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Dopaminergic mechanisms in the conditioned and unconditioned fear as assessed by the two-way avoidance and light switch-off tests

Fernanda L.V. Reis, Sueli Masson, Amanda R. de Oliveira, Marcus L. Brandão*

Laboratório de Psicobiologia, Departamento de Psicologia, FFCLRP-USP, Campus, Av Bandeirantes, 3900, 14049-901 Ribeirão Preto, Brasil

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Abstract

The involvement of dopaminergic mechanisms in fear and anxiety is still unclear. Behavioral studies aimed to disclose the involvement of dopamine in anxiety have reported anxiolytic-like, anxiogenic-like and lack of effects with the use of dopaminergic agonists and antagonists in animal models of anxiety. This work was an attempt to contribute to this field by providing evidence that these discrepancies may be due to the kind of aversive situation the animals experience in these models. The present study examined the effects of a dopaminergic agonist apomorphine, a dopaminergic D_1 antagonist SCH 23390 and a D_2 receptor antagonist sulpiride on the two-way avoidance response test (CAR) and on the switch-off responses to light (SOR). In both tests, learning was assessed by the performance of the animals across four blocks of 10 trials in which light was paired to footshocks (CAR) or only light was presented to the animals (SOR). The obtained data show that rats learn to make a shuttling response to avoid the shock in the CAR test and maintain a regular pace of switch-off responses in the SOR. While sulpiride and SCH 23390 administrations prevented learning of the avoidance responses, apomorphine injections produced a dose-dependent enhancement in the conditioned learning in the CAR test. The number of escape responses was unchanged by these drugs. In the light-induced switch-off test, apomorphine reduced the number of switch-off responses whereas sulpiride increased these responses. These findings suggest that the involvement of dopaminergic mechanisms in threatening situations depends on the nature of the aversive stimulus. Activation of D_1 and D_2 receptors seems to be implicated in the heightened aversiveness to conditioned stressful situations, as assessed by the CAR test. Thus, blockade of D_1 and D_2 receptors may be necessary for attenuating the aversiveness triggered by these conditioned fear stimuli. In contrast, mechanisms mediated by D_2 receptors seem to be involved in the setting up of adaptive responses to innate fear reactions. Therefore, the signal of the modulatory dopaminergic mechanisms on defensive behavior will depend on the type of emotional stimuli triggering the coping reaction.

Keywords: Dopamine; Apomorphine; Sulpiride; SCH 23390; Two-way avoidance; Light switch-off; Anxiety; Fear

1. Introduction

GABAergic, serotonergic, neuropeptides, glutamate-mediated and several other mechanisms have long been shown to be implicated in the modulation of fear and anxiety (see Brandão et al., 1994, 1999, 2003; Millan, 2003, for reviews). However, studies implicating dopaminergic mechanisms in the elaboration and production of aversive states in the brain are relatively recent. It seems that changes in dopaminergic transmission occur in response to particular threatening challenges. For instance, dopaminergic mechanisms have been reported to mediate conditioned avoidance behaviors but not the escape responses in two-way avoidance tests (Baldessarini, 1996). Although the precise neural circuit of the dopamine transmission involved in aversive states remains unclear, pharmacological and neurochemical studies have pointed to dopamine prefrontal neurons (Espejo, 1997; Morrow et al., 1999). Indeed,

* Corresponding author. Tel.: +55 16 602 3638; fax: +55 16 633 0619.
E-mail address: mbrandao@usp.br (M.L. Brandão).

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cortical dopamine projections are activated by several types of aversive stimulation (Anisman et al., 1991; Feenstra et al., 1995; Feenstra and Botterblom, 1996; Goldstein et al., 1996; Cuadra et al., 1999). In this way, it has been reported that benzodiazepines counteract the increase in dopamine in the prefrontal cortex during context conditioned freezing (Inoue et al., 1996). Besides, recent evidence has shown that aversive stimulation of structures belonging to the so-called brain aversion system, such as dPAG and inferior colliculus, enhances dopamine release in the prefrontal cortex (Cuadra et al., 2000).

The abovementioned findings could be taken as evidence for a secondary involvement of dopamine in aversive states elicited by stressful conditions. However, behavioral studies aimed to disclose the involvement of dopamine in anxiety have reported anxiolytic-like, anxiogenic-like and lack of effects with the use of dopaminergic agonists and antagonists in animal models of anxiety (Rodgers et al., 1994). Taking into account these reports, we thought that these conflicting results could be due to differences in the nature of the aversive stimuli currently used in the laboratory models of anxiety. In order to assess the dopaminergic mediation of aversive states, we examined the effects of dopaminergic agents in two tests: the two-way avoidance test (CAR) and the light-induced switch-off test (SOR). In the CAR, the animals make a conditioned avoidance response to a learned sensory cue that signals the onset of punishing shock avoidable by moving to a safe place in an experimental chamber. Under the influence of neuroleptics, animals tend to ignore the warning signals but still attempt to escape once the shock is applied (Wadenberg and Hicks, 1999). The avoidance response of this test is considered to represent a complex response of conditioned reaction (Gray and McNaughton, 2000). In the second case, the escape from illuminated areas is considered to be an innate response with an evolutionary basis; that is, rodents are nocturnal and are more vulnerable in the light (Crawley and Goodwin, 1980; Bourin and Hascoet, 2003). In this test, the animals could switch off the light by moving to the other compartment so that the light-induced switch-off response could be compared to the avoidance behavior of the CAR test. Although at least five DA receptor subtypes are now recognized (see Vallone et al., 2000; Millan, 2003, for reviews), the initial identification of D_1 and D_2 receptors (Kebabian and Calne, 1979) provided the major motivation for research aimed at defining particular functional roles for DA subtypes. In both tests, the drugs used were a selective D_2 dopaminergic agonist—apomorphine (Ljungberg and Ungerstedt, 1977; Creese et al., 1983), a D_1 selective antagonist SCH 23390 (Hytell, 1983; Fletcher and Starr, 1988; Ozer et al., 1997; Greba and Kokkinidis, 2000) and a D_2 selective antagonist sulpiride (Standish-Barry et al., 1983; White and Wang, 1984; Guarraci et al., 2000).

2. Materials and methods

2.1. Animals

One-hundred and forty-eight male Wistar rats from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were used. These animals, weighing 230–250 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 given free access to food and drinking water throughout the experiment. All animals were experimentally naive. The experiments reported in this article were performed in compliance with the recommendations of SBNeC (Brazilian Society of Neuroscience and Behavior), which are based on the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Apparatus

The experimental chamber consisted of a shuttle box comprising two compartments 30×25×25 cm (Insight, Brazil). The ceiling, side and back walls of the chamber were constructed of black Plexiglas and the front door was made of transparent Plexiglas covered with opaque paper. The experimental chamber was equipped with a compartmentalized flip-flop electrifiable grid floor with 15 stainless steel rods with 2.0 mm diameter spaced 1.2 mm apart. Thus, the shuttle behavior of test animals was quantitatively measured during the session by counting the number of times the floor moved over the fulcrum in the shuttle box. This arrangement allowed detecting the shuttle locomotion of the rat as well as its gross locomotor activity within each compartment. The foot-shocks were delivered through the test cage floor by a constant current generator built with a scrambler (Albarsh Instruments, Brazil). Two 28-V light bulbs were centered on each side of the rear of the chamber, 12 cm from the floor. 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 by a video camera (Everfocus, USA) positioned in the lateral wall of the observation chamber, thus allowing the discrimination of all behavior, with the signal relayed to a monitor located in an adjacent room via a closed circuit.

2.3. Procedure

The animals were placed inside the shuttle box and left for 5 min for acclimatization to the experimental context before the beginning of the session. Each session consisted of 40 associations of CS (light) and US (footshock—0.6 mA, 1 s) when each animal was submitted to 20 s of CS

and the US was presented for 10 s, always at the end of each CS presentation. The stimulus light produced an illumination level of approximately 120 lx, measured at the floor level of the cage with a Lutron luxmeter (LX 103; USA). Two successive trials were separated by a random interval from 10 to 50 s. Whenever a rat passed from one compartment to the other during the illumination, it avoided the footshock; if it changed compartments during the footshock, then the stimulation was automatically terminated. Then, avoidance and escape responses always had latencies below 10 and 20 s, respectively. The software and an appropriate interface connected to a PC provided by the manufacturer of the equipment (Insight) allowed for recording and analysis of the frequencies of avoidance and escape responses as well as the intertrial locomotor activity. The presentation and sequencing of the acoustic stimuli were also controlled by the same software, which also allowed collecting data in blocks of 10 trials during the whole session.

The same procedure was followed for the light-induced switch-off test with the only exception that no footshock was applied. Whenever a rat passed from one compartment to the other during the illumination, it switched off the light (switch-off responses = responses within 20 s). There was no escape component in this test. Like the CAR test, here, also two successive trials were separated by a random interval from 10 to 50 s. In this case, an additional control group of animals was only placed inside the box without any light presentations and the transitions between compartments were similarly scored. The same software and interface of the CAR test controlled the presentation and termination of the stimuli, along with all data collection. In both test conditions, each animal was submitted to only one session.

2.4. Drugs

Apomorphine hydrochloride (Sigma, USA), (±) sulpiride (Sigma) and SCH 23390 [*R*-(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine, Sigma] were dissolved in physiological saline (0.9%) shortly before use. For the CAR test, the animals were randomly assigned to seven groups: (a) saline (n=10), (b) 0.5 mg/kg of apomorphine (n=10), (c) 1.0 mg/kg of apomorphine (n=10), (d) 20 mg/kg of sulpiride (n=8), (e) 40 mg/kg of sulpiride (n=8), (f) 0.025 mg/kg of SCH 23390 (n=12) and (g) 0.05 mg/kg (n=10) of SCH 23390. In the switch-off test, a similar distribution of groups was followed. The number of animals used in this latter experiment was equal to 10 for all groups, except for SCH 23390 (0.025 mg/kg) which had n=12 and sulpiride 40 mg/kg with n=8. An additional group (n=10), which did not receive light or footshock presentations, was added to serve as an additional control group. The injections of apomorphine were given immediately before the sessions. Sulpiride and SCH 23390 were given 10 and 30 min before the sessions, respectively. The doses of the drugs were administered in a constant volume of 1 ml/kg, IP. Drug doses and time of injections were based on previous studies from this and other laboratories and also based on pilot experiments, in which apomorphine 2 mg/kg caused stereotyped behaviors, sulpiride 10 mg/kg did not cause any significant effect and of SCH 23390 0.1 mg/kg caused a motor deficit (Guarraci et al., 2000; Furlan and Brandão, 2001; Garcia et al., submitted for publication).

2.5. Analysis of results

Data are reported as mean±S.E.M. In the CAR test, the effects of the treatments on the number of avoidance, escape and intertrial responses was subjected to a one-way analysis of variance (ANOVA). Frequencies of avoidance responses across the four blocks of trials were subjected to a two-way analysis of variance with repeated measures using drug doses as the between factor and blocks of 10 trials each as the within-group repeated-measure factor. Similar analyses were performed for the shuttling responses in the light-induced switch-off test. The treatment effects on the locomotor activity measured during the intertrial period of this test was subjected to one-way ANOVA. Post hoc differences between group means were tested with Newman–Keuls test. A p value lower than 0.05 was considered significant.

3. Results

Fig. 1A presents the mean frequency of avoidance responses of the groups under the various treatments in the whole sessions. One-way ANOVA showed significant effects of treatments [$F(6,61)=10.48$, $p<0.05$]. Post hoc comparisons revealed that while apomorphine caused a dose-dependent increase in the avoidance responses, the dopamine D_1 and D_2 receptor antagonists, SCH 23390 and sulpiride caused significant decreases in these responses. The effects of these DA antagonists were due to both doses of SCH 23390 and 20 mg/kg of sulpiride. However, the effects of 40 mg/kg of sulpiride reached marginal significance ($p<0.06$). Fig. 1B presents the mean frequency of avoidance responses of the seven groups across the session blocks. Two-way ANOVA showed that significant differences emerged between treatments: drug effects [$F(6,61)=10.56$, $p<0.05$]. Overall, the number of avoidance responses significantly increased across blocks of 10 trials: block effect [$F(3,183)=15.39$, $p<0.05$]. These changes across blocks varied as a function of drug treatment: treatment×blocks interaction [$F(18,183)=2.97$, $p<0.05$]. Post hoc Newman–Keuls method shows that control rats presented increased avoidance responses across blocks indicating significant learning of the light/footshock associations. Post hoc comparisons also showed that this learning was significantly enhanced by

apomorphine 1.0 mg/kg. These effects of apomorphine vanished by the fourth block probably because of the short duration of action (30 min) of this dopaminergic agonist (Furlan and Brandão, 2001). Sulpiride and SCH 23390 inhibited the increase in avoidance responses across blocks.

One-way ANOVA applied to the escape frequencies in the whole session showed significant effects of treatments [$F(6,61)=3.59$, $p<0.05$]. Post hoc comparisons showed that these effects were due to differences between 1.0 mg/kg of apomorphine and both doses of sulpiride but no significant differences could be observed between these drug treatments and saline. These data are illustrated in Fig. 1C.

Analysis of the locomotor activity during the conditioned sessions showed significant changes during the intertrial period [$F(6,61)=21.51$, $p<0.05$]. Post hoc comparison

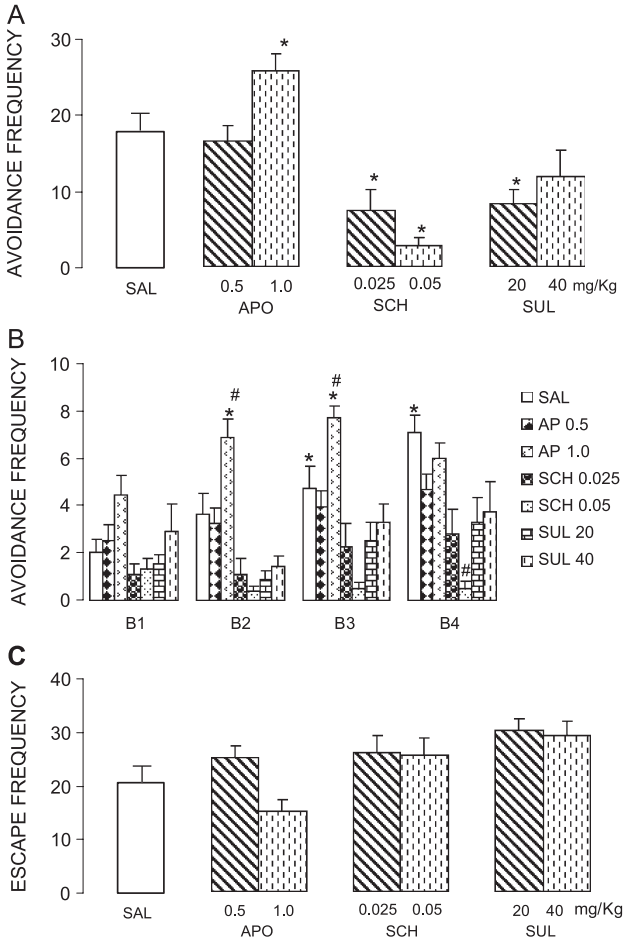

Fig. 1. Mean (±S.E.M.) of total avoidance (A), avoidance responses across blocks of 10 trials (B) and total escape (C) during sessions with independent groups of rats injected with saline, 0.5 and 1.0 mg/kg of apomorphine, 20 and 40 mg/kg of sulpiride, 0.025 and 0.05 mg/kg of SCH 23390 and submitted to 40 trials of conditioning with pairing footshocks with neutral conditioned stimulus (box illumination). *$p<0.05$ in relation to the same treatment in the first block (B1) and $^{\#}p<0.05$ in relation to saline group in the same block, Newman–Keuls comparisons. $n=10$ for all groups, except for SCH 23390 0.025 with $n=12$ and both groups of sulpiride with $n=8$.

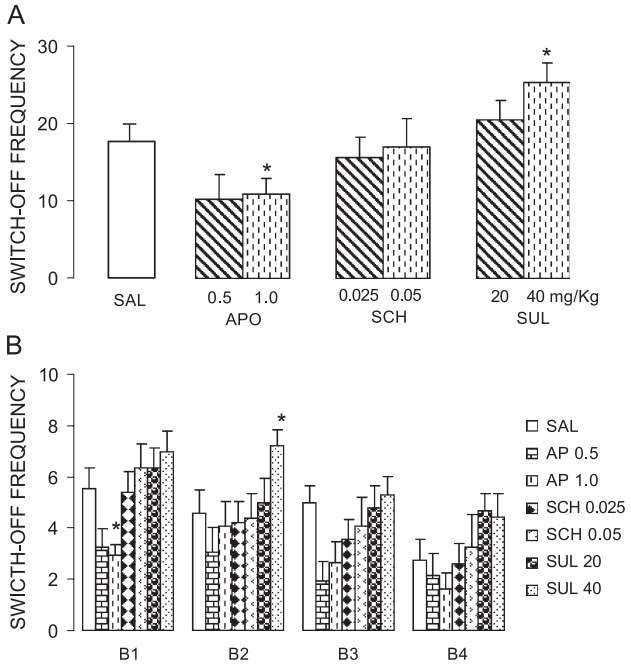

Fig. 2. Mean (±S.E.M.) of total (A) and across blocks of 10 trials (B) of switch-off responses during sessions with independent groups of rats injected with saline, 0.5 and 1.0 mg/kg of apomorphine, 20 and 40 mg/kg of sulpiride and 0.025 and 0.05 mg/kg of SCH 23390 and submitted to 40 trials of light presentations. *$p<0.05$, Newman–Keuls comparisons. $n=10$ for all groups, except for SCH 23390 (0.025 mg/kg) with $n=12$ and sulpiride 40 mg/kg with $n=8$.

revealed that these effects were due to increases caused by the dose of 1.0 mg/kg of apomorphine.

Fig. 2A presents the mean frequency of switch-off responses recorded for the groups of animals under the seven treatments. One-way ANOVA showed significant effects of treatments [$F(6,63)=3.27$, $p<0.05$]. Post hoc comparisons revealed that these effects were due to the higher doses of apomorphine and sulpiride. Fig. 2B presents the mean frequency of light-induced switch-off responses of the seven groups across the four blocks of the sessions. Significant differences emerged between treatments: drug effects [$F(6,63)=2.74$, $p<0.05$]. Overall, the number of switch-off responses significantly changed across blocks of 10 trials: block effect [$F(3,189)=8.68$, $p<0.05$]. These changes across blocks, however, did not vary as a function of drug treatment: treatment×blocks interaction [$F(18,189)=1.04$, $p>0.05$].

Comparisons of the groups exposed and not exposed to light showed that light-induced switch-off responses were significantly higher (17.60±2.32) than the responses of the no-light group (1.90±0.72) during the corresponding period of the session [$t(1,18)=41.91$, $p<0.001$].

4. Discussion

The present results with the CAR test show that rats learn to make a shuttling response in order to avoid the US

footshocks. Therefore, in this test, rats increased their rate of responding in the presence of the CS (cage illumination) over a number of trials.

The association of changes in dopaminergic transmission and threatening challenges has already been demonstrated by numerous reports. It has been suggested that dopaminergic mechanisms mediate conditioned avoidance behaviors but not unconditioned escape of the two-way avoidance test, probably because of the increase in sensitivity to conditioned aversive stimuli (Baldessarini, 1996). In support of this, in the present study, apomorphine clearly enhanced the aversiveness of the light-CS, which signaled the incoming footshocks by increasing the frequencies of responses to avoid the US footshocks. On the other hand, no change in frequencies of escape responses could be observed following apomorphine injections in relation to the control group. Apomorphine has been considered to be a nonselective dopaminergic agent (Ljungberg and Ungerstedt, 1977; Creese et al., 1983). The association of changes in dopaminergic transmission and threatening challenges has already been demonstrated by numerous reports. Indeed, some studies show that dopaminergic agonists enhance anxiety-related behaviors, such as hyperdefensiveness toward aggressive partners in previously defeated mice (Puglisi-Allegra and Cabib, 1988; Belzung et al., 1991). In agreement with these data, in a recent study with the same CAR test but using midbrain tectum stimulation as US instead of footshocks, it was shown that apomorphine produced a dose-dependent increase in the number of avoidance responses and a decrease in the latency of these responses, whereas chlorpromazine administration promoted dose-dependent reduction of the conditioned avoidance responses (Troncoso et al., 2003). Several lines of evidence suggest that there is a primary involvement of the DA mesocorticolimbic system in the neural substrate of CAR (Wadenberg and Hicks, 1999). In fact, alterations of dopamine transmission always occur following the exposure to a wide variety of acute stressors and cortical dopamine projections are also activated by diverse types of aversive stimulation (Anisman et al., 1991; Feenstra et al., 1995; Feenstra and Botterblom, 1996; Goldstein et al., 1996, Cuadra et al., 2000). Experimental studies have shown that conditioned fear elicits an activation of VTA-derived dopaminergic pathways to the amygdala and adjacent bed nucleus of the stria terminalis and also to the nucleus accumbens (Kalivas and Duffy, 1995; Inglis and Moghaddam, 1999; Greba et al., 2001; Pezze et al., 2003). Although the precise neural circuit of the DA transmission in aversive states remains unclear, pharmacological and neurochemical studies also point to the involvement of DA prefrontal neurons (Espejo and Minãno, 1999; Morrow et al., 1999). Support for a functional link between the activation of DA prefrontal neurons and the behavioral response induced by footshocks has been reported recently (Cuadra et al., 1999).

The analyses of 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). Sulpiride decreased the frequency of avoidance responses in relation to saline-injected animals. The observed effects of this dopaminergic D_2-receptor antagonist cannot be attributed to nonspecific effects, as it did not affect the intertrial locomotor activity. These effects could well be due to an action at the level of the nucleus accumbens since local application of this DA D_2-receptor antagonist into the ventral, but not the dorsal neostriatum in the rat produces complete suppression of CAR (Wadenberg et al., 1990). It is still a point of concern to clarify the extent at 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 the mouse hyperdefensiveness test (Puglisi-Allegra and Cabib, 1988).

SCH 23390 caused a significant decrease in the frequency of avoidance responses. The observed effects of this dopaminergic D_1-receptor antagonist cannot be attributed to nonspecific effects, as it did not affect the intertrial locomotor responses of the animals in the two-way avoidance procedure. These findings are in line with the reported anxiogenic action of SKF 38393, a D_1-receptor agonist, which is blocked by SCH 23390 in a modified mouse light–dark exploration test (Simon et al., 1993). Other studies have also reported an anxiogenic role for the dopaminergic D_1 receptors in situations where responses, such as grooming, to mild stressors are studied (Arnt et al., 1987; Clark and White, 1987). More specifically, our findings suggest that an activation of D_1 receptors seem to be involved in the acquisition of conditioned avoidance responses.

Taken together, the data obtained in the CAR test suggest that a combined activation of D_1 and D_2 receptors seem to be involved in the acquisition of conditioned avoidance responses. Thus, dopamine, through these receptors, may strengthen the aversiveness triggered by conditioned threatening conditions, as assessed by the present conditioned avoidance model of anxiety. In line with this view, there have been several studies showing that DA mechanisms of the ventral tegmental area are involved in fear arousal to shock and 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 fear arousal is a possible explanation of the avoidance results of the present work, particularly if we admit that avoidance behavior has an element of Pavlovian conditioned fear arousal. Similar synergistic interactions of dopamine D_1 and D_2 receptors have been observed in other behavioral studies (Arnt et al., 1987; Kamei et al., 1995).

The results obtained in this work show that the switch-off responses to light is a good index to measure innate fear

reactions to illuminated areas as rats promptly make a shuttling response in order to avoid this stimulus. Indeed, rats emitted a higher number of switch-off responses in the presence of the light in relation to the number of shuttling responses of a group of rats submitted to a similar paradigm but without light presentations. The present test may be considered an unconditioned (or spontaneous/ethological) animal model of anxiety similar to the light–dark test or the elevated plus-maze test in that the animals are exposed to an aversive environment from which they can easily escape (Treit, 1985; Lister, 1990; Chaouloff et al., 1997; Bourin and Hascoet, 2003). In contrast with the increase in the CAR, apomorphine caused a reduction in the switch-off responses in this study. SCH 23390 did not change the number of switch-off responses but an increase in these responses was observed with the administration of sulpiride. Similar results were also reported in the literature and also in a recent study from this laboratory with the use of the elevated plus-maze, a test also considered to model unconditioned fear, in which apomorphine caused a selective increase in entries and time spent into the open arms and sulpiride, in the same doses as those used here, caused opposite effects (Rodgers et al., 1994; Garcia et al., submitted for publication).

The inhibitory role of D_2 mechanisms in the light-induced switch-off responses contrasts with its heightened effect on the avoidance conditioned responses. Thus, it might be argued that the differences of action of the dopaminergic agents in the two tests used here may be related to specificity of the test situation. Considering the differences in the eliciting stimuli, time course of the response between CAR and switch-off test, these paradigms might indeed model different states of fear and that the light-induced switch-off behavior and the avoidance responses of the CAR are subserved by distinct neurochemical mechanisms.

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 and SCH 23390 administrations. The number of escape responses was unchanged by these drugs. In the switch-off test, apomorphine reduced the number of responses whereas sulpiride increased them. These findings suggest that the involvement of dopaminergic mechanisms in threatening situations depends on the nature of the aversive stimulus. Activation of D_2 receptors occurs in the setting up of adaptive responses to unconditioned responses to innate fear stimuli while a combined activation of D_1 and D_2 receptors seem to be involved in the acquisition of conditioned avoidance responses. Thus, dopamine, through D_1 and D_2 receptors, may strengthen the aversiveness triggered by conditioned threatening conditions as assessed by the CAR. The use of both CAR and light-induced switch-off paradigms in parallel seems to be a valuable approach for studying the underlying neuroanatomy of fear and anxiety and for screening drugs that selectively target either fear or anxiety. Much study is still needed to clarify the involvement of other dopaminergic receptors in the effects reported in the present work. For instance, D_2-dopamine receptor agonists and antagonists also bind to D_3 receptors (Sokoloff et al., 1990).

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