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Dopamine D2 receptor mechanisms in the expression of conditioned fear

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Abstract

The increase in the startle reflex in the presence of a stimulus that has been previously paired to foot shock is taken as an index of anxiety and named fear potentiated startle (FPS). Freezing behavior, defined as a cessation of all observable movements except those associated with respiration, has also been used as an index of fear in rats. Recently, a growing body of evidence has suggested that dopaminergic mechanisms are significant for different aspects of affective memory, namely its formation, expression or retrieval. However, the results of studies that have directly examined the ability of the dopaminergic system to influence fear have been inconsistent. This work is aimed at examining the involvement of D1 and D2 receptors in the acquisition and expression of conditioned fear using the fear potentiated startle test and the freezing behavior. We evaluated the effects of systemic administration of the D1 antagonist SCH 23390, the D1 agonist SKF 38393, the D2 antagonist sulpiride and the D2 agonist quinpirole before and after conditioning on FPS and freezing as indices of fear memory. The motor activity of the animals was also evaluated in an open field test. Injections of SCH 23390, SKF 38393, sulpiride and quinpirole before conditioning sessions did not produce any significant effect on FPS, but SCH 23390 decreased freezing. Injections of SCH 23390, SKF 38393 and sulpiride before testing session did not produce any significant effect on FPS or freezing. Quinpirole caused significant reduction in FPS and freezing when injected before testing. Drugs' actions were not due to nonspecific impairments in the ability to respond to the CS because the identical drug treatments had no effect in an open field test. Our findings indicate that DA mechanisms mediated by D2 receptors are mainly involved in the expression rather than in the acquisition of fear.

Keywords: SCH 23390; SKF 38393; Sulpiride; Quinpirole; Fear potentiated startle; Freezing

1. Introduction

The Pavlovian fear conditioning is one of the most common paradigms used to study the biological basis of emotion, as well as of learning and memory. In the acquisition phase of a fear conditioning experiment, an emotionally neutral stimulus is paired with an aversive unconditioned stimulus (US), for example a foot shock. As a result, the neutral stimulus becomes a conditioned stimulus (CS) that elicits conditioned fear responses when subsequently presented alone during the expression phase of the experiment.

A sudden and unexpected burst of noise triggers a whole-body response, which consists of a skeletal muscle contraction, known as the acoustic startle reflex (Fleshler, 1965; Kock, 1999). When the startle-inducing noise is shown in the presence of a light CS, the startle response is enhanced. This increase in the startle reflex is taken as an index of fear and named fear potentiated startle (FPS) (Brown et al., 1951). This method has been considered to be a valid and reliable tool for measuring anxiety, based on the extensive investigation analyzing several of its behavioral, physiological and pharmacological aspects (Silva et al., 2002, 2004; Walker et al., 1997; Yeomans and Frankland, 1996).

Freezing behavior, defined as a cessation of all observable movements except those associated with respiration, has also been used as an index of fear in rats (Fanselow, 1991; Fendt and Fanselow, 1999). Many studies have shown that freezing is an important response to cues associated with shock and that this behavior is useful as a measure of conditioned fear (Fanselow, 1991; Maren and Holt, 2000).

While considerable work has been done relating specific circuits of the brain to fear conditioning, less is known about its regulation by neuromodulators; the understanding of which

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would be of therapeutic relevance for fear related diseases (Brandão et al., 1994, 1999, 2003; Millan, 2003). Dopamine is one of the neuromodulators most active on the mechanisms underlying states of fear and anxiety. Recently, a growing body of evidence has suggested that dopaminergic mechanisms are significant for different aspects of affective memory, namely its formation, expression or retrieval (Pezze and Feldon, 2004). Dopaminergic mechanisms have been related to the production and elaboration of acute and chronic stress (Feenstra et al., 1995). Indeed, current knowledge indicates that cortical dopamine projections are activated by a wide variety of aversive stimuli (Feenstra and Botterblom, 1996; Goldstein et al., 1996). In this context, it has been shown that acute environmental stressors cause the release of dopamine from the cortical dopaminergic terminals (Biggio et al., 1990; Feenstra et al., 1995; Feenstra and Botterblom, 1996). Support for the notion that dopamine release is associated to the aversive properties of stimuli comes from the fact that such release is inhibited by diazepam (Feenstra et al., 1995).

The above mentioned findings could be taken as evidence for a secondary involvement of dopamine in fear-like states elicited by acute aversive stimuli. However, behavioral studies aimed to assess 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 (Davis et al., 1993; Garcia et al., 2005; Greba and Kokkinidis, 2000; Inoue et al., 2000; Reis et al., 2004; Rodgers et al., 1994). It has been shown that these effects depend on the nature of the aversive stimulus, i.e., the signal of the modulatory dopaminergic mechanism on defensive behavior will depend on the type of emotional stimuli triggering the coping reaction. Although at least five DA receptor subtypes are now recognized (Millan, 2003; Vallone et al., 2000), the initial identification of D1 and D2 receptors (Kebabian and Calne, 1979) provided the major motivation for research aimed at defining particular functional roles for DA receptor subtypes.

In order to assess the dopaminergic mediation of aversive states, we examined the involvement of D1 and D2 receptors in acquisition and expression of conditioned fear using the fear potentiated startle test and the freezing behavior. This pharmacological assessment was done with systemic administrations of the D1 antagonist SCH 23390 (Fletcher and Starr, 1988; Greba and Kokkinidis, 2000; Hytell, 1983; Ozer et al., 1997), the D1 agonist SKF 38393 (Borowski and Kokkinidis, 1998; Kamei et al., 1995; Rodgers et al., 1994), the D2 antagonist sulpiride (Guarraci et al., 2000; Standish-Barry et al., 1983; White and Wang, 1984a,b) and the D2 agonist quinpirole (Levant et al., 1992; Seeman and Schaus, 1991).

2. Materials and methods

2.1. Animals

Four-hundred and forty-seven naive male Wistar rats from the animal house of the Campus of Ribeirão Preto of the University of São Paulo were used. The animals, weighing 220 to 280 g each, were housed in groups of five per cage, under a 12:12 dark/light cycle (lights on at 07:00 h) at 23±1 °C, and given free access to food and water. The experiments were carried out during the light phase of the cycle and were performed in compliance with the recommendations of SBNeC (Brazilian Society of Neuroscience and Behavior), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.2. Apparatus and procedure

2.2.1. Fear potentiated startle

2.2.1.1. Matching. To record the amplitude of the acoustic startle response, two separated stabilimeter devices were used simultaneously. The rats were placed into a stabilimeter, which consisted of a wire mesh cage (16.5×7.5×7.5 cm) suspended within a PVC frame, which was firmly placed on a response platform by four thumb screws. The floor of the stabilimeter consisted of six 5.0 mm diameter stainless steel bars spaced 1.5 cm apart. Each stabilimeter and platform were located independently inside ventilated plywood sound-attenuating chambers (64×60×40 cm). The startle reaction of the rats generated a pressure on the response platform and analog signals were amplified, digitized and analyzed by a software (Startle Reflex, version 4.10, Med Associates Inc., VT, USA) provided by the manufacturer of the equipment. The presentation and sequencing of the acoustic stimuli were also controlled by the same software and an appropriate interface (Med Associates Inc., VT). A loudspeaker, located 10 cm behind the wire mesh cage, was used to deliver both the acoustic startle stimulus, which was a 100 dB, 50 ms burst of white noise, having a rise-decay time of 5 ms, and a continuous background noise (55 dB). The startle reaction was recorded within a time window of 100 ms after the startle stimulus onset. Calibration procedures were conducted before the experiments to ensure statistically equivalent sensitivities of the response platforms. The behavior of the animals was recorded by a video camera (Everfocus, USA) positioned behind the stabilimeter, with the signal being relayed to a monitor in another room via a closed circuit. A red light bulb (6.0 W) located inside the isolation chamber provided illumination for the camera. On the first 2 days, the animals were placed in the stabilimeter for 5 min for habituation and, afterwards, received a total of 30 startle stimuli at an interstimulus interval of 30 s. Each matching session was 20 min in duration. For each experiment, the animals were matched into equivalent groups based on their mean startle amplitude across the 30 noise bursts on the last matching day before training began.

2.2.1.2. Training. Animals were conditioned to light CS in a cage (20×20×25 cm) with side and back walls being constructed of stainless steel and the ceiling and front door made of transparent Plexiglas. The grid floor of this cage consisted of stainless-steel rods spaced 1.5 cm apart. The cage was located within a ventilated and sound-attenuated chamber (45×45×45 cm). On each of two consecutive days, animals were placed in the training cage and, 5 min later, each rat

received 10 CS–US pairings, using a 4 s light CS coterminating with a 1 s, 0.6 mA, foot shock US. The shocks were delivered through the training cage floor by a constant current generator built with a scrambler (Albarsh Instruments, Brazil). The CS was a white light presented through a bulb (6.0 W, 127 V) located in the ceiling of the chamber. Stimulus presentation was controlled by a microprocessor and an I/O board (Insight Equipment, Brazil). The inter-trial interval varied randomly between 60 and 180 s. The duration of each training session was about 25 min.

2.2.1.3. Testing. The testing sessions were conducted in the same cages used for matching. The behavior of the animals was recorded by a video camera (Everfocus) positioned behind the cages, with the signal being relayed to a monitor in another room via a closed circuit. The testing session was conducted without presentation of foot shocks. The animals were placed into the startle testing cages and, after 5 min of habituation, were presented with 60 startle stimuli (noise bursts) at a 30 s interstimulus interval. The startle stimulus intensity used was 100 dB. Half of the startle stimuli were presented in the absence of the CS (noise-alone trials) to provide a baseline, and the other half were presented in the presence of the CS (light-noise trials). In the light-noise trials, the startle stimulus was presented in the last second of a 4 s presentation of light, similar to the training sessions. The duration of testing session was 32 min. Startle response amplitudes collected from this experiment were stored on the hard disk of the computer, and transferred to Excel (Microsoft) tables for the offline analysis.

2.2.2. Freezing behavior

An additional measure used to assess conditioned fear was the time rats spent freezing during the testing session. Freezing was operationally defined as the total absence of movement of the body and vibrissa, except the ones required for respiration, for at least 6 s. Freezing behavior was monitored during the testing and the freezing behavior was subsequently scored from videotapes. The results are presented as total time of freezing or in 4 blocks of 8 min each. To separate unconditioned freezing to noise alone and conditioned freezing to light CS we included an additional group of rats submitted to similar testing sessions in which the noise+light CS component was replaced with noise alone presentations.

2.2.3. Open-field

The experiment for assessment of locomotor activity was conducted in an arena that was a circular enclosure made of Plexiglas (60 cm in diameter and 50 cm height), with the floor divided into 12 sections. The following behavioral responses were recorded every minute for 30 min: number of crossings (number of floor sections traversed), number of rearing (standing with the forelegs raised in the middle of the arena or against the walls) and grooming (cleaning the head and/or the body with the forelegs for more than 10 s). The behavior of the animals was recorded by a video camera positioned in front of the arena. The animals were first habituated in the arena for 10 min prior to treatments. After drug injection, animals were placed again in the middle of the arena and left for a 30 min period of free exploration.

2.3. Drugs

Drugs used were: the D1 antagonist R-(+)-SCH-23390 hydrochloride, the D1 agonist (±)-SKF-38393 hydrochloride, the D2 antagonist (−)-sulpiride and the D2 agonist (−)-quinpirole hydrochloride. All drugs were purchased from Sigma, USA. All drugs, except sulpiride that was first mixed to Tween 80, 2% (Sigma), were dissolved in physiological saline (0.9%) shortly before use. Physiological saline also served as vehicle control. The doses of the drugs were administered in a constant volume of 1 ml/kg, intraperitoneally (IP). For experiments I (acquisition) and II (expression), rats received saline, saline+Tween, SCH 23390 (0.05 or 0.1 mg/kg), sulpiride (20 or 40 mg/kg), SKF 38393 (5 or 10 mg/kg) or quinpirole (0.25 or 0.5 mg/kg for experiment I and 0.1 or 0.25 mg/kg for experiment II). In experiment I, sulpiride, SKF 38393 and quinpirole were injected 10 min before each of the two training sessions and SCH 23390, 30 min before these sessions. It has been shown that higher doses of SCH cause a deficit in the motor activity (Reis et al., 2004). In experiment II, sulpiride, SKF 38393 and quinpirole were injected 10 min before testing session, and SCH 23390, 30 min before this session. For experiment III (locomotor activity), the same drugs and time of injections were used. However, only one dose of SCH 23390 (0.1 mg/kg), sulpiride (40 mg/kg), SKF 38393 (10 mg/kg) and quinpirole (0.25 mg/kg) were tested. Drug doses and time of injections were based on previous studies from this and other laboratories (Garcia et al., 2005; Kamei et al., 1995; Reis et al., 2004; Rodgers et al., 1994).

2.4. Analysis of results

Data are reported as mean+S.E.M. For each drug treatment, one-way analysis of variance (ANOVA) was conducted on the time of freezing. A two-way ANOVA with repeated measures was conducted on the mean startle response, with treatments (drug and its respective control) as a between-subjects factor and trial-type (noise-alone and light-noise) as a within-subjects factor. For analysis of locomotor activity, one-way ANOVA was conducted on the number of crossings, rearings and grooming. Significant comparisons were tested with Newman–Keuls test. Significance level was set at $p<0.05$. In experiment I, $N=15$ per group. In experiment II, $N=20$ for SKF 38393 and SCH 23390 groups and $N=15$ for the other groups. In experiment III, $N=9$ for the control group and $N=7$ for all other groups.

3. Results

3.1. Experiment I: acquisition of conditioned fear

3.1.1. SCH 23390 and SKF 38393

Fig. 1A displays the mean startle amplitude for animals that received IP administrations of saline or SCH 23390 (0.05 or

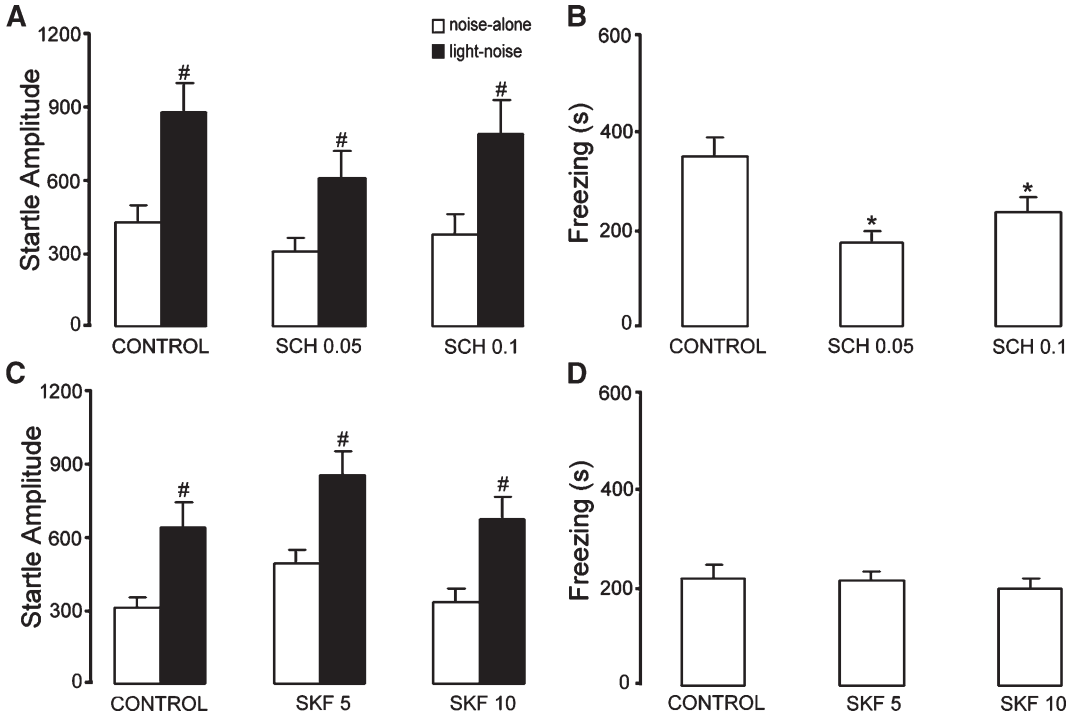

Fig. 1. Effects of IP injections of saline or D1 dopaminergic drugs before training sessions on the acquisition of conditioned aversive information. (A) Effects of SCH 23390 0.05 and 0.1 mg/kg (SCH 0.05 and SCH 0.1) on mean startle amplitude. (B) Effects of SCH 23390 0.05 and 0.1 mg/kg on freezing behavior. (C) Effects of SKF 38393 5 and 10 mg/kg (SKF 5 and SKF 10) on mean startle amplitude. (D) Effects of SKF 38393 5 and 10 mg/kg on freezing behavior. #Different from noise-alone condition. *Different from control group ($p<0.05$, Newman-Keuls test). Mean+S.E.M. $N=15$ for all groups.

0.1 mg/kg) before training sessions. Two-way ANOVA with repeated measures revealed that there was no effect of treatments ($F(2,89)=1.05$; $p>0.05$), but there was a main effect for trial type ($F(1,89)=79.50$; $p<0.001$). There was no significant treatments vs. trial-type interaction ($F(2,89)=1.16$; $p>0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light CS enhanced the startle response to noise in all groups ($p<0.05$). Startle reflex to noise alone was not statistically different in all groups ($p>0.05$). Fig. 1B displays the mean freezing for animals that received IP administrations of saline or SCH 23390 (0.05 or 0.1 mg/kg) before training sessions. One-way ANOVA showed significant differences among groups ($F(2,42)=8.41$; $p<0.001$). Post-hoc comparisons by the Newman–Keuls test revealed that SCH 23390, in both doses, caused a significant decrease in the time of freezing spent by these rats compared with controls ($p<0.05$).

Fig. 1C shows the mean startle amplitude for animals that received IP injections of saline or SKF 38393 (5 or 10 mg/kg) before training sessions. Two-way ANOVA with repeated measures revealed that there was no effect of treatments ($F(2,89)=2.24$; $p>0.05$), but there was a main effect for trial type ($F(1,89)=97.98$; $p<0.001$). There was no significant treatments vs. trial-type interaction ($F(2,89)=0.09$; $p>0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light-CS enhanced the startle response to noise in all groups ($p<0.05$). Startle reflex to noise alone was not statistically different in all groups ($p>0.05$). Fig. 1D shows the mean freezing for animals that received IP administrations of saline or SKF 38393 (5 or 10 mg/kg) before training sessions. One-way ANOVA showed no significant differences among groups ($F(2,42)=0.26$; $p>0.05$).

3.1.2. Sulpiride and quinpirole

Fig. 2A depicts the mean startle amplitude for animals that received IP injections of saline+Tween 2% or sulpiride (20 or 40 mg/kg) before training sessions. Two-way ANOVA with repeated measures showed that there was no effect of treatments ($F(2,89)=1.97$; $p>0.05$). There was a main effect for trial type ($F(1,89)=135.86$; $p<0.001$). There was no significant treatments vs. trial-type interaction ($F(2,89)=0.25$; $p>0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light CS enhanced the startle response to noise in all groups ($p<0.05$). Startle reflex to noise alone was not statistically different in all groups ($p>0.05$). Fig. 2B displays the mean freezing for animals that received IP administrations of saline+Tween 2% or sulpiride (20 or 40 mg/kg) before training sessions. One-way ANOVA showed no significant differences among groups ($F(2,42)=1.75$; $p>0.05$).

Fig. 2C displays the mean startle amplitude for animals that received IP administrations of saline or quinpirole (0.25 or 0.5 mg/kg) before training sessions. Two-way ANOVA with repeated measures revealed that there was no effect of treatments ($F(2,89)=0.16$; $p>0.05$). There was a main effect for trial type ($F(1,89)=65.62$; $p<0.001$). There was no significant treatments vs. trial-type interaction ($F(2.89)=2.98$; $p>0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light-CS enhanced the startle response to noise in all groups ($p<0.05$). Startle reflex to noise alone was not

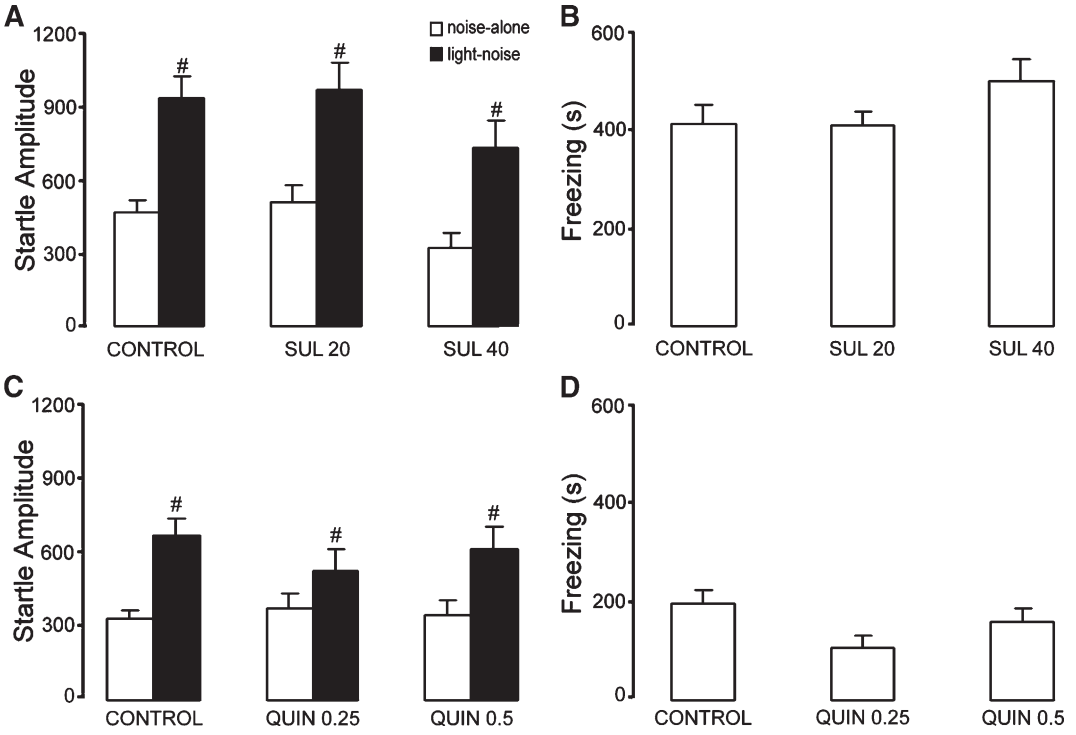

Fig. 2. Effects of IP injections of saline or D2 dopaminergic drugs before training sessions on the acquisition of conditioned aversive information. (A) Effects of sulpiride 20 and 40 mg/kg (SUL 20 and SUL 40) on mean startle amplitude. (B) Effects of sulpiride 20 and 40 mg/kg on freezing behavior. (C) Effects of quinpirole 0.25 and 0.5 mg/kg (QUIN 0.25 and QUIN 0.5) on mean startle amplitude. (D) Effects of quinpirole 0.25 and 0.5 mg/kg on freezing behavior. #Different from noise-alone condition ($p<0.05$, Newman–Keuls test). Mean+S.E.M. $N=15$ for all groups.

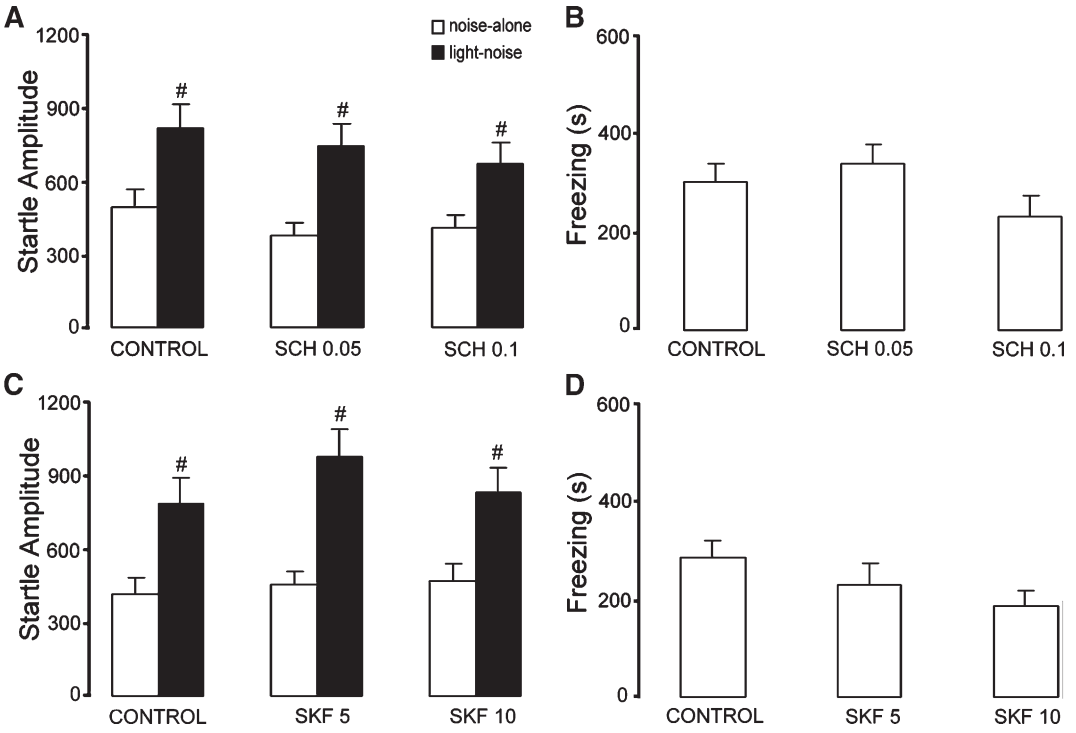

Fig. 3. Effects of IP injections of saline or D1 dopaminergic drugs before testing sessions on the expression of conditioned defensive responses. (A) Effects of SCH 23390 0.05 and 0.1 mg/kg (SCH 0.05 and SCH 0.1) on mean startle amplitude. (B) Effects of SCH 23390 0.05 and 0.1 mg/kg on freezing behavior. (C) Effects of SKF 38393 5 and 10 mg/kg (SKF 5 and SKF 10) on mean startle amplitude. (D) Effects of SKF 38393 5 and 10 mg/kg on freezing behavior. #Different from noise-alone condition ($p<0.05$, Newman–Keuls test). Mean+S.E.M. $N=20$ for all groups.

statistically different in all groups ($p>0.05$). Fig. 2D displays the mean freezing for animals that received IP administrations of saline or quinpirole (0.25 or 0.5 mg/kg) before training sessions. One-way ANOVA showed no significant differences among groups ($F(2,42)=2.95$; $p>0.05$).

3.2. Experiment II: expression of conditioned fear

3.2.1. SCH 23390 and SKF 38393

Fig. 3A displays the mean startle amplitude for animals that received IP administration of saline or SCH 23390 (0.05 or 0.1 mg/kg) before testing session. Two-way ANOVA with repeated measures revealed that there was no significant effect of treatments ($F(2,119)=0.72$; $p>0.05$). There was a main effect for trial type ($F(1,119)=98.03$; $p<0.001$). There was no significant treatments vs. trial-type interaction ($F(2,119)=0.86$; $p>0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light-CS enhanced the startle response to noise in all groups ($p<0.05$). Startle reflex to noise alone was not statistically different between groups ($p>0.05$). Fig. 3B displays the mean freezing for animals that received IP injection of saline or SCH 23390 (0.05 or 0.1 mg/kg) before testing session. One-way ANOVA showed no significant differences between groups ($F(2,57)=1.92$; $p>0.05$).

Fig. 3C shows the mean startle amplitude for animals that received IP administration of saline or SKF 38393 (5 or 10 mg/kg) before testing session. Two-way ANOVA with repeated measures revealed that there was no significant effect of treatments ($F(2,119)=0.50$; $p>0.05$). There was a main effect for trial type ($F(1,119)=169.15$; $p<0.001$). There was no significant treatments vs. trial-type interaction ($F(2,119)=2.72$; $p>0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light CS enhanced the startle response to noise in all groups ($p<0.05$). Startle reflex to noise alone was not statistically different between groups ($p>0.05$). Fig. 3D shows the freezing behavior in groups of animals that received IP administration of saline or SKF 38393 (5 or 10 mg/kg) before testing sessions. One-way ANOVA showed no significant differences between groups ($F(2,57)=1.94$; $p>0.05$).

3.2.2. Sulpiride and quinpirole

Fig. 4A shows the mean startle amplitude for animals that received IP administration of saline+Tween 2% or sulpiride (20 or 40 mg/kg) before testing session. Two-way ANOVA with repeated measures revealed that there was no effect of treatments ($F(2,89)=1.75$; $p>0.05$). There was a main effect for trial type ($F(1,89)=126.65$; $p<0.001$). There was significant treatments vs. trial-type interaction ($F(2.89)=3.33$; $p<0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light CS enhanced the startle response to noise in all groups ($p<0.05$). Startle reflex to noise alone was not statistically different between groups ($p>0.05$). Fig. 4B displays the mean freezing for animals that received IP administration of saline+Tween 2% or sulpiride (20 or 40 mg/kg) before testing session. One-way ANOVA showed no significant differences between groups ($F(2,42)=2.56$; $p>0.05$).

Fig. 4C depicts the mean startle amplitude for animals that received IP administration of saline or quinpirole (0.1 or

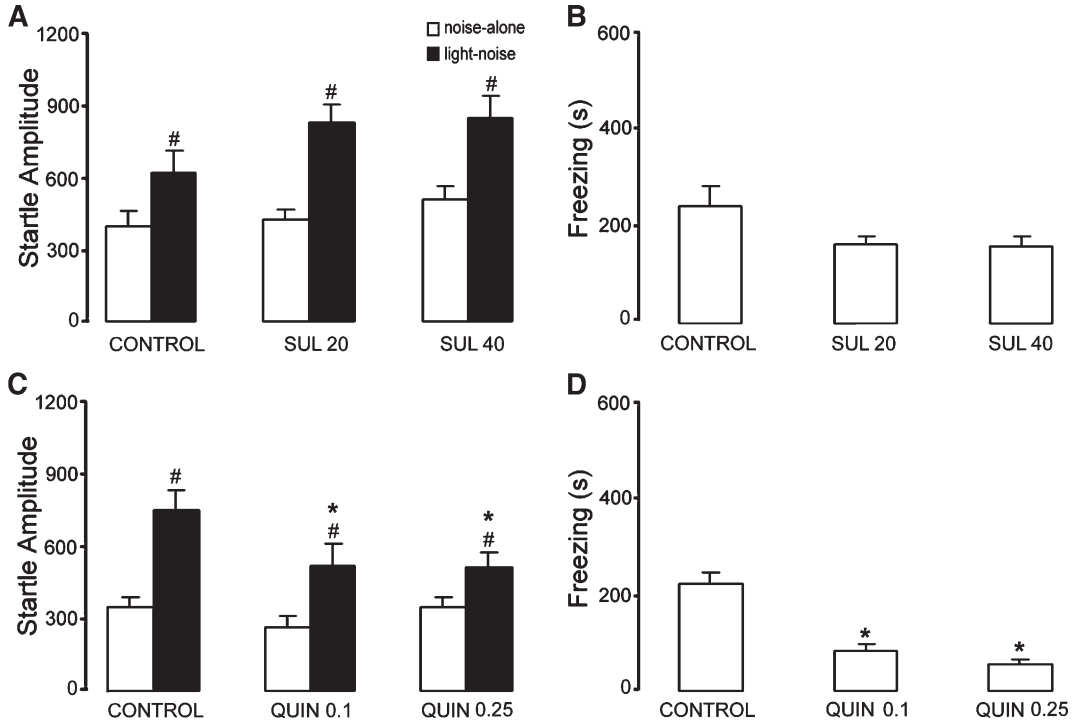

Fig. 4. Effects of IP injections of saline or D2 dopaminergic drugs before testing sessions on the expression of conditioned defensive responses. (A) Effects of sulpiride 20 and 40 mg/kg (SUL 20 and SUL 40) on mean startle amplitude. (B) Effects of sulpiride 20 and 40 mg/kg on freezing behavior. (C) Effects of quinpirole 0.1 and 0.25 mg/kg (QUIN 0.1 and QUIN 0.25) on mean startle amplitude. (D) Effects of quinpirole 0.1 and 0.25 mg/kg on freezing behavior. #Different from noise-alone condition. *Different from control group ($p<0.05$, Newman–Keuls test). Mean+S.E.M. $N=15$ for all groups.

0.25 mg/kg) before testing sessions. Two-way ANOVA with repeated measures revealed that there was no significant effect of treatments ($F(2,89)=1.84$; $p>0.05$). There was a main effect for trial type ($F(1,89)=84.65$; $p<0.001$). There was significant treatments vs. trial-type interaction ($F(2,89)=5.40$; $p<0.05$). Post-hoc comparisons with Newman–Keuls test revealed that light CS enhanced the startle response to noise in all groups ($p<0.05$). Post-hoc comparisons also showed that quinpirole, at both doses, decreased the startle response to light-noise compared with controls ($p<0.05$). Startle reflex to noise alone was not statistically different between groups ($p>0.05$). Fig. 4D displays the mean freezing for animals that received IP administration of saline or quinpirole (0.1 or 0.25 mg/kg) before testing session. One-way ANOVA showed significant differences between groups ($F(2,42)=29.23$; $p<0.001$). Post-hoc comparisons with the Newman–Keuls test revealed that quinpirole, at both doses, caused a significant decrease in the time of freezing compared with controls ($p<0.05$).

Given that freezing behavior was used as an index of fear during the potentiated startle paradigm the unconditioned response to noise could be a confound factor in the analysis of the conditioned freezing to light CS in the testing sessions, which had both light CS+noise and noise-alone trials. For this reason an additional control group of rats (Noise-alone Group, $N=20$) was included in this study. For these animals, the noise +light CS component of the testing sessions was replaced with noise-alone presentations. The control groups for experiment I (acquisition) were pooled together to constitute the Fear Acquisition Group ($N=60$) and the control groups for experiment II (expression) were pooled together to constitute the Fear Expression Group ($N=70$). The freezing behavior was scored in 4 blocks of 8 min each across the session of 32 min duration. Two-way ANOVA with repeated measures across the four blocks performed on these data showed significant effects of groups ($F(2,42)=29.23$; $p<0.001$) and blocks ($F(2,42)=29.23$; $p<0.001$). Post-hoc comparisons of Newman–Keuls revealed that all groups of animals conditioned to light CS presented significantly higher freezing response than the control group exposed to noise-alone trials. The freezing behavior was concentrated in the first half and declined appreciably in the second half of the testing sessions (Table 1).

Table 1
Mean±S.E.M. of time of freezing as an index of fear in a startle response paradigm using light as CS

	Noise-alone group	Acquisition group	Expression group
1st Block	75.76±15.24	184.48±10.58*	167.62±9.23*
2nd Block	16.60±8.25$^{\#}$	64.93±7.71*$^{\#}$	60.42±5.99*$^{\#}$
3rd Block	2.10±1.21	29.25±4.15*$^{\#}$	28.32±5.48*$^{\#}$
4th Block	0.00±0.00	10.33±2.96*$^{\#}$	13.28±3.55*

The animals conditioned to light were submitted to training sessions in which light CS cues were previously associated with foot shocks. 24 h later they were exposed to 30 noise alone plus 30 light+noise presentations (30-s inter-trial interval) in the testing sessions. The noise-alone group ($N=20$) received 60 noise alone presentations (30-s inter-trial interval) in the testing sessions. The freezing behavior of each animal was scored continuously for a 32 min testing period, which was divided into four 8-min periods. The control groups for experiment I (acquisition) were pooled together and constitute the Fear Acquisition Group and the control groups for experiment II (expression) were pooled together and constitute the Fear Expression Group. *$p<0.05$ in relation to the corresponding values in the noise-alone group. $^{\#}p<0.05$ in relation to the precedent block in the same group. Two-way ANOVA with repeated measures followed by the Newman–Keuls test.

3.3. Experiment III: locomotor activity

In this experiment we evaluated whether or not the dopaminergic agonists, SKF 38393 and quinpirole, and antagonists, SCH 23390 and sulpiride, would affect the motor activity of rats in the open field test. One-way ANOVA applied on the number of crossings did not reveal any significant effects of treatments ($F(4,32)=2.341$; $p>0.05$). Similarly, one-way ANOVA performed on the number of rearing also showed no significant differences between treatments ($F(4,32)=2.94$; $p>0.05$). The number of grooming recorded during the sessions for the treatment groups was not statistically different from the control group ($p>0.05$).

4. Discussion

The present data show that the magnitude of freezing response to light CS+noise was higher than to noise alone presentations in the testing sessions. This finding demonstrates that the anxiety-like state of these animals, and not just the aversiveness of the loud noise, contributes to the fear potentiated startle in rats previously submitted to the association of light and foot shock.

The association of changes in dopaminergic transmission and threatening challenges has already been demonstrated by numerous reports. In fact, alterations of DA transmission always occur following the exposure to a wide variety of acute stressors (Anisman et al., 1991; Goldstein et al., 1996). Activation of ventral tegmental area (VTA) neurons by threatening environmental stimuli likely modulates fear and anxiety through their ascending forebrain projections. For example, electrical stimulation of the VTA potentiates acoustic startle reflex (Borowski and Kokkinidis, 1996). The findings reported here help to understand at what extent dopaminergic mechanisms mediate the acquisition of information and expression of responses in the conditioned fear to an explicit cue, light CS.

The present results stress the importance of DA neurons in the fear-activating effects of the Pavlovian conditioning. Animals that received pre-training injections of D1 antagonist, SCH 23390, had a disruption of the acquisition of freezing while FPS remained unchanged. Therefore, as recently suggested, distinct neural substrates could underlie the fear responses generated in these two types of tests (Borelli et al., 2005). SCH 23390, at doses used here, did not affect the pain threshold induced by foot shocks (Inoue et al., 2000). Therefore, the inhibitory effect of this drug in our study cannot be attributed to a reduction of animals' sensitivity to the shock. Also, we can discard an eventual effect of this drug on the motor activity since the doses of SCH 23390 used here were lower than those reported to reduce motor behavior (Díaz-Véliz et al., 1999; Mizuki et al., 1996; Ushijima et al., 1995a,b). However,

higher doses of SCH 23390 than those used here may cause motor deficit (Reis et al., 2004). On the other hand, the D1 agonist, SKF 38393, did not cause effects on FPS or freezing. We can suggest that the unexpected lack of an anxiogenic effect in freezing can be attributed to the fact that intrinsic dopamine are activating all D1 receptors during training sessions. In this way, the drug's action does not result in more activation. Quinpirole and sulpiride, D2 agonist and antagonist respectively, did not change the FPS or the freezing response produced by light CS previously paired with foot shock. Altogether, these data point out to the implication of D1 receptor-mediated mechanism in the acquisition of conditioned freezing while D2 DA receptors are not involved in the acquisition of conditioned freezing and FPS.

The most important contribution of the present findings is, however, that systemic pre-testing injections of quinpirole dose dependently inhibited fear potentiated startle and freezing behavior produced by light CS previously paired with foot shocks. Rats showed normal baseline acoustic startle responding after quinpirole administration, indicating that the inhibition of the FPS and freezing was not associated with impaired sensory gating. Besides, the observed effects of quinpirole cannot be attributed to nonspecific effects, as this drug did not affect the motor activity of the animals in the open field test. The selectivity of the effects produced by the D2 agonist quinpirole is further attested by the lack of effects of pre-testing injections of the D1 agonist, SKF 38393, on conditioned fear.

The doses of quinpirole chosen (0.1 and 0.25 mg/kg) were an order of magnitude lower than the doses that activate postsynaptic receptors (Nader and LeDoux, 1999). Although D2 receptors are found both pre- and postsynaptically, it has been reported that the presynaptic sites are between 6 and 10 times more sensitive to apomorphine than the postsynaptic sites (Skirboll et al., 1979). Thus, it seems reasonable to suppose from these findings that quinpirole's ability to decrease fear is the result of an action on presynaptic dopaminergic receptors that decreases dopamine levels in the terminal fields. These effects might have been due to an action on D2 presynaptic autoreceptors located in the VTA. Indeed, DA D2 autoreceptors located on cell bodies and dendrites of the VTA tonically inhibit DA neural activity in rats exposed to aversive situations (White and Wang, 1984a,b). Doses of quinpirole higher than 0.25 mg/kg, acting in post-synaptic receptors, were not used here because they increase locomotor activity and cause stereotyped behavior like sniffing and licking (Kurashima et al., 1995).

Quinpirole has been reported to be a full agonist at D2 receptor sites (Levant et al., 1992; Seeman and Schaus, 1991). Stimulation of these inhibitory autoreceptors with postconditioning injections of the specific DA D2 receptor agonist quinpirole into the VTA suppresses fear potentiated startle (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997). Intra-VTA quinpirole infusion also interferes with Pavlovian conditioning of defensive freezing (Nader and LeDoux, 1999). Thus, these findings are consistent with the notion that activation of D2-mediated mechanisms decreases fear by impairing the retrieval of a learned association between a light CS and a foot shock US (Nader and LeDoux, 1999). Unexpectedly, sulpiride did not produce an increase in animal reactivity to the light CS associated to loud sounds or an increase in freezing behavior. So, it is probable that dopamine exerts a phasic modulation on the expression of conditioned fear through D2 receptors. These findings taken together, plus the fact that D1 DA receptors do not seem to be involved in the expression of fear, supports the view that D2 autoreceptors at the level of VTA regulate the FPS caused by light/foot shock association, where D1 receptors do not seem to act preferentially (Meador-Woodruff et al., 1991; Mengod et al., 1992; Schambra et al., 1994).

A growing body of evidence is converging on the idea that excitation of the mesoamygdaloid pathway originating from dopamine neurons in the VTA are particularly sensitive to fear-arousing environmental stimuli and may be important for the development of exaggerated fear responding (Deutch et al., 1985; Greba et al., 2001; Guarraci and Kapp, 1999; Guarraci et al., 2000). Previous research found that VTA DA neural excitation is necessary for fear arousal produced by exposing animals to an explicit conditioned stimulus after Pavlovian fear conditioning (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997). Further studies using local injections into this structure of selective D2 receptor antagonists, like sulpiride, on the aversive associative learning will help to elucidate this point.

It is important to mention here that several procedural confounds may underlie the conflicting results in the literature in studies looking at dopaminergic systems and fear. For example, the dopamine antagonist metoclopramide has been reported to potentiate the acquisition of freezing behavior in a fear-conditioning paradigm (Blackburn and Phillips, 1990). Conversely, pretreatment with a number of typical or atypical dopamine antagonists has been reported to decrease the acquisition of freezing (Inoue et al., 1996). Specifically, pharmacological or anatomical manipulations of the dopaminergic system on the test day could interfere with conditioned responding by altering CS processing. Arguing against this possibility in our study is the fact that quinpirole did not change the light processing as a conditioned cue when given to rats submitted to pre-training sessions. Also, quinpirole did not change the baseline response to noise-alone when given in pre-testing sessions. Another source of faulty interpretation would be that most dopaminergic manipulations cause unconditioned changes in behavior. Thus, these changes could indirectly compete with the expression of fear responses, leading to the mistaken conclusion that these dopaminergic manipulations directly affected fear. Again, speaking against this argument is the lack of effects of quinpirole on the motor activity as assessed in the present study by the open field test.

The inhibitory role of D2 mechanisms in the FPS and freezing contrasts with its heightened effect on the conditioned avoidance response (CAR) (Reis et al., 2004) and contextual conditioned freezing (Masson et al., 2003). Thus, it might be argued that the differences of action of the dopaminergic agents in these experimental conditions may be related to specifics of the test situations. Considering the differences in the eliciting stimuli and the time course of the responses between CAR and

FPS and between contextual and light-CS conditioned freezing, these paradigms might indeed model different states of fear. Therefore, the FPS, contextual conditioned fear and avoidance responses of the CAR might be subserved by distinct neurochemical mechanisms.

In summary, the selective effect of post-training injections of the DA D2 agonist quinpirole on the expression of conditioned fear consequent of light/foot shock association is consistent with the notion that this drug decreases fear by impairing the retrieval of a learned association between a CS and a foot shock US. It has been proposed that heightened attentional and cognitive functions of dopaminergic mechanisms of the mesocorticolimbic systems are called into play in the setting up of adaptive responses aimed at coping with or signaling the presence of stimuli of aversive nature. Probably, these findings have clinical implications for understanding how stress affects and influences anxiety-related behaviors. The contribution of agents acting as agonists at D2 receptors as adjunctive agents in controlling exaggerated fear states may involve the ability of these anxiolytics to impede stress-induced activation of VTA neurons and associated increases in emotionality. In an attempt to find specific dopaminergic neural substrates for the drug actions reported here, drug injections into the VTA of rats submitted also to the fear potentiated startle paradigm are being conducted in a current study of this laboratory. In addition to demonstrating the importance of dopaminergic mechanisms mediated by D2 receptors in the fear motivational consequences of foot shock, the present results also indicate that these neural circuits are mainly involved in the expression of fear states rather than in the acquisition of fear, where postsynaptic D1 receptors appear to be more involved.

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