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A comparative study on the effects of the benzodiazepine midazolam and the dopamine agents, apomorphine and sulpiride, on rat behavior in the two-way avoidance test

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ABSTRACT

In recent years, studies in behavioral pharmacology have shown the involvement of dopaminergic mechanisms in avoidance behavior as assessed by the two-way active avoidance test (CAR). Changes in dopaminergic transmission also occur in response to particularly threatening challenges. However, studies on the effects of benzodiazepine (BZD) drugs in this test are still unclear. Given the interplay of dopamine and other neurotransmitters in the neurobiology of anxiety and schizophrenia the aim of this work was to evaluate the effects of systemic administration of midazolam, the dopaminergic agonist apomorphine, and the D_2 receptor antagonist sulpiride using the CAR, a test that shows good sensitivity to typical neuroleptic drugs. Whereas midazolam did not alter the avoidance response, apomorphine increased and sulpiride reduced them in this test. Escape was not affected by any drug treatments. Heightened avoidance was not associated with the increased motor activity caused by apomorphine. In contrast with the benzodiazepine midazolam, activation of post-synaptic D_2 receptors with apomorphine facilitates, whereas the D_2 receptor antagonism with sulpiride inhibited the acquisition of the avoidance behavior. Together, these results bring additional evidence for a role of D_2 mechanisms in the acquisition of the active avoidance.

1. Introduction

Changes in the neural system that mediate fear cognition may lead to psychiatric disturbances associated with anxiety and schizophrenia. In this respect, there has recently been an increase in the number of mechanisms suggested to explain the pathogenesis, modulation, and treatment of mental disorders, particularly anxiety and schizophrenia. Interest in the modulation of fear and anxiety by dopaminergic mechanisms has grown steadily in recent years (see Millan, 2003, for review). It seems that changes in dopaminergic transmission occur in response to particularly threatening challenges. For example, with the use of the conditioned avoidance response test (CAR) it has been shown that dopaminergic mechanisms are involved in avoidance behavior, but not in escape responses (Baldessarini, 1996; Wadenberg and Hicks, 1999).

One issue of potential therapeutic relevance is the interaction between benzodiazepine and dopaminergic mechanisms, given the involvement of both in the neurobiology of stress, which has been reported to underlie processes involved in anxiety and schizophrenia. In this respect, there is evidence that benzodiazepines counteract the increase in dopamine in the prefrontal cortex during context-conditioned freezing (Ida et al., 1989; Wedzony et al., 1996) and that aversive stimulation of structures belonging to the so-called brain aversion system, such as the dorsal periaqueductal gray (dPAG) and inferior colliculus, enhances dopamine release in the prefrontal cortex (Cuadra et al., 2000, 2001). The importance of such findings is linked to the fact that whereas benzodiazepines show "anxiolytic-like effects" in many animal models of anxiety, only a few studies have clearly shown such effects with the use of agonists or antagonists of dopamine (DA) receptors (Pich and Samanin, 1986; Puglisi-Allegra and Cabib, 1988; Oliveira et al., 2006).

Considering that several animal models based in the aversive conditioning are sensitive to the "anxiolytic-like action" of benzodiazepines it is of interest to examine the extent to which anxiety may underlie the acquisition of CAR, a test that shows good sensitivity to typical neuroleptic drugs. In this test, animals have a conditioned avoidance response to a learned sensory cue that signals the onset of punishing shock, which is avoidable by moving to a safe place in an experimental chamber. The avoidance response of this test is considered to represent a complex response of the conditioned reaction and appear to have an element of Pavlovian-conditioned fear arousal (Gray and Mcnaughton, 2000). Under the influence of neuroleptics animals tend to ignore the warning signals but still attempt

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to escape once the shock is applied (Baldessarini, 1996). Thus, this study examines the extent to which rats in the two-way avoidance test, which measures learned fear reactions to neutral stimuli associated with unconditioned fear stimuli, respond to the anxiolytic compound midazolam and to the dopaminergic agonist apomorphine (Ljungberg and Ungerstedt, 1977; Creese et al., 1983), and the D_2-selective antagonist sulpiride (Standish-Barry et al., 1983; White and Wang, 1984; Guarraci et al., 2000) in the two-way active avoidance test.

2. Materials and methods

2.1. Animals

Sixty-four male Wistar rats from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were used. These animals, weighing 230–260 g, were housed in groups of four in Plexiglas-walled cages. They were maintained under a 12-h dark/light cycle (lights on at 0700 h) in a temperature-controlled environment (22±1 °C) and were given free access to food and drinking water throughout the experiment. All animals were experimentally naive. The experiments reported in this article were carried out according to protocols approved by the ethical review committee of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, complied with the recommendations of the Brazilian Society for Neuroscience and Behavior, which are based on the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Two-way active avoidance test

The experimental chamber consisted of a shuttle box comprising two compartments 57×35×31 cm (Insight Brazil), which were divided by a Plexiglas bar of 3 cm. The side and back walls of the chamber were constructed of gray Plexiglas and the ceiling and the front wall were made of transparent Plexiglas. The chamber was equipped with a compartmentalized electrifiable grid floor with 15 stainless steel rods, 2.0 mm in diameter and spaced 1.2 mm apart, and infrared beams placed in the center of the rear wall and sensors in the front wall. Thus, the shuttle behavior of test animals was measured quantitatively during the session by counting the number of times the beam was interrupted by the passage of the animals from one side of the shuttle box to the other. This arrangement allowed detection of the shuttle locomotion of the rat in addition to its gross locomotor activity within each compartment. The foot shocks were delivered through the floor of the test cage by a constant current generator built with a scrambler (Insight Instruments, Brazil). Two 30 W light bulbs were centered on each side of the rear of the chamber. The light was turned on and off noiselessly. The experimental chamber was located within a small, ventilated room (2.5×2.5×1 m). The behavior of the animals during the testing sessions was recorded with a video camera (Everfocus, Duarte, CA) positioned in the lateral wall of the observation chamber, thus allowing the discrimination of all behavior. The signal was relayed to a monitor located in an adjacent room via a closed circuit.

2.3. Procedure

The procedure and tests used in this study were as currently used in this laboratory (Reis et al., 2004). The animals were placed inside the shuttle box and left for 5 min for acclimatization to the experimental context before the start of the session. Each session consisted of 40 associations of a conditioned stimulus (light-CS) and an unconditioned stimulus (foot shock-US, 0.6 mA) during which each animal was submitted to 20 s of CS with the US presented for 10 s, always at the end of each CS presentation. The light stimulus produced an illumination level of approximately 120 lx, as measured at the level of the floor of the cage with a Lutron luxmeter (LX 103; Lutron, Coopersburg, PA). Two successive trials were separated by a random interval of 10 to 50 s, with an average of 30 s. Whenever a rat passed from one compartment to the other during the illumination, it avoided the foot shock; if it changed compartments during the foot shock, then the stimulation was automatically terminated. Thus, avoidance and escape responses always had latencies below 10 s. The software and an appropriate interface connected to a PC provided by the manufacturer of the equipment (Insight) permitted recording and analysis of the frequencies of avoidance and escape responses and also the inter-trial locomotor activity. The presentation and sequencing of the light stimuli were controlled by the same software, which allowed data to be collected in blocks of 10 trials during the session.

2.4. Drugs

Midazolam hydrochloride (Merck, Brazil) and apomorphine hydrochloride (Sigma) were dissolved in physiological saline (0.9%) shortly before use. (±) sulpiride (Sigma) was dissolved in saline containing Tween 2%. For each drug, the animals were randomly assigned to three groups: midazolam (saline, 0.5 mg/kg and 1.0 mg/kg); apomorphine (saline, 0.5 mg/kg and 1.0 mg/kg) and sulpiride (saline, 10 and 20 mg/kg). The injections of midazolam and sulpiride were given 15 min before the sessions. Apomorphine was given 5 min before the sessions. The doses of the drugs were administered at a constant volume of 1 ml/kg intraperitoneally (i.p.). Drug doses and times of injections were based on previous studies from this and other laboratories (Guarraci et al., 2000; Furlan and Brandão, 2001; Garcia et al., 2005). Each animal was subjected to only one of the treatments and to a single test session.

2.5. Analysis of results

Data are reported as mean+S.E.M. Frequencies of avoidance or interruptions of shocks (escape), and inter-trial responses across the four blocks of trials were subjected to a two-way ANOVA with repeated measures (RM two-way ANOVA) using drugs as the between factor and blocks of 10 trials each as the within-group repeated-measures factor. RM two-way ANOVA was applied on the data of the saline, lower apomorphine dose (0.5 mg/kg) and lower sulpiride dose (10 mg/kg) groups. RM two-way ANOVA was also performed on the data of the saline, higher apomorphine dose (1.0 mg/kg) and higher sulpiride dose (20 mg/kg) groups. Statistical significance for the data obtained from the midazolam groups (saline, 0.5 and 1.0 mg/kg) were determined by similar analysis. Escape ratios were calculated as the number of interruptions of shocks divided by the sum of escape and no-escape [escape/(escape+failure to escape)]. Post hoc differences between group means were tested with the Bonferroni test. The number of animals was 8 per group. A p-value of less than 0.05 was considered significant. Relationships between avoidance responses and intertrial activity were examined with the Pearson's correlation test.

3. Results

In experiments with all three drugs the control rats demonstrated increased avoidance responses across blocks, indicating significant learning of the light/foot shock association. Fig. 1A shows the mean frequency of avoidance responses of the three groups injected with saline, apomorphine 0.5 mg/kg and sulpiride 10 mg/kg across the blocks. Two-way ANOVA showed that there were significant differences between treatments ($F_{2,63}=3.59$; $p<0.05$) and blocks ($F_{2,63}=14.96$; $p<0.05$). These changes across blocks varied as a function of drug treatment ($F_{6,63}=2.35$; $p<0.05$). Post hoc comparisons showed that learning was significantly enhanced by apomorphine 0.5 mg/kg. As can be seen in Fig. 1B, treatments did not change the escape ratio ($F=0.52$, 1.59, and 1.14 for treatments, blocks, and interaction between treatments and blocks, respectively; $p<0.05$ in all cases). Under our experimental conditions, there were only a few

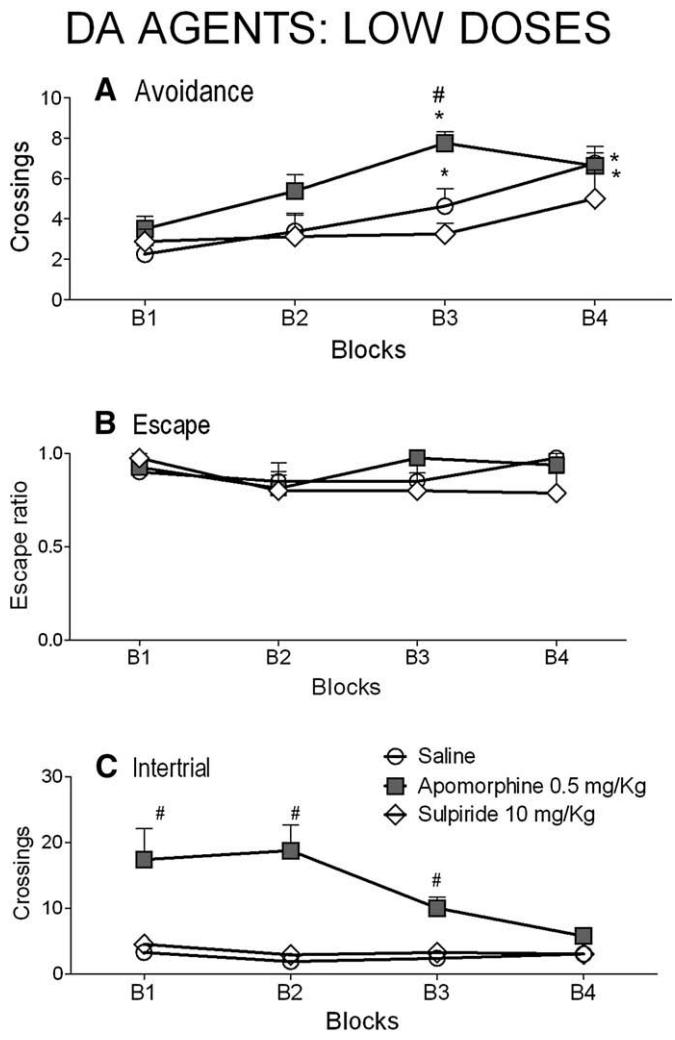

Fig. 1. Numbers of avoidance responses (A—Avoidance), escape ratio (B—Escape), and inter-trial periods (C) across B1–B2–B3–B4 (blocks of 10 trials each) during sessions with independent groups of rats injected with saline, apomorphine 0.5 mg/kg or sulpiride 10 mg/kg and submitted to 40 trials of conditioning with foot shocks paired with a neutral conditioned stimulus (box illumination). Escape ratios were calculated as the number of interruptions of shocks divided by the sum of escape and failure to escape [escape/(escape+no-escape)]. Columns represent the means and bars the SEM. $^{*}p<0.05$ in relation to the same treatment in the first block (B1) and $^{\#}p<0.05$ in relation to saline group in the same block; Bonferroni post hoc comparisons. $N=8$ for all groups.

failures to respond to both light and foot shocks. Apomorphine also caused significant increases in motor activity in the first two blocks ($F=16.10$, 5.57, and 5.21 for treatments, blocks, and interaction between treatments and blocks, respectively; $p<0.05$ in all cases). The effects of apomorphine vanished by the fourth block, probably because of the short duration of action (30 min) of this dopaminergic agonist (Fig. 1C). There was no correlation between inter-trial crossing and avoidance responses in the first, third and fourth blocks ($r=0.42$, 0.54, 0.51; N.S.) but there was a trend towards a positive correlation in the third block ($r=0.65$, $p=0.08$). Sulpiride 20 mg/kg did not have any significant effects on motor activity.

Fig. 2A shows the mean frequency of avoidance responses of the groups injected with saline, apomorphine 1.0 mg/kg and sulpiride 20 mg/kg, across the session blocks. Two-way ANOVA showed that there were significant differences between treatments ($F_{2,63}=12.15$; $p<0.05$) and blocks of 10 trials ($F_{2,63}=9.85$; $p<0.05$). These changes across blocks varied as a function of drug treatment ($F_{6,63}=5.70$; $p<0.05$). Post hoc comparisons showed that learning was significantly increased by apomorphine 1.0 mg/kg and reduced by treatment with 20 mg/kg of sulpiride. As can be seen in Fig. 2B, treatments did not cause significant changes in the escape ratio ($F=0.39$, 0.73, and 0.47 for treatments, blocks, and interaction between treatments and blocks, respectively; N.S. in all cases). Apomorphine also caused significant increases in motor activity in the first two blocks ($F=20.63$, 4.18, and 4.20 for treatments, blocks, and interaction between treatments and blocks, respectively; $p<0.05$ in all cases). There was no correlation between inter-trial crossing and avoidance responses in all four blocks ($r=0.02$, 0.22, 0.32 and 0.16; N.S.). The effects of apomorphine vanished by the fourth block, probably because of the short duration of action (30 min) of this dopaminergic agonist (Fig. 2C). Sulpiride 20 mg/kg did not have any significant effects on motor activity.

Fig. 3A shows the mean frequency of avoidance responses of the groups treated with midazolam. Two-way ANOVA with repeated measures revealed that there was no significant effect of treatments ($F_{2,63}=0.56$; N.S.). However, there was a significant effect of blocks ($F_{3,63}=14.20$; $p<0.05$), without any significant interaction between

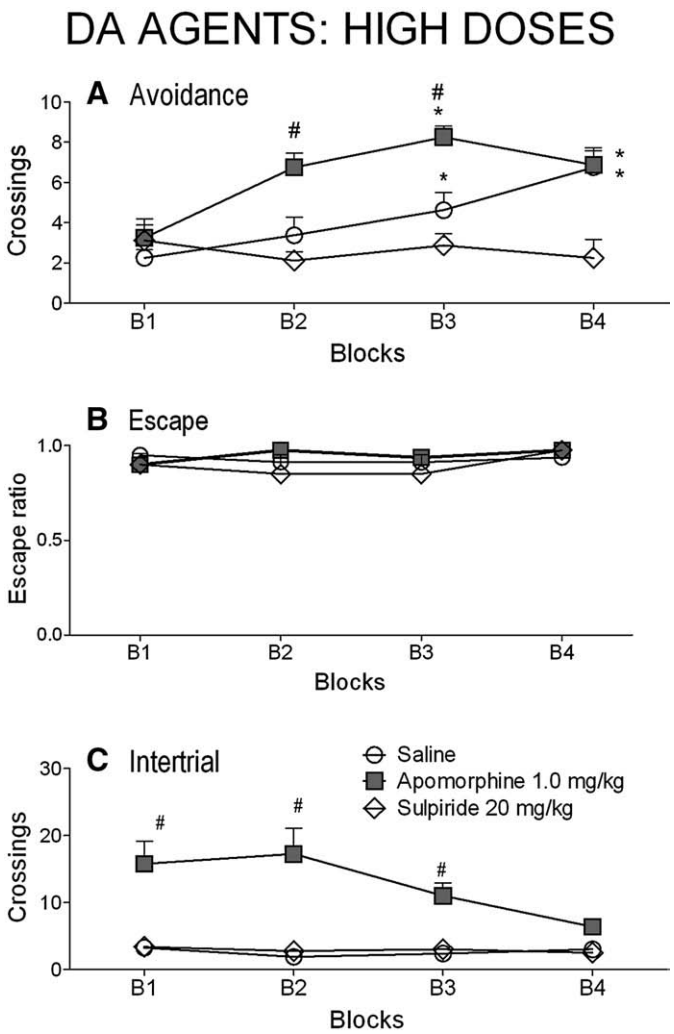

Fig. 2. Mean±SEM of number of crossings during light-CS (A—Avoidance), escape ratio (B—Escape), and inter-trial periods (C) across B1–B2–B3–B4 (blocks of 10 trials each) during sessions with independent groups of rats injected with saline or apomorphine 1.0 mg/kg and sulpiride 20 mg/kg and submitted to 40 trials of conditioning with foot shocks paired with a neutral conditioned stimulus (box illumination). Escape ratios were calculated as the number of interruptions of shocks divided by the sum of escape and failure to escape [escape/(escape+no-escape)]. Columns represent the means and bars the SEM.$^{*}p<0.05$ in relation to the same treatment in the first block (B1) and $^{\#}p<0.05$ in relation to saline group in the same block; Bonferroni post hoc comparisons. $N=8$ for all groups.

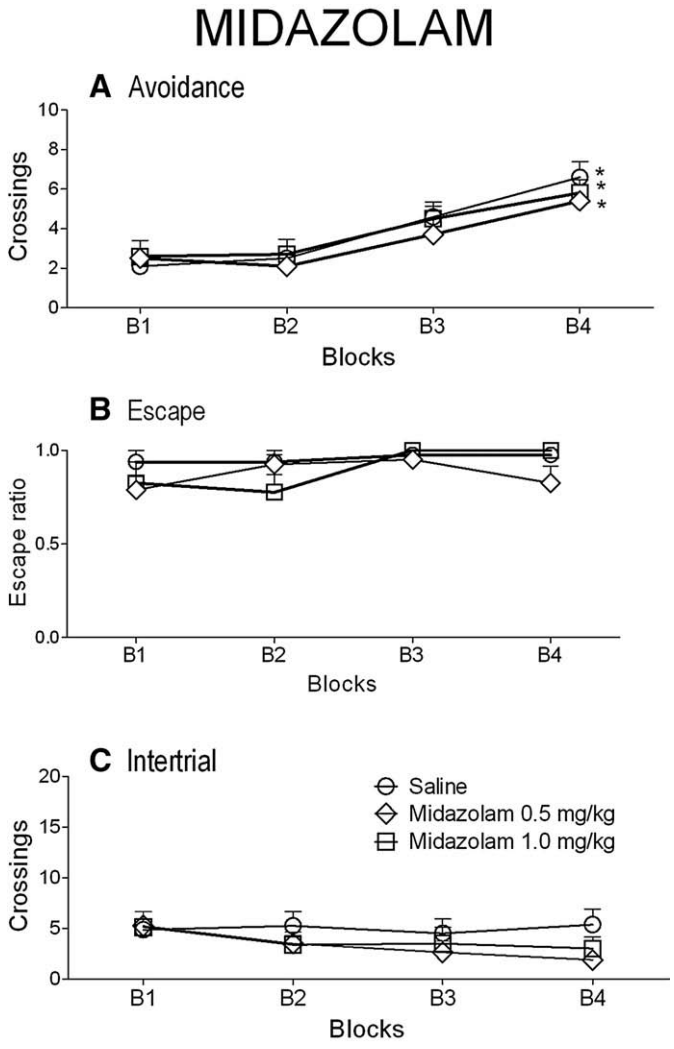

Fig. 3. Mean ± SEM of number of crossings during light-CS (A—Avoidance), escape ratio (B—Escape), and inter-trial periods (C) across B1–B2–B3–B4 (blocks of 10 trials each) during sessions with independent groups of rats injected with saline or midazolam 0.5 mg/kg or 1.0 mg/kg and submitted to 40 trials of conditioning with foot shocks paired with a neutral conditioned stimulus (box illumination). Escape ratios were calculated as the number of interruptions of shocks divided by the sum of escape and failure to escape [escape/(escape+no-escape)]. Columns represent the means and bars the SEM. $^{*}p<0.05$ in relation to the same treatment in the first block (B1); Bonferroni post hoc comparisons. $N=8$ for all groups.

treatments and blocks ($F_{6,63}=0.78$; N.S.). Post hoc comparisons showed that the significant effects were due to increases in avoidance responses in all three groups in the fourth block. As can be seen in Fig. 3B, treatments did not cause significant changes in the escape ratio ($F=1.37$, 2.12, and 1.55 for treatments, blocks, and interaction between treatments and blocks, respectively; N.S. in all cases). Midazolam did not have any significant effects on motor activity (Fig. 3C).

4. Discussion

Recent evidence suggests that dopaminergic mechanisms are significant for different aspects of affective memory, namely its formation, expression, and retrieval (Pezze and Feldon, 2004). Thus, whereas it is known that DA-mediated mechanisms underlie CAR an involvement of anxiety-related processes in the acquisition of this response is still a matter of debate. This study was an attempt to go further in this direction, through comparison of the effects of single acute systemic administration of the benzodiazepine agent midazolam and the dopaminergic compounds apomorphine and sulpiride in rats submitted to single sessions of the CAR test. Rats of the control groups injected with saline showed movement to the safe compartment of the shuttle box on presentation of the light, indicating that they acquired the CAR. Apomorphine clearly enhanced the avoidance responses to the light-CS, which signaled the incoming foot shocks, by increasing the frequencies of responses to avoid them. Heightened attentional and cognitive function of dopaminergic mechanisms have been proposed to set up adaptive responses to cope with or signal the presence of aversive stimuli. In fact, alterations in DA transmission always occur following exposure to a wide variety of acute stressors (Anisman et al., 1991; Goldstein et al., 1996). Interestingly, the number of crossings during the intertrial period also increased following administration of apomorphine, indicating that enhanced motor activity could underlie its effects on avoidance. However, whereas the heightened motor activity was significant in the first block of the session, the avoidance behavior started to be augmented from the second block onwards. In the fourth block, both effects disappeared in accordance with the average duration of action of apomorphine — of about 30 min (Furlan and Brandão, 2001; Mattingly et al., 2001; Reis et al., 2004). Other studies have also shown that dopaminergic mechanisms are associated with increased CAR learning independently of locomotor activity (Reis et al., 2004; Da Cunha et al., 2001).

The data obtained in the CAR test are consistent with the assertion that dopaminergic agonists strengthen while dopaminergic antagonists impair the acquisition of conditioned avoidance responses (Wadenberg and Hicks, 1999; Troncoso et al., 2003; Reis et al., 2004). Sulpiride reduced the frequency of avoidance responses relative to saline-injected animals. The observed effects of this dopaminergic D_2-receptor antagonist cannot be attributed to unspecific effects, as it did not affect the inter-trial locomotor activity. It is still a point of concern to determine the extent to which these effects of sulpiride can be related to the reported anxiolytic-like effects of this drug in the elevated plus-maze test (Rodgers et al., 1994), in punished drinking behavior (Pich and Samanin, 1986), and in the mouse hyperdefensiveness test (Puglisi-Allegra and Cabib, 1988). The combined activation of D_1 and D_2 receptors in the acquisition of conditioned avoidance responses has also been reported, since SCH 23390 — a selective D_1 receptor antagonist — was found to cause a significant decrease in the frequency of avoidance responses in a CAR test similar to the one used in the present study (Reis et al., 2004).

It has been suggested that dopaminergic mechanisms mediate conditioned avoidance behaviors — but not unconditioned escape — in the CAR test, probably because of the increase in sensitivity to conditioned aversive stimuli (Baldessarini, 1996). Neither apomorphine nor sulpiride changed the escape responses. Thus, an increase in the sensitivity or enhanced response to footshocks following the administration of these dopaminergic agents can be discarded. These findings confirm the results reported in other studies also using CAR (Troncoso et al., 2003; Reis et al., 2004) and come in support of the assertion that under the influence of neuroleptics, animals tend to ignore the warning signals but still attempt to escape once the shock is applied (Baldessarini, 1996).

The association between changes in DA transmission and threatening challenges has been demonstrated by numerous reports. Dopaminergic mechanisms have been related to the production and elaboration of acute and chronic stress (Feenstra et al., 1995; Kamei et al., 1995; Greba et al., 2001; Troncoso et al., 2003). However, instead of enhancing the acquisition of avoidance responses as shown with both doses of the DA agonist apomorphine in the present study an opposite pattern of results may be yielded depending on the conditioned fear paradigm. For instance, it has been reported that activation of dopaminergic-mediated mechanisms may decrease fear by impairing the retrieval of a learned association between a light-CS and a footshock-US (see Pezze and Feldon, 2004 for a review). Moreover, in contrast with the increase in the CAR, it has been reported that apomorphine causes “anxiolytic-like” effects in the

elevated plus-maze test, which has been considered a mixed model of conditioned and unconditioned fear. This DA agonist caused a selective increase in entries and time spent into the open arms and sulpiride, at the same doses as those used here, caused opposite effects (Rodgers et al., 1994; Garcia et al., 2005). Thus, the inhibitory role of D_2 mechanisms in this animal model of anxiety contrasts with its heightened effect on the avoidance conditioned responses. Considering the differences in the eliciting stimuli in the CAR and elevated plus-maze test, it has been suggested that these paradigms might indeed model different aversive states. In the case of the elevated plus-maze the increased exploratory activity produced by activation of the D_2-mediated mechanisms was attributed to several factors including enhanced drive to explore, heightened of the approach over the avoidance component in such conflict paradigm or even heightened motor activity (Garcia et al., 2005).

In a recent study in this laboratory, systemic administration of quinpirole — a specific D_2 receptor agonist — was found to reduce the expression of conditioned fear as a result of an association between light/foot shocks in the FPS paradigm (Oliveira et al., 2006). The complex picture emerging from studies using systemic manipulations may reflect that systemic manipulations act on DA mechanisms in different brain areas. Studies with local injections of DA agents into specific brain regions have helped to clarify the mechanisms relevant for the acquisition and expression of conditioned fear. In this respect, a recent study from this laboratory showed that quinpirole produced similar effects to those reported above when injected into the ventral tegmental area (VTA) only before the test session, but not before the conditioned sessions (Oliveira et al., 2009). Several other studies have shown that DA mechanisms of the VTA are involved in fear and that D_1 and D_2 receptors mediate the acquisition of Pavlovian-conditioned fear (Nader and LeDoux, 1999; Gifkins et al., 2002). Involvement of mesoamygdaloid DA in the organization of fear responses was suggested as an explanation of these results, based on the fact that avoidance behavior has an element of Pavlovian-conditioned fear arousal. Similar synergistic interactions between dopamine D_1 and D_2 receptors have been observed in other behavioral studies (Arnt et al., 1987; Kamei et al., 1995, Mattingly et al., 1998; Reis et al., 2004).

The anxiolytic-like actions of midazolam have been demonstrated in several studies using fear conditioning procedures (Hijzen and Slangen, 1989; Brodkin et al., 2002; Santos et al., 2005). However, the effects of this drug in the two-way avoidance are still unclear (Sanger, 1985; Fernandez-Teruel et al., 1991; Escorihuela et al., 1993; Çelik et al., 1999; Li et al., 2004; Miroslav et al., 2005). In the present study, contrasting with the dopaminergic drugs the benzodiazepine midazolam did not cause any significant effects on conditioned avoidance or escape responses. These results indicate that the underlying DA mechanisms implicated in the acquisition of conditioned avoidance are not shared with benzodiazepine-mediated processes.

In conclusion, apomorphine injections produced a dose-dependent increase in the number of avoidance responses in the CAR test. On the other hand, a reduction in these responses was observed with sulpiride administration. These findings have important implications for our understanding of the neurochemical mechanisms underlying the acquisition of avoidance behavior. They also shed some light on the mediation of affective states by dopaminergic mechanisms. In contrast with the benzodiazepine midazolam, activation of D_2 post-synaptic receptors with apomorphine facilitates, whereas the D_2 receptors antagonism with sulpiride inhibited the acquisition of the avoidance behavior. Together, these results bring additional evidence for a role of D_2 mechanisms in the expression of conditioned avoidance response.

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