The roles of the nucleus accumbens core, dorsomedial striatum, and dorsolateral striatum in learning: performance and extinction of Pavlovian fear-conditioned responses and instrumental avoidance responses

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Abstract

This study examined the effects of bilateral excitotoxic lesions of the nucleus accumbens core (NAc-co), dorsomedial striatum (DMS) or dorsolateral striatum (DLS) of rats on the learning and extinction of Pavlovian and instrumental components of conditioned avoidance responses (CARs). None of the lesions caused sensorimotor deficits that could affect locomotion. Lesions of the NAc-co, but not DMS or DLS, decreased unconditioned and conditioned freezing. The NAc-co and DLS lesioned rats learned the 2-way active avoidance task more slowly. These results suggest: (i) CARs depend on both Pavlovian and instrumental learning; (ii) learning the Pavlovian component of CARs depends on the NAc-co; learning the instrumental component of CARs depends on the DLS, NAc and DMS; (iii) although the NAc-co is also needed for learning the instrumental component, it is not clear whether it plays a role in learning the instrumental component *per se* or if it simply allows learning of the Pavlovian component which is a pre-condition for learning the instrumental component; (iv) we did not find evidence that the DMS and DLS play the same roles in habit and goal-directed aspects of the instrumental component of CARs as observed in appetitive motivated instrumental responding.

Keywords: Pavlovian fear conditioning; conditioned avoidance responses; ventral striatum; neostriatum; caudate-putamen; procedural memory.

1. Introduction

During dangerous situations animals express species-specific fear behaviors. Freezing, fleeing (escape) and fighting are common unconditioned fear responses in rodents (Blanchard and Blanchard, 1989; Martinez, Oliveira, Macedo, Molina, and Brandao, 2008). Pavlovian conditioning significantly increases the chances of survival by allowing animals to anticipate a threatening event and respond preemptively (Blanchard and Blanchard, 1969; Fanselow and Bolles, 1979). Animals can also learn responses that are instrumental in avoiding danger (Bolles, 1970) and can learn conditioned avoidance responses (CARs) when responding to a Pavlovian stimulus in order to avoid a threatening event that would otherwise follow (Mowrer, 1956; Maia, 2010). Unconditioned fear responses, Pavlovian conditioned fear responses and CARs are also critical for human beings to deal with situations involving physical risks or aversive social challenges, and deficits in these processes are implicated in anxiety disorders (Deakin and Graeff 1991; Graybiel, 2008; Levita, Hoskin, and Champi, 2012; Lovibond, Chen, Mitchell, and Weidemann, 2012).

CARs depend on both Pavlovian and instrumental conditioning (Mowrer, 1956; Maia, 2010). In order to know when to act in order to avoid an aversive event signaled by a cue, the subject must first learn that the specific cue predicts the aversive event. Knowing this, one can choose an instrumental action to avoid the announced aversive event. In rodents, such learning is modeled by the 2-way active avoidance task in which rats can avoid a cued (announced) footshock by crossing to the opposite side of a shuttle box. Performance of this task depends on selecting this action in response to a specific predictive stimulus.

There is compelling evidence that the striatum and other regions of the basal ganglia play a role in learning how to select actions that result in rewarding outcomes (Schultz ,Dayan, and Montague, 1997; Alderson, Latimer, Blaha, Phillips, and Winn, 2004; Yin, Knowlton, and Balleine, 2004; Yin, Knowlton, and Balleine, 2006; Da Cunha,

Wietzikoski, Dombrowski, Santos, Bortolanza, Boschen, , and Miyoshi, 2009; Wilson, MacLaren, and Winn, 2009; Haber and Knutson, 2010; Redgrave, Rodriguez, Smith, Rodriguez-Oroz, Lehericy, Bergman, Agid, DeLong, and Obeso, 2010; Flagel, Robinson, Mayo, Czuj, Willuhn, Akers, Clinton, Phillips, and Akil, 2011; Da Cunha, Gomes, and Blaha, 2012; Dezfouli and Balleine, 2012; Kravitz, Tye, and Kreitzer, 2012; Liljeholm and O'Doherty, 2012) as well as learning how to select actions instrumental to avoid aversive stimuli (Wadenberg, Ericson, Magnusson, and Ahlenius, 1990; Prado-Alcala, Galindo, Aguilar, Guante, and Quirarte 2004; La Lumiere, Nawar, and McGaugh,2005; Izquierdo, Bevilaqua, Rossato, Bonini, Da Silva, Medina, and Cammarota, 2006; Manago, Castellano, Oliverio, Mele, and De Leonibus, 2009; Darvas, Fadok and Palmiter, 2011; Dombrowski, Maia, Boschen, Bortolanza, Wendler, Schwarting, Brandao, Winn, Blaha, and Da Cunha, 2012). Subregions of the dorsal and ventral striatum are known to play differential roles in learning appetitive motivated actions (Yin et al., 2004; Yin and Knowlton, 2006; Yin et al., 2006; Redgrave et al., 2010; Dezfouli and Balleine, 2012). During instrumental conditioning learned under appetitive motivation, early responding appears to be goal-directed and slowly progresses to habitual responding (Mishkin, Malamut, and Bachevalier, 1982; Knowlton, Mangels, and Squire, 1996; Packard and Knowlton 2002; but see Broadbent, Squire, and Clark 2009). Conversely, during extinction (when a response is no longer rewarded), goal-directed responding of appetitive motivated actions rapidly fade while habitual responses persist for a relatively longer time (Devan and White, 1999; Yin et al., 2006; Balleine and O'Doherty, 2010). An action is considered to be goal-directed if it is sensitive to outcome devaluation; for example, by pre-feeding the animal (Dickinson and Balleine, 1994). In contrast, stimulus-response (S-R) habits are considered to be insensitive to outcome devaluation, being performed not with an intended goal but as an automatic response to a stimulus that precedes the response's outcome (Yin, Ostlund, and Balleine, 2008). The dorsomedial striatum (DMS) and the dorsolateral striatum (DLS) are thought to be needed for selection of, respectively,

goal-directed (that is, action-outcome, A-O) and S-R habits learned under appetitive reinforcement (Yin et al., 2006; Ikemoto, 2007). Although this is well established for appetitive motivated learning, it is not clear whether the same striatal regions play equivalent roles in aversively motivated learning. There is evidence that the nucleus accumbens core (NAc-co) plays a role in Pavlovian conditioning (Riedel, Harrington, Hall, and Macphail, 1997; Ikemoto and Panksepp, 1999; Di Chiara, 2002; Belin, Jonkman, Dickinson, Robbins, and Everitt, 2009; Berridge, 2012; Bossert, Stern, Theberge, Marchant, Wang, Morales, and Shaham, 2012; Klucken, Schweckendiek, Koppe, Merz, Kagerer, Walter, Sammer, Vaitl, and Stark, 2012) but there is some uncertainty about the specific roles the NAc-co and other limbic structures have in Pavlovian conditioning (for a review see Da Cunha et al., 2012).

The present study tested whether lesions in the NAc-co, DMS or DLS would produce deficits in learning and extinction of the Pavlovian and instrumental components of 2-way active avoidance that are compatible with a role in Pavlovian, goal-directed, and habitual aspects of CARs. First, sham-operated rats and rats bearing lesions in the NAc-co, DMS, and DLS were trained to predict inescapable footshocks by use of a sound cue. Pavlovian learning and extinction was inferred from scores of conditioned freezing measured under extinction in 3 sessions. Next, instrumental CARs were measured in 6 sessions in which rats were trained to avoid cued footshocks by crossing to the opposite side of a shuttle box. Finally, CAR extinction was evaluated in the next 4 sessions in which the rats were exposed to the same footshock and sound stimuli, but presented in an unpredictable (non-contingent), inescapable and unavoidable manner.

2. **Methods and Materials**

2.1 Subjects

Adult male Wistar rats from the colony of the Universidade Federal do Paraná, weighing 200-260 g at the beginning of the experiments were used. The rats were maintained in a temperature-controlled room (22 ± 2ºC) on a 12-h light/dark cycle (lights on, 7:00 a.m.) with water and food available *ad libitum*. These procedures were approved by the Animal Care and Use Committee of the Universidade Federal do Parana (protocol number 545) and are consistent with the Brazilian (11.794/ 8 October 2008) and European (EC Council Directive, 24 November 1986; 86/609/EEC) legislation.

Forty four rats were randomly assigned to 4 experimental groups and given lesions in the: NAc-co (n = 9), DMS (n = 10), DLS (n = 10) and an additional group was sham-operated (n=15). Five of the sham-operated group were given sham lesions of the DMS, NAc-co, and DLS. From these rats, 5 died and 5 were tested but eventually discarded because of inappropriate lesion location. Deaths were caused probably to respiratory arrest during long-duration surgery. Only the remaining rats had their behavioral data analyzed: 7 NAc-co, 8 DMS, 8 DLS, and 11 sham rats.

2.2 Surgery

The rats received atropine sulfate (0.4 mg/kg, i.p.) and penicillin G-procaine (20,000U in 0.1 mL, i.m.) and were anesthetized with 3 mL/kg equithesin (1% sodium thiopental, 4.25% chloral hydrate, 2.13% magnesium sulfate, 42.8% propylene glycol, and 3.7% ethanol in water), placed in the stereotaxic frame with the nose bar adjusted to -3.3 mm. Burr holes were drilled in the skull and the neurotoxin quinolinic acid (20 µg/µL) infused with a Hamilton syringe fitted to a microinfusion pump (Stoelting, QSI-quintessential Stereotaxic Injector, Wood Dale, IL) into the NAc-co, DMS, and DLS according to coordinates adapted from Castañé, Theobald, and Robbins (2010) (shown in Table S2). Sham-lesioned rats received vehicle (PBS solution composed of phosphate buffer 0.1 M, 0.9% NaCl, pH 7.4) in the NAc-co, DMS or DLS instead of quinolinic acid (5 rats per group). After surgery, rats were allowed to recover from

anesthesia in a temperature-controlled chamber and then placed back in their home cages.

2.3 Behavioural procedures

Fifteen days after surgery, rats underwent a Pavlovian fear conditioning training session followed by 3 sessions under extinction over the next 3 days. Three days later, rats were trained in the 2-way active avoidance task for 3 days and tested under extinction for 2 additional days (2 training or extinction sessions per day).

2.4 Behavioural apparatus

Pavlovian fear conditioning and 2-way active avoidance were carried out in an automated shuttle box (Insight Instruments, Ribeirao Preto, Brazil). The box (23 x 50 x 70 cm) has walls made of Plexiglas and a floor made of parallel 5 mm caliber stainless-steel bars, 15 mm apart. The floor was divided (unmarked) into six 12.5 x 10 cm rectangles. The number of tones (conditioned stimulus, CS), footshocks (unconditioned stimulus, US), and crossings between the two sides of the box were recorded automatically. The sessions were videotaped and the time of freezing (see below) was scored manually. We used the same box for the two tasks in order to study the contribution of both Pavlovian and instrumental-learning processes on the learning and extinction of conditioned avoidance. Using different stimuli or different groups of rats would have impaired such analysis.

2.5 Pavlovian fear conditioning

The training session was carried out immediately after the 10 min in which the rats were habituated to the shuttle box. Ten tones (1.5 KHz, 60 dB, 10 s) were delivered, each of them paired with a 0.4 mA inescapable footshock delivered in the last second of the tone presentation. The interval between each pair of stimuli varied randomly between 30 and 120 s. The rats returned to their home cages immediately

after delivery of the last pair of stimuli. The test sessions were carried out in the same box; during 10 min, the same 10 s tones were presented 10 times, separated by the same random intervals, without presentation of the US. The time during which rats were exhibiting freezing (no movements, except for respiratory and vibrissal movement) in the test sessions was recorded by an observer blind with respect to treatment condition. This protocol was adapted from the study of Albrechet-Souza, Borelli, Almada, and Brandao (2011).

2.6 Two-way active avoidance

Three days after Pavlovian fear conditioning, the animals were trained in the 2-way active avoidance task for 3 days and tested under extinction for 2 additional days (2 training/extinction sessions per day). Training was carried out according to Da Cunha, Gevaerd, Vital, Miyoshi, Andreatini, Silveira, Takahashi, and Canteras (2001). The 2-way active avoidance training sessions started immediately after the rat was placed in the shuttle-box and consisted of 40 pairings of the same tone (maximum duration of 20 s) with a 0.4 mA footshock (maximum of 10 s) that started 10 s after the beginning of the tone. The rat could interrupt the tone and avoid the shock by the instrumental action of crossing to the opposite side of the shuttle box. In the extinction sessions, the same mean number and duration of footshocks and tones were presented but in an inescapable, unpredictable and unavoidable manner: stimuli of different durations (varying from 1-10 s for the shocks and from 1-20 s for the tones) were presented in a random order and at random intervals; the tones and shocks were presented in a temporally non-contiguous manner except for 2 times in order to not allow the animal to learn that the tone was a safe signal. This protocol was adapted from that used by Dombrowski et al. (2012). We used it because (in contrast to extinction of an appetitive instrumental response) after the rat has learned the instrumental action to avoid the US, omission of the US contingent to the instrumental response represents a reward, thus reinforcing behaviour. Three measures of

behaviour were taken: (i) avoidance: during presentation of the CS, the rats could turn off the sound and actively avoid the shock by crossing to the opposite side of the box; (ii) response failure: a trial in which the rat did not cross to the opposite side during either the CS or US presentation; (iii) inter-trial crossing (ITC): the number of crossings between the two sides of the box during the intervals between CS-US pairings. Locomotor activity during 10 min of habituation to the shuttle box (before the Pavlovian fear conditioning training session) was also evaluated. The number of transitions between the six 12.5 x 10 cm rectangles into which the floor was mentally divided (there were no actual markings on the cage floor) was counted for 10 min. The time during which rats were exhibiting freezing was scored in the first 10 min of the first training day.

2.7 Histology

At the end of the experimental procedures, histological analysis was carried out. Rats were killed by an overdose of pentobarbital and brains were fixed *in situ* using transcardial perfusion at room temperature of saline solution (0.9%) followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were placed in the same fixative containing 20% sucrose for 72 h at 4ºC. A series of 40 µm sections were cut in the frontal plane with a vibrating-blade microtome (Leica, VT1000 S, Bensheim, Germany). Some sections were immediately mounted on gelatin-coated slides and, after 48 h stained with thionin, before being examined under a light microscope (DM 2500, Leica, Heerbrugg, Switzerland) in order to evaluate lesions in the NAc-co, DMS and DLS groups and the intact tissue in sham operated rats. A parallel series of sections were processed free floating to demonstrate neuronal nuclear protein (NeuN) using immunohistochemical techniques. The sections were incubated for 45 min in goat serum-based blocking solution (20% serum, 0.1% Triton, in PBS). Primary antibody was mouse anti-NeuN (1:20.000/ overnight; Chemicon International Inc., Temecula, CA, USA) followed by IgG anti-mouse secondary antibody (1:10.000/ 90

min) and Elite Peroxidase ABC kits (Vector Labs, Peterborough, UK) and Sigma fast DAB substrate (Sigma Chemical Co., St Louis, MO, USA). After the NeuN stained sections were mounted, they were examined using light microscopy in order to estimate damage in the NAc-co, DMS and DLS.

2.8 Statistical analysis

The results were analyzed by one- or two-way ANOVA with repeated measures (lesion group as independent factor and session as repeated factor) followed by post-hoc Dunnett's test. A co-variant was added in cases in which the lesion factor affected both training and test of related scores. Correlations were analyzed using the Pearson test. Differences were considered to be statistically significant at the level of $p < 0.05$.

3. **Results**

3.1 Histology

Data of 5 rats showing damage outside the target region were excluded from the analyses. The remaining numbers of rats in each group were: sham (n= 11), NAc-co (n= 7), DMS (n= 8), and DLS (n= 8). Acceptable lesions for statistical analysis included bilateral damage of the investigated areas (NAc-co; DMS and DLS) throughout most of its extent with minor damage to surrounding areas. Typical thionin- and NeuN immunostained sections of control and lesioned brains and the maximum and minimum damage resulting from the lesions for the animals included in the behavioral analyses are shown in Fig. 1 (NAc-co), Fig. 2 (DMS), and Fig. 3 (DLS). NAc-co rats showed substantial neuronal loss bilaterally that typically extended from 10.0 mm to 11.3 mm anterior to the interaural line (IA). DMS lesions were confined to the part of the caudate-putamen close to the lateral ventricle; they typically extended

from 10.1 mm to 11.3 mm anterior to the IA. DLS lesions were also substantial and confined to the area between the DMS to the corpus callosum; typically, they extended from 10.0 mm to 11.1 mm anterior to the IA.

Additionally, we analyzed all sham rats by surgery group (SHAM-DMS, SHAM-DLS, SHAM-NAc-co) to evaluate if there were any differences between them. Two-way ANOVA showed non-significant group effect ($F(2,7) = 0.02$; $p= 0.97$); a significant session effect ($F(4,28) = 33.63$; $p < 0.001$) and a non-significant group x session interaction ($F(8,28) = 0.41$; $p = 0.89$) to the Pavlovian fear conditioning sessions (pre-training, training, test 1, test 2 and test 3). Also, we did not observe differences between these groups when analyzing the instrumental-conditioning training (1-6) sessions. A two-way ANOVA showed non-significant group effect ($F(2,8) = 0.89$; $p= 0.44$), a significant session effect ($F(5,40) = 26.40$; $p < 0.001$), and a non-significant group x session interaction ($F(10,40) = 1.21$; $p = 0.31$). Instrumental conditioning extinction (7-10) sessions showed non-significant group effect ($F(2,8) = 2.29$; $p= 0.16$), a significant session effect ($F(3,24) = 22.13$; $p < 0.001$), and a non-significant group x session interaction ($F(6,24) = 0.83$; $p = 0.55$).

3.2 Post-surgery evaluation of health and sensorimotor parameters

Behavioral testing began after all the rats had recovered their body weight after surgery (Table S1); they did this quickly regardless of the location of lesions: no significant difference in body weight was observed between groups (ANOVA: group effect $F(3,30) = 0.16$; $p = 0.91$). Control experiments were carried out to evaluate whether the lesions caused sensorimotor alterations that could affect performance in fear conditioning or in the 2-way active avoidance task. As shown in Table 1, when the rats were exposed to the shuttle box for the first time (during habituation, just before the fear conditioning training session), locomotor activity of the lesioned groups did not

significantly differ from the sham group. ANOVA showed no significant effect $F(3,30) = 1.32$, $p = 0.28$). In addition, during 2-way active avoidance sessions, no significant difference between groups was observed in the inter-trial crossings between shuttle box compartments ($F(3,30) = 0.52$, $p = 0.66$). These findings suggest that the lesions did not cause motor deficits which could affect performance in fear conditioning and 2-way active avoidance tasks. Decreased sensitivity to the footshock – or decreased fear during exposure to it – is also unlikely to have occurred in the lesioned rats: all of them reacted to footshock by (for example) a startle reaction, jumping or running with a latency of less than one second. As shown in Table 2, no significant effects were observed in relation to the latency to escape from the first footshock ($F(3,30) = 0.60$, $p = 0.61$) or in the latency to cross in response to the last tone ($F(3,30) = 2.06$, $p = 0.12$). In agreement with these findings, only occasionally did the lesioned rats fail to respond to the tone/footshock – no more than twice in each 40-trial session; a one-way ANOVA showed no significant difference between groups ($F(3,30) = 0.52$; $p = 0.66$).

3.3 Unconditioned responses to the footshock

The lesions did not alter unconditioned motor and emotional responses to the footshock. As shown in Fig. 4, in all groups the time of freezing elicited by the tone/footshock pairing in the training session was significantly longer compared to the pre-training session in which the animals habituated to the apparatus $F(1,24) = 234.37$, $p < 0.001$). No significant difference between the groups was observed in pre-training $F(3,24) = 2.10$, $p = 0.12$). However, on the training day the NAc-co group showed freezing times that were significantly lower compared to the sham group $F(3,25) = 2.99$, $p < 0.05$, ANOVA; $p < 0.05$ Dunnett's test). It is important to note that the training did not produce place preference. The rats could freely move between the two sides of the shuttle box while the 10 tone-footshocks stimuli were presented. ANOVA of the number of times the rats received shocks in the right or left side of the cage revealed

no group effect ($F(3,22) = 1.12$, $p = 0.35$), side effect ($F(1,22) = 0.47$, $p = 0.49$) or interaction effect ($F(3,22) = 0.23$, $p = 0.87$).

3.4 Conditioned fear responses to the tone

Data are shown in Fig. 4. ANOVA shows that only the NAc-co group presented significant reduced freezing in the first test session compared to the sham group $F(3,29) = 6.30$, $p < 0.01$; $p < 0.05$, Dunnett's test). ANOVA showed significant differences among groups in the test 2 session $F(3,29) = 3.91$, $p < 0.05$), but the Dunnett's test detected no significant difference between the sham group and any of the lesioned groups. No significant effect among groups was observed in the test 3 session $F(3,29) = 1.75$, $p = 0.17$). Although the lesion of the NAc-co decreased the unconditioned response to the US (see above), this cannot completely account for the reduced response to the CS observed in the NAc-co rats. ANOVA of the freezing scores in the first session test, considering freezing scores in the training session as covariate, this statistical outcome also showed a significant difference between the NAc-co and sham groups ($F(1,14) = 14.13$, $p < 0.01$). This statistical outcome did not change analyzing all the lesioned and the sham groups data ($F(3,24) = 8.79$; $p < 0.01$) and comparing sham and NAc-co groups with the Dunnett's test ($p < 0.05$).

3.5 Instrumental avoidance responses to the tone

A two-way ANOVA of the number of avoidances in the training (sessions 1-6, Fig 5A) showed significant group ($F(3,30) = 5.78$; $p < 0.01$) and session effects ($F(5,150) = 81.56$; $p < 0.001$) and a significant group X session interaction effect ($F(15,150) = 3.83$; $p < 0.001$). Separate ANOVA for each session showed significant effects in all of the 6 sessions ($p < 0.05$). Post-hoc Dunnett's tests showed different patterns of impairment among the lesioned groups. In the first 3-4 sessions the NAc-co

and DLS groups learned the 2-way active avoidance task significantly more slowly than the sham-lesioned group ($p < 0.05$), but they achieved avoidance scores not significantly different compared to the sham group in the last 2-3 training sessions. In contrast, the DMS group presented scores not significantly different compared to the sham group until the third session, but presented significantly lower scores in the last 3 training sessions ($p < 0.05$). A two-way ANOVA of the number of avoidances in the sessions carried out under extinction (sessions 7-10, Fig 5B) showed a significant group $F(3,30) = 4.77$; $p < 0.01$) and session effects $F(3,90) = 88.59$; $p < 0.001$) and a non-significant group X session interaction effect $F(9,90) = 1.08$; $p = 0.38$). Separate ANOVA for each session showed significant effects in all sessions ($p < 0.05$). Post-hoc Dunnett's tests showed different patterns of impairment among the lesioned groups. Compared to the sham group, rats of the DLS lesioned group responded significantly less to the CS in the first 3 sessions ($p < 0.05$). Significantly fewer CARs were also observed in the DMS group, but only in the last 2 extinction sessions. NAc-co lesioned rats scored significantly less than the sham-lesioned group only in the third extinction session ($p < 0.05$).

Although the same tone was used as CS in the fear conditioning and in the 2-way active avoidance tasks, training in the first task appeared to have not affected performance in the second. As described above, during the fear conditioning training session, footshocks were delivered on both sides of the shuttle box. Pearson tests showed no significant correlation between: freezing scores on day 1 and avoidance scores on day 1 ($r = 0.19$, $p = 0.27$); freezing scores on day 1 and avoidance on day 2 ($r = 0.08$, $p = 0.62$); and freezing on day 3 (after extinction) and avoidance on day 2 ($r = 0.21$, $p = 0.22$). The same analysis restricted to sham group data also showed low correlation among these variables (varying from -0.47 to -0.003) which were not significant. In addition, even taking the conditioned fear in test 1 session as covariate, two-way ANOVA showed that the lesion of the NAc-co significantly impaired CAR learning: group effect, $F(1,15) = 21.26$, $p < 0.001$; session effect, $F(5,80) = 69.90$, $p <$

0.001; and group X session interaction, $F(5,80) = 6.49$, $p < 0.001$. Separate ANOVA for each session taking the conditioned fear in test 1 session as covariate also showed significant effects ($p < 0.05$).

Nevertheless, the inability of the NAc-co lesioned rats to learn conditioned fear seems to affect CAR learning. As shown in Fig. 6, compared to the sham group animals of this group, but not of the DMS and DLS groups, spent less time in freezing during the first 10 min of the first 2-way active avoidance session ($F(3,30) = 5.05$; $p < 0.01$, ; $p < 0.05$, Dunnett's test).

4. **Discussion**

The main results are summarized in Table 3. The NAc-co, but not the DMS and DLS, decreased the conditioned and unconditioned fear response; none of the lesions affected extinction. The three structures seem to play different roles in the instrumental component of the CARs: Lesion of the NAc-co and DLS delayed, but did not prevent, learning; lesion of the DMS did not affect the early phases of learning, but decreased CARs after extensive training; under extinction, CARs were decreased in the first sessions in the DLS, only in the third session in the NAc-co, and in the last sessions in the DMS groups.

The current data suggest that during CAR learning the NAc-co plays a role in expression of unconditioned fear and acquisition of aversive properties by the CS. The reduction in conditioned and unconditioned freezing observed in the NAc-co lesioned rats is unlikely attributable to either altered locomotor activity – the lesioned rats did not show higher locomotor activity during the 10 min habituation preceding the fear conditioning training session – or to a lower sensitivity to footshock, to which all animals reacted instantly. In addition to the possible impact of reduced fear during the training sessions, two statistical analyses suggest that lesion of the NAc-co also impaired acquisition and/or consolidation of conditioned fear: (i) lack of significant

correlation between unconditioned and conditioned fear; (ii) covariance analysis showed that the significant effect of the NAc-co lesion is independent of its effect on unconditioned fear. It is also possible that the lesions could have affected memory recall, whereby these animals acquired the tone-footshock-freezing associations, but could not recall them in the first test session. Therefore, the present results suggest that the NAc-co plays a role in learning and/or memory in the Pavlovian component of CARs. In addition, the present results suggest that the NAc-co does not play a role in extinction of conditioned fear.

The present study contests the behavioral neuroscience literature bias that emphasizes the role of the NAc only in appetitive aspects of learning and motivation (Schultz et al. 1997; Alderson et al. 2004; Yin et al. 2004; Yin et al. 2006; Da Cunha et al. 2009, 2012; Wilson et al. 2009; Haber and Knutson 2010; Redgrave et al. 2010; Flagel et al. 2011; Dezfouli and Balleine 2012; Kravitz et al. 2012; Liljeholm and O'Doherty 2012). Although we recognize that this bias still exists, other previous studies have suggested that the NAc also plays a role in aversive aspects of learning and motivation, which is in agreement with the present study. Previous studies have shown that lesions of the NAc-co (but not shell) or infusion of lidocaine into the rat NAc-co decreased acquisition of conditioned freezing (Parkinson, Robbins, and Everitt 1999; Haralambous and Westbrook 1999; Levita, Dalley and Robbins 2002). In addition, it has been known for some years that dopamine antagonists impair active avoidance responding (see review by Salamone 1994), and that NAc-co dopamine depletion impairs Sidman avoidance (McCullough, Sokolowski and Salamone 1993). Furthermore, NAc dopamine release is activated during avoidance responding (McCullough et al. 1993; Dombrowski et all, 2012), and also is activated in response to several aversive conditions, including anxiogenic drugs (McCullough and Salamone 1992) and footshock (Sorg and Kalivas 1991). Neurochemical measures of NAc dopamine transmission are also elevated in response to aversive conditions as diverse as tailshock, tailpinch, restraint stress, instrumental avoidance, conditioned aversive

stimuli, and social stress (Salamone 1994, 1996; McCullough et al. 1993; Tidey and Miczek 1996; Salamone, Cousins and Snyder 1997; Young 2004; Pezze and Feldon 2004; Martinez et al,. 2008). Electrophysiological studies have shown that ventral tegmental dopamine neurons, which project to the NAc-co, can respond to aversive stimuli (Anstrom and Woodward, 2005). Finally, several imaging studies show activation of the human ventral striatum is responsive to aversive stimuli (Jensen, Crawley, Mikulis, Remington, Kapur, S.. 2003; Phan, Taylor, Welsh, Ho, Britton, Liberzon 2004; Delgado, Li, Schiller, Phelps, 2008; Delgado and Tricomi, 2011; Klucken et al., 2012).

A study by Schenberg, Ferreira, Figueredo, Hipolide, Nobrega, and Oliveira (2006) reported increased NR2A glutamate receptor subunits in the dorsal striatum of rats presenting lower fear conditioning performance. Reports that the dorsal striatum affects learning of inhibitory avoidance (Wyers, Peeke, Williston, Herz 1968; Prado-Alcala, Fernandez-Samblancat, and Solodkinherrera, 1985; Packard, Introini-Collison, and McGaugh, 1996; Roozendaal, De Quervain, Ferry, Setlow, and McGaugh, 2001; Cammarota, Bevilaqua, Kohler, Medina, and Izquierdo, 2005) might be taken as evidence that it plays a role in Pavlovian fear conditioning. However, none of these studies have addressed the question of which sub-region of the striatum has a role in the Pavlovian fear conditioning component of CARs as we have done here.

Ferreira, Moreira, Ikeda, Bueno, Gabriela, and Oliveira (2003) observed impaired tone fear conditioning (when the CS is a tone) but not contextual fear conditioning (when the CS is the box in which the animals received the footshocks) in rats with lesions in the dorsal striatum. Consistent with this, White and Salinas (2003) reported that post-training infusion of amphetamine into the dorsal striatum improved memory consolidation of tone, but not contextual fear conditioning. In the present study, we cannot be certain whether conditioned freezing was a response to the context or the tone CS.

The present study also suggests that the NAc-co and the DLS play important roles in learning of the instrumental component. This conclusion agrees with a recent study reporting that the deficit in learning 2-way active avoidance seen in dopamine-deficient KO mice was reversed by restoration of dopamine signaling in the NAc, dorsal striatum, and amygdala, but not by restoration of DA signaling restricted to the ventral striatum and amygdala (Darvas et al. 2011). We observed impaired learning of 2-way active avoidance by NAc-co lesioned rats, a finding consistent with previous studies showing dopamine release during the first training sessions of this task (Wietzikoski, Boschen, Miyoshi, Bortolanza, Santos, Frank, Brandao, Winn, and Da Cunha, 2012), and that infusion of D1 (Wietzikoski et al. 2012) or D2 (Boschen, Wietzikoski, Winn, and Da Cunha, 2011) dopamine receptor antagonists into the rat NAc impaired learning of this task.

The fact that lesion of the NAc-co impaired instrumental avoidance responding does not necessarily mean that the NAc-co is needed for the instrumental learning *per se*. Conceivably, lesioned rats may not have responded simply because they did not fear it or could not predict the imminence of the cued footshock (that is, because the lesion affected the Pavlovian component of the task). The fact that rats of the NAc-co, but not of the other lesioned groups, expressed less freezing behavior during the beginning of the 2-way active avoidance training supports this view. As such, although the NAc-co is also needed for learning the instrumental component, it is not clear whether it plays a role in learning the instrumental component *per se* or if it simply allows learning of the Pavlovian component which is a pre-condition for learning the instrumental component.

Actions instrumental to obtain positive (appetitive) reinforcement are thought to be learned both as goal-directed actions and S-R habits (Dickinson and Balleine, 1994). Evidence from studies in which instrumental responding was reinforced by appetitive stimuli supports the view that learning and performance of instrumental goal-directed actions and S-R habits in rodents depends, respectively, on the dorsomedial

and dorsolateral parts of the striatum (Yin et al., 2004; Yin et al., 2006; Redgrave et al., 2010; Dezfouli and Balleine, 2012). These studies showed that bar-pressing for an appetitive reward is sensitive to outcome devaluation in rats after lesion or inactivation of the DLS (Yin et al., 2004; Yin et al., 2006), but not in rats with a lesion in the DMS (Yin et al., 2004). However, it is not clear whether the same regions of the striatum are also involved in learning habitual responses and goal-directed actions motivated by aversive stimuli. The present study provides a clue to resolve this.

Goal-directed actions are learned and extinguish quickly, while S-R habits are learned and extinguish slowly (Balleine, Delgado, and Hikosaka, 2007). If the DMS and the DLS play the same role for learning and extinction of goal-directed and S-R habits motivated by appetitive and aversive stimuli, it would be expected that lesion of the DMS would affect the early phases of learning and extinction of the 2-way active avoidance and expect lesion of the DLS would affect the late phases. However, we observed the opposite: late phases of learning and extinction affected by lesion of the DMS and early phases of learning and extinction affected by lesion of the DLS. Such results might be related to the opposite effect of appetitive and aversive stimuli on reinforcement of instrumental actions: while presentation of unexpected appetitive stimuli reinforces instrumental action, it is the omission of expected aversive outcome that reinforces the instrumental response. Therefore, presentation of the rewarding stimulus contingent to an instrumental response increases with extension of the training, while presentation of the aversive stimulus decreases with the extension of the training. It has been shown that appetitive reinforcement instrumental learning is more affected by lesion of the DMS when the animals are trained under a ratio interval schedule, while lesion of the DLS affects more instrumental conditioning under a variable interval schedule (Yin et al., 2006). Reinforcement is presented more frequently under fixed ratio than under variable interval schedules. Therefore, the frequency of presentation of appetitive and aversive stimuli might be a factor determining that the DMS and DLS play asymmetric roles in different times of learning

and extinction. Nevertheless, a role for the DLS in slowly learned (putatively habitual) conditional avoidance responding is supported by two recent studies showing that the pre-training infusion of D1 (Wietzikoski et al., 2012) or D2 (Boschen et al., 2011) receptor antagonists into the rat DLS did not affect avoidance responding during training for 2-way active avoidance but decreased avoidance responses in the test session carried out 24 h later. These pieces of evidence are consistent with the DLS having a role in slowly learned (putatively habitual) avoidance responding.

In summary, the present study supports the following conclusions: (i) CAR learning depends on both Pavlovian and instrumental learning; (ii) learning the Pavlovian component depends on the NAc-co, while learning the instrumental component depends on the DLS, NAc-co, and DMS; (iii) although the NAc-co is also needed for learning of the instrumental component, it is not clear whether it plays a role in learning the instrumental component *per se* or if it simply allows learning of the Pavlovian component, since it is a pre-condition for learning of the instrumental component; (iv) the NAc-co, DMS, and DLS do not play a role in extinction of the Pavlovian component; and (v) the NAc-co, DMS, and DLS play a role in extinction of the instrumental component.

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Figure legends

Figure 1. Excitotoxic lesions of the nucleus accumbens core (NAc-core). (A) Shaded areas represent the maximum (black) and minimum (gray) extent of the lesions for the animals included in the behavioral analyses. (Silhouettes adapted from Paxinos and Watson, 2005.) Examples of NeuN immunostained and thionin stained brain slices of sham-lesioned rats are presented in B and F; sections of NAc-co lesioned rats are presented in C, D, E, and G.

Figure 2. Excitotoxic lesions of the dorsomedial striatum (DMS). (A) Shaded areas represent the maximum (black) and minimum (gray) extent of the lesions for the animals included in the behavioral analyses. (Silhouettes adapted from Paxinos and Watson, 2005.) Examples of NeuN immunostained and thionin stained brain slices of sham-lesioned rats are presented in B and F; slices of DMS lesioned rats are presented in C, D, E, and G.

Figure 3. Excitotoxic lesions of the dorsolateral striatum (DLS). (A) Shaded areas represent the maximum (black) and minimum (gray) extent of the lesions for the animals included in the behavioral analyses. (Silhouettes adapted from Paxinos and Watson, 2005.) Examples of NeuN immunostained and thionin stained brain slices of sham-lesioned rats are presented in B and F; slices of DLS lesioned rats are presented in C, D, E, and G.

Figure 4. Effects of lesions in the rat nucleus accumbens core (NAc-co), dorsomedial striatum (DMS), and dorsolateral striatum (DLS) on fear conditioning. The rats were submitted to 10 tone-footshock pairings and the duration of freezing in test sessions carried out 1, 2, or 3 days after training was scored. Freezing times are expressed as

means ± S.E.M.* $p < 0.05$ compared to scores of the sham group in the training and first test day (Dunnett's test after ANOVA).

Figure 5. Effects of lesions in the rat nucleus accumbens core (NAc-co), dorsomedial striatum (DMS), and dorsolateral striatum (DLS) on 2-way active avoidance learning (A) and extinction (B). For training, rats underwent 6 sessions of 40 tone-footshock pairings; avoidance responses were automatically computed. For extinction, the rats underwent 4 sessions of 40 non-pairing tone-footshocks in which avoidance responses were automatically computed. The number of CARs are expressed as means ± S.E.M. * $p < 0.05$ compared to the NAc-co group; # $p < 0.05$, compared to the DLS group, + $p < 0.05$, compared to the DMS group (Dunnett's test after ANOVA).

Figure 6. Effects of lesions in the rat nucleus accumbens core (NAc-co), dorsomedial striatum (DMS), and dorsolateral striatum (DLS) on fear expression during learning of the 2-way active avoidance task. Freezing times were scored in the first 10 min of the first training session and are expressed as means ± S.E.M.* $p < 0.05$ compared to scores of the sham group (Dunnett's test after ANOVA).

The rats were submitted to 10 tone-footshock pairings and the duration of freezing in test sessions carried out 1, 2, or 3 days after training was scored. Freezing times are expressed as means ± S.E.M.* $p < 0.05$ compared to scores of the sham group in the training and first test day (Dunnett's test after ANOVA).

Table 1: Locomotor activity in the shuttle box

	Number of crossings					
	Habituation period (before fear conditioning)	Inter-trials (avoidance sessions 1 to 6)				
Sham	96,4 ± 6	28,8 ± 4	22,8 ± 3	28,0 ±6	28,0 ± 6	20 ±4 23,5 ± 5
NAc-co	112,80 ± 9	17,4 ± 3	19,7 ± 6	27,0 ± 8	22,5 ± 5	17,2 8 26,2 ± 11
DMS	97,7 ± 7	24,0 ± 4	19,2 ± 4	20,6 ± 4	16,6± 2	15,8 3 14,1 ±2
DLS	107,2 ±4	25,7 ± 8	22,3 ± 8	28,0 ±11	27,7 ± 7	29 ±10 33,0± 12

Free locomotor activity of the rats was scored: (i) during the 10 min of habituation to the shuttle box (before the fear conditioning training session) and (ii) during the inter-trial intervals between CS-US presentations in the first training session of the 2-way active avoidance task. In habituation, locomotor activity was scored as the number of times the rat crossed the imaginary lines dividing the shuttle box floor into equal areas. Inter-trial crossings were scored as the number of times rats crossed from the left to the right areas of the shuttle box. Data are expressed as means ± S.E.M. No significant difference between the sham and lesioned groups was observed (ANOVA followed by post-hoc Dunnett's test).

Table 2: Reaction times to the footshock and sound cue

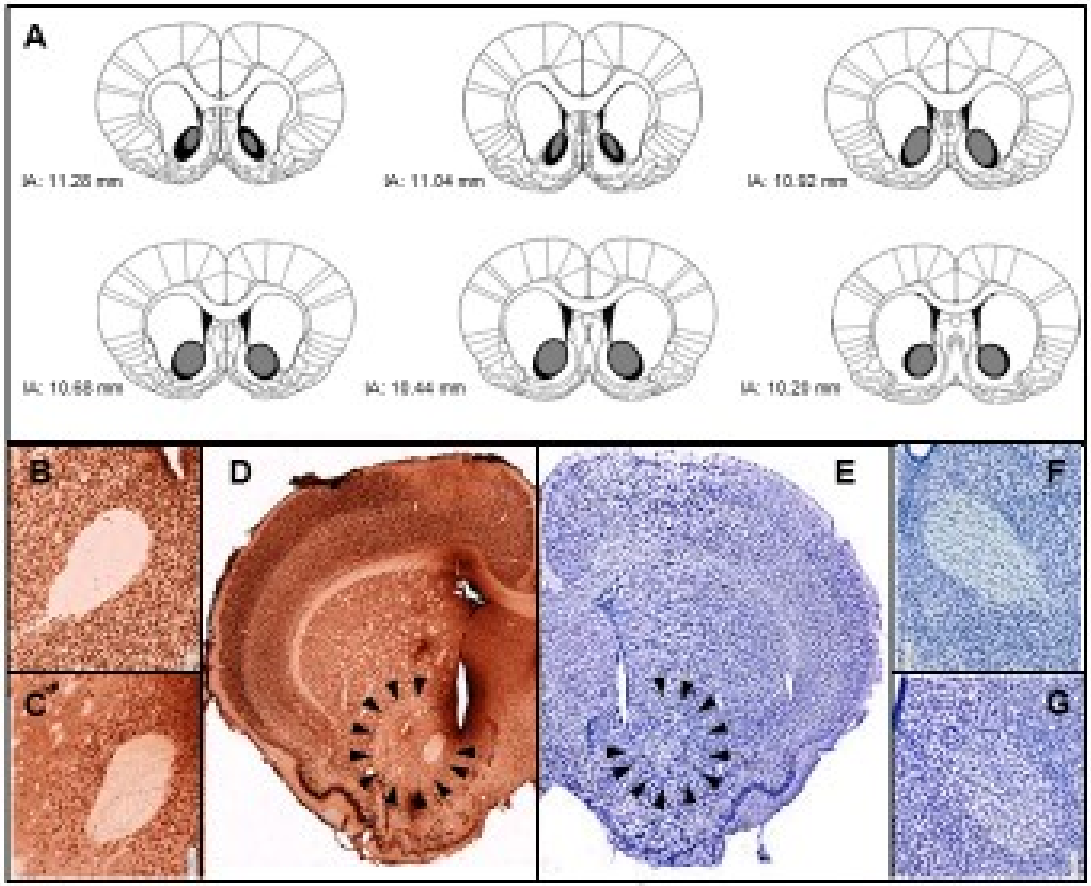
	Latency (s)	
	Footshock	Sound cue
Sham	1,9 ± 0	3,0 ± 0
NAc-co	1,2 ± 0	2,0 ± 0
DMS	2,1 ± 0	2,4 ± 0
DLS	1,4 ± 0	3,2 ± 0

All rats reacted to the footshock with a startle, running or jump behavior in less than one second. Data above represent mean + S.E.M. latencies to cross to the opposite side of the shuttle box in response to the first footshock presented in training session 1 or in response to the last sound cue presented in the training session 6 of the 2-way active avoidance. Data are expressed as means ± S.E.M. No significant difference among groups was observed (ANOVA followed by post-hoc Dunnett's test).

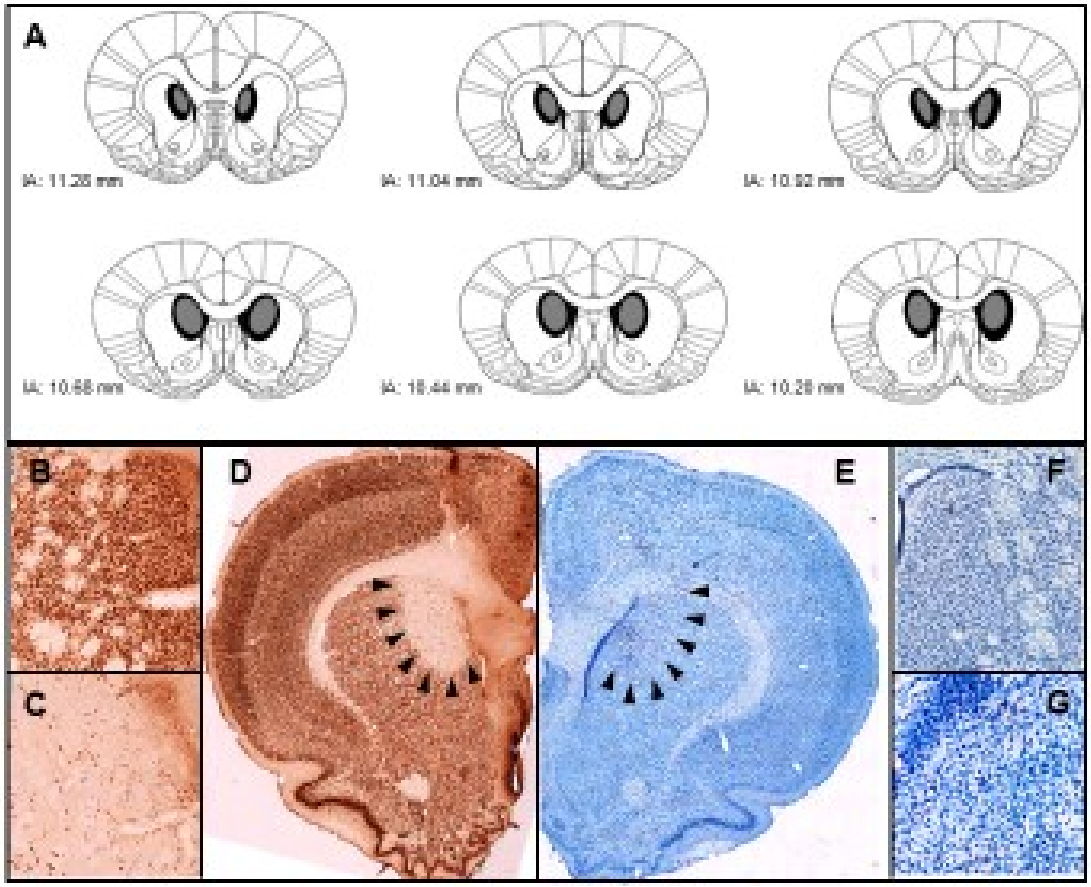
Table 3: Summary of the main lesion effects.

	UF	CF learning	CF extinction	2-WAA training	2-WAA extinction
NAc-co	↓	↓	---	↓ (*)	↑ (+)
DMS	---	---	---	↓ (#)	↑ (#)
DLS	---	---	---	↓(*)	↑ (*)

Arrows indicate significant differences and --- lack of significant difference between lesion and control groups. UF, Unconditioned fear; CF, conditioned fear; 2-WAA, two-way active avoidance; NAc-co, nucleus accumbens core; DMS, dorsomedial striatum; DLS, dorsolateral striatum; ↑, increase; ↓, decrease; ---, no significant difference; * only in the first sessions; # only in the last sessions; +, only in the third session.



Flg. 1



Flg. 2

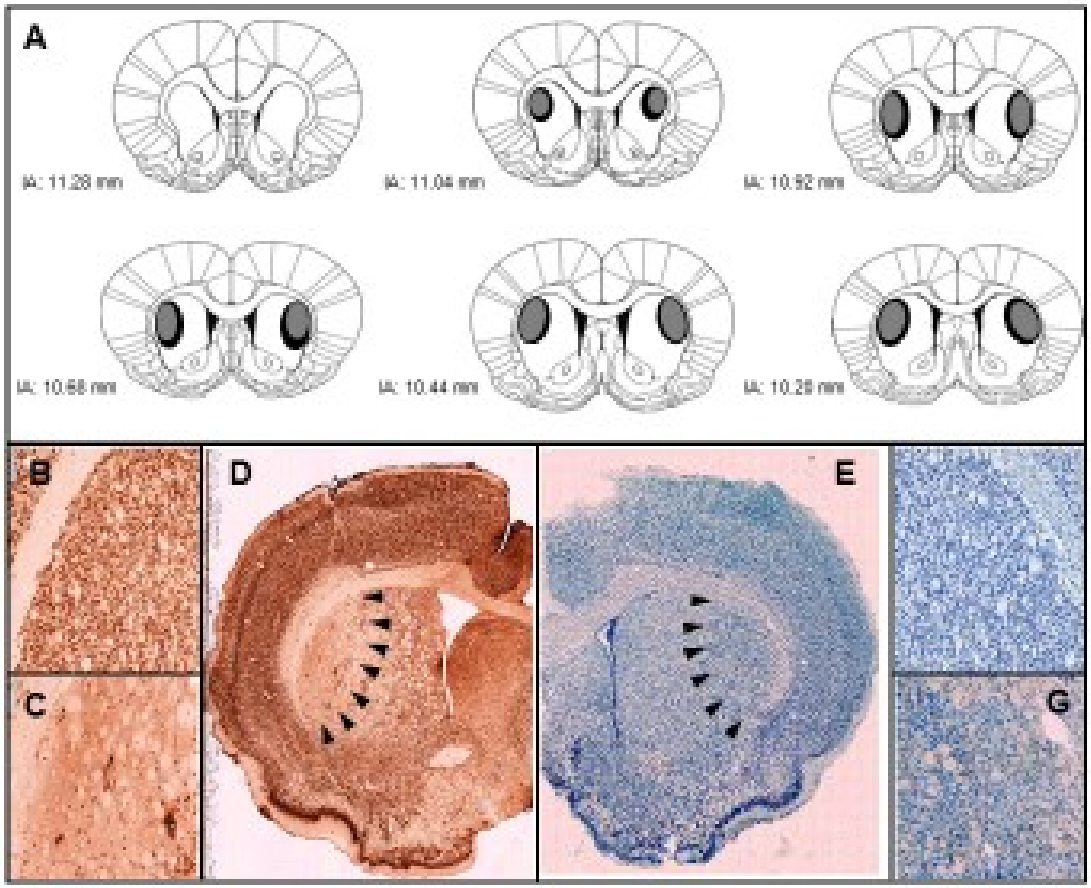

A
IA: 11.28 mm
IA: 11.04 mm
IA: 10.92 mm
IA: 10.68 mm
IA: 10.44 mm
IA: 10.28 mm
B
C
D
E
G

Fig. 3

Figure 4

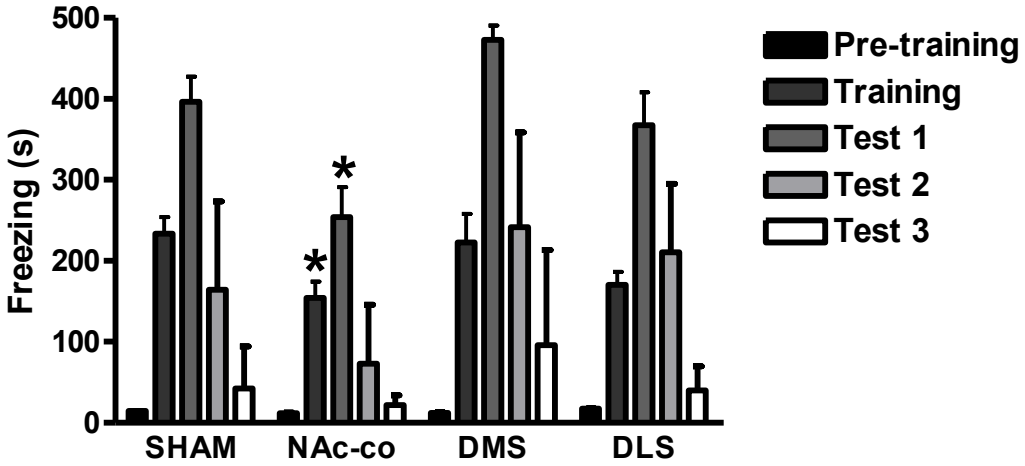


Figure 5

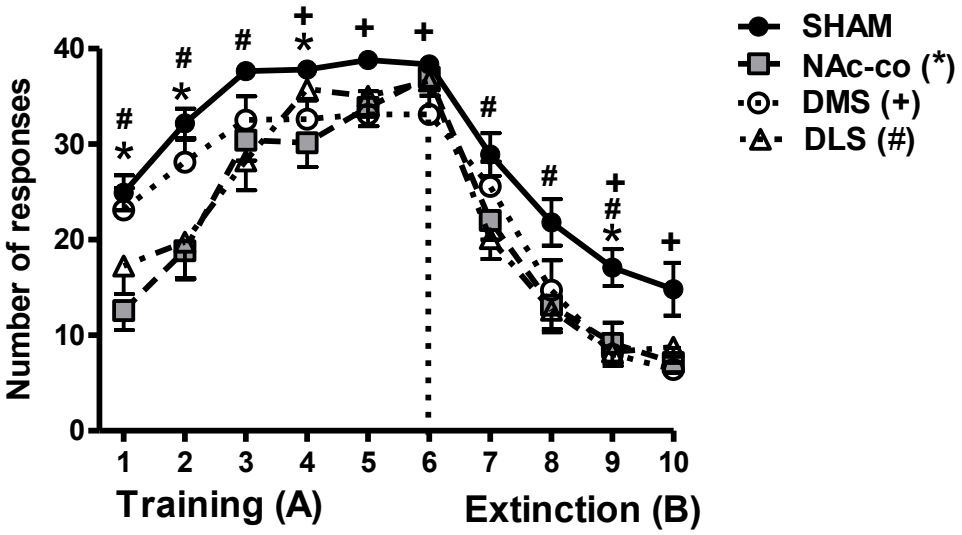


Figure 6