; Matheus and Guimarães, 1997; Matheus et al., 1994). However, different from these previous studies, AP7 in the DMH produced an important interference with locomotor activity, precluding any conclusion about possible changes in anxiety measured by the EPM. The mechanisms of this interference are not known, but suggest that glutamatergic neurotransmission in this area may influence locomotor behavior. Actually, it has been shown that an injection of glutamate into the DMH can initiate reflexive locomotor activity in anesthetized rats (Marciello and Sinnamon, 1990). Nevertheless, considering that the change in locomotor activity was seen only after a single relatively high dose of AP7, it is not possible to rule out non-specific effects outside the DMH.

5. Conclusion

The present results show that benzodiazepine receptors located in the DMH of rats can modulate anxiety elicited by exposure to the EPM. The results also suggest that NMDA-mediated neurotransmission in this area may influence locomotor behavior.

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