D₁-like receptors in the nucleus accumbens shell regulate the expression of contextual fear conditioning and activity of the anterior cingulate cortex in rats

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Abstract

Although dopamine-related circuits are best known for their roles in appetitive motivation, consistent data have implicated this catecholamine in some forms of response to stressful situations. In fact, projection areas of the ventral tegmental area, such as the amygdala and hippocampus, are well established to be involved in the acquisition and expression of fear conditioning, while less is known about the role of the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc) in these processes. In the present study, we initially investigated the involvement of the mPFC and NAc in the expression of conditioned fear, assessing freezing behaviour and Fos protein expression in the brains of rats exposed to a context, light or tone previously paired with footshocks. Contextual and cued stimuli were able to increase the time of the freezing response while only the contextual fear promoted a significant increase in Fos protein expression in the mPFC and caudal NAc. We then examined the effects of specific dopaminergic agonists and antagonists injected bilaterally into the posterior medioventral shell subregion of the NAc (NAcSh) on the expression of contextual fear. SKF38393, quinpirole and sulpiride induced no behavioural changes, but the D₁-like receptor antagonist SCH23390 increased the freezing response of the rats and selectively reduced Fos protein expression in the anterior cingulate cortex and rostral NAcSh. These findings confirm the involvement of the NAcSh in the expression of contextual fear memories and indicate the selective role of NAcSh D₁-like receptors and anterior cingulate cortex in this process.

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Introduction

The fear system is characterized by a set of processing circuits that detect and respond to danger, rather than as a mechanism by which subjective states are experienced. With this approach, one of the most common paradigms used to study the biological bases of fear in animal models is Pavlovian fear conditioning. An aversive unconditioned stimulus, usually an electrical footshock, is paired with an initially neutral cue, such as a tone, a light or a background context. After a few pairings, the conditioned stimulus (CS) begins to evoke a conditioned fear reaction that consists of behavioural and autonomic responses, including freezing, urination, increased arterial blood pressure and ultrasonic vocalization (Antoniadis & McDonald, 1999; Bouton & Bolles, 1980; Fanselow, 1984; Fanselow & Tighe, 1988; LeDoux et al. 1988; Pezze & Feldon, 2004; Wöhr et al. 2005).

In the last decades, evidence has accumulated that dopamine (DA) neurons in the ventral tegmental area (VTA) – which project to limbic regions including the amygdala, hippocampus, nucleus accumbens (NAc) and medial prefrontal cortex (mPFC) – react strongly to stressful situations (Herman et al. 1982; Thierry et al. 1976; Tidey & Miczek, 1996; Trainor, 2011). Additionally, further investigation revealed that distinct populations of DA VTA neurons may differentially respond to reward or aversive stimuli (Brischoux et al. 2009). DA exerts its actions through five G protein-coupled receptors, classified as D₁-like (D₁ and D₅) and D₂-like (D₂, D₃ and D₄) receptors, widely, but distinctly, distributed within the central nervous system (Gerfen, 2000; Missale et al. 1998; Rankin et al. 2010; Seeman, 2006; Vallone et al. 2000).

The amygdala has been extensively investigated and emerged as a crucial structure for the formation and expression of conditioned fear memories (Davis, 1992; LeDoux, 2000; LeDoux et al. 1988). The hippocampus, in turn, has been demonstrated to be more related to the acquisition and expression of associative fear memories evoked by contextual stimuli (LeDoux, 2000;
Maren, 2001). The mPFC, the other target structure of the mesolimbic DA system, has long been implicated in emotional processes (Albrechet-Souza et al. 2008, 2009; Devinsky et al. 1995; Kalisch et al. 2004; Mobbs et al. 2007; Pezze et al. 2003; Singewald, 2007), including conditioned fear (Kalisch et al. 2004; Milad et al. 2004; Singewald, 2007). However, the specific role of the mPFC subregions on defensive reaction is not completely well defined and divergent results suggest that these areas may be functionally dissociable (Etkin et al. 2011; Hedou et al. 2001; Heidbreder & Groenewegen, 2003; Morgan & LeDoux, 1995).

Although the involvement of the NAc in the formation/expression of fear memories is not clearly established (Josselyn et al. 2004), stressful experiences such as restraint (Abercrombie et al. 1989; Copeland et al. 2005; Ling et al. 2009), footshocks (Herman et al. 1982; Kalivas & Duffy, 1995; Thierry et al. 1976) and conditioned aversive stimuli (Martinez et al. 2008; Murphy et al. 2000; Wilkinson et al. 1998; Young et al. 1993, 1998) induce DA release/turnover in this area. The NAc contains two anatomically and functionally distinct subregions: a medioventral shell (NAcSh); a dorsolateral core (NAcC). The NAcSh predominantly receives limbic connections, whereas the NAcC has been described as a ventral extension of the striatum that plays a role in sensorimotor integration (Alheid & Heimer, 1996; Zahm, 1999; Zahm & Heimer, 1990). Moreover, recent formulations further divide the NAcSh into additional subregions, namely, the vertex, arch, cone and the intermediate and ventrolateral zones (Brenhouse & Stellar, 2006; Todtenkopf & Stellar, 2000).

In general, less is known about the involvement of the mesolimbic DA system in aversive compared to reward situations (Mirennowicz & Schultz, 1996; Ungless et al. 2010). In addition, the majority of studies that investigated this issue have focused on the participation of the amygdala and the hippocampus in fear-related circuits (Davis, 1992; LeDoux, 2000; LeDoux et al. 1988; Maren, 2001). In order to clarify the involvement of other target structures of the mesolimbic DA system in fear conditioning, the distribution of Fos protein (a neuronal activity marker) was assessed in the mPFC and NAc of Wistar rats after exposure to contextual or cued conditioned fear. Furthermore, the effects of selective DA drugs locally administered into the NAcSh on the expression of contextual freezing were examined, followed by measurements of Fos-immunoreactive cells.

Method

Animals

A total of 165 male Wistar rats from the animal house of the University of São Paulo, Ribeirão Preto campus, weighing 280–300 g, were used. They were housed in groups of four per cage with food and water available ad libitum in a temperature-controlled room (23 ± 1 °C) under a 12 h/12 h light/dark cycle (lights on 07:00 hours) for 72 h. The rats were transported to the experimental room in their home cages and left undisturbed for 1 h prior to testing. All efforts were made to minimize animal suffering and reduce the number of rats used. The experiments reported in this article were performed in accordance with the recommendations of the Brazilian Society of Neuroscience and Behavior and complied with the United States National Institutes of Health Guide for Care and Use of Laboratory Animals. The procedures were approved by the Committee for Animal Care and Use, University of São Paulo (No. 11.1.140.53.0).

Expt 1: Involvement of the mPFC and NAc in the expression of contextual and cued conditioned fear

Altogether, 60 rats were tested to reveal the involvement of the mPFC and NAc in the expression of conditioned fear elicited by a context, light or tone previously paired with footshock. These distinct paradigms of fear conditioning were used in view of evidence from neurobiological studies that pointed to the participation of different neural systems in cued and contextual fear conditioning (Davis, 1998; LeDoux, 1998). Fos protein expression was evaluated in four mPFC subregions [cingulate cortex area 1 (Cg1) and area 2 (Cg2), prelimbic (PrL) and infralimbic (IfL) cortices] and caudal NAc. The choice of the more posterior portion of the NAc was based on previous evidence of a rostrocaudal gradient in this structure, in which caudal NAcSh has been described to be more related to aversive states, whereas rostral NAcSh has been associated with appetite and reward (Kerfoot et al. 2007; Reynolds & Berridge, 2002).

The rats were randomly assigned into the following groups: non-conditioned group (control N group; n = 8, 5 and 5 for context, light and tone conditioning controls, respectively); different context-conditioned group (DC group; n = 8); same context-conditioned group (SC group; n = 8); light-conditioned group (L group; n = 13); tone-conditioned group (T group; n = 13). Twenty-four hours after conditioning, the rats were tested and time of the freezing response was scored for 16 min. Two hours after the beginning of the test session, they were deeply anaesthetized with urethane (3 g/kg i.p.; Sigma-Aldrich, USA) and intracardially perfused with 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). The brains were then removed from the skulls for the immunohistochemical detection of Fos protein.

Expt 2: Effects of dopaminergic drugs locally administered into the caudal NAcSh in the expression of contextual fear conditioning

A total of 105 rats were used to assess the involvement of NAcSh dopaminergic mechanisms in the expression of contextual fear conditioning. The choice of this area was
based on its activation found in expt 1 and its connections with limbic structures, assumed to be involved in integrating motivational information to modulate behaviours. The NAcC and its connections with motor structures seem to be more related to motor performance (Allheid & Heimer, 1996; Pezze & Feldon, 2004; Zahm, 1999; Zahm & Heimer, 1990).

The rats were subjected to stereotaxic surgery for bilateral cannula implantation in the caudal NAcSh. After 7 d recovery, they were subjected to contextual fear conditioning. Before the test session, the rats received central injections of saline (Sal) or drugs and were exposed to the context previously paired with footshock (chamber A) or a distinct context (chamber B). The time of the contextual freezing was scored during the test session. After 2 h, the rats were anaesthetized and intracardially perfused. The brains were removed from the skulls for histological analysis of the cannulae positions and Fos protein immunohistochemistry. Separate cohorts of rats were subjected to the rotarod test for locomotor activity assessment or received drug into the caudal NAcC to evaluate the specificity of the injection site.

**Behavioural procedures**

Two distinct chambers, chamber A and chamber B, were used for the fear conditioning procedures. Chamber A (48 × 26 × 25 cm) was used for the conditioning sessions. The side and back walls were made of grey acrylic and the ceiling and front door were made of transparent Plexiglas. The grid consisted of 36 stainless steel rods, spaced 1.5 cm apart, through which computer-controlled footshocks could be delivered. The chamber had a loudspeaker and a 15 W red lamp mounted on the rear panel. A sound generator produced a 1 kHz tone (72 dB; Insight Instruments, Brazil). The chamber was cleaned with 20% alcohol after each session. Chamber B (31 × 21 × 21 cm) was used for the test sessions. The side and back walls were made of steel and the ceiling and front door were made of transparent Plexiglas. The grid was covered with durable plastic. The chamber had a sound generator, loudspeaker and a 15 W white lamp mounted on a different position compared to chamber A (Insight Instruments). The chamber was cleaned with 20% alcohol and scented with 0.5% acetic acid after each session. Both chambers were enclosed in wooden sound-attenuating boxes.

Individual rats were placed in chamber A. Following a 5 min acclimatization period, the rat received 10 light-or tone-paired footshocks. In each pairing, a light (40 lux) or tone (72 dB, 1 kHz) was presented for 10 s, co-terminating with a footshock (0.6 mA, 1 s). The inter-trial interval varied randomly between 30 and 120 s. In the contextual conditioning sessions, no explicit cue was presented between footshocks. The rat was removed 3 min after the last shock and returned to its home cage. The conditioning session lasted approximately 16 min. Non-conditioning control groups were subjected to this same conditioning procedure, with the exception that the electrical stimulation was switched off during the session. After 24 h, the test session was conducted similar to the training session, but without the presentation of footshocks. All of the rats were tested in chamber B, with the exception that the SC and its respective N group were tested in chamber A. The experiments were conducted in the morning, between 09:00 and 11:00 hours. The criterion used to assess fear conditioning was the time of the freezing response over a period of 16 min. Freezing was operationally defined as the total absence of movement, with the exception of respiration (Bouton & Bolles, 1980; Fanselow, 1984). All of the experiments were monitored in real time by a trained investigator, who manually scored the freezing response through a video camera mounted 12 cm above the door and situated at the upper side of the box.

**Immunohistochemical analysis**

After removal from the skull, the brains were immersed overnight in paraformaldehyde and stored for 72 h in 30% sucrose in 0.1 M PBS for cryoprotection. The brains were quickly frozen in isopentane (−40 °C) and sliced in a cryostat (−19 °C). Sections (40 μm) were collected in antifreeze solution and Fos protein immunoreactivity was detected using standard techniques as previously described (Albrechet-Souza et al. 2008, 2009, 2011). Briefly, the sections were first treated with 1% hydrogen peroxide in 0.1 M PBS for 10 min and incubated with primary c-fos antibody (1:400; rabbit polyclonal; Santa Cruz Biotechnology, USA) in 0.1 M PBS enriched with 0.2% Triton-X and 0.1% bovine serum albumin overnight at 23 ± 1°C. The sections were then incubated in biotinylated goat anti-rabbit secondary antibody (1:400; Vector Laboratories, USA) and avidin-biotin complex (1:200; Vector Laboratories) for 1 h each. Fos immunoreactivity was revealed by the addition of the chromogen 3,3'-diaminobenzidine (DAB; 0.02%; Sigma-Aldrich) in 0.1 M PBS, to which 0.04% peroxide hydrogen was added before use. In expt 2, DAB was intensified with a 2.5% nickel solution in 0.2 M sodium acetate buffer (pH 6.5). After 7–8 min, the tissue sections were rinsed with buffer solution, mounted on gelatine-coated slides and dehydrated. Fos-positive neurons were visualized and counted under a 10× objective using a bright-field microscope (Olympus BX-50; Olympus Corporation, Japan) equipped with a video camera module (Leica DFC320; Leica Camera AG, Germany). The analysed brain areas, as represented in Fig. 1, were the mPFC, comprising the PrL, IFL (anterior/posterior coordinates from bregma: 3.24–3.00 mm), Cg1 and Cg2 (2.16–1.68 mm) and the NAc (NAcSh and NAcC; 1.56–1.20 mm in expt 1 and 2.16–2.04 mm in expt 2), according to Paxinos & Watson (2005). The representative area measured in the NAcSh corresponds to the cone and
intermediate zone (Brenhouse & Stellar, 2006; Todtenkopf & Stellar, 2000). In expt 1, Fos protein was counted in the caudal NAcSh. In expt 2, however, due to tissue damage produced by drug injections into this portion, immunohistochemical analysis was performed in the rostral NAcSh. Dark objects with areas between 10 and 80 $\mu m^2$ were identified and bilaterally counted using a computerized image analysis system (Image Pro Plus 6.2; Media Cybernetics, USA). Nuclei were counted individually and are expressed as the mean number of Fos-positive cells per 0.2 mm$^2$ tissue.

Surgery

Before the experimental sessions, the rats used in expt 2 were anaesthetized with ketamine/xylazine (100/7.5 mg/kg i.p.) and fixed in a stereotaxic apparatus (David Kopf, USA). The upper incisor bar was set 3.3 mm below the interaural line so that the skull was horizontal between bregma and lambda. After scalp anaesthesia with 2% lidocaine, the skull was surgically exposed and stainless steel guide cannulae (10 mm length, 0.6 mm outer diameter, 0.4 mm inner diameter) were bilaterally implanted in the caudal NAcSh (coordinates: anterior/posterior, 1.8 mm; medial/lateral, $\pm 0.6$ mm; dorsal/ventral: $-3.0$ mm) or caudal NAcC (coordinates: anterior/posterior, 1.8 mm; medial/lateral, $\pm 1.7$ mm; dorsal/ventral: $-3.0$ mm), according to Paxinos & Watson (2005). The cannulae were fixed to the skull with dental cement and two stainless steel screws. At the end of surgery, each guide cannula was sealed with a stainless steel wire to prevent obstruction and the rats received an i.m. injection of penicillin G benzathin (Pentabiotic, 600 000 IU, 0.2 ml; Fort Dodge, Brazil) and a subcutaneous injection of the anti-inflammatory and analgesic Banamine [flunixin meglumine, 2.5 mg/kg (10 mg/ml, 0.2 ml); Schering-Plough, Brazil]. After surgery, the rats were returned to their home cages in groups of four.

Drugs and drug administration

The following drugs were administered intra-NAcSh in expt 2: D$_1$-like agonist (±)-SKF38393 hydrochloride ($n=7$); D$_1$-like antagonist R(+)-SCH23390 hydrochloride ($n=7$ for both, same and different context); D$_2$-like agonist (−)-quinpirole hydrochloride ($n=7$); D$_2$-like antagonist (−)-sulpiride ($n=6$). All of the drugs were purchased from Sigma-Aldrich and dissolved in 0.9% physiological Sal shortly before use until a final concentration of 1 µg/0.2 µl for SKF38393, SCH23390 and quinpirole or 2 µg/0.2 µl for sulpiride. Physiological Sal also served as the vehicle control ($n=8$ and 7 for same and different context, respectively). The drugs were administered bilaterally 3 min prior to testing. The doses and times of the injections were based on previous studies (de Oliveira et al. 2009, 2011; Macedo et al. 2007). Infusions were delivered using an infusion pump (Harvard Apparatus, USA) in a constant volume of 0.2 µl for 30 s (speed of injection = 0.4 µl/min). A thin dental needle (0.3 mm outer diameter) attached by polyethylene tubing to a 5 µl Hamilton syringe was introduced through each guide cannula. The injection needle extended 4.5 mm below the ventral tip of the implanted guide cannula and bilateral infusions were administered simultaneously. The displacement of an air bubble inside the polyethylene tubing that connected the syringe to the injection needle was used to monitor the microinjections. The injection needles were left in place for 1 min after the end of the infusion to allow for diffusion.

Motor performance

The rats were placed on a rotarod (Rota-Rod 7750; Ugo Basile, Italy) and each time the animal fell off the rotarod (four rotations per min), it was immediately placed on it again until it achieved 2 min of continuous stability. The next day, the time spent on the rotarod was measured 5, 10 and 15 min after bilateral injections of Sal ($n=6$) or...
SCH23390 (n = 6) into the NAcSh. Rotarod performance was measured for 2 min or until the rat fell from the rod.

**SCH23390 administration intra-NAcC to test site specificity of the drug effect**

To assess the site specificity of the SCH23390 effect on freezing behaviour, rats were implanted with cannulae directed to the caudal NAcC. After recovery, they were subjected to contextual fear conditioning as previously described. Before the test session, they received bilateral injections of Sal (n = 7) or SCH23390 (n = 9) and were exposed to the context previously paired with footshock (chamber A).

**Histology**

Coronal brain slices (40 μm) that contained the NAc (1.92–0.96 mm from bregma) were stained with Cresyl Violet (5%, Sigma-Aldrich) to localize the microinjection sites by microscopic examination according to Paxinos & Watson (2005). Twenty-eight rats were excluded from exp 2 because of inappropriate cannula placement in the NAcSh or NAcC.

**Data analysis**

The software used for all of the statistical analysis was STATISTICA version 6.0. The data are expressed as mean ± S.E.M. In exp 1, the time spent freezing and the number of Fos-positive cells were analysed by one-way analysis of variance (ANOVA) for contextual fear or Student’s t test for cued fear. In exp 2, freezing response was analysed using one-way ANOVA for same context conditioning or Student’s t test for different context conditioning. The analysis of Fos-positive cells was performed using Student’s t test as well as the time spent freezing in rats treated with Sal or drug intra-NAcC. The rotarod data were analysed by two-way repeated-measures ANOVA. Newman–Keuls post-hoc test was used when appropriate. Values of p < 0.05 were considered statistically significant.

**Results**

**Exp 1**

The time spent freezing and number of Fos-positive cells in the rats subjected to context, light or tone conditioned fear are shown in Fig. 2a–c, respectively. The three CSs significantly increased conditioned freezing. The ANOVA revealed a significant main effect of group on contextual conditioning (F_{2,21} = 59.21, p < 0.0001). Subsequent post-hoc comparisons showed that the SC group exhibited significantly higher freezing compared with the DC group and N group. Student’s t test revealed increased freezing in the L group (t = 8.27, p < 0.0001) and T group (t = 10.21, p < 0.0001) compared to their respective control groups.

With regard to Fos immunohistochemistry, the ANOVA followed by subsequent post-hoc comparisons showed that contextual conditioning promoted a significant increase in the number of Fos-positive cells in the mPFC (Cg1: F_{1,17} = 5.12; Cg2: F_{1,19} = 4.03; PrL: F_{1,19} = 3.72; IFL: F_{1,19} = 3.65, p < 0.05 in all cases), compared to the N group and in the caudal NAc (NAcC: F_{1,18} = 7.50; NAcSh: F_{1,18} = 7.79, p < 0.005 in both cases), compared to the DC group and N group. Light conditioning and tone conditioning promoted a decrease in Fos expression in the IFL (t = 2.99 and 2.42, respectively, p < 0.05 in both cases) and caudal NAcSh (t = 3.38 and 2.87, respectively, p < 0.05 in both cases) compared to the respective N group. Cued conditioning did not produce significant changes in the activation of the other areas assessed (light conditioning: Cg1: t = 0.08; Cg2: t = 0.30; PrL: t = 1.88; NAcC: t = 0.91; tone conditioning: Cg1: t = 0.32; Cg2: t = 0.19; PrL: t = 1.61; NAcC: t = 1.82, p > 0.05 in all cases).

Representative photomicrographs that illustrate Fos immunoreactivity in the caudal NAcC and NAcSh in rats after the expression of contextual conditioned fear are shown in Fig. 3.

**Exp 2**

Diagrammatic representations of the Sal and drug injection sites in the NAcSh and NAcC are shown in Fig. 4. A representative photomicrograph that shows an injection site in the caudal NAcSh is presented in Fig. 5a. As shown in Fig. 5b, one-way ANOVA followed by the Newman–Keuls post-hoc test revealed that SCH23390 was the only drug that significantly increased the time of the freezing response when administered bilaterally into the caudal NAcSh before exposure to contextual fearful stimuli compared to all of the other treatments (F_{4,38} = 13.65, p < 0.00001).

However, Student’s t test revealed that SCH23390 intra-caudal NAcSh did not change the freezing response when administered to rats that were subjected to a context that was distinct from the conditioning context (t = 0.59, p > 0.05; Table 1).

As shown in Fig. 6a, Student’s t test revealed that SCH23390 injected bilaterally into the caudal NAcSh of rats before exposure to contextual fear significantly decreased the number of Fos-positive cells in the Cg1 (t = 3.23, p < 0.01), Cg2 (t = 2.65, p < 0.05) and rostral NAcSh (t = 2.15, p < 0.05). The drug did not produce immunoreactivity changes in the PrL (t = 1.51, p > 0.05), IFL (t = 1.03, p > 0.05) and rostral NAcC (t = 1.46, p > 0.05). Representative photomicrographs that illustrate Fos immunoreactivity in the Cg1 are shown in Fig. 6b.

Rotarod activity was not significantly influenced by intra-caudal NAcSh SCH23390 treatment, as revealed by two-way repeated-measures ANOVA (F_{1,10} = 1.86, p > 0.05). No significant effect of trials (F_{2,20} = 2.59, p > 0.05) nor treatment x trials interaction were found (F_{2,28} = 1.87, p > 0.05; Table 2).
**Fig. 2.** Time spent freezing and number of Fos-positive cells in the medial prefrontal cortex and nucleus accumbens of rats subjected to (a) contextual conditioning, (b) light conditioning or (c) tone conditioning test sessions. The data are expressed as mean ± S.E.M. *p < 0.05, compared to respective non-conditioned group (N); #p < 0.05, compared to different context-conditioned group (DC; n = 5–13 per group). SC, Same context-conditioned group; Cg1, cingulate cortex, area 1; Cg2, cingulate cortex, area 2; PrL, prelimbic cortex; IfL, infralimbic cortex; cNAcC, caudal nucleus accumbens core; cNAcSh, caudal nucleus accumbens shell; L, light-conditioned group; T, tone-conditioned group.

**Fig. 3.** Representative photomicrographs that illustrate Fos immunoreactivity (dark dots) in the caudal portions of the core (cNAcC) and shell (cNAcSh) subregions of the nucleus accumbens in rats after the expression of contextual fear conditioning. Scale bar = 150 μm. aca, Anterior commissure; N, non-conditioned group; DC, different context-conditioned group; SC, same context-conditioned group.
Finally, Student’s $t$ test revealed that injections of intra-caudal NAc SCH23390 did not change the time of freezing response in rats subjected to the contextual fear procedure ($t = 0.62, p > 0.05$; Table 3).

Discussion

In the present study, contextual and cued CSs (light and tone) consistently increased the freezing response during the test session. However, distinct neural activation patterns for contextual and cued fear conditioning were found, probably associated with the predictability of the threat. During cued fear – in which the CSs reliably signal a footshock – freezing response was negatively correlated with ventral mPFC activation. In contrast, during contextual fear – in which the trigger of the response is a set of unspecified environmental stimuli – there was a positive correlation between freezing response and mPFC activation. Distinct patterns of cortical activation in rats subjected to contextual or cued fear conditioning were previously demonstrated (Albrechet-Souza et al. 2011). Furthermore, dynamically changing brain activation in the mPFC has also been proposed in a study that used a manipulation of proximal vs. distal threat (Mobbs et al. 2007). In humans, mPFC activation has been demonstrated to depend upon the threat level during anxiety states (Straube et al. 2009).

Our results also showed that contextual fear increased the number of Fos-positive cells in caudal portions of the NAcC and NAcSh, whereas cued fear decreased Fos activation in the caudal NAcSh. In fact, the NAc has been considered necessary for the acquisition of conditioned taste aversion (Fenu et al. 2001) and expression of fear conditioning (Jackson & Moghaddam, 2001; Martinez et al. 2008; Pezze & Feldon, 2004; Riedel et al. 1997), although emotional responses to contextual CSs seem to be more susceptible to NAc manipulations than responses to explicit CSs (Gal et al. 2005; Lavoie & Mizumori, 1998; Levita et al. 2002; Pezze & Feldon, 2004; Riedel et al. 1997; Westbrook et al. 1997, 2000). Prior studies have already shown that exposure to fearful contexts increased the expression of the genes c-fos (Beck & Fibiger, 1995) and zif268 (Thomas et al. 2002) in the NAc. More recently, Muschamp et al. (2011) demonstrated that footshocks activate the transcription factor cAMP response element binding protein within the NAcSh. These genomic responses appear to reflect interactions between the hippocampal formation and NAc in the storage and retrieval of contextual fear memories (Groenewegen et al. 1987).

Possibly, cued and contextual fear conditioning may elicit distinct emotional states. Cued fear has been viewed as a model of fear-related disorders, such as phobias, in which the animals learn to fear a clear threat signal that predicts imminent danger (Ohman & Mineka, 2001). Then, the presentation of the CS induces a brief period of fear that subsides shortly after the offset of threat. In this way, cued fear does not model the essential features of anxiety, which is activated in a less discriminative way and focuses on future potential threat. The symptoms that characterize anxiety may be better modelled by contextual conditioning, since anxiety is neither triggered nor suppressed by explicit cues (Grillon, 2002).

Additional studies focusing on discrete subregions into the major subdivisions of the NAc may help clarify the participation of this area in fear conditioning (Brenhouse & Stellar, 2006; Todtenkopf & Stellar, 2000). Moreover, a possible explanation for the discrepancy in the literature about the involvement of the NAc in aversive situations is that the majority of the studies did not take into account the distinct rostral/caudal inputs of the NAcSh, which appear to be more susceptible to NAc manipulations than responses to explicit CSs (Gal et al. 2005; Lavoie & Mizumori, 1998; Levita et al. 2002; Pezze & Feldon, 2004; Riedel et al. 1997; Westbrook et al. 1997, 2000). Prior studies have already shown that exposure to fearful contexts increased the expression of the genes c-fos (Beck & Fibiger, 1995) and zif268 (Thomas et al. 2002) in the NAc. More recently, Muschamp et al. (2011) demonstrated that footshocks activate the transcription factor cAMP response element binding protein within the NAcSh. These genomic responses appear to reflect interactions between the hippocampal formation and NAc in the storage and retrieval of contextual fear memories (Groenewegen et al. 1987).

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Possibly, cued and contextual fear conditioning may elicit distinct emotional states. Cued fear has been viewed as a model of fear-related disorders, such as phobias, in which the animals learn to fear a clear threat signal that predicts imminent danger (Ohman & Mineka, 2001). Then, the presentation of the CS induces a brief period of fear that subsides shortly after the offset of threat. In this way, cued fear does not model the essential features of anxiety, which is activated in a less discriminative way and focuses on future potential threat. The symptoms that characterize anxiety may be better modelled by contextual conditioning, since anxiety is neither triggered nor suppressed by explicit cues (Grillon, 2002).

Additional studies focusing on discrete subregions into the major subdivisions of the NAc may help clarify the participation of this area in fear conditioning (Brenhouse & Stellar, 2006; Todtenkopf & Stellar, 2000). Moreover, a possible explanation for the discrepancy in the literature about the involvement of the NAc in aversive situations is that the majority of the studies did not take into account the distinct rostral/caudal inputs of the NAcSh, which appear to be more susceptible to NAc manipulations than responses to explicit CSs (Gal et al. 2005; Lavoie & Mizumori, 1998; Levita et al. 2002; Pezze & Feldon, 2004; Riedel et al. 1997; Westbrook et al. 1997, 2000). Prior studies have already shown that exposure to fearful contexts increased the expression of the genes c-fos (Beck & Fibiger, 1995) and zif268 (Thomas et al. 2002) in the NAc. More recently, Muschamp et al. (2011) demonstrated that footshocks activate the transcription factor cAMP response element binding protein within the NAcSh. These genomic responses appear to reflect interactions between the hippocampal formation and NAc in the storage and retrieval of contextual fear memories (Groenewegen et al. 1987).
expression in the posterior NAcSh than in its anterior portion (Kerfoot et al. 2007). Thus, the rostrocaudal gradient of the NAcSh may help clarify the involvement of this structure in both positive and negative motivational functions.

Once DA is considered one of the most potent neurotransmitters that modulate mechanisms underlying states of fear and anxiety (de Oliveira et al. 2006; Millan, 2003; Oei & King, 1980; Pezze & Felson, 2004), the role of the NAcSh in the expression of contextual aversive memories was further investigated through the local administration of selective dopaminergic drugs. The D1-like receptor antagonist SCH23390 injected into the caudal NAcSh produced an increase in freezing response; whereas, at the doses tested, the D2-like receptor agonist SKF38393, the D2-like receptor agonist quinpirole and the D2-like receptor antagonist sulpiride (Sul, 2 µg/side) did not alter this conditioned fear response.

Dopaminergic modulation at the level of the NAc has been suggested to play a key role in the ability to switch between behavioural repertoires in response to changing environmental contingencies (Howland et al. 2002; Kelley, 1999; Parkinson et al. 1999, 2000). More specifically, the NAcSh appears to mediate the motivational insignificance of stimuli (Gal et al. 2005). Therefore, antagonism of D1-like receptors in the caudal NACsh may produce a deficit in the recognition of a change in the aversive stimulus valence during the test session – in which the context, unlike during conditioning, no longer predicts footshock – resulting in an inappropriately high level of conditioned fear. Corroborating this idea, it has been demonstrated that the blockade of DA activity in the NAc impaired the learning extinction of conditioned fear (Holtzman-Assif et al. 2010) and a previous report found that DA is released in the NAcSh during the extinction of conditioned memories (Pezze et al. 2002). Interestingly, some symptoms of schizophrenia might be caused partly by selective abnormal recruitment of NAc microcircuits, causing abnormally valenced affect or motivational salience (Gray et al. 1999; Kapur & Remington, 2001; Taylor & Liberonz, 1999).

An alternative interpretation of the present results may be that the effects of SCH23390 could be associated with its spreading to the NAcC. Contrary to this possibility, however, SCH23390 administered intra-NAcC did not produce changes in contextual freezing. Also, in agreement with these results, it has been demonstrated that NAcSh-lesioned rats showed reduced conditioned freezing to context and a tendency toward reduced freezing to discrete stimuli, whereas NAC lesions do not impair cued freezing (Jongen-Reilo et al. 2003), nor conditioned suppression (McDannald & Galarce, 2011). Non-specific motor effects of the drug can also be discarded because SCH23390 did not alter the performance of rats in the rotarod test. Moreover, the D2-like receptor antagonist did not promote behavioural changes when administered to rats before exposure to a context that was distinct from the conditioning context.

The lack of the D1-like receptor agonist SK38393 effect may be attributed to the fact that endogenous DA released in the NAcSh during the expression of contextual conditioned fear already leads to a maximum activation of these receptors, in a way that the presence

| Table 1. Effects (mean ± S.E.M.) of intra-caudal nucleus accumbens shell injections of saline (Sal, n=7) or the D1-like receptor antagonist SCH23390 (1 µg/side, n=7) on the time spent freezing in rats before exposure to a context that was distinct from the conditioning context |
|---------------------------------------------------|------------------|
| Freezing time (s)                                 |                 |
| Sal                                               | 136.6 ± 63.2     |
| SCH23390                                          | 182.7 ± 46.0     |

Fig. 5. (a) Cresyl Violet-stained tissue showing the location of the cannula tip into the caudal nucleus accumbens shell; (b) effects of intra-nucleus accumbens shell infusion of saline (Sal), the D1-like receptor agonist SKF38393 (Skf, 1 µg/side), the D1-like receptor antagonist SCH23390 (Sch, 1 µg/side), the D2-like receptor agonist quinpirole (Quin, 1 µg/side) or the D2-like receptor antagonist sulpiride (Sul, 2 µg/side) before the test session on the freezing response in rats subjected to contextual fear conditioning. The data are expressed as mean ± S.E.M. * p < 0.05, compared to all other groups (n = 7–8 per group). Scale bar = 1 mm. cc, Corpus callosum; LV, lateral ventricle; aca, anterior commissure.
of the agonist does not result in further activation. In fact, neurochemical experiments have demonstrated that conditioned aversive stimuli evoke DA release in the NAc (Martinez et al. 2008; Murphy et al. 2000; Wilkinson et al. 1998; Young et al. 1993, 1998), and an in vivo microdialysis study showed an increase in DA release in NAcC and NAcSh during the retrieval of contextual fear (Martinez et al. 2008). Moreover, in a recent study, intra-NAc administration of the D₁-like

![Graph showing Fos-positive cells](http://ijnp.oxfordjournals.org/)

**Fig. 6.** (a) Effects of intra-caudal nucleus accumbens shell injection of saline (Sal) or the D₁-like receptor antagonist SCH23390 (Sch, 1 μg/side) on the number of Fos-positive cells in the medial prefrontal cortex and rostral nucleus accumbens of rats before exposure to contextual fear conditioning; (b) Representative photomicrographs illustrating Fos immunoreactivity (dark dots) in the cingulate cortex, area 1 (Cg1). The data are expressed as the mean ± S.E.M. of immunoreactive neurons in 0.2 mm² tissue in the indicated structure. *p < 0.05, compared to Sal (n = 5–7 per group). Scale bar = 100 μm. Cg2, Cingulate cortex, area 2; PrL, prelimbic cortex; IfL, infralimbic cortex; rNAcC, rostral nucleus accumbens core; rNAcSh, rostral nucleus accumbens shell.

**Table 2.** Effects (mean ± S.E.M.) of intra-caudal nucleus accumbens shell injections of saline (Sal, n = 6) or the D₁-like receptor antagonist SCH23390 (1 μg/side, n = 6) on time spent on the rotarod

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal</td>
<td>102.6 ± 11.5 s</td>
<td>119.8 ± 0.2 s</td>
<td>117.8 ± 2.2 s</td>
</tr>
<tr>
<td>SCH23390</td>
<td>118.7 ± 1.3 s</td>
<td>120.0 ± 0.0 s</td>
<td>120.0 ± 0.0 s</td>
</tr>
</tbody>
</table>

Motor performance was measured 5, 10 and 15 min after the injections.

of the agonist does not result in further activation. In fact, neurochemical experiments have demonstrated that conditioned aversive stimuli evoke DA release in

**Table 3.** Effects (mean ± S.E.M.) of intra-caudal nucleus accumbens core injections of saline (Sal, n = 7) or the D₁-like receptor antagonist SCH23390 (1 μg/side, n = 9) on the time spent freezing in rats subjected to contextual fear

<table>
<thead>
<tr>
<th></th>
<th>Freezing time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sal</td>
<td>414.3 ± 78.0</td>
</tr>
<tr>
<td>SCH23390</td>
<td>356.1 ± 56.0</td>
</tr>
</tbody>
</table>

the NAc (Martinez et al. 2008; Murphy et al. 2000; Wilkinson et al. 1998; Young et al. 1993, 1998), and an in vivo microdialysis study showed an increase in DA release in NAcC and NAcSh during the retrieval of contextual fear (Martinez et al. 2008). Moreover, in a recent study, intra-NAc administration of the D₁-like
receptor agonist SKF81297 did not affect the learning of conditioned avoidance responses (Wietzikoski et al. 2012).

In this study, while exposure to a context previously paired with footshocks was responsible for a generalized activation of the mPFC and caudal NAc, the administration of SCH23390 into the caudal NAcSh promoted a selective decrease in Fos protein expression in the rostral NAcSh and anterior cingulate cortex (ACC). Although the NAc does not send direct projections to the mPFC, this bottom-up processing of information may occur via amygdala, which shares reciprocal connections with the mPFC (St Onge et al. 2012). Thereby, the deactivation of the rostral NAcSh – the portion more related to appetite and reward (Kerfoot et al. 2007; Reynolds & Berridge, 2002) – associated with a reduction in ACC activity, which, in turn, disrupts its inhibitory control over limbic structures such as the amygdala and periaqueductal grey (Albrechet-Souza et al. 2009; Brandão et al. 2005; LeDoux, 2000; Sesack & Grace, 2010) may cause a strong negative affective reaction, leading to even higher levels of fear in an aversive environment.

Consistent with these results, ACC lesions have been demonstrated to increase conditioned freezing response in mice (Vouimba et al. 2000). In addition, a study that used a strain of rats with high innate anxiety-like behaviour showed that these animals presented ACC hyporeactivity when subjected to different threatening challenges, such as open arms, open field and social defeat (Singewald, 2007). In humans, individuals at ultra-high risk for psychosis have been shown to present cortical thickness in the ACC (Jung et al. 2011) and post-mortem and functional imaging studies have pivotally implicated the ACC in the generation or expression of psychotic symptoms in schizophrenia (Benes & Bird, 1992; Benes et al. 1992; Carter et al. 1997; Tamminga et al. 2000).

Altogether, the present data confirmed the involvement of the NAcSh in the expression of contextual fear memories. We also demonstrated that, whereas NAcSh D1-like receptors appear not to be involved in the expression of contextual fear, the D1-like receptors antagonist administered into the caudal NAcSh promoted the deactivation of rostral NAcSh and ACC neurons, leading to an inappropriately high level of freezing response in a context previously paired with footshocks. Acting as a modulatory area, the NAcSh can allow the fine tuning of the final behavioural response, rendering the fear system more plastic and defensive responses to external demands more appropriate. In humans, dysfunction of the mesolimbic DA pathway and morphological and functional alterations of the ACC are related to a series of psychiatric disorders, such as schizophrenia. Animal models may prove useful for unveiling the regulation of this limbic-motor interface and ultimately aid in the development of evidence-based selective drugs.

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Statement of Interest
None.

References


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