



Dopamine D2 receptors in the expression and extinction of contextual and cued conditioned fear in rats

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Abstract

Dopamine seems to mediate fear conditioning through its action on D2 receptors in the mesolimbic pathway. Systemic and local injections of dopaminergic agents showed that D2 receptors are preferentially involved in the expression, rather than in the acquisition, of conditioned fear. To further examine this issue, we evaluated the effects of systemic administration of the dopamine D2-like receptor antagonists sulpiride and haloperidol on the expression and extinction of contextual and cued conditioned fear in rats. Rats were trained to a context-CS or a light-CS using footshocks as unconditioned stimuli. After 24 h, rats received injections of sulpiride or haloperidol and were exposed to the context-CS or light-CS for evaluation of freezing expression (test session). After another 24 h, rats were re-exposed to the context-CS or light-CS, to evaluate the extinction recall (retest session). Motor performance was assessed with the open-field and catalepsy tests. Sulpiride, but not haloperidol, significantly reduced the expression of contextual and cued conditioned fear without affecting extinction recall. In contrast, haloperidol, but not sulpiride, had cataleptic and motor-impairing effects. The results reinforce the importance of D2 receptors in fear conditioning and suggest that dopaminergic mechanisms mediated by D2 receptors are mainly involved in the expression rather than in the extinction of conditioned freezing.

Keywords Dopamine · D2 receptors · Fear conditioning · Expression · Extinction · Freezing

Introduction

Fear and anxiety have been commonly investigated through Pavlovian aversive conditioning (Pavlov 1927; Bolles and Collier 1976; Fendt and Fanselow 1999; Reimer et al. 2012; LeDoux 2014). In this procedure, during the acquisition phase, an emotionally neutral stimulus, such as a tone, light or the experimental context itself, is paired with an unconditioned aversive stimulus (US), for example, a mild electric shock. As a result, the initially neutral stimulus acquires the function of eliciting conditioned defensive responses when

subsequently presented alone during the expression phase of the experiment, thus becoming a conditioned stimulus (CS). The conditioned responses can then be progressively diminished if the CS is repeatedly presented in the absence of the US, a process that inhibits rather than erases the original association and known as extinction (Milad et al. 2009; Milad and Quirk 2012).

The study of the neural bases involved in fear conditioning in rodents is of great interest, since it may contribute to a better understanding of different aspects of several human mental disorders (Davis 1990; Phelps and LeDoux 2005; Milad et al. 2009, 2013; Mobbs et al. 2009; Indovina et al. 2011; Reimer et al. 2015). Besides, the roots of exposure-based interventions are firmly planted in fear-learning research. Cued fear, in which an animal learns to fear an explicit threat signal that predicts imminent danger, has been sometimes viewed as a model for fear-related disorders, such as phobias. In contrast, anxiety-related disorders seem to be better modeled by contextual conditioning, since anxiety is triggered in a less discriminatory manner, with a high degree of uncertainty and conflict (Phillips and LeDoux 1992; Ohman and Mineka 2001; Grillon 2002; Albrechet-Souza

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et al. 2011, 2013). Nevertheless, freezing behavior—a disruption of all observable movements except those associated to respiration—is the main response observed in the laboratory when rats are exposed to both cues associated with footshocks (cued fear conditioning) or the same chamber in which footshocks were previously experienced (contextual fear conditioning) (Bolles and Collier 1976; Fendt and Fanselow 1999; Carrive 2000; Reimer et al. 2018).

The understanding underlying dopamine's role in appetitive conditioning has progressed dramatically in the last decades (Datla et al. 2002; Burgdorf and Panksepp 2006; Schultz 2015). In contrast, although the involvement of dopaminergic mechanisms in fear/anxiety has been under intense investigation lately and the participation of dopaminergic mechanisms in aversive conditioning is currently well documented, less consensus exists on the exact nature of such participation (Miller et al. 1957; Posluns 1962; Brandão et al. 2015; Lloyd and Dayan 2016; Brandão and Coimbra 2018). Changes in dopaminergic neurotransmission by environmental and pharmacological stressors or by stimulation of areas of the brain aversion system has been consistently demonstrated (Feenstra et al. 1995; Goldstein et al. 1996; Cuadra et al. 2000; Carvalho et al. 2005; Macedo et al. 2005). Additionally, a growing body of evidence supports the hypothesis that the mesolimbic dopaminergic pathway, originating from the ventral tegmental area (VTA), is recruited during fear conditioning (Deutch et al. 1985; Guarraci and Kapp 1999; Nader and LeDoux 1999b; Pezze and Feldon 2004; Fadok et al. 2010; Zweifel et al. 2011).

VTA neurons likely modulate fear and anxiety through their ascending projections. Studies from our laboratory examining the neurochemical aspects of fear conditioning revealed an increase in the extracellular concentration of dopamine in the nucleus accumbens and basolateral amygdala during the expression of contextual and cued conditioned fear, respectively (Martinez et al. 2008; de Oliveira et al. 2011, 2013, 2014b). Using systemic and local injections of dopaminergic agents, we showed that D2 receptors are preferentially involved in the expression, rather than in the acquisition, of conditioned fear (de Oliveira et al. 2006, 2009). Both systemic administrations of the D2 agonist quinpirole and D2 antagonist sulpiride have been shown to significantly reduce the expression of conditioned fear responses to light-CS and context-CS (de Oliveira et al. 2006, 2013; de Souza Caetano et al. 2013). We also demonstrated that the action of those compounds occurred in dopaminergic receptors located in distinct brain areas: the VTA in the case of the D2 agonist and the basolateral amygdala for the D2 antagonist (de Oliveira et al. 2009, 2011, 2017; de Souza Caetano et al. 2013). Therefore, in general, dopamine seems to mediate fear expression through its action on D2 receptors in the mesolimbic pathway (de Oliveira et al. 2014a; Brandão et al. 2015).

Dopaminergic activity has also been reported to play a role in fear extinction (Abraham et al. 2014; Madsen et al. 2017; Shi et al. 2017; Luo et al. 2018; Kalisch et al. 2019). Likely, D2 receptors can also modulate extinction, since they mediate the expression of conditioned fear and extinction is a learning process triggered by the expression of a previously acquired association in the absence of the US. In fact, systemic blockade of D2 receptors with sulpiride has been shown to facilitate the extinction of cued fear conditioning using a tone-CS in mice (Ponnusamy et al. 2005).

Another important D2 antagonist, mostly used for the treatment of schizophrenic symptoms, is haloperidol (Creese et al. 1976; Reynolds 1992; Di Lorenzo et al. 2019). Haloperidol is widely used due to its effectiveness and relatively low cost. However, the presence of important motor side effects, such as Parkinsonism, akathisia, and acute dystonia, is frequently observed (Tran et al. 1997; Adams et al. 2013). In rodents, haloperidol has been shown to reduce conditioned avoidance behaviors (Arnt 1982; Wadenberg and Hicks 1999; Wadenberg 2010), but it can also induce catalepsy, a state in which individuals fail to correct externally imposed postures (de Ryck et al. 1980; Sanberg 1980; Lorenc-Koci et al. 1996; Vasconcelos et al. 2003). Exploring the relationship between haloperidol-induced catalepsy and emotional states, we showed that haloperidol reduced distress calls emitted during contextual fear conditioning (Colombo et al. 2013). An antiaversive effect of haloperidol in the conditioned freezing, however, was not observed (Colombo et al. 2013).

To further examine the involvement of dopaminergic mechanisms in fear conditioning, we assessed the effects of sulpiride and haloperidol, two D2 receptors antagonists, in the expression and extinction of contextual and cued conditioned freezing in rats. We aimed at reproducing previous findings regarding the effects of sulpiride on the reduction of fear conditioning expression, to test the hypothesis that haloperidol would have similar effects to sulpiride, and to expand the characterization of the involvement of dopamine in conditioned fear by evaluating the effects of these two drugs on the extinction process.

Methods

Animals

One hundred and sixty naive male Wistar rats ($n = 10$ – 12 per group) from the animal facility of the Federal University of São Carlos (UFSCar) were used. The animals, weighing 300 g on average (ranging from 260 to 320 g), were housed in groups of four per cage (polypropylene boxes, $40 \times 33 \times 26$ cm), under a 12/12 h dark/light cycle (lights on at 07:00 h), at 23 ± 2 °C, and given free access to food and

water. Experiments were carried out during the light phase of the cycle. All procedures were performed in accordance with the National Council for Animal Experimentation Control and were approved by the Committee for Animal Care and Use of Federal University of São Carlos (Protocol No. 3627090915 and 9143060617).

Drugs

Drugs used were the dopaminergic D2 antagonists sulpiride and haloperidol. Both drugs were purchased from Tocris Bioscience (Bristol, UK), first mixed to 2% Tween 80, and then dissolved in physiological saline (0.9%). Physiological saline with Tween 80, 2%, served as vehicle control. The drugs were administered in a constant volume of 1 ml/kg, intraperitoneally (ip). Rats received vehicle, sulpiride (40 mg/kg) or haloperidol (0.1 or 0.25 mg/kg) 15 min before the start of the experiments. The drugs, doses and injection times were based on previous studies (de Oliveira et al. 2006, 2013; Colombo et al. 2013; de Souza Caetano et al. 2013; Barroca et al. 2019). The investigator was blind to the treatment condition of each rat. To achieve this, the drugs were prepared and stored in vials labeled with codes by a different investigator than the one who performed the behavioral experiments and data analysis.

Contextual fear conditioning

The experimental protocol for contextual fear conditioning was based on de Souza Caetano et al. (2013) and Reimer et al. (2018). Rats were submitted to three consecutive sessions, spaced by 24 h each: training, test and retest. Rats were conditioned to the context in a cage (26 × 20 × 20 cm) with the back wall, the two side walls and the ceiling made of metallic white material. The front door of the cage was made of transparent glass and the floor consisted of 13 stainless steel bars, 5 mm in diameter, spaced 1.5 cm apart. This cage was housed in a sound-attenuation chamber (66 × 43 × 45 cm) to avoid interference of environmental stimuli during the execution of the procedures. During the training session, a rat was placed in the experimental cage and, after a habituation phase of 5 min, it received ten unsignaled presentations of 1 s, 0.6 mA, footshocks-US. The intertrial interval varied randomly between 30 and 90 s. The footshocks were delivered through the cage floor by a constant current generator built with a scrambler (Insight Equipment, Ribeirão Preto, Brazil). The cage environment itself served as the CS. Each animal was removed 2 min after the last footshock and returned to its home-cage. The training session lasted approximately 15 min. The contextual conditioned fear test and retest sessions were conducted, without footshock presentation, in the same cage used for training. Twenty-four hours after training, the rats received

the intraperitoneal administration of drug or vehicle and, after 15 min, were re-exposed to the experimental cage for 10 min (test session) for evaluation of freezing expression. Twenty-four hours after the test, the rats were once again placed in the experimental cage for 10 min (retest session), for evaluation of extinction recall. The time rats spent freezing during the sessions was the outcome used to assess conditioned fear. Freezing was operationally defined as the total absence of movements, except those required for respiration, for at least 6 s per episode. The results are presented as a temporal analysis for each assay stage (percentage of freezing exhibited in two minutes blocks) and also reported for the total duration of test and retest (CS exposure). The fear extinction index (FEI) was computed subtracting the percentage of freezing exhibited during the retest session from the percentage of freezing during the final 2 min of the training session (in which the animals showed the peak freezing response).

To attest the efficacy of the conditioning on the expression of the contextual conditioned freezing response, two additional groups of animals were used, without drug administration. One group (same-context group) was submitted to the same training and test procedures described above. The other group (different-context group) was exposed to an identical training procedure to the first group, but the conditioned fear test was conducted in a different cage from the one used for training. This cage (32 × 30 × 30 cm) had transparent acrylic back wall, ceiling and front door, silver metallic side walls and a white plastic floor.

Cued fear conditioning

The experimental protocol for cued fear conditioning was based on de Oliveira et al. (2013) and Reimer et al. (2018). A different group of rats was submitted to three consecutive sessions—training, test, and retest—with a 24-h interval between sessions. During the training session, rats were conditioned to a light-CS in the same cage used for contextual fear conditioning. Each rat was placed in this training cage and, after a habituation phase of 5 min, it received eight CS–US pairings using a 20-s, 6-W, light-CS coterminating with a 1 s, 0.6 mA, footshock-US. The intertrial interval varied randomly between 60 and 120 s. The duration of each training session was about 20 min. The cued fear test and retest sessions were conducted in a different cage (26 × 25 × 20 cm) that had a gray metallic back and side walls, a transparent acrylic door and ceiling, and the floor consisting of 18 metal bars, 3 mm in diameter, spaced 1.5 cm apart. Twenty-four hours after training, the rats received the intraperitoneal administration of drug or vehicle and, after 15 min, were placed in the test cage for evaluation of freezing expression. After 5 min of habituation, eight light-CS with 20-s duration and interstimulus interval

varying between 60 and 120 s were presented (test session). Twenty-four hours after the test, rats were once again placed in the experimental cage and were presented with eight light-CS (retest session), for evaluation of extinction recall. The behavioral measure used to assess conditioned fear was the time that the rats spent freezing during the light-CS presentations. The results are presented as a temporal analysis for each assay stage (percentage of freezing exhibited for each 20-s light-CS presentation) and also reported for the total duration of the eight light-CS presentations for the test and retest sessions. The FEI was computed subtracting the percentage of freezing exhibited during the light-CS presentations in the retest session from the percentage of freezing during the final light-CS presentation of the training session (in which the animals showed the peak freezing response).

To attest the efficacy of the cued conditioning, two additional drug-free groups of animals were used. One group (paired group) was submitted to the exact same training and test procedures described above. The second group (non-paired group) was exposed to similar training and test procedures, but the eight presentations of the light and footshocks during the training session occurred in a non-paired fashion.

Motor performance

Two days after the retest session, the same rats used for the contextual fear conditioning experiment were used for the motor performance evaluation, consisting of the catalepsy and open-field tests.

Catalepsy test

The experimental protocol for the catalepsy test was based on Colombo et al. (2013) and Barroca et al. (2019). The rats were tested for catalepsy 15 and 45 min after administrations. During the interval between catalepsy tests, the animals were submitted to the open-field test. The catalepsy test was performed in a cage (41 × 33 × 17 cm), in which there was a horizontal acrylic bar (30-cm long and 1 cm in diameter), positioned 8 cm above the floor. The test consisted of carefully placing the animal's forepaws on the bar while their hind paws were kept on the floor. The cataleptic behavior is recognized as the failure to correct externally imposed postures; thus, the duration of catalepsy was measured as the latency to step-down from the horizontal bar.

Open-field test

The experimental protocol for the open-field test was based on de Oliveira et al. (2006) and Reimer et al. (2018). Locomotor activity and exploration were evaluated in an arena consisting of a circular enclosure made of transparent acrylic (60 cm in diameter, 50-cm height, floor divided into

12 sections). Twenty-five minutes after drug injection, rats were placed in the middle of the arena and left for a 15-min period of free exploration. For the course of the session, total number of crossings (number of floor sections traversed) and total number of rearings (standing with the forelegs raised in the middle of the arena or against the walls) were evaluated.

Analysis of results

Data are reported as mean ± SEM. For each experiment and drug treatment, two-way ANOVA for repeated measures was conducted on the percentage of freezing (contextual fear: time spent freezing during blocks of 2-min/2-min*100 or time spent freezing during session/total session duration*100; cued fear: time spent freezing during each cue presentation/cue presentation duration*100 or time spent freezing during cue presentation/total cue presentation duration*100), with treatments (drug doses and respective control) as a between-subjects factor and trials (2-min blocks/cue presentation or test and retest) as a within-subjects factor. Fear extinction (FEI: % freezing exhibited during the final phase of the training session—% freezing exhibited during the retest session) was analyzed with Student's *t* tests or one-way ANOVA. Motor performance was evaluated on the open-field test with Student *t* tests or one-way ANOVA and on the catalepsy test with two-way ANOVA for repeated measures, with treatment (drug doses and control) as a between-subjects factor and trials (15 and 45 min) as a within-subjects factor. Significant comparisons were followed by Newman–Keuls post hoc test. Values of $p < 0.05$ were considered statistically significant.

Results

Contextual fear conditioning

Rats submitted to the training and testing procedures in the same cage (same context) spent more time freezing than rats submitted to the test session in a cage different from the one used for training (different context; $t_{18} = 2.99$; $p < 0.05$; Table 1).

Sulpiride impaired the expression of contextual conditioned freezing (Fig. 1a, b). For the within-session analysis (Fig. 1a), freezing response increased across the training session ($F_{1,22} = 64.16$; $p < 0.05$); no differences were observed for treatments ($F_{1,22} = 0.75$; $p > 0.05$) or interaction between factors ($F_{1,22} = 0.13$; $p > 0.05$). Within the test session, sulpiride group exhibited less freezing response than the control group ($F_{1,22} = 20.64$; $p < 0.05$); no differences were observed for blocks ($F_{4,88} = 1.75$; $p > 0.05$) or interaction between factors ($F_{4,88} = 1.11$; $p > 0.05$). Within the retest session, no significant differences were observed

Table 1 Time of freezing during the test session in animals submitted to contextual or cued fear conditioning

	Groups	Freezing (%)
Contextual fear conditioning	Different context	36.07 ± 6.04
	Same context	62.07 ± 6.23*
Cued fear conditioning	Non-paired	9.44 ± 2.77
	Paired	37.81 ± 6.90#

Results are expressed as mean ± SEM. *n* = 10 for all groups

**p* < 0.05, different from the different-context group

#*p* < 0.05, different from the non-paired group

for blocks ($F_{4,88} = 2.25$; $p > 0.05$), treatments ($F_{1,22} = 0.16$; $p > 0.05$) or interaction between factors ($F_{4,88} = 1.62$; $p > 0.05$). For the between-session analysis (Fig. 1b), there was a significant effect for treatment ($F_{1,22} = 6.33$; $p < 0.05$) and a significant treatment vs. session interaction ($F_{1,47} = 10.76$; $p < 0.05$), but no significant effect for session ($F_{1,47} = 0.77$; $p > 0.05$). Post hoc comparisons revealed that sulpiride reduced freezing during the test session and that freezing was reduced between sessions in the control group ($p < 0.05$). No significant difference between treatments was observed for the fear extinction index (FEI; $t_{22} = 0.33$; $p > 0.05$; Fig. 1c).

Haloperidol did not affect the expression or extinction of contextual conditioned freezing (Fig. 1d–f). For

the within-session analysis (Fig. 1d), freezing response increased across the training session ($F_{1,33} = 159.74$; $p < 0.05$); no differences were observed for treatment ($F_{2,33} = 0.19$; $p > 0.05$) or interaction between factors ($F_{2,33} = 0.03$; $p > 0.05$). Within the test session, there was no difference for treatment ($F_{2,33} = 2.04$; $p > 0.05$); but significant differences for blocks ($F_{4,132} = 3.54$; $p < 0.05$) and interaction between factors ($F_{8,132} = 2.57$; $p < 0.05$) were observed. There were no relevant differences in the post hoc findings. Within the retest session, there was a difference for blocks ($F_{4,132} = 6.28$; $p < 0.05$), but not for treatment ($F_{2,33} = 1.45$; $p > 0.05$) or interaction between factors ($F_{8,132} = 1.15$; $p > 0.05$). Post hoc comparisons revealed a decrease in freezing in blocks 4 and 5 compared to block 2. For the between-session analysis (Fig. 1e), there was a significant treatment vs. session interaction ($F_{2,71} = 4.99$; $p < 0.05$), but no significant effect for treatment ($F_{2,33} = 0.11$; $p > 0.05$) or session ($F_{1,71} = 1.12$; $p > 0.05$). Post hoc comparisons revealed a reduction of freezing behavior between test and retest sessions for the control group ($p < 0.05$). No significant difference among treatments was observed for the fear extinction index (FEI; $F_{2,33} = 0.29$; $p > 0.05$; Fig. 1f).

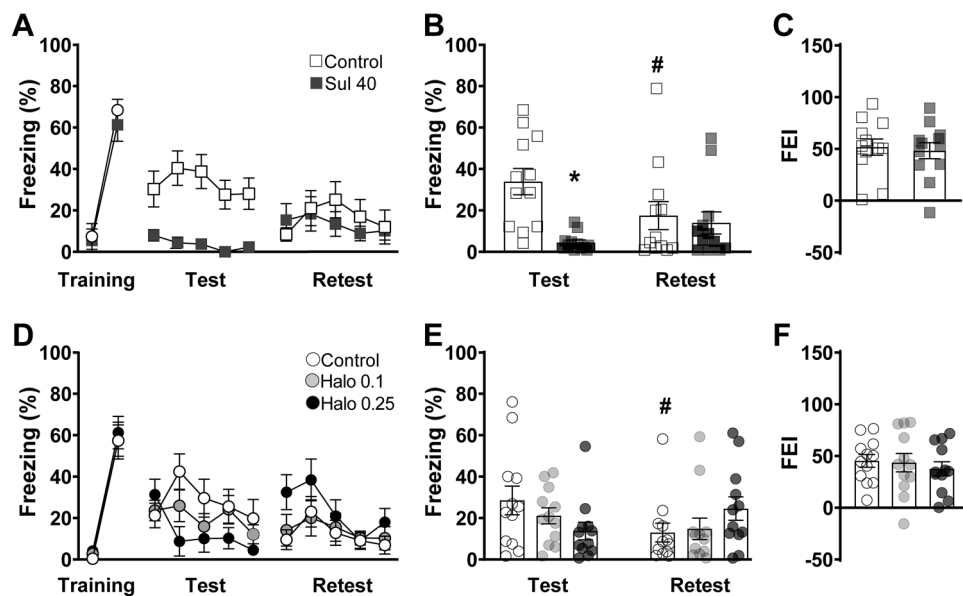


Fig. 1 Effects of sulpiride 40 mg/kg and haloperidol 0.1 and 0.25 mg/kg on the expression (test) and extinction (retest) of contextual conditioned freezing. **a** and **d** Mean percentage of freezing (blocks of 2 min) for training (initial and final blocks), test and retest sessions (time spent freezing during the block/block duration*100). **b** and **e** Mean percentage of freezing during test and retest sessions (time spent freezing during session/session duration*100). **c** and **f** Fear

extinction index (FEI; % freezing exhibited during the final block of the training session—% freezing exhibited during retest session). §*p* < 0.05: different from the initial phase of training session; **p* < 0.05: different from the control group in the same session; #*p* < 0.05: different from the same group during test session. *n* = 12 for all groups

Cued fear conditioning

Rats submitted to paired CS–US presentations (paired group) froze more than rats submitted to unpaired presentations (non-paired group; $t_{18} = 3.82$; $p < 0.05$; Table 1).

Sulpiride decreased the expression of cued conditioned freezing (Fig. 2a, b). For the within-session analysis (Fig. 2a), freezing response increased across the training session ($F_{1,22} = 54.1$; $p < 0.05$); no differences were observed for treatment ($F_{1,22} = 1.93$; $p > 0.05$) or interaction between factors ($F_{1,22} = 1.58$; $p > 0.05$). Within the test session, there was a difference for trial ($F_{7,154} = 6.64$; $p < 0.05$), but not for treatment ($F_{1,22} = 2.81$; $p > 0.05$) or interaction between factors ($F_{7,154} = 0.28$; $p > 0.05$). Post hoc comparisons revealed a decrease in freezing in trials 6, 7, and 8 compared to trial 2, and trial 7 and 8 compared to trials 3 and 4. Within the retest session, there were differences for trial ($F_{7,154} = 2.72$; $p < 0.05$) and interaction between factors ($F_{7,154} = 2.70$; $p < 0.05$), but no difference between treatments ($F_{1,22} = 0.02$; $p > 0.05$). There were no relevant differences in the post hoc findings. For the between-session analysis (Fig. 2b), there were significant differences for sessions ($F_{1,47} = 13.57$; $p < 0.05$) and treatment vs. session interaction ($F_{1,47} = 4.78$; $p < 0.05$), but not for treatments ($F_{1,22} = 1.30$; $p > 0.05$). Post hoc comparisons revealed that sulpiride reduced freezing during the test session and that freezing was reduced between sessions in the control group

($p < 0.05$). No significant difference between treatments was observed for the fear extinction index (FEI; $t_{22} = 1.19$; $p > 0.05$; Fig. 2c).

Haloperidol did not affect the expression or extinction of cued conditioned freezing (Fig. 2d–f). For the within-session analysis (Fig. 2d), freezing response increased across the training session ($F_{1,33} = 70.38$; $p < 0.05$) and no difference was observed for interaction between factors ($F_{2,33} = 1.37$; $p > 0.05$). Differences were observed for treatments ($F_{2,33} = 4.43$; $p < 0.05$); post hoc comparisons revealed differences in freezing response for the groups haloperidol 0.1 versus haloperidol 0.25 ($p < 0.05$), but no differences between drug treatments and control group were observed. Within the test session, there was a difference for trial ($F_{7,231} = 5.78$; $p < 0.05$), but not for treatment ($F_{2,33} = 2.14$; $p > 0.05$) or interaction between factors ($F_{14,231} = 1.48$; $p > 0.05$). Post hoc comparisons revealed a decrease in freezing in trials 8 compared to trials 1–4, and trial 6 compared to trials 2 and 3. Within the retest session, there were differences for trial ($F_{7,231} = 2.52$; $p < 0.05$) and interaction between factors ($F_{14,231} = 2.63$; $p < 0.05$), but not for treatment ($F_{2,33} = 1.71$; $p > 0.05$). There were no relevant differences in the post hoc findings. For the between-session analysis (Fig. 2e), there was a significant difference for session ($F_{1,71} = 9.20$; $p < 0.05$), but not for treatment ($F_{2,33} = 2.28$; $p > 0.05$) or treatment vs. session interaction ($F_{2,71} = 1.25$; $p > 0.05$). Post hoc comparisons

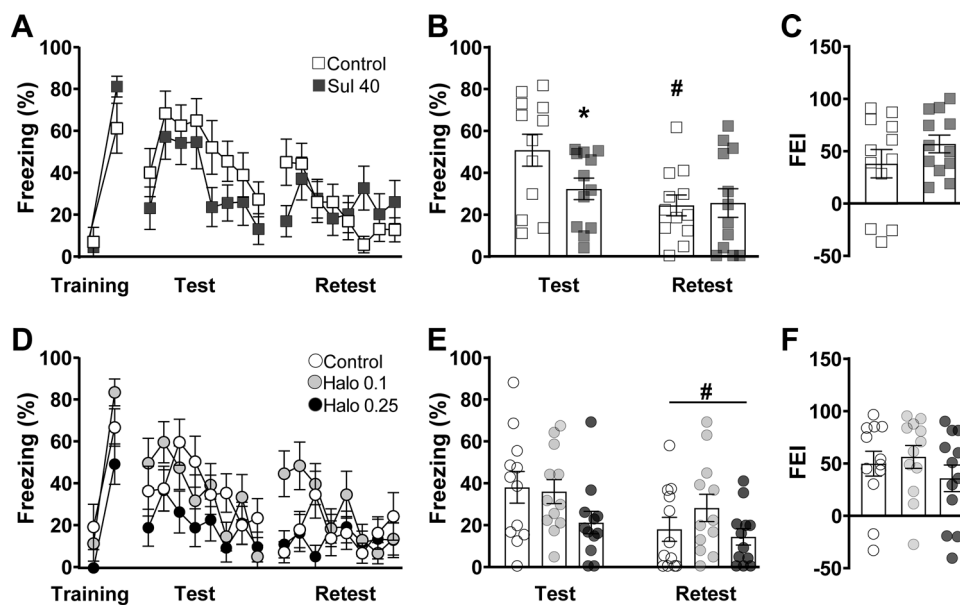


Fig. 2 Effects of sulpiride 40 mg/kg and haloperidol 0.1 and 0.25 mg/kg on the expression (test) and extinction (retest) of cued conditioned freezing. **a** and **d** Mean percentage of freezing in 20-s trials (corresponding to each light-CS presentation) for training (initial and final trials), test and retest sessions (time spent freezing during trial/trial duration*100). **b** and **e** Mean percentage of freezing during test

and retest sessions (time spent freezing during cue/total cue duration*100). **c** and **f** Fear extinction index (FEI; % freezing exhibited during the final light of training session—% freezing exhibited during retest session). § $p < 0.05$: different from the initial trial of the training session; * $p < 0.05$: different from the control group in the same session; # $p < 0.05$: different from the test session. $n = 12$ for all groups

showed a significant reduction of the freezing behavior during the retest in relation to the test session ($p < 0.05$). No significant difference among treatments was observed for the fear extinction index (FEI; $F_{2,33} = 0.78$; $p > 0.05$; Fig. 2f).

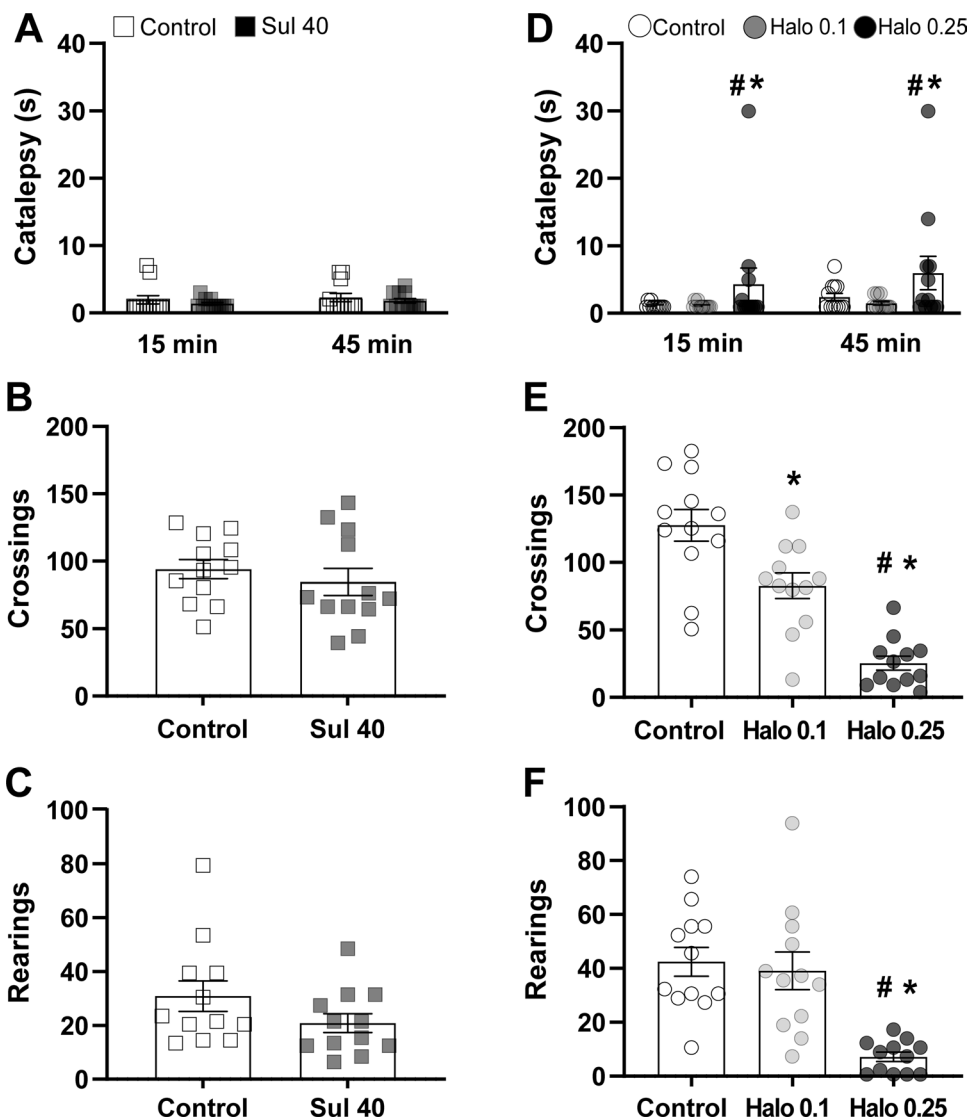
Motor performance

Sulpiride did not affect motor performance evaluated with the open-field and catalepsy tests (Fig. 3a–c). For the catalepsy test, the two-way ANOVA for repeated measures revealed no significant effect for treatments ($F_{1,22} = 0.74$; $p > 0.05$), trials ($F_{1,47} = 2.90$; $p > 0.05$) or treatment vs. trial interaction ($F_{1,47} = 0.04$; $p > 0.05$). For the open-field test, Student t tests revealed no significant differences for treatments on the number of crossings ($t_{22} = 0.77$; $p > 0.05$) or number of rearings ($t_{22} = 1.50$; $p > 0.05$).

Haloperidol-induced catalepsy and decreased the exploration of the open-field (Fig. 3d–f). For the catalepsy test,

the two-way ANOVA for repeated measures revealed a significant effect for treatments ($F_{2,33} = 3.61$; $p < 0.05$), but not for trials ($F_{1,71} = 1.07$; $p > 0.05$) or treatment vs. trial interaction ($F_{2,71} = 0.14$; $p > 0.05$). Newman–Keuls test revealed that haloperidol 0.25 mg/kg increased the latency to step-down the bar in relation to control and haloperidol 0.1 groups ($p < 0.05$). For the open-field test, one-way ANOVA revealed significant differences for treatments on the number of crossings ($F_{2,33} = 30.91$; $p < 0.05$) and number of rearings ($F_{2,33} = 14.18$; $p < 0.05$). Newman–Keuls test revealed that haloperidol 0.25 mg/kg decreased the number of crossings and rearings ($p < 0.05$), while haloperidol 0.1 mg/kg decreased the total crossings ($p < 0.05$) but did not affect rearings ($p > 0.05$).

Fig. 3 Effects of sulpiride 40 mg/kg and haloperidol 0.1 and 0.25 mg/kg on the motor performance in the catalepsy and open-field tests. **a** and **d** Latency to step-down in the catalepsy test 15 and 45 min after treatments. **b** and **e** Total number of crossings in the open-field test. **c** and **f** Total number of rearings in the open-field test. * $p < 0.05$: different from the control group; # $p < 0.05$: different from halo 0.1 group. $n = 12$ for all groups



Discussion

The ability to recognize and react properly to negative valence stimuli is critical for survival and mental health, and it may be dysfunctional in psychopathologies such as fear/anxiety-related disorders. The present study aimed to broaden the evaluation of the involvement of D2 dopaminergic receptors in the expression and extinction of cued and contextual conditioned fear. We replicated previous findings for sulpiride concerning the decrease in the expression of conditioned freezing response. The effects of sulpiride do not appear to be related to nonspecific action of the drug on motility. Although similar effects for haloperidol on the expression of freezing responses were expected, they have not been presented in a statistically significant way. The main effect of haloperidol was the motor impairment, with rats presenting a cataleptic-like state and reduced activity in the open-field. Both sulpiride and haloperidol had no significant effect on the extinction recall of conditioned fear. Taken together, the present results confirm the involvement of D2 receptor-mediated mechanisms in the expression of conditioned fear and suggest that D2 receptors are not involved in conditioned freezing extinction.

The results of the present study show the efficacy of the protocols used for the study of cued and contextual fear conditioning (Bolles and Collier 1976; Phillips and LeDoux 1992; Milad et al. 2009; Reimer et al. 2018). For the control experiments, reexposure to the aversive context or paired light-CS resulted in increased freezing compared with the different-context and non-paired groups, respectively. For the main experiments, freezing response increased along the training session, indicating that the initially neutral stimuli (context or light) became aversive CSs in all groups. For the control groups (which received vehicle), cued and contextual stimuli consistently kept freezing response high in the initial phase of the test, providing further evidence of learning during the training. Also, the results show freezing response declining throughout the test session, indicating extinction learning (supplementary information). Finally, the retest results allow us to conclude that our protocol promoted extinction since animals in the control groups retrieved and expressed the learned extinction memory after a 24-h delay.

The involvement of dopamine in aversive conditioning has been proposed by numerous studies using diverse paradigms (Nader and LeDoux 1999a, b; Pezze and Feldon 2004; Reis et al. 2004; Carvalho et al. 2009; Fadok et al. 2009; Zweifel et al. 2011; Brandão et al. 2015). Systemic injections of the D2 antagonist sulpiride have been shown to reduce the expression of conditioned fear (de Oliveira et al. 2006, 2013; de Souza Caetano et al. 2013). The

present study replicates this effect. Here, systemic injections of sulpiride attenuated freezing expression elicited by either a light-CS or a contextual-CS during the test session. We also show that sulpiride did not affect motor activity, so that nonspecific effects of this drug that could indirectly compete with the expression of conditioned fear can be discarded. Therefore, the present data indicate that D2 receptors play an important role in the expression of conditioned freezing.

Systemic injections of sulpiride in mice have also been shown to facilitate the extinction of cued fear conditioning, in this case using a tone-CS (Ponnusamy et al. 2005). In our study, however, no differences between sulpiride and control rats were observed during the retest session. Also, when exploring the effects of sulpiride with the fear extinction index (Milad et al. 2005; Reimer et al. 2018; Lonsdorf et al. 2019), by comparing freezing in training and retest sessions—both drug-free states that allow evaluation of fear while avoiding nonspecific effects of the drugs—no significant effects could be observed. These different results may be associated with variations in the protocols used. In Ponnusamy et al. (2005)'s study, sulpiride decreased freezing only in a protocol that did not promote extinction; with enough CS presentations, all groups extinguished equally, regardless of treatment, similar to what we observed in our study. Additional differences may occur because of the particularities of the dopaminergic mechanisms between rodent's species. During the extinction process in mice, which takes place slowly, D2 receptors would be maximally activated, while in rats, in which extinction is faster, less D2 activation might normally be present (Ponnusamy et al. 2005). This suggests that D2 receptors may have a modulatory rather than an essential role for extinction.

Haloperidol has also been shown to affect conditioned responses (Arnt 1982; Wadenberg and Hicks 1999; Wadenberg 2010) and, most evidently, to induce catalepsy in rodents (de Ryck et al. 1980; Sanberg 1980; Lorenc-Koci et al. 1996; Vasconcelos et al. 2003). Under our present experimental conditions, haloperidol did not seem to influence the expression or extinction of cued or contextual conditioned freezing behavior. Although based on previous studies (Colombo et al. 2013; Barroca et al. 2019), we adjusted haloperidol doses in an attempt to avoid motor impairments, we noted that haloperidol induced some degree of catalepsy, increased immobility and reduced ambulation in the open field. Therefore, motor effects influencing freezing expression during the test session cannot be discarded. Using the fear extinction index to avoid the impact of motor effects, we were able to verify that haloperidol did not affect freezing extinction. Haloperidol motor effects are associated with a higher affinity of the drug for D2 receptors in the nigrostriatal pathway (Creese et al. 1976; Sanberg 1980; Wadenberg et al. 2001; Vasconcelos et al. 2003). Thus, since

hypolocomotion was the primary effect observed with haloperidol treatment, we can suggest that the lack of an anxiolytic-like effect in freezing expression may be attributed to a higher modulation of the nigrostriatal dopaminergic pathway. On the other hand, blockade of D2 receptors at the terminals of the mesolimbic pathway may have been responsible for the decreased expression of conditioned fear caused by sulpiride.

Previous research found that VTA dopaminergic neural excitation is necessary for fear arousal produced by exposing animals to a CS during Pavlovian fear conditioning (Borowski and Kokkinidis 1996; Munro and Kokkinidis 1997; Zweifel et al. 2011). Activation of VTA neurons by threatening environmental stimuli likely modulates fear and anxiety through their ascending projections to structures such as the amygdala, nucleus accumbens, and prefrontal cortex (Oades and Halliday 1987; Pezze and Feldon 2004). Local injections of dopaminergic agents into diverse brain regions are helping to clarify the mechanisms relevant to fear conditioning. For example, both intra-VTA injection of quinpirole and intra-basolateral amygdala injection of sulpiride significantly reduce the expression of conditioned fear (de Oliveira et al. 2009, 2011, 2017; de Souza Caetano et al. 2013). These findings suggest that interference with the ability of the aversive CS to activate mesolimbic dopaminergic neurons reduces fear. This hypothesis is further supported by microdialysis data showing that an aversive contextual-CS increases dopamine release in the nucleus accumbens core and shell subregions (Martinez et al. 2008) and that an aversive light-CS increases the release of dopamine in the basolateral amygdala (de Oliveira et al. 2011, 2013, 2014b). Quinpirole and sulpiride, however, did not affect the contextual conditioned freezing response when injected into the nucleus accumbens (Albrechet-Souza et al. 2013). Indeed, VTA-nucleus accumbens signaling seems to be of critical importance for the formation of long-term extinction memories, rather than fear expression (Kalisch et al. 2019). A specific role for additional dopaminergic areas that receive projections from VTA in conditioned fear remains to be investigated.

The present results, together with previous findings from our laboratory (de Oliveira et al. 2006, 2009, 2011; de Souza Caetano et al. 2013), may have clinical implications for a better understanding of the contributions of agents targeting dopaminergic D2 receptors as adjunctive treatments in controlling exaggerated fear states. It may involve, for example, the ability of these drugs to block stress-induced activation of basolateral amygdala neurons and associated increases in emotionality. The present findings also support that dopaminergic D2 receptors are important for the expression of conditioned freezing regardless of the CS used. Contextual and cued fear conditioning has been considered to model distinct aspects of

fear/anxiety. Synaptic plasticity in the hippocampus seems to be especially involved in contextual, rather than in cued fear conditioning, whereas lesions of the amygdala have been shown to reduce both contextual and cued conditioned fear (Blanchard and Blanchard 1972a, b; Hitchcock and Davis 1987; Kim and Fanselow 1992; Phillips and LeDoux 1992). Since in the present study, contextual and cued responses were similarly affected by the dopaminergic pharmacological manipulation, the idea of an involvement of the amygdala in sulpiride's effects is reinforced.

Taken together, in addition to supporting the importance of dopaminergic mechanisms mediated by D2 receptors in the Pavlovian fear conditioning, the present results also indicate that these receptors are mainly involved in the expression of conditioned freezing rather than in the extinction of conditioned fear. A stronger action of sulpiride on the mesolimbic dopaminergic pathway is suggested, whereas haloperidol seems to preferentially act on the nigrostriatal dopaminergic pathway. In a study using amisulpride, an atypical neuroleptic with great similarity to sulpiride, an important feature of this drug was its limbic selectivity, that is, greater selectivity for limbic projections than for striatal projections (Schoemaker et al. 1997). Haloperidol, in contrast, showed more pronounced effects on dopamine receptors in striatal tissues (Schoemaker et al. 1997). Therefore, such affinity specificities may explain why sulpiride had a more significant effect on the expression of conditioned fear, while haloperidol had more effects on motor functions throughout our experiments.

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Data availability statement The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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