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Role of glutamate ionotropic receptors in the dorsomedial hypothalamic nucleus on anxiety and locomotor behavior

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Abstract

The medial hypothalamus is proposed to play an important role in the modulation of defensive responses. Administration of a NMDA receptor antagonist (AP7) into the dorsomedial hypothalamic nucleus (DMH) of rats reduced exploratory behavior in the open field and elevated plus-maze (EPM), but failed to produce anxiolytic effects in the latter test. The objectives of the present work were to test the hypotheses that (i) AP7 injections into the DMH would also fail to induce anxiolytic effects in another model of anxiety, the Vogel's punished licking test; (ii) injection into the DMH of other glutamate ionotropic antagonists would also decreased exploratory behavior; and (iii) the decrease in exploratory activity found after AP7 administration into the DMH does not involve any gross locomotor impairment. Male Wistar rats (n=5–16/group) with cannulas aimed at the DMH were submitted to the following behavioral tests: EPM, Vogel, catalepsy and rota-rod. Diazepam (3 mg/kg) and haloperidol (2.5 mg/kg) were used as positive controls in the Vogel, rota-rod and catalepsy tests. AP7 failed to modify the number of punished licks in the Vogel test. It also did not induce any change on the rota-rod and catalepsy tests. Diazepam increased the number of punished licks and reduced the latency to fall in the rota-rod. Both 7-chlorokynurenic acid (4–8 nmol), an antagonist of the glycine competitive site in the NMDA receptor and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[*f*]-quinoxaline-7-sulphonamide (NBQX, 1–10 nmol), a non-NMDA receptor antagonist, decreased the total distance moved in the EPM. The former compound also decreased open arm exploration at the dose of 4 nmol. The results suggest that the antagonism of ionotropic glutamate receptors in the DMH does not induce anxiolytic effects in the EPM or Vogel tests, but decreases exploratory behavior in a new environment.

Keywords: Exploratory behavior; Elevated plus-maze; NMDA; Aversion; Vogel

1. Introduction

Stimulation of regions such as the amygdala or the periaqueductal grey (PAG) results in behavioral and autonomic changes that resemble responses exhibited by animals facing natural threats (Hilton, 1979; Lammers et al., 1988; Silveira and Graeff, 1992; DiMicco et al., 2002). Similar changes are also seen after electrical or chemical stimulation of the dorsomedial hypothalamic nucleus (DMH). Together, these regions are proposed to be part of a brain aversive system that mediates defensive reactions (Graeff, 1990).

Glutamate is the main excitatory neurotransmission in the brain, acting through ionotropic and metabotropic receptor subtypes (Stone and Burton, 1988) Three glutamate ionotropic receptor subtypes, the *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, have been proposed based on preferential agonist compounds. AMPA and kainate receptors are sometimes grouped as non-NMDA. The NMDA receptor has a complex pharmacology, with binding sites for glutamate, glycine, cation channel blockers and polyamines (Nakanishi, 1992). Glycine binding potentiates NMDA receptor activation (Johnson and Archer, 1987) and drugs acting on this site have been proposed to produce more selective effects than NMDA receptor competitive antagonists (Kemp and Leeson, 1993).

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Systemic or intra-dorsolateral PAG administration of NMDA-receptor antagonists produces anxiolytic-like effects in several animal models of anxiety, including the elevated plus-maze (EPM), brain aversive electrical stimulation and Vogel's punished licking test (Stephens et al., 1986; Matheus et al., 1994; Russo et al., 1993; Molchanov and Guimarães, 2002). Similar effects have also been reported after intra-amygdaloid administration (Maren et al., 1996).

The DMH has significant levels of glutamate receptors (Meeker et al., 1994). However, although anxiolytic effects of midazolam, a benzodiazepine agonist, have been showed after intra-DMH injection, 2-amino-7-phosphonoheptanoic acid (AP7), a NMDA receptor antagonist, failed to decrease anxiety after injection into this nucleus (Jardim and Guimarães, 2001). Instead, it decreased exploratory activity in the EPM. The mechanisms involved in this decreased exploratory activity have not been explored but it may have prevented the uncovering of anxiolytic effects in the EPM (File, 1992).

The objectives of the present work were to verify if (i) AP7 injections into the DMH would also fail to induce anxiolytic effects in another model of anxiety, the Vogel's punished licking test; (ii) injection into the DMH of other glutamate ionotropic antagonists would also decreased exploratory behavior in the EPM; and (iii) the decrease in exploratory activity found after AP7 administration into the DMH does not involve any gross locomotor impairment.

2. Methods

2.1. Subjects

Male Wistar rats weighing 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.). Independent groups of animals were used for each drug or dose tested. 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.

2.2. Drugs

AP7 (2 nmol, Ciba-Geigy), 7-chlorokynurenic acid (7ClKy), a competitive antagonist of NMDA-linked glycine sites (Stone and Burton, 1988, 4–8 nmol, Tocris, USA) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[*f*]-quinoxaline-7-sulphonamide (NBQX), an antagonist of non-NMDA glutamate ionotropic receptors (1–10 nmol, Tocris, USA) were dissolved in sterile saline or DMSO. The doses were chosen based on previous results obtained after intracerebral injections (Jardim and Guimarães, 2001; Guimarães et al., 1991; Matheus et al., 1994; Matheus and Guimarães, 1997; Molchanov and Guimarães, 2002). Diazepam (3 mg/kg i.p., Roche) was used as a positive control in the Vogel and rota-rod tests and haloperidol (2.5 mg/kg i.p., Janssen) as a positive control in the catalepsy test.

2.3. Behavioral tests

The EPM experiments were 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 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 prevent falls. Rodents naturally avoid the open arms of the elevated plus maze and anxiolytic compounds typically increase the exploration of these arms without changing the number of enclosed arm entries (File, 1992). The Ethovision software (V. 9, Noldus, Netherlands) was employed for behavioral analysis in the EPM. It detects the position of the animal in the maze and calculates the number of entries and time spent in open and enclosed arms. For these calculations, a 6-cm large "exclusion" zone was added between the center of the maze and each arm so that most of the animal's body should be in the open or enclosed arm for an entry to be registered. The software also calculated the total distance moved by the rat in the maze. Each session lasted for 5 min and after each trial the maze was cleaned with an alcohol solution.

The Vogel test (Vogel et al., 1971) was performed in an apparatus placed in a sound-attenuated cage. It consisted of a Plexiglas box (42×25×20 cm) with a grid floor made of stainless steel bars. The metallic spout of a drinking bottle containing water projected into the box. The contact of the animal with the spout and the grid floor closed an electrical circuit controlled by a sensor (Anxio-Meter model 102, Columbus, USA), which produced 7 pulses/s whenever the animal was in contact with both components. Each pulse was considered as a lick, and every 20 licks the animal received a 0.5-mA shock for 2 s. The number of licks were recorded for 3 min. Catalepsy was evaluated by placing the animal with both forelegs over a horizontal glass bar (diameter: 0.5 cm), elevated 9.0 cm from floor. The time (in sec) during which the rat maintained this position was recorded up to 180 s (Zarindast et al., 1993). The Rota-rod test was performed in a constant speed (8 rpm) model (Ugo-Basile 7700, Italy). The animals were submitted to three consecutive training trials 5 min before drug injection. The latency to fall from the apparatus was recorded for each animal up to a maximum of 120 s.

2.4. Surgery

Rats were anesthetized with 2.5% 2,2,2-tribromoethanol (10 ml/kg i.p.) and fixed in a stereotaxic frame. The position of the incisor bar was −3.5 mm from the horizontal plane. A

unilateral stainless steel guide cannula (0.7 mm OD) aimed at the DMH (coordinates: A: −3.0 mm from bregma, L: 0.6 mm, D: 7.2 mm bellow the surface of skull) was introduced. The cannula was attached to the bones with stainless steel screws and acrylic cement. An obturator inside the guide cannulae prevented obstruction.

2.5. Procedure

Seven days after the surgery, the animals were randomly assigned to one of the treatment groups. In the punished licking, catalepsy and rota-rod tests the animals received intra-DMH injection of isotonic saline or AP7 (2 nmol) and were tested 10 min later. The number of animals receiving saline or AP7 in each test was as follows: punished licking: 12 and 14, respectively; rota-rod: 8 and 9, respectively; catalepsy: 4 and 5, respectively. As positive controls, additional groups received i.p. injections of vehicle (punished licking test, n=10; rota-rod, n=8, catalepsy, n=5), diazepam (3 mg/kg, punished licking test, n=9; rota-rod, n=8) or haloperidol (0.5 mg/kg, n=7) and were tested 20 min later.

In the experiments using the EPM, animals received an intra-DMH injection of 7ClKy (4 or 8 nmol, n=9 and 5, respectively), NBQX (1, 3 or 10 nmol, n=16, 10 and 7, respectively) or vehicle (DMSO, n=12 and 13, respectively). Ten minutes later, they were placed in the center of the EPM facing an enclosed arm. The number of entries and time spent on open and enclosed arms were recorded during 5 min.

Intra-cerebral 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 0.25 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 apparatus was cleaned with an alcohol solution. Vehicle and drug-treated groups were always run in parallel.

2.6. Histology

After the 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, the dental needle was inserted through the guide cannula and a 0.2-μl micro-injection of 1% 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 40 μm were obtained in a cryostat (Cryocut 1800). Injection sites were localized with the help of the Paxinos and Watson's (1997) rat brain atlas. Except for Experiment 1, the number of animals that received drug injections outside the DMH was small (less than four per group) and these animals were discarded from analysis.

2.7. Statistical analysis

In 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, the number of enclosed arm entries and total distance moved, were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan test for multiple comparisons. In the punished licking test, the groups were compared by Student's t-test. Catalepsy data were analyzed by a repeated measure MANOVA. Data from the rota-rod were analyzed by Mann–Whitney test. The significance level was set at $p<0.05$.

3. Results

Representative injection sites in the DMH can be seen in Fig. 1. In the Vogel test, diazepam significantly increased the number of punished licks ($t_{10.21}$=2.79, p=0.02, Fig. 2). AP7, however, failed to do so (t_{24}=0.69, NS, Fig. 2). AP7 injections outside the DMH also did not change the number of punished licks (t_{17}=0.25, NS).

In the EPM, 7ClKy (4 nmol) significantly reduced the percentages of entries ($F_{2,23}$=4.79, $p<0.05$) and time spent ($F_{2,23}$=4.12, $p<0.05$) in the open arms of the EPM (Fig. 3). The higher dose failed to change these parameters. Although no change in the number of enclosed arm entries was found ($F_{2,23}$=1.99, p=0.16), both doses of 7ClKy decreased the total distance moved in the apparatus ($F_{2,23}$=10.44, $p<0.05$, Fig. 3).

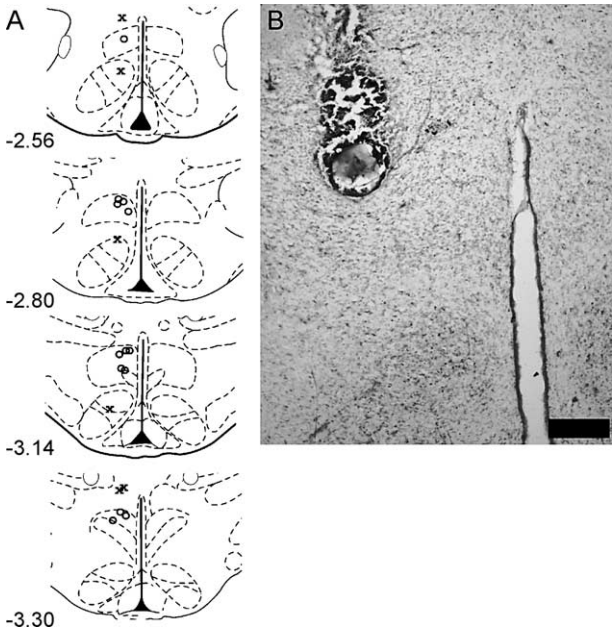

Fig. 1. (A) Histological localization of representative injection sites (circles) in diagrams based on the atlas of Paxinos and Watson (1997). Injection sites outside (×) the dorsomedial hypothalamic nucleus were also displayed. (B) Photomicrography of an injection site in the dorsomedial hypothalamic nucleus (bar=250 μm).

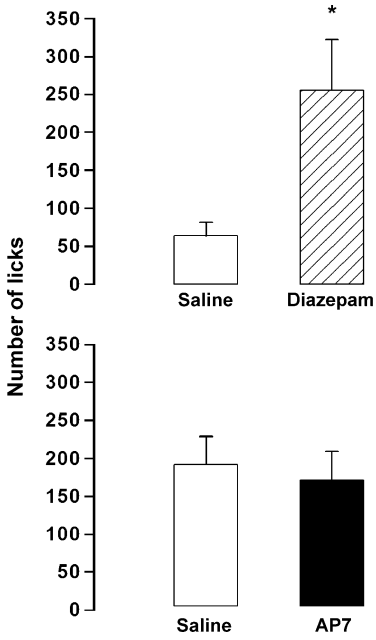

Fig. 2. Effects of diazepam (upper panel) or AP7 (lower panel) on the number of licks in the punished licking test. Animals received i.p. injection of saline (n=10) or diazepam (3 mg/kg, n=9), or intra-DMH injection of saline (0.25 μl, n=12) or AP7 (2 nmol, n=14), and were tested 30 or 10 min later, respectively. Results were expressed as mean (+S.E.M.) number of licks in the whole 3-min session. * indicates significant different (p<0.05, t-test).

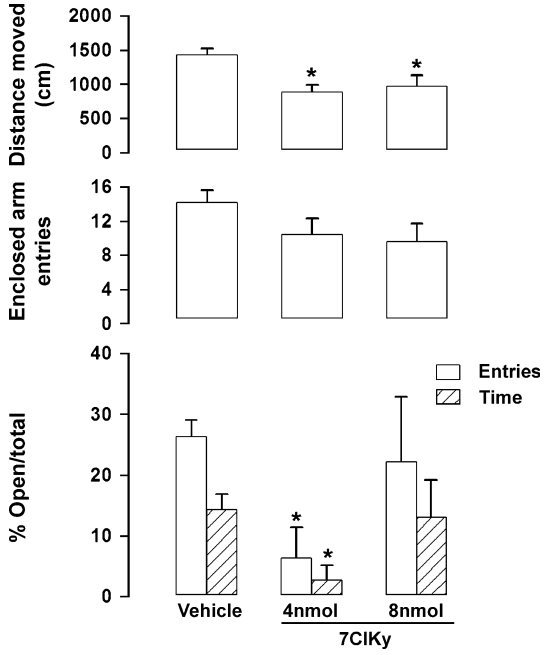

Fig. 3. Effect of 7ClKy (4 nmol, n=9; 8 nmol, n=5) or vehicle (0.25 μl, n=12) microinjected into the dorsomedial hypothalamus of rats tested on the elevated plus-maze. Columns represent the mean (+S.E.M.). In the upper panel, open columns refer to the total distance moved in the maze. In the middle panel, 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 on open arms. * indicates significant difference from vehicle-treated group (ANOVA followed by the Duncan test, p<0.05).

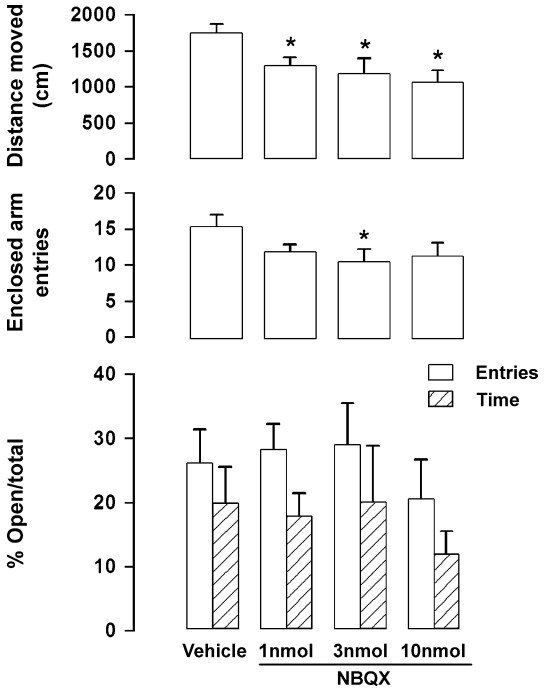

Fig. 4. Effect of NBQX (1 nmol, n=16; 3 nmol, n=10; 10 nmol, n=7) or vehicle (0.25 μl, n=13) microinjected into the dorsomedial hypothalamus of rats tested on the elevated plus-maze. Columns represent the mean (+S.E.M.). Further specifications as in Fig. 3.

NBQX did not change the percentage of entries and time spent in the open arms ($F_{3,42}$=0.36, p=0.78 and $F_{3,42}$=0.32, p=0.81, respectively, Fig. 4). The dose of 3 nmol, however, reduced the number of entries into the enclosed arms

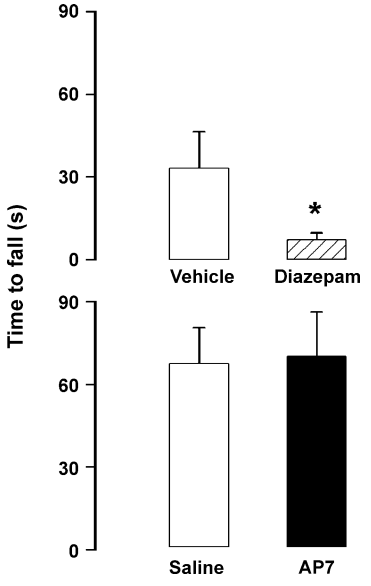

Fig. 5. Effects of diazepam (upper panel) or AP7 (lower panel) on the latency to fall from the rota-rod. Animals received i.p. injection of vehicle (n=8) or diazepam (3 mg/kg, n=8), or intra-DMH injection of saline (0.25 μl, n=8) or AP7 (2 nmol, n=9), and were tested 30 or 10 min later, respectively. Results were expressed as means (+S.E.M.). * indicates significant different from control (p<0.05, t-test).

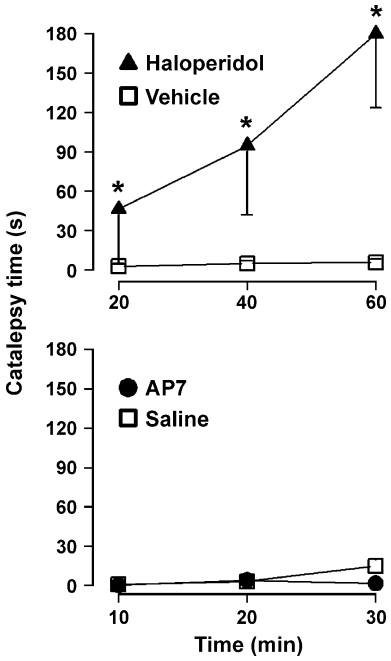

Fig. 6. Effects of haloperidol or AP7 (lower panel) on catalepsy time. Animals received i.p. injection of vehicle (n=5) or haloperidol (2.5 mg/kg, n=7), or intra-DMH injection of saline (0.25 μl, n=4) or AP7 (2 nmol, n=5), and were tested 30 or 10 min later for a period of 30–60 min, respectively. Results were expressed as means (+S.E.M.). * indicates significant different from control (p<0.05, t-test).

($F_{3,42}$=2.07, p<0.05). All doses reduced the total distanced moved in the maze ($F_{3,42}$=3.7, p<0.01, Fig. 4).

Diazepam (3 mg/kg) decreased the latency to fall in the rota-rod (p=0.01, Mann–Whitney, Fig. 5), whereas haloperidol increased catalepsy time (p<0.05, Fig. 6). AP7 did not produce any effect in the two tests (p>0.05, Figs. 5 and 6).

4. Discussion

The present results showed that intra-DMH administration of AP7, a selective NMDA-receptor antagonist, did not change the number of punished licks in the Vogel's punished licking test, an animal model of anxiety (Vogel et al., 1971). The test, however, was sensitive to detect anxiolytic-like effects of systemically injected diazepam, used as a positive control. These results contrast with the anxiolytic effects of NMDA-receptor antagonists detected after either systemic (Stephens et al., 1986) or intra-PAG or amygdala administration (Guimarães et al., 1991; Matheus et al., 1994, Maren et al., 1996). They agree, however, with the lack of anxiolytic results using the EPM found after intra-DMH injection of AP7 (Jardim and Guimarães, 2001). Moreover, in the present study, 7ClKy, a NMDA-receptor glycine-site antagonist, and NBQX, a non-NMDA receptor antagonist, also failed to induce any anxiolytic effect in the EPM.

Factors such as the AP7 dose used (2 nmol) and the interval between injection and testing (10 min) could have contributed to the lack of drug effect. However, AP7, at the same dose and interval employed in the present work, produced anxiolytic-like effects in the Vogel and EPM models after intra-cerebral injection into the dorsolateral PAG (Molchanov and Guimarães, 2002; Guimarães et al., 1991). Moreover, a lower dose of AP5, a less potent NMDA-receptor antagonist, was able to block NMDA-induced cardiovascular changes in the DMH (Soltis and DiMicco, 1992).

Another factor that might have contributed to the lack of anxiolytic drug effect was the unilateral administration employed, since lack of NMDA effects have been previously reported in this situation (De Novellis et al., 1995). However, in a previous study using similar unilateral DMH injection, the drug was able to decrease exploratory activity (Jardim and Guimarães, 2001). Finally, although the rota-rod and catalepsy tests were sensitive to detect the effects of the positive controls diazepam and haloperidol, no AP7 effect was demonstrated in these models. This suggests that gross motor impairment was not responsible for the lack of anxiolytic effect.

Several pieces of evidence suggest that the DMH plays an important role in the generation of behavioral, autonomic and neuroendocrine responses to threatening stimuli (Bailey and DiMicco, 2001). Defensive responses are seen after DMH stimulation by glutamate agonists (Silveira and Graeff, 1992; Bailey and DiMicco, 2001) and high concentrations of all major glutamate receptors are present in this region (Meeker et al., 1994). GABA antagonism in the DMH also induces defensive reactions, indicating a tonic inhibitory role of this neurotransmitter in this region (Soltis and DiMicco, 1991; De Novellis et al., 1995). Chronic inhibition of GABA synthesis in the DMH has recently been shown to facilitate panic-like lactate-induced changes in animals, an effect that was prevented by systemic administration of a selective agonist of group II metabotropic glutamate receptor, which inhibits glutamate release (Shekhar and Keim, 2000).

It has been suggested that distinct subsets of neurons could mediate different aspects of the defensive responses mediated by the DMH (Bailey and DiMicco, 2001). The present results, showing lack of anxiolytic effects of glutamate antagonists injected into the DMH, coupled with the reported anxiolytic effect of midazolam under similar conditions (Jardim and Guimarães, 2001), favor this possibility.

Besides not inducing any anxiolytic effects in the EPM, 7ClKy and NBQX decreased the total distance moved in this apparatus. Moreover, the former compound, at the lower dose used, decreased the percentage of time spent in the open arms, which is usually interpreted as an anxiogenic effect (File, 1992). Although an anxiogenic effect can clearly not be ruled out by the present study, the interpretation of this result is complicated by the lack of a dose–response relationship and the sensitivity of the EPM model to interference with general exploratory activity (File,

1992). The number of enclosed arm entries is usually employed to measure this latter parameter (File, 1992). Our results, however, suggest that the total distance moved could be a better index of this activity.

The mechanisms of this decreased exploratory activity are still not clear. Glutamate injection into the DMH can initiate locomotion in anesthetized rats (Marciello and Sinnamon, 1990) and kainic acid increased locomotor activity in this region (Bailey and DiMicco, 2001). Carrive (2002) has recently reported that temporary inactivation of the DMH and adjacent perifornical region, besides blocking fear responses, produce a quiescent state suggesting that level of arousal of the animals had been dramatically reduced. In addition to defensive responses, the DMH has been linked to several other brain functions, and hypoactivity, hypophagia and hypodipsia have been reported after DMH lesions (Bernardis and Bellinger, 1998). Interference with other motivational drives, therefore, could be involved.

In conclusion, our results indicate that antagonism of NMDA and non-NMDA glutamate receptors in the DMH fails to produce anxiolytic-like effects evaluated in the EPM or the punished licking test, but decreases exploration of new environments. This latter effect does not involve gross locomotor dysfunction detectable by the catalepsy and rotarod tests.

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