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Brain Structure and Function

Repeated cocaine exposure prior to fear conditioning induces persistency of PTSD-like symptoms and enhancement of hippocampal and amygdala cell density in male rats --Manuscript Draft--

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Abstract:	<p>Pre- and post-trauma drug use can interfere with recovery from post-traumatic stress disorder (PTSD). However, the biological underpinnings of this interference are still poorly understood. Here we examined, in male rats, the effect of pre-fear conditioning cocaine self-administration on PTSD-like symptoms and defined impairment of fear extinction as difficulty to recover from PTSD. We also examined cell density changes in brain regions suspected of being involved in resistance to PTSD recovery. Rats for footshock stress testing were trained to self-administer cocaine during 20 consecutive days, after which they were exposed to a 1.5-mA footshock, while other rats continued to self-administer cocaine until the end of the experiment. Upon assessment of three PTSD-like symptoms (fear during situational reminders, anxiety-like behavior, and impairment of recognition memory) and fear extinction learning and memory, changes in cell density were measured in the medial prefrontal cortex, hippocampus, and amygdala. Results show that pre-footshock cocaine exposure did not affect fear during situational reminders. Fear conditioning did not lead to an increase in cocaine consumption. However, cocaine, in footshock stressed rats, induced a reduction of anxiety-like behavior, an aggravation of recognition memory decline, and an impairment of extinction memory. These behavioral alterations were associated with increased cell density in the hippocampal CA1, CA2 and CA3 regions and basolateral amygdala, but not in the medial prefrontal cortex. Our findings suggest that enhancement of cell density in the hippocampus and amygdala may be part of changes associated to drug use, interfering with PTSD recovery.</p>	

Replies to Reviewer # 1

Reviewer # 1 has raised 10 points. Please find below our corresponding replies.

POINT 1. While it is understandable and even desirable to relate the procedures and outcomes in this study to PTSD, these rats have not undergone anything remotely close to trauma. The terms "PTSD" and "trauma" should not appear in the Methods or Results sections at all. Instead, please use a more concrete description of the actual testing conditions. For example, "shock" and "no shock" groups as opposed to "PTSD" and "no PTSD".

REPLY TO POINT 1: As requested by the reviewer, we have replaced 'PTSD' and 'trauma' with either 'fear conditioning' or 'fear related behavior/symptoms'. 'PTSD' and 'no PTSD' groups have been changed to 'shock' and 'no shock' groups, respectively.

POINT 2: The description of results depicted in Figure 8A states that there were no significant effects of "trauma", but the p-value is reported as 0.044 and alpha was set at 0.05. Isn't that significant?

REPLY TO POINT 2: The p-value is indeed significant. We have corrected the text accordingly.

POINT 3: The first sentence on page 19 is confusing. "Furthermore, we showed that a history of cocaine consumption did not induce any sensitization to future cocaine use." Was there an attempt to measure sensitization? If so, what exact comparison is this sentence alluding to?

REPLY TO POINT 3: We did not specifically measure sensitization (cocaine-induced behavioral sensitization to the locomotor response). Our intention was to indicate that fear conditioning after cocaine use (20 sessions) did not alter subsequent cocaine use. To avoid confusion, the corresponding sentence has been removed.

POINT 4: The last sentence of that paragraph is equally confusing. "Taken together, one can conclude that a history of cocaine use does not increase cocaine intake following stress or trauma." Was there a comparison somewhere of cocaine use between animals with and without a prior history of cocaine use? I think I'm just genuinely not following what point the authors are trying to make here.

REPLY TO POINT 4: We did not analyze the differences of cocaine intake between rats with and without a history of cocaine use. Therefore, the mentioned sentence has been replaced with "Taken together, one can conclude that exposure to stress is less likely to induce any increase in cocaine consumption."

POINT 5: The paragraph in the Discussion on "anxiety-like behavior" mentions several studies that were "not based on trauma". Are the authors contrasting those studies to the present study? If so, please describe more precisely, which aspects of trauma are recapitulated by the procedures used in this study as opposed to the ones described as "not based on trauma".

REPLY TO POINT 5: These studies exclusively depicted the effect of cocaine on anxiety-like behavior, hence the 'not based on trauma'.

POINT 6: Cocaine is described here as having an "anxiolytic effect" based on increased open arm entries on the EPM test. Could this effect also be explained by increased locomotor activity?

REPLY TO POINT 6: A new version of Figure 8 has been provided. The analysis of locomotor activity, represented as the distance travelled (Fig. 8A) and total arm entries (Fig. 8B) did not reveal any significant difference between the groups. The observed anxiolytic-like effect cannot therefore be explained by increased locomotor activity.

POINT 7: The paragraph on page 21 concludes that "cocaine and trauma probably induced dysfunction in this prefrontal area." This is a highly speculative conclusion, especially given that the only anatomically specific outcome measure in this study showed no group differences in the prefrontal regions. I would be ok with "might", but "probably" is too strong a word to use here.

REPLY TO POINT 7: The requested change has been done.

POINT 8: On page 22, it again goes a bit too far to say that reinstatement of conditioned fear responses "strongly depends on the hypofunction of the mPFC (Bentefour et al., 2016, 2018)." It can safely be said that inactivation of the mPFC can facilitate fear reinstatement under some conditions, but as written this sentence implies that the mPFC is almost completely responsible for fear reinstatement. I think that has yet to be proven.

REPLY TO POINT 8: The reviewer is right. The requested change has been done.

POINT 9: The authors mostly describe the findings depicted in Figure 9 in terms of a failure of extinction recall in the "Coc-PTSD" group, but they also acknowledge on page 23 the possibility that cocaine facilitated the fear priming effect in this group. I think this possibility should be emphasized earlier in the discussion. Especially since there was no evidence of a priming effect at all in the "NaCl-PTSD" group, it actually might be a more likely explanation than an extinction recall deficit, though either one is possible.

REPLY TO POINT 9: The requested change has been made (see at the beginning of the Discussion section).

POINT 10: The second paragraph of the introduction states "Most studies carried out so far have investigated how PTSD could affect vulnerability to addiction, but none investigated how regular use of drugs could affect vulnerability to PTSD." While it is true that investigations most commonly center on the effects of PTSD on drug use rather than the reverse, it is not true that "none" have investigated the effects of drug use on PTSD-related behaviors, e.g. (Gordon and Rosen 1999; Blanchard and Blanchard 1999; Burke et al. 2006; Wood et al. 2007; Kaag et al. 2016). It might be useful to discuss some of this published literature as a way of contextualizing the current study.

REPLY TO POINT 10: The requested change has been made. We have included the suggested references.

Reply to Reviewer # 2

Reviewer # 2 has raised 7 points. Please find below our corresponding replies.

POINT 1: This study only uses male rats. Justification for not using females should be included and this limitation should be acknowledged.

REPLY TO POINT 1: We have indicated in the Material and Methods section that we chose to use only male rats for convenience reasons in our animal facility. We have also acknowledged the fact that despite the hormonal fluctuations associated with the female reproductive cycle, it appears that behavioral data obtained with female rats are not more variable than those of male rats (Becker et al., 2016).

POINT 2: In the Introduction, an attempt is made to discuss animal models of PTSD, but the authors fail to discuss that fear conditioning is not a model of trauma but rather reflects normal learning to aversive stimuli. The use of fear conditioning in these studies is appropriate but should be put in context and rationales provided to draw similarities to human studies and other animal models of traumatic stress.

REPLY TO POINT 2: The requested changes have been made (see the Introduction section).

POINT 3: The authors fail to report published studies that have investigated the effects of cocaine on stress-related outcomes, including a published study that specifically models cocaine pre-trauma exposure on anxiety-like behaviors following traumatic stress exposure in rats (PMID: 28750215). The authors inaccurately claim that their study is the first to show that cocaine has anxiolytic effects in traumatic stress exposed rats.

REPLY TO POINT 3: Changes has been made as requested by the Reviewer.

POINT 4: In the Discussion, the effects of stress on later cocaine taking are discussed, and the authors conclude that trauma does not change cocaine intake following stress or trauma. A lot of literature that could be referenced is omitted here, and specifically the authors need to consider a few reports that have tested the effects of traumatic-like stress on cocaine taking and related behaviors (PMIDs: 25712697, 26115790, 23664123, 30291225, 29061385, 27181613).

REPLY TO POINT 4: We thank the Reviewer for guiding us to these valuable references. We have now reviewed them all in the Discussion section of the revised manuscript.

POINT 5: The authors claim that hypofunction of the infralimbic cortex facilitates return of PTSD-like symptoms, but only provides 1 reference that this occurs in human or animals.

REPLY TO POINT 5: We have added an example of a human study.

POINT 6: The justification for why hippocampal cell density is increasing is poor and not put in perspective to the many human PTSD-related studies and animal stress-models showing decreases in HC volume and cell density. The published literature needs to be referenced and better integrated into the current findings.

REPLY TO POINT 6: We have further discussed this point by acknowledging that PTSD is characterized by a reduced hippocampal volume.

POINT 7: The suggestion that cell densities are increased because of inflammatory markers is interesting but overly speculative. In order to justify this claim, the authors should identify the cell type(s) that they are measuring. Identifying neurons from glial will increase the value of the data and provide more evidence that inflammatory response is playing a role in the increased cell density, particularly in the HC.

REPLY TO POINT 7: Unfortunately, we were not able to identify neurons from glial cells.

However, we have rephrased our point.

[Click here to view linked References](#)

Repeated cocaine exposure prior to fear conditioning induces persistency of PTSD-like symptoms and enhancement of hippocampal and amygdala cell density in **male** rats

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ABSTRACT

Pre- and post-trauma drug use can interfere with recovery from post-traumatic stress disorder (PTSD). However, the biological underpinnings of this interference are poorly understood. Here we examined the effect of pre-fear conditioning cocaine self-administration on PTSD-like symptoms in male rats, and defined impairment of fear extinction as difficulty to recover from PTSD. We also examined cell density changes in brain regions suspected of being involved in resistance to PTSD recovery. Before footshock stress testing, rats were trained to self-administer cocaine during 20 consecutive days, after which they were exposed to **footshocks**, while other rats continued to self-administer cocaine until the end of the experiment. Upon assessment of three PTSD-like symptoms (fear during situational reminders, anxiety-like behavior, and **impairment of recognition memory**) and **fear extinction learning and memory**, changes in cell density were measured in the medial prefrontal cortex, hippocampus, and amygdala. Results show that **pre-footshock cocaine exposure did not affect fear during situational reminders. Fear conditioning did not lead to an increase in cocaine consumption.** However, **in footshock stressed rats, cocaine induced a reduction of anxiety-like behavior, an aggravation of recognition memory decline, and an impairment of extinction memory.** These behavioral alterations were associated with increased cell density in the hippocampal CA1, CA2, and CA3 regions and basolateral amygdala, but not in the medial prefrontal cortex. Our findings suggest that enhancement of cell density in the hippocampus and amygdala may be changes associated with drug use, interfering with PTSD recovery.

Keywords **Footshock stress.** Rats. Cocaine self-administration. **Extinction memory impairment**

INTRODUCTION

Post-traumatic stress disorder (PTSD) develops after a very stressful, frightening, or distressing event, or a prolonged traumatic experience. Symptoms may improve with treatment, but unfortunately not in everyone (Zohar *et al.*, 2011). Although it is not clear exactly why some people develop chronic PTSD while others do not, a few possible reasons have been suggested such as co-occurring substance use disorders. Indeed, PTSD is common among people with substance use disorders (McFarlane, 1988; Breslau and Davis, 1991; Saladin *et al.*, 1995; Bremner *et al.*, 1996; Cottler *et al.*, 2001). Approximately half of individuals seeking treatment for substance use disorders meet current criteria for PTSD (Berenz and Coffey, 2012). For example, up to 43% of cocaine-dependent individuals meet criteria for lifetime PTSD (Back *et al.*, 2000). In most cases, one disorder worsens the other, increasing the possibility of relapse and decreasing the chances of recovery (Berenz and Coffey, 2012; McGovern *et al.*, 2015). For instance, cocaine addicts with comorbid PTSD are more resistant to treatment than those without a dual diagnosis (Coffey *et al.*, 2002). **An explanation for PTSD treatment failure in cocaine users could be drug-induced stronger fear conditioning and impaired extinction learning (Kaag *et al.*, 2016).**

Animal models based on cocaine self-administration and fear conditioning/extinction may be useful for improving our understanding of (i) how PTSD and substance use disorders interact and (ii) which biological disruptions contribute to resistance to treatment. Most studies carried out so far have investigated how PTSD could affect vulnerability to addiction (María-Ríos and Morrow, 2020), **while studies investigating the effects of drug use on PTSD-like phenotype mostly included drug injections (Gordon and Rosen 1999; Blanchard and Blanchard 1999; Burke *et al.* 2006; Wood *et al.* 2007).** However, none of these studies investigated how regular use of cocaine (in the form of cocaine self-administration) could affect vulnerability to PTSD-like symptoms. In addition, while many animal models of PTSD not

based on footshock stress are commonly used [including predator stress (**Adamec et al., 2006**), single prolonged stress (**Librezon et al., 1997**), underwater trauma (**Richter-Levin, 1998**)], those based on footshock stress are particularly popular given their convenience in assessing learning and emotional memory processes following exposure to aversive stimuli (**Anagnostaras et al., 2000**). Footshock is also a powerful tool to reveal the neurobiological underpinnings of psychiatric disorders in which a strong emotional memory component is present, such as in PTSD (**Careaga et al., 2016**). However, confusion between paradigms based on footshock-induced long-lasting PTSD-like symptoms and footshock-induced normal conditioned fear without PTSD-like symptoms must be avoided. Indeed, a strong footshock (e.g., a 1.5-mA footshock) has been reported to produce both conditioned fear memory and non-conditioned PTSD-like symptoms (e.g. fear incubation and sensitization, social withdrawal, neophobia and depression-like behavior; **Siegmund and Wotjak, 2007**). On the other hand, a mild footshock (e.g., a 0.4-mA footshock) is known to induce conditioned fear memory without PTSD-like symptoms (**Bentefour et al., 2015**).

Concerning fear conditioning, it is known that in PTSD the fear responses triggered by the initial trauma are repetitively reexperienced, often through flashbacks and recurring nightmares (**Goswami et al., 2013**). Regarding extinction, PTSD patients have been reported to be deficient at learning that stimuli previously associated with adverse outcomes no longer presents a threat (**Milad et al., 2008; Jovanovic et al., 2012**). These findings suggest that deficits in fear extinction underlie resistance to PTSD recovery (**Wessa & Flor, 2007; Fenster et al., 2018**). Based on this idea, we have recently found that ethanol exposure does not interfere with avoidance, which is a core symptom of PTSD (**Sheynin et al., 2017**), but impairs avoidance extinction (**Lguensat et al., submitted manuscript**). More importantly, we have previously demonstrated with the same mouse model (**Bentefour et al., 2015**) that avoidance extinction training suppresses not only avoidance behavior but all other PTSD-like symptoms

(Bentefour et al, 2016). In other words, resistance to extinction indicates impairment of recovery from PTSD-like symptoms.

Fear conditioning requires gene expression and protein synthesis in both the hippocampus and the basolateral amygdala alongside activation of various metabolic signaling pathways **(Vianna et al., 2004)**. Interestingly, these requirements are like those for fear extinction **(Vianna et al., 2004)**. For example, in the hippocampus it has been shown that fear conditioning activates a fear engram, while extinction training inhibits these engram cells and activates a second extinction engram **(Lacagnina et al., 2019)**. It is therefore possible that a comorbidity with a substance use disorder will potentiate fear engram in the hippocampus and amygdala, which may contribute to resistance to fear extinction. Moreover, we recently found that, in mice, post-footshock exposure to ethanol induces both resistance to avoidance extinction and increases in dendritic density in the prelimbic area of the medial prefrontal cortex (PrL) **(Lguensat et al., submitted manuscript)**. This finding suggests that, in addition to the hippocampus and amygdala, the PrL may also be one of the brain sites displaying changes that correlate with resistance to fear extinction.

Using a fear conditioning paradigm in male rats, we here examined the effects of prior cocaine exposure on conditioned fear memory (which may be altered: **Wood et al., 2007**), post-footshock anxiety-like behavior (which may not be altered: **Lisieski and Perrine, 2017**), post-footshock recognition memory (which may be altered: **Mottarlini et al., 2020**) and fear extinction (which may also be altered: **Burke et al., 2006**). To identify associated brain changes, we examined cortical thickness in the medial prefrontal cortex and cellular density changes in the hippocampal CA1, CA2, and CA3 regions; basolateral amygdala; and medial prefrontal cortex.

MATERIAL AND METHODS

Animals and catheterization surgery

Fifty-three outbred male Lister Hooded rats (Charles River, Saint- Germain- sur- l'Arbresle, France) were used in the present study. **Despite the hormonal fluctuations associated with the female reproductive cycle, it appears that behavioral data obtained with female rats are not more variable than those of male rats (Becker *et al.*, 2016). However, we chose to use only male rats for convenience reasons in our animal facility.** Three rats were eliminated from the study because of their blocked catheters and therefore their measures were not considered. Half of the remaining rats were submitted to self-administration sessions for 20 days while the other half were submitted to self-administration sessions for 52 days (until the end of the experiment). Animals were maintained in the animal facility, housed in pair, and handled 2 to 3 times per week before surgery. They were submitted to a 12h light/dark cycle (lights on at 7PM) at a constant temperature ($22 \pm 1^{\circ}\text{C}$). The rats had free access to food pellets (Scientific Animal Food & Engineering, Augy, France) and water except during the experiments. All animal care and use conformed to the French regulation (Decree 2013-118) and were approved by the local ethics committee and the Ministère de l'Agriculture (Saisine #3129.01).

Intra-jugular catheter implantation was performed on day 0. Rats were anesthetized using a mixture of ketamine (Imalgene, Merial, Lyon, France) and medetomidine (Domitor, Janssen, Issy- les-Moulineaux, France). Later, the extremity of a catheter, made out of silicone tubing, was inserted in the right jugular vein. The catheter was placed so that it followed a subcutaneous path above the scapula and exited at the level of the back of the rat.

Self-administration of cocaine or NaCl

Apparatus: Self-administration (SA) sessions were conducted in standard SA chambers (operant boxes from Camden Inst and Med Associates, Georgia, VT) placed in sound-

attenuating boxes. Each chamber had a central light, two retractable levers (Camden Inst boxes) or two holes (Med Associates boxes) with two cue lights placed above them and a grid floor. At the beginning of each session, the rats were connected to the chamber tubing, which was protected by a stainless-steel spring and connected to a 10 ml syringe containing either Cocaine or NaCl (0.9%). Each syringe was placed on a pump (Razel Scientific Instruments, St. Albans, VT). Both the chambers and the pumps were run by either a home-built software (Camden Inst boxes) or MedPC IV software (Med Associates boxes).

Procedure: SA sessions began 7 days post-surgery. Twenty-seven animals were assigned to self-administer cocaine, while 23 animals self-administered NaCl. The SA sessions lasted 1 hour per day for 20 or 52 days, according to the group. The aim of continuing cocaine self-administration for 52 days was to assess any eventual effect of **the fear conditioning procedure** on the progression of cocaine self-administration.

Before the beginning of the sessions, each rat was taken from its home-cage and had its catheter tested and flushed using an injection of saline solution containing heparin (Heparin Sodium, Sanofi, Paris, France; 5000 U.I/ml) and gentamecin (Pangram, Vibrac, France) to prevent formation of blood clot and infection. The animal was then connected to the chamber tubing, and the 1-hour sessions were launched on the software. When the session started, the central light was turned on. For each rat, an active lever or an active hole was available. When the active lever was pressed or the active nosepoke was performed, the cue light above it lit up for 5 seconds and an intravenous infusion of either cocaine (250 μ g/90 μ l) or NaCl (90 μ l) was delivered (FR1 schedule: every active lever press or active nosepoke resulted in the delivery of an intravenous infusion). A time-out period of 20 seconds following the infusion was imposed, during which a press on the active lever or an active nosepoke was counted as a preservative response and had no consequence. By the end of the session, each rat was disconnected, and the catheter re-tested before returning the animal to its home-cage. The total number of rewards,

corresponding to the total number of infusions (either cocaine or NaCl), was considered for the result analysis.

Fear conditioning and assessment of conditioned fear response

Apparatus: **Fear conditioning and testing** took place in an operant box different from the ones used during the self-administration sessions. The floor of the box was made of a metal grid with bars separated by 1 cm and connected to an electrical shock generator (Med associates, Georgia, VT). A camera was attached to the ceiling of the box to record the behavior of the rat. The box was controlled by a MedPC IV interface that we used to deliver the shocks.

Procedure: Twenty-four hours following the last SA session (day 28), rats were submitted to the first session of **footshock exposure**. During this session, animals from the shocked groups (**NaCl-Shock**: n=12 and **Coc-Shock**: n=14) were placed in the operant box for 180 seconds, where two footshocks (1.5 mA, 2 seconds), were delivered 6 seconds apart. Sixty seconds following the last footshock, rats were removed from the box. Four days later, they were submitted to a second conditioning session. During this session they were put back in the same operant box, and after 120 seconds, received a footshock (2 mA, 10 seconds). The no-shock groups (**NaCl-No shock**: n=11 and **Coc-No shock**: n=13) were submitted to the same procedure, but with the shock mechanism inactivated. The **situational reminder (SR) sessions for conditioned fear memory testing** started the following day. These sessions involved placing the rats in the conditioning box for 30 seconds, on days 34, 40, and 47 (SR1, SR2 and SR3), without any footshock delivery. This procedure, based on short context re-exposures (less than 1 minute/session), has been reported to induce behavioral sensitization (expressed by increased freezing behavior) without causing fear extinction (**Pynoos et al, 1996; Louvart et al, 2006**). Here, conditioned fear memory was assessed by analyzing freezing behavior during each SR session based on the video recordings.

Assessment of the other footshock exposure-related symptoms

The elevated plus-maze test (EPM): This test is designed to assess the anxiety state of rodents. The apparatus used consisted of four arms of the same size (49.4 cm × 49.4 cm) at 80 cm from the floor and opened on a central square platform (10.5 cm × 10.5 cm). Two open arms (OA) had no walls, while the two other arms were closed (CA) with walls delimiting the corridors. The maze was illuminated by a 50-watt bulb placed above it.

On day 48, each rat was put in the apparatus for 5 minutes. We initially put the animal at the junction of the four arms of the maze, facing an open arm. The animal was free to explore the maze. The animal's behavior was recorded to determine the number of arm entries and time spent in each open arm. The anxiety index was calculated as follows (**Rao and Sadananda, 2016**):

$$Anxiety\ index = 1 - \left(\frac{\frac{Time\ spent\ in\ the\ OA}{Total\ time} + \frac{Number\ of\ enteries\ to\ the\ OA}{Total\ enteries}}{2} \right)$$

Locomotor activity measures were obtained using ToxTrac software (**Rodriguez et al., 2017, 2018**).

The Novel Object Recognition test (NOR): The NOR test was done in a circular arena (diameter: 65cm) and performed over 2 days. During the first day (day 49), the rats freely explored the arena for 15 minutes. The next day (day 50), they underwent the NOR procedure, which consisted of placing each animal in the arena with two objects A and B. The procedure consisted of 3 phases separated by 15 minutes each (**Eagle et al., 2013**):

1- *Acclimatization phase (duration: 5 min)*: the rat explored the arena without objects.

2- *Exploration of the two identical objects A and B (duration: 5 min)*: The rat was placed in the arena with objects A and B and was expected to explore and become familiar with the objects.

3- *Exploration of a novel object (duration 5min)*: the rat was placed back in the arena with objects A (identical to A used in 2) and C (novel object). The animal was expected to spend more time at exploring the new object. The discrimination ratio was calculated as follows:

Discrimination ratio

$$= \frac{\text{Time spent exploring the new object} - \text{Time spent exploring the familiar object}}{\text{Total time}}$$

Assessment of extinction learning and strength of extinction memory

Extinction learning: For fear extinction training, rats were put back in the conditioning box for a longer duration per day compared to the SR session (10 minutes/day vs. 30 seconds/day). This was done on five consecutive days (from days 51 to 55) without footshock application. Fear behavior (freezing) was assessed during each session. We manually measured freezing time (the time that the animal spends in a fixed position) for at least one second. The cumulative freezing time is expressed in percentage of total time.

Strength of extinction memory: To test the strength of extinction memory, on day 56 rats were submitted to the same regimen as during the first conditioning session, except that the footshock was applied with a much lower intensity (0.15 mA) which served as footshock priming **to facilitate fear return in case of weak extinction memory**. The freezing behavior of the rats was evaluated prior to the footshock priming application (day 56) and 24 hours later (day 57).

All behavioral protocol steps are summed up in **Figure 1**.

Assessment of cortical thickness and cellular density changes

Tissue preparation and Cresyl violet staining: At the end of the protocol, the animals were deeply anaesthetized by an injection of pentobarbital (Dolethal, Vetoquinol, Lure, France, 73 mg/mL, 1.5 mL i.p.). They were then perfused intracardially with PBS solution (pH=7.4) followed by 4% paraformaldehyde solution in PBS (pH=7.4). The brains were collected and post-fixed overnight in the same paraformaldehyde solution used for the perfusion with stirring at 4°C. These brains were then post-fixed in a 30% sucrose solution overnight. Each brain was then cut in cryostat (Leica Microsystems, Germany) into 50µm sections and mounted on gelatinized slides. These slides were kept in cold until staining.

For Nissl staining, the following baths were used for the slides: a 95° alcohol bath and a water bath before being placed in the Cresyl violet bath until the desired coloration is obtained. Then the slides were rinsed with water and the dehydration stage was carried out by submerging them into the 70° and then 95° alcohol baths. Finally, the slides were passed through xylene bath and mounted with Eukitt (Sigma-Aldrich).

Image acquisition and quantification: These analyses were carried out on three adjacent brain sections. The regions of interest were determined using the atlas by **Paxinos and Watson (1998)**. The images were taken using a photomicroscope (Olympus CX2) equipped with a camera (Olympus, DP71), connected to a computer with the acquisition software "DP Controller" and analyzed using the ImageJ software (NIH). Analyses were done on 3 adjacent sections/region/rat and by choosing 3 zones of 1 mm²/section. The measurements obtained on the 3 zones were then averaged and the average obtained was considered in statistical analyses.

Statistical analysis

Data was analyzed using GraphPad Prism v8 software. One-way ANOVAs were used to assess the effect of a single parameter, while the effect of independent variables was assessed using

two-way ANOVAs (regular and repeated measures) or three-way ANOVAs, followed by Holm-Sidak *post-hoc* for multiple comparisons. The significance was set at $p < 0.05$.

RESULTS

Self-administration during the first 20 days:

Cocaine vs. NaCl groups: Data showed that rats that self-administered cocaine (Cocaine group) received more rewards (injections) compared to those in the NaCl group (12.04 ± 1.10 vs. 3.70 ± 0.63) (**Figure 2**).

Two-way repeated measures ANOVA (2 groups, 20 sessions) revealed a significant effect of group ($F_{(1, 48)} = 875$; $p < 0.001$), but there was no effect of session ($F_{(19, 30)} = 0.37$; $p = 0.992$). The group x session interaction was not significant ($F_{(19, 30)} = 0.514$; $p = 0.957$). The Holm-Sidak *post-hoc* test showed that the Cocaine group received significantly more rewards compared to the NaCl group during all sessions (**Table 1**).

No shock and Shock groups: Data from the Cocaine sub-groups (**Coc-No shock and Coc-Shock**) showed that their number of rewards were similar (**Figure 3A-B**). Two-way repeated measures ANOVA on data from all sessions (**Figure 3A**) showed no significant effect of either group or session ($F_{(1, 25)} = 0.026$; $p = 0.871$ and $F_{(19, 5)} = 0.343$; $p = 0.996$, respectively), or any group x session interaction ($F_{(19, 25)} = 0.139$; $p = 0.993$). Two-way ANOVA analysis of data on blocks (**Figure 3B**) revealed no significant effect of either group or block ($F_{(1, 25)} = 0.011$; $p = 0.916$ and $F_{(19, 7)} = 0.042$; $p = 0.837$, respectively) and no significant group x block interaction ($F_{(19, 25)} = 0.005$; $p = 0.941$).

Data from the NaCl sub-groups (**NaCl-No shock and NaCl-Shock**) did not show any difference regarding the number of rewards (**Figure 4A-B**). Two-way repeated measures

ANOVA on data from all sessions (**Figure 4A**) did not indicate any significance of group or session ($F_{(1; 21)}=0.024; p=0.875$ and $F_{(19; 3)}=0.017; p=0.896$, respectively). The group x session interaction was not significant ($F_{(19; 21)}=0.444; p=0.980$). Two-way ANOVA analysis of data on blocks (**Figure 4B**) did not reveal any significance effect of group or block ($F_{(1; 21)}=0.016; p=0.896$ and $F_{(19; 3)}=0.437; p=0.512$, respectively). The group x block interaction was not significant ($F_{(19; 11)}=0.008; p=0.926$).

Self-administration during 52 days:

Data from the Cocaine sub-groups (**Coc-No shock and Coc-Shock**) did not reveal any change in the total number of rewards after the trauma exposure (**Figure 5A-B**). Two-way repeated measures ANOVA on data from all sessions (**Figure 5A**) showed no significant effects of group nor session ($F_{(1; 12)}=1.134; p=0.998$ and $F_{(51; 38)}=0.239; p=0.999$, respectively). The group x session interaction was not significant ($F_{(51; 38)}=0.280; p=0.999$). Two-way ANOVA analysis of data on blocks (**Figure 5B**) did not reveal any significant effect of group or block ($F_{(1; 12)}=0.020; p=0.887$ and $F_{(4; 9)}=0.367; p=0.830$, respectively). The interaction between these two factors was not significant ($F_{(4; 12)}=1.735; p=0.157$).

Data from the NaCl sub-groups (**NaCl-No shock and NaCl-Shock**) were similar regarding the total number of rewards received during the sessions (**Figure 6A-B**). Two-way repeated measures ANOVA on all sessions (**Figure 6A**) did not reveal a significant effect of group ($F_{(1; 9)}=0.276, p=0.599$), while there was a significant effect of session ($F_{(51; 41)}=2.740, p=0.01$). The interaction between these factors was not significant ($F_{(51; 41)}=0.478, p=0.999$). Two-way ANOVA analysis of data on blocks (**Figure 6B**) did not reveal a significant effect of group ($F_{(1; 9)}=0.358, p=0.552$). The effect of block was significant ($F_{(4; 6)}=5.545, p=0.001$). The group x block interaction was not significant ($F_{(4; 9)}=0.144; p=0.964$).

Footshock exposure-related behavioral changes

Conditioned fear response: Before conditioning, all rats exhibited a similar lack of fear response (freezing). However, after the 1.5-mA footshocks, all **Shock** groups (**NaCl-Shock** and **Coc-Shock**) showed freezing behavior, both immediately and during each of the subsequent SR tests, while no freezing was expressed in any of the **No-Shock** groups (**NaCl-No Shock** and **Coc-No Shock**) (**Figure 7**).

Three-way ANOVA repeated measures (session x **shock** x self-administration) on these data showed significant effects of session and **shock** ($F_{(4; 44)} = 25.68; p < 0.001$ and $F_{(1; 47)} = 316.9; p < 0.001$, respectively). There was no significant effect of self-administration ($F_{(1; 47)} = 0.389; p = 0.533$). The analysis of pairwise interactions revealed that the session x **shock** interaction was significant ($F_{(4; 47)} = 24.58; p < 0.001$), while the other interactions were not significant (session x self-administration: $F_{(4; 47)} = 0.467; p = 0.474$ and **shock** x self-administration: $F_{(1; 47)} = 0.085; p = 0.420$). The Holm-Sidak *post-hoc* test revealed that the **NaCl-Shock** and **Coc-Shock** groups showed a significantly longer freezing time compared to the **NaCl-No shock** group and to the **Coc-No shock** group (**Table 2**).

Anxiety-like behavior and recognition memory: Data from the EPM test showed that **footshock exposure** increased the anxiety index in rats that did not self-administer cocaine (the **NaCl-Shock** group). However, this effect was abolished in rats that self-administered cocaine (**Figure 8A**). Two-way ANOVA analysis (**shock** x self-administration) on these **data revealed a significant effect of shock** ($F_{(1; 47)} = 4,545; p = 0.044$) while no effect of self-administration was observed ($F_{(1; 47)} = 0.171; p = 0.196$). Moreover, the interaction between the two factors was significant ($F_{(1; 47)} = 5.376; p = 0.024$). The Holm-Sidak *post-hoc* analysis showed that the **NaCl-Shock** group expressed a significant increase of the anxiety index compared to the **NaCl-No shock** group ($t = 2.931, p = 0.031$). **There was no effect of either footshock exposure or cocaine self-administration on locomotor activity (Figure 8B)**. Two-way ANOVA analysis (**shock** x self-administration) did not reveal any significant effect of both shock ($F_{(1, 47)} = 1.452, p = 0.234$)

and self-administration ($F_{(1, 47)} = 0.029, p=0.863$). There was no significant interaction between these two factors ($F_{(1; 47)} = 1.098; p=0.300$).

The NOR data showed fear conditioning by itself (**NaCl-Shock group**) reduced the recognition index. This effect was augmented when cocaine exposure was added (**Coc-Shock group**) (**Figure 8C**). The two-factor ANOVA test (**shock** x self-administration) indicated significant effects of **shock** and self-administration ($F_{(1; 47)} = 32.14; p < 0.001$ and $F_{(1; 47)} = 6.571; p = 0.013$, respectively). However, the interaction between these two factors was not significant ($F_{(1; 47)} = 0.934; p = 0.338$). On one hand, the Holm-Sidak *post-hoc* test revealed, ~~on the one~~ ~~hand,~~ that the **NaCl-Shock** group had a reduced recognition index compared to the **NaCl-No shock** group ($t = 3.199, p = 0.014$). On the other hand, the **Coc-Shock** group expressed a significantly reduced recognition index compared to the **NaCl-No shock** and **Coc-No shock** groups ($t = 5.803, p < 0.001$ and $t = 4.893, p < 0.001$).

Fear extinction learning and memory

Fear extinction learning: Extinction data showed that the **Shock** groups expressed a progressive reduction of time spent freezing during extinction training (**Figure 9**). Three-way ANOVA repeated measures analysis (session x **shock** x self-administration) indicated a significant effect of session and **shock** ($F_{(6; 18)} = 23.92; p < 0.001$ and $F_{(1; 24)} = 3.476; p = 0.003$ respectively). There was no significant effect of self-administration ($F_{(1; 24)} = 0.396; p = 0.529$). The analysis of interactions in pairs revealed the significance of the following interactions: session x **shock** and session x self-administration ($F_{(6; 24)} = 17.57; p < 0.001$ and $F_{(6; 24)} = 3.476; p = 0.003$), while the **shock** x self-administration interaction was not significant ($F_{(1; 24)} = 0.003; p = 0.952$). The Holm-Sidak *post-hoc* test showed that between Ext 1 (D51) and Ext 4 (D54), freezing time expressed by the **NaCl-Shock** and **Coc-Shock** groups significantly exceeded fear response expressed by the **NaCl-No shock** and **Coc-No shock** groups. During Ext 5 (D55), only rats in

the **Coc-Shock** group continued to show a significant difference compared to the previous groups and to the **NaCl-Shock** group.

Extinction memory strength: Data indicate that before footshock priming (with 0.15 mA; D56) all groups displayed low levels of freezing. However, after footshock priming (D57) only the **Coc-Shock** group showed **fear return, indicating a weak extinction memory (Table 3)**.

Cortical thickness and cellular density assessment

Cortical thickness: The analysis of total cortical thickness in the cingulate cortex (Cg), pre-limbic (PrL), and infralimbic (IL) regions of the mPFC did not show any significant difference between the groups (**Figure 10A**). Three-way ANOVA test (**shock** x self-administration x mPFC regions) revealed a significant effect of mPFC region ($F_{(2; 21)} = 118.4; p < 0.001$), while no significant effect was seen for **shock** or self-administration ($F_{(3; 20)} = 1.916; p = 0.196$ and $F_{(3; 20)} = 0.147; p = 0.171$, respectively). No significant interaction between each pair of factors was noted.

The analysis of layer thickness in the Cg did not reveal any difference between the groups for all layers (**Figure 10B**). Three-way ANOVA test (**shock** x self-administration x layers) revealed a significant effect of layer ($F_{(2; 21)} = 213.2; p < 0.001$), while no significant effect was seen for **shock** or self-administration ($F_{(3; 20)} = 0.567; p = 0.638$ and $F_{(3; 20)} = 0.903; p = 0.345$, respectively). No significant interaction between each pair of factors was found.

In the PrL, no significant variation was detected between the layers (**Figure 10C**). Three-way ANOVA test (**shock** x self-administration x layers) demonstrated a significant effect of layer ($F_{(2; 21)} = 150.7; p < 0.001$). The other factors (**shock** and self-administration) were not significant ($F_{(3; 20)} = 1.436; p = 0.242$ and $F_{(3; 20)} = 0.817; p = 0.206$, respectively). No significant interaction between pairs was seen.

In the IL, as noted for the PrL, no significant variation was found (**Figure 10D**). Three-way ANOVA test (**shock** x self-administration x layers) showed a significant effect of layer ($F_{(2; 21)} = 104.2$; $p < 0.001$), while the other factors (**shock** and self-administration) were not significant ($F_{(3; 20)} = 1.149$; $p = 0.336$ and $F_{(3; 20)} = 0.355$; $p = 0.556$, respectively).

Cell density:

In the medial prefrontal cortex (mPFC): Cell density was evaluated in the three regions of the mPFC (Cg, PrL and IL). Data from the Cg showed that there was no significant difference between the groups (**Figure 11A**). Two-way ANOVA test (**shock** x self-administration) performed on these data did not reveal any significant effect of **shock** or self-administration ($F_{(3; 20)} = 2.032$; $p = 0.141$ and $F_{(3; 20)} = 1.092$; $p = 0.102$, respectively). In the PrL, no significant difference between the groups was recorded (**Figure 11B**). Two-way ANOVA test did not show any significant effect of **shock** or self-administration ($F_{(3; 20)} = 2.178$; $p = 0.122$ and $F_{(3; 20)} = 1.928$; $p = 0.098$, respectively). Similarly, in the IL region, no significant difference was observed (**Figure 11C**). Two-way ANOVA test did not reveal any significant effect of the studied factors ($F_{(3; 20)} = 0.5945$; $p = 0.625$ and $F_{(3; 20)} = 0.182$; $p = 0.762$, respectively).

In the hippocampus: The analysis targeting the hippocampus revealed increases in cell density in CA1, CA2, and CA3 regions for the Coc-PTSD group (**Figure 12A**).

Two-way ANOVA analysis (**shock** x self-administration) on data from CA1 did not show any significant difference of the two factors ($F_{(1; 19)} = 3.817$; $p = 0.065$ and $F_{(1; 19)} = 2.939$; $p = 0.102$, respectively). However, the interaction between these two factors was significant ($F_{(1; 19)} = 6.001$; $p = 0.024$). The Holm-Sidak *post-hoc* test demonstrated that rats from the **Coc-Shock** group expressed a significantly higher cell density compared to those from the **Coc-No shock** group ($t = 3.190$, $p = 0.028$) and those from the **NaCl- Shock** group ($t = 2.870$, $p = 0.047$).

Two-way ANOVA analysis (**shock** x self-administration) on data from CA2 indicated significant effects of both **shock** and substance ($F_{(1; 19)} = 5.206$; $p = 0.034$ and $F_{(1; 19)} = 4.410$; $p = 0.049$, respectively). The interaction between the two factors was not significant ($F_{(1; 19)} = 1.925$; $p = 0.181$). The Holm-Sidak *post-hoc* test showed a significant increase of cell density in the **Coc-Shock** group compared to the **NaCl-No shock** ($t = 3.175$, $p = 0.029$).

Two-way ANOVA analysis (**shock** x self-administration) on data from CA3 revealed significant effects of both shock and self-administration ($F_{(1; 19)} = 5.099$; $p = 0.035$ and $F_{(1; 19)} = 7.795$; $p = 0.011$, respectively). The **shock** x self-administration interaction was not significant ($F_{(1; 19)} = 0.785$; $p = 0.387$). The Holm-Sidak *post-hoc* test revealed a significant increase in cell density for rats in the **Coc-Shock** group compared to those in the **NaCl-No shock** group ($t = 3.659$, $p = 0.010$).

In the DG, no significant effect of the factors was recorded (**shock**: $F_{(1; 19)} = 2.092$; $p = 0.164$; treatment: $F_{(1; 19)} = 1.068$; $p = 0.314$). The **shock** x self-administration interaction failed to reach significance ($F_{(1; 19)} = 4.119$; $p = 0.058$).

In the basolateral nucleus of the amygdala (BLA): The analysis targeting the amygdala demonstrated increases in cell density in the BLA for both the **NaCl-Shock** and **Coc-Shock** groups (**Figure 13B**). Two-way ANOVA test (**shock** x self-administration) revealed significant effects of both **shock** and self-administration ($F_{(1; 19)} = 12.36$; $p = 0.002$ and $F_{(1; 19)} = 7.792$; $p = 0.011$, respectively). However, the **shock** x self-administration interaction was not significant ($F_{(1; 19)} = 0.686$; $p = 0.168$). The Holm-Sidak *post-hoc* test revealed a significant increase in the cell density of the NaCl Shock group compared to the **NaCl-No shock** group ($t = 2.713$, $p = 0.047$). Similarly, the **Coc-Shock** group showed a significant increase in cell density compared to the **NaCl-No shock** group ($t = 4.570$, $p = 0.001$).

DISCUSSION

In this study, we first investigated whether a history of cocaine self-administration would interfere with (i) **footshock exposure-related behavioral changes** (**conditioned fear**, anxiety-like behavior, and **recognition memory impairment**), (ii) **fear extinction learning and/or memory**, and (iii) the progression of cocaine self-administration following **footshock exposure**. We found that cocaine experience did not alter expression of fear **during situational reminders**, but it reduced anxiety-like behavior and potentiated cognitive decline. Behavioral data also show **that cocaine facilitated the fear priming effect**, suggesting difficulty to recover from a core PTSD-like symptom. Moreover, **cocaine intake remained unchanged after footshock exposure**. Examination of cortical thickness and cellular density in structures of interest (i.e., the hippocampus, amygdala and mPFC) revealed that **pre-fear conditioning cocaine intake provoked** an elevated cellular density in the hippocampal CA1, CA2 and CA3 regions and the BLA.

Behavioral data

Cocaine self-administration: Several hypotheses have been developed to explain the interactions between substance use disorders and PTSD. The most investigated is the self-medication hypothesis, according to which individuals with PTSD use psychoactive substances in an attempt to cope with their PTSD symptoms (**Khantzian, 1985; Brown and Wolfe, 1994**). Another interesting but less studied explanation for these behaviors is the high risk/susceptibility hypothesis, according to which, a prior history of substance use increases the risk of exposure to dangerous and threatening situations, and therefore increasing their risk of developing PTSD (**Cordovilla-Guardia et al., 2014**). Additionally, drug users become more susceptible to PTSD following trauma exposure (**Chilcoat & Breslau, 1998**). Based on the latter hypothesis, the present rodent study tested the effect of an extended (20 session), but

moderate (1h/session), intake of cocaine on **footshock exposure-associated behavioral changes and fear extinction learning and memory.**

On a separate group of animals, we were interested in assessing the evolution of cocaine self-administration in rats **after footshock exposure.** Our data did not reveal **any effect of footshock stress on cocaine consumption.** Our findings agree with multiple reports that show short access to cocaine, self-administered through a FR1 schedule, remains unchanged after predator-scent stress (**Hadad et al., 2016**) or single prolonged stress (SPS) (**Eagle et al., 2015; Enman et al., 2015**). In contrast, modified SPS reduced cocaine self-administration only during initial acquisition (**Hofford et al., 2018**), suggesting that the type of stress may play a role in determining how drug self-administration evolves. In addition to the type of stress, interindividual differences in cocaine self-administration might be detected within stress-exposed animals. In this regard, **Schwendt and colleagues (2018)** reported that stress-resilient animals self-administered less cocaine compared to stress-susceptible and control animals. This suggests that there might be interindividual differences in cocaine self-administration within shocked animals of our study, and that we need a larger sample size, which would allow us to distinguish between stress-susceptible and stress-resilient animals. Beyond regular self-administration procedures, footshock stress seems to have no effect on drug-seeking behavior, although it promotes relapse (**Pizzimenti et al., 2017**). Indeed, many studies in rodents have highlighted the phenomenon of relapse to substances under the effect of stress (**Wang et al., 2000; Shaham et al., 2003**). However, the effect of the latter on substance use and substance-seeking behavior is less clear (**Liu and Weiss, 2002; Mcfarland, 2004**). In humans, it has been shown individuals suffering from comorbid PTSD and substance use disorder do not differ in terms of consumption from individuals suffering from substance abuse disorder alone (**Brown et al., 1999; Eggleston et al., 2009**), but they are more likely to relapse after long periods of

abstinence (**Kubiak, 2004**). Taken together, one can conclude that exposure to stress is less likely to induce any increase in cocaine consumption.

Traumatic fear during situational reminders. Reexperiencing trauma is a hallmark symptom of PTSD (**Ehlers and Clark, 2000**). It can occur intrusively (such as post-traumatic flashbacks) or during exposure to conditioned stimuli (such as places and people). In rodent experiments, the model used for reexperiencing corresponds to brief re-exposure to the trauma context without further footshock application (**Pynoos et al., 1996; Louvart et al., 2005**). This procedure is also known to maintain, rather than decrease, the expression of fear over time (**Korem & Akirav, 2014**). Here, we confirmed the maintenance of traumatic fear over sessions and showed that this fear was not altered by the experience of cocaine. According to **Bauer (2014)**, in order to detect the effect of an addictive substance on fear learning or memory, it must be administered immediately before or after training, respectively. In our case, the last cocaine self-administration session took place hours before the first footshock exposure session, explaining why no effect of cocaine was observed on traumatic fear.

Anxiety-like behavior. High anxiety levels are also a hallmark of PTSD (**Pitman et al., 2012**). As in humans, studies with rodent models have revealed exaggerated levels of anxiety among other PTSD-like symptoms (**Blanchard et al., 1990; Pynoos et al., 1996; Bentefour et al., 2015; Lguensat et al., 2019**). In the current study, we observed this effect and found that it was abolished in rats with a history of cocaine self-administration. Previous rodent studies, which were not based on stress, have provided contradictory results, with some showing an increase (**Basso et al., 1999; Perrine et al., 2008; Aujla et al., 2008**) or a decrease (**Erhardt et al., 2006; Mueller et al., 2008; Lisieski et al., 2019**) in anxiety levels, and others indicating any of these effects (**Sarnyai et al., 1995 ; Mantsch et al., 2008**). In a study modeling cocaine pre-trauma exposure, **Lisieski and Perrine, (2017)** reported that the drug decreased anxiety while increasing the overall locomotor activity and exploration of the animal. In contrast, our data

show that the observed effect of cocaine on stress-induced anxiety is related to a decreased anxiety index, without affecting activity, indicating a specific attenuation of anxiety.

Cognitive impairment. PTSD is associated with deficits in various cognitive domains including attention, processing speed, memory, and executive functioning (**Morgan *et al.*, 2006; Poljac *et al.*, 2011; Stricker *et al.*, 2017**). Similarly, impairment of cognitive abilities are observed in cocaine users (**Kumar *et al.*, 2019**). Regarding rodent models trauma is known to induce cognitive function impairment (for example: recognition memory impairment: **Eagle *et al.*, 2013; Perez-Urrutia *et al.*, 2017**; alterations in cognitive flexibility: **George *et al.*, 2015**). Furthermore, cocaine intake alone has been reported to alter cognitive functions (**Briand *et al.*, 2009; Gobin *et al.*, 2019; Preston *et al.*, 2019**). This effect is long-lasting. For instance, **Briand *et al.*, (2009)** showed that extended access to cocaine produced deficits in recognition memory that persisted for at least 2 weeks after the cessation of drug use. In the present study, we found effects of trauma alone and cocaine use alone on recognition memory. The cognitive impairment was amplified in **footshock exposed rats with a history of cocaine, suggesting that the presence of both experiences (cocaine use and stress)** leads to a more potentiated cognitive decline. It has been shown that the hippocampus is one of the key structures involved in object recognition processes (**Reger *et al.*, 2009**). This led us to speculate that, in our study, cocaine **and footshock stress** probably induced a cumulative dysfunction in the hippocampus.

Suppression of conditioned fear. One of the core problems in PTSD is the reduced ability to suppress fear under safe conditions (**Davidson *et al.*, 2004; Jovanovic *et al.*, 2011**). One way to test this deficiency is to conduct fear extinction experiments with PTSD patients. Such studies have shown either impaired extinction learning (**Blechert *et al.*, 2007**) or normal extinction learning, but impaired extinction memory (**Milad *et al.*, 2008**). Different results may depend upon study conditions. In rodent studies, behavioral blockade of extinction memory recall is commonly obtained using the footshock priming procedure (**Deschaux *et al.*, 2011**;

Zheng et al., 2013; Bentefour et al., 2016). Interestingly, this procedure can also provoke the relapse of PTSD-like symptoms in mice (**Bentefour et al., 2016, 2018**). Here, we noticed that extinction of traumatic fear occurred in rats exposed to cocaine. However, footshock priming led to a return of traumatic fear in these rats. We have recently reported a similar finding with the use of alcohol (**Lguensat et al., submitted**). **In humans, PTSD is associated with prefrontal cortical dysfunction (Zubieta et al., 1999; Bremner et al., 2000) and antidepressant-induced activation of the prefrontal cortex prevents PTSD relapse (Davidson et al., 2001; Martenyi et al., 2002; Martenyi and Soldarenkova 2006; Yuan et al., 2018)**. Similarly, hypofunction of the infralimbic area of the mPFC facilitates the return of PTSD-like symptoms in animals (**Bentefour et al., 2016, 2018**). This leads us to suggest, based on our findings, that cocaine and footshock stress might induce dysfunction in this prefrontal area, reducing the ability of inputs from the prefrontal cortex to inhibit amygdala activity, knowing that increased activation of the amygdala characterizes PTSD state (**Nutt and Malizia, 2004**).

Brain data

Cortical measures: thickness and cellular density. In humans, a prolonged history of cocaine use has been reported to be associated with reduced gray matter volume in different cortical regions, including the prefrontal cortex (**Sim et al., 2007; Makris et al., 2008; Crunelle et al., 2014**). A similar result has also been reported in rats (**Cannella et al., 2018**). Here, we did not observe any significant change in cortical thickness or cell density in the mPFC of rats exposed to cocaine alone or to cocaine and trauma. In the study of **Cannella and colleagues (2018)**, rats were separated into two groups showing either addict-like or non-addict-like behavior based on their inability to refrain from drug-seeking and taking, high motivation for the drug, and maintenance of drug use despite negative consequences. Cortical changes were found only in rats displaying addict-like behavior (**Cannella et al., 2018**). Although in human studies structural changes have been reported in non-dependent cocaine users (**Mackey et al., 2014**), it

is more likely that the lack of cocaine effect in the present study resulted from our protocol of cocaine intake, which did not model cocaine addiction.

Contradictory data have been obtained regarding cortical changes in PTSD, with some studies indicating decreased thickness (e.g. Kühn & Gallinat, 2013; O'Doherty *et al.*, 2015) and activity (Hughes and Shin, 2011), while others showing no changes (Landré *et al.*, 2010; Rinne-Albers *et al.*, 2020) or an increase in microglial cell number and density (Smith *et al.*, 2019). Consistent with a previous study in rats (Gao *et al.*, 2014), we did not observe any effect of footshock exposure on thickness or cell density. However, we cannot conclude that our experiment protocol, which failed to induce alteration in the mPFC cytoarchitecture, also failed to induce alteration in the mPFC functioning. This is, in part, corroborated by our behavioral data revealing that a history of cocaine use can facilitate the return of extinguished fear, which has been observed in case of hypofunction of the mPFC (e.g. in humans: Milad *et al.*, 2005; e.g. in mice: Bentefour *et al.*, 2016, 2018).

Cellular density in the hippocampus and amygdala. Studies of patients with PTSD have shown structural abnormalities in limbic areas, including two key regions: the hippocampus and the amygdala (Shin *et al.*, 2006). Reduced hippocampal volume is frequently observed in PTSD (Bremner *et al.*, 1995; Gurvits *et al.*, 1996; Karl *et al.*, 2006). When assessing the effect of stress on hippocampal integrity, the volume reduction could be explained by the loss of dendrites and their synapses (Kassem *et al.*, 2013; O'Doherty *et al.*, 2015). However, some reports point out the possibility that smaller hippocampal volume is a vulnerability factor rather than a result of exposure to trauma (Gilberston *et al.*, 2002; Rubin *et al.*, 2016). On a cellular level, it has been reported that hippocampal pyramidal neurons are vulnerable to damage caused by stress through the action of corticosterone in rodents (e.g. McEwen *et al.*, 1986; Stein-Behrens *et al.*, 1994) and cortisol in primates including humans (e.g. Uno *et al.*, 1989; Wingenfeld and Wolf, 2014). The damage is mainly represented as dendritic spine loss

(Radley *et al.*, 2006; Clarke *et al.*, 2018; Smith *et al.*, 2019) and/or microglia activation and cell changes (Milior *et al.*, 2016; Smith *et al.*, 2019; Li *et al.*, 2021). The latter could eventually have repercussion on cell density leading to its increase.

Concerning the amygdala volume, the findings are more controversial, with some studies showing no significant changes (e.g. Woon and Hedges, 2009), whereas others indicate significantly smaller left amygdala volumes in with PTSD patients as compared to both healthy and trauma-exposed controls (e.g. Karl *et al.*, 2006). At a cellular level, rodent models of PTSD indicate that stress affects the cytoarchitecture of the amygdala either greatly (e.g. Cohen *et al.*, 2014) or poorly (e.g. Smith *et al.*, 2019). In the present rodent study, we found that footshock exposure induced an increase in cell density in the amygdala (in the BLA), but not in the hippocampus. However, in stressed rats previously exposed to cocaine self-administration, there was an increase in cell density in the hippocampus CA1, CA2, and CA3 regions and amplification of the BLA change. Changes in these structures could have resulted from two major causes. First, we observed that extinction memory was weak in rats with a history of cocaine intake. It has been demonstrated that activity of hippocampal and BLA ensembles regulates the expression of extinction memory, with high activity of fear engrams facilitating the return of the extinguished fear (Lacagnina *et al.*, 2019). Activation of the BLA has also been demonstrated to be essential for both persistence of newly acquired and reactivated fear memories (Nakayama *et al.*, 2016). It is therefore possible that cocaine self-administration experience facilitated reactivation of fear memories by increasing fear engram activity in the hippocampus and amygdala. Second, it is well known that the neuroimmune response to various threats (including stress and drug use) operates through neuroinflammation and manifests as proliferation as well as morphological and functional changes of microglia and astrocytes (Ransohoff & Brown, 2012). PTSD is known to be associated with neuroinflammation through the expression of proinflammatory cytokines (Hammad *et al.*, 2012; Lindqvist *et al.*, 2014;

Dennis *et al.*, 2016; Wang *et al.*, 2019). Rodent models of PTSD have also provided similar observations (Aga-Mizrachi *et al.*, 2014; Wohleb *et al.*, 2014). Furthermore, PTSD-associated changes in proinflammatory cytokines were observed in the hippocampus (Peng *et al.*, 2013; Lee *et al.*, 2016) and amygdala (Wilson *et al.*, 2013). Furthermore, microglial cell changes and hyper-ramification have been recently reported in the hippocampus (Smith *et al.*, 2019; Li *et al.*, 2021). Therefore, we could conclude a possible link between stress-induced microglial changes in these regions and an eventual elevated cell density. However, elevated cell density in the hippocampus was not found because of footshock exposure itself, but because of cocaine self-administration experience. In this context, it is interesting to note that the repeated use of cocaine promotes inflammation and activation of microglia in both humans (Zaparte *et al.*, 2019) and rodents (López-Pedrajas *et al.*, 2015; Cotto *et al.*, 2018; Burkovetskaya *et al.*, 2020). Moreover, since drug use induces an excessive release of dopamine and glutamate, these neurotransmitters binding to receptors expressed on microglia and astrocytes, could amplify inflammatory signaling via the additional release of cytokines. As a result, there is greater activation of the microglia (Kohno *et al.*, 2019) and potentially, more cell density, as seen here.

In summary, the repeated use of cocaine before footshock stress induction is implicated in amplification of stress-associated impairment of recognition memory and weakening of extinction memory. Our data also show that the repeated use of cocaine before footshock stress induction increased cell density in the hippocampus and amygdala. According to our interpretation, this reflects increases in neuronal and/or glial cells, which may contribute to the observed reactivation of conditioned fear memory and, by extrapolation, the impaired recovery from PTSD.

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Compliance with ethical standards

All experimental procedures were conducted in agreement with the French regulation (Decree 2013-118) and were approved by local ethic committee and the Ministère de l'Agriculture (Saisine #3129.01).

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Figure 1. The behavioral experiments timeline.

Figure 2. The mean number of cocaine and/or NaCl rewards (injections) per session and per blocks of sessions: A comparison between cocaine and NaCl rats. Bars represent mean \pm SEM.

Holm-Sidak *post-hoc*: ** $p < 0.01$, *** $p < 0.001$: significant difference compared to NaCl group.

Figure 3. The mean number of rewards (cocaine injections) per session and per blocks of sessions. **A-** Mean number of rewards: comparison between the Coc-No shock and Coc-Shock to-be groups. **B-** Block analysis of rewards: comparison between the Coc-No shock and Coc-Shock to-be groups. Bars represent mean \pm SEM.

Figure 4. The mean number of rewards (NaCl injections) per session and per block of sessions. **A-** Mean number of rewards: comparison between the NaCl-No shock and NaCl-Shock to-be groups. **B-** Block analysis of rewards: comparison between the NaCl-No shock and NaCl-Shock to-be groups. Bars represent mean \pm SEM.

Figure 5. The mean number of rewards (cocaine injections) per session and per block of sessions. A- Mean number of rewards recorded for the Coc-No shock and Coc-Shock groups before (session 1-20) and after **fear conditioning** (session 21-52). B- Block analysis of the number of rewards during different blocks of the 52 sessions. Bars represent mean \pm SEM.

Figure 6. The mean number of rewards (NaCl injections) per session and per block of sessions. A- Mean number of rewards recorded for the NaCl-No shock and NaCl-Shock groups before

(session 1-20) and after PTSD induction (session 21-52). B- Block analysis of the number of rewards during different blocks of the 52 sessions. Bars represent mean \pm SEM.

Figure 7. The evolution of freezing behavior during **fear conditioning** and Situational Reminders. Bars represent mean \pm SEM. Holm-Sidak *post-hoc*: ** $p < 0.01$, *** $p < 0.001$: significant difference compared to the NaCl-No shock group; ### $p < 0.001$: significant difference compared to the Coc-No shock group.

Figure 8. Data recorded during the Elevated Plus Maze (EPM) and the Novel Object Recognition (NOR) tests **A**-The anxiety index recorded during EPM test. **B**-The **locomotor activity of different groups**. **C**-The recognition index recorded during the NOR test. Bars represent mean \pm SEM. Holm-Sidak *post-hoc*: * $p < 0.05$, *** $p < 0.001$: significant difference compared to the NaCl-No shock group; ### $p < 0.001$: significant difference compared to the Coc-No shock group.

Figure 9. The evolution of freezing behavior recorded during extinction and reinstatement sessions. Bars represent mean \pm SEM. Holm-Sidak *post-hoc*: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$: significant difference compared to the NaCl-No shock group; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$: significant difference compared to the Coc-No shock group. †† $p < 0.01$: significant difference compared to the NaCl-Shock group.

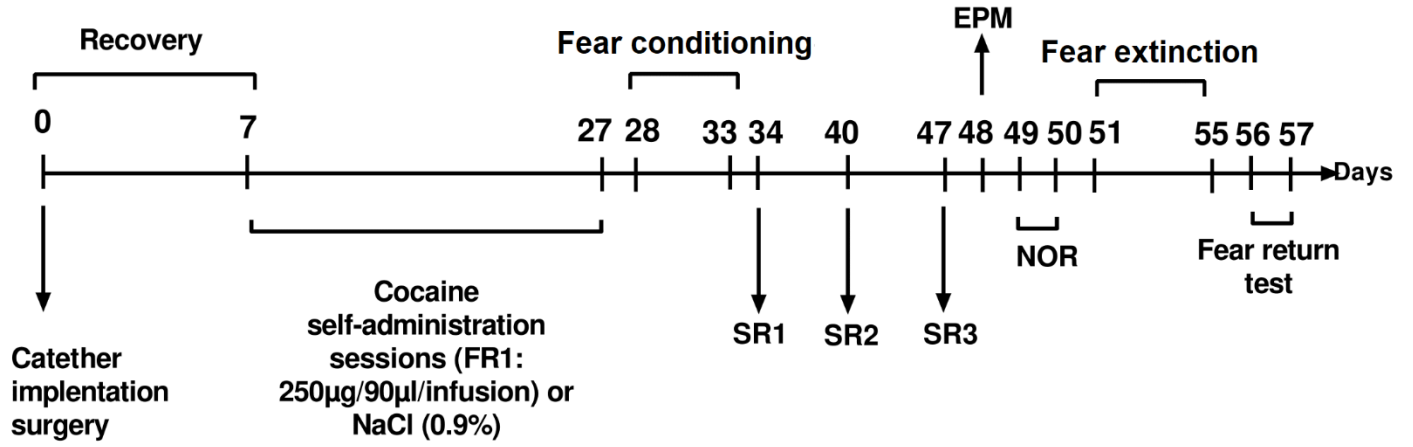
Figure 10. Cortical thickness assessment across the groups. **A**- Total cortical thickness in the Cg, PrL and IL regions of the mPFC. Layers thickness in the Cg (**B**), PrL (**C**) and IL (**D**). Bars represent mean \pm SEM.

Figure 11. Mean of cell density measures in the Cg, PrL and IL regions of the mPFC (A-C). Bars represent mean \pm SEM.

Figure 12. Cell density measures in different regions of the hippocampus. **A-** Representative photomicrographs of the CA1, CA2, CA3 and DG regions of the hippocampi of rats belonging to different groups. **B-** Mean of cell density within the CA1 region. **C-** Mean of cell density within the CA2 region. **D-** Mean of cell density within the CA3 region. **E-** Mean of cell density within the DG region. Bars represent mean \pm SEM. Holm-Sidak *post-hoc*: * $p < 0.05$ and ** $p < 0.01$: significant difference compared to the NaCl-No shock group; # $p < 0.05$: significant difference compared to the Coc-No shock group. † $p < 0.05$: significant difference compared to the NaCl-Shock group. Hipp photomicrographs: scale bar= 500 μ m. CA1, CA2, CA3 and DG photomicrographs: scale bar= 100 μ m

Figure 13. Cell density measures in the BLA region of the amygdala. **A-** Representative photomicrographs of the BLA region of rats belonging to different groups. **B-** The mean of cell density in the BLA of different groups. Bars represent mean \pm SEM. Holm-Sidak *post-hoc*: * $p < 0.05$ and ** $p < 0.01$: significant differences compared to the NaCl-No shock group. AMY photomicrographs: scale bar= 500 μ m. BLA photomicrographs: scale bar= 100 μ m.

Figure 1



FR: Fixed Ratio
EPM: Elevated Plus Maze test
NOR: Novel Object Recognition test
SR: Situational Reminder

Figure 2

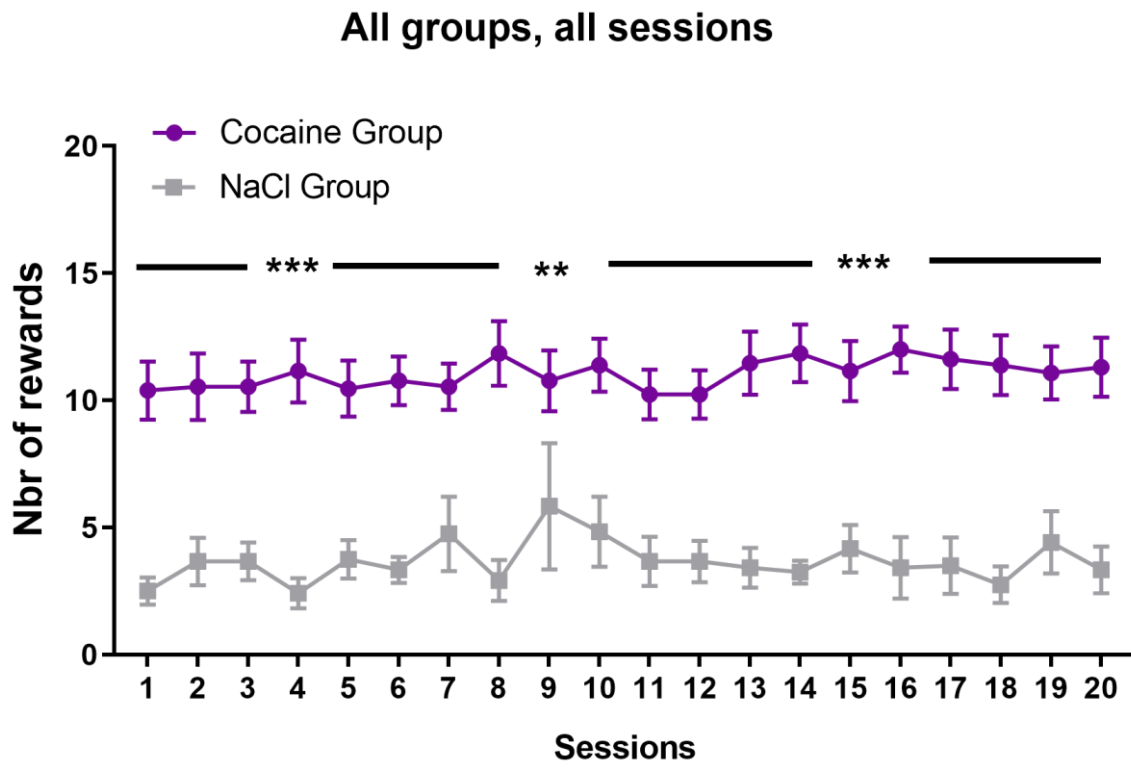


Figure 3

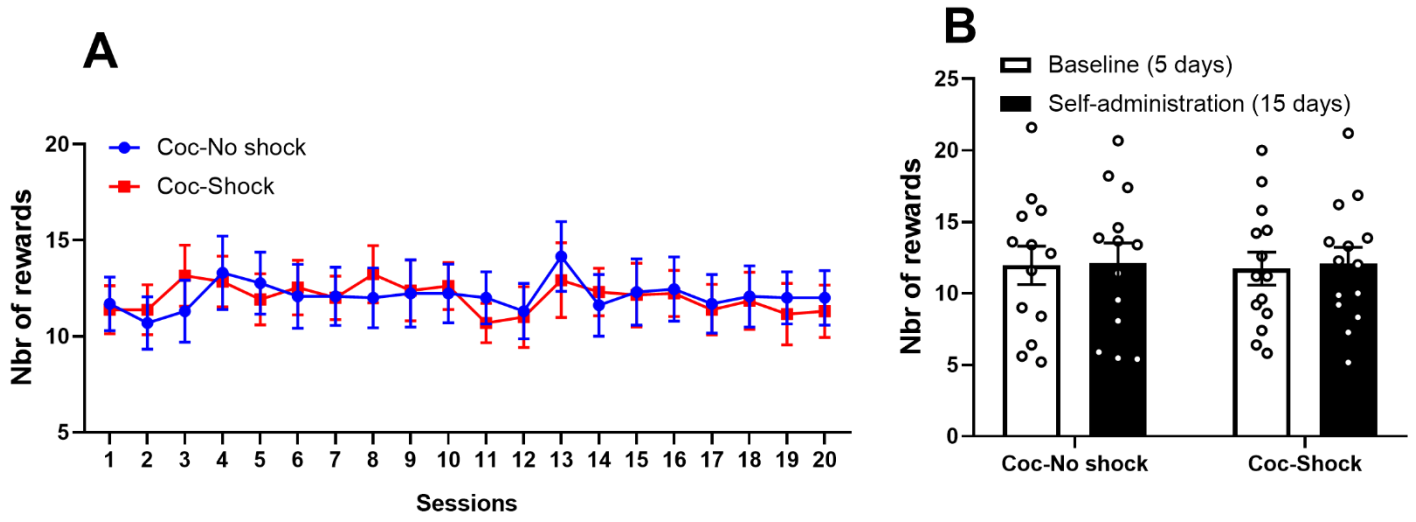


Figure 4

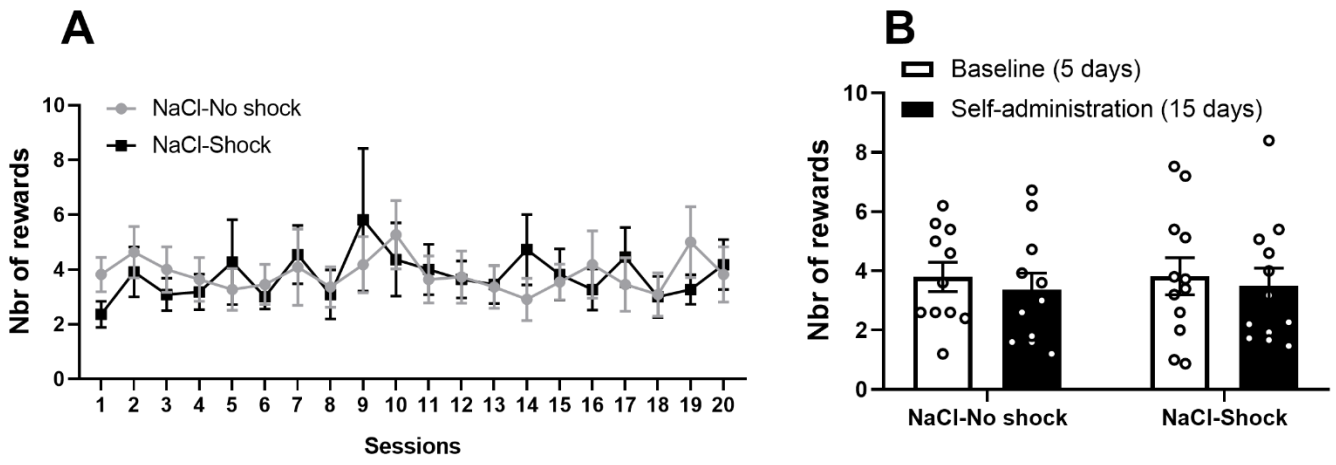


Figure 5

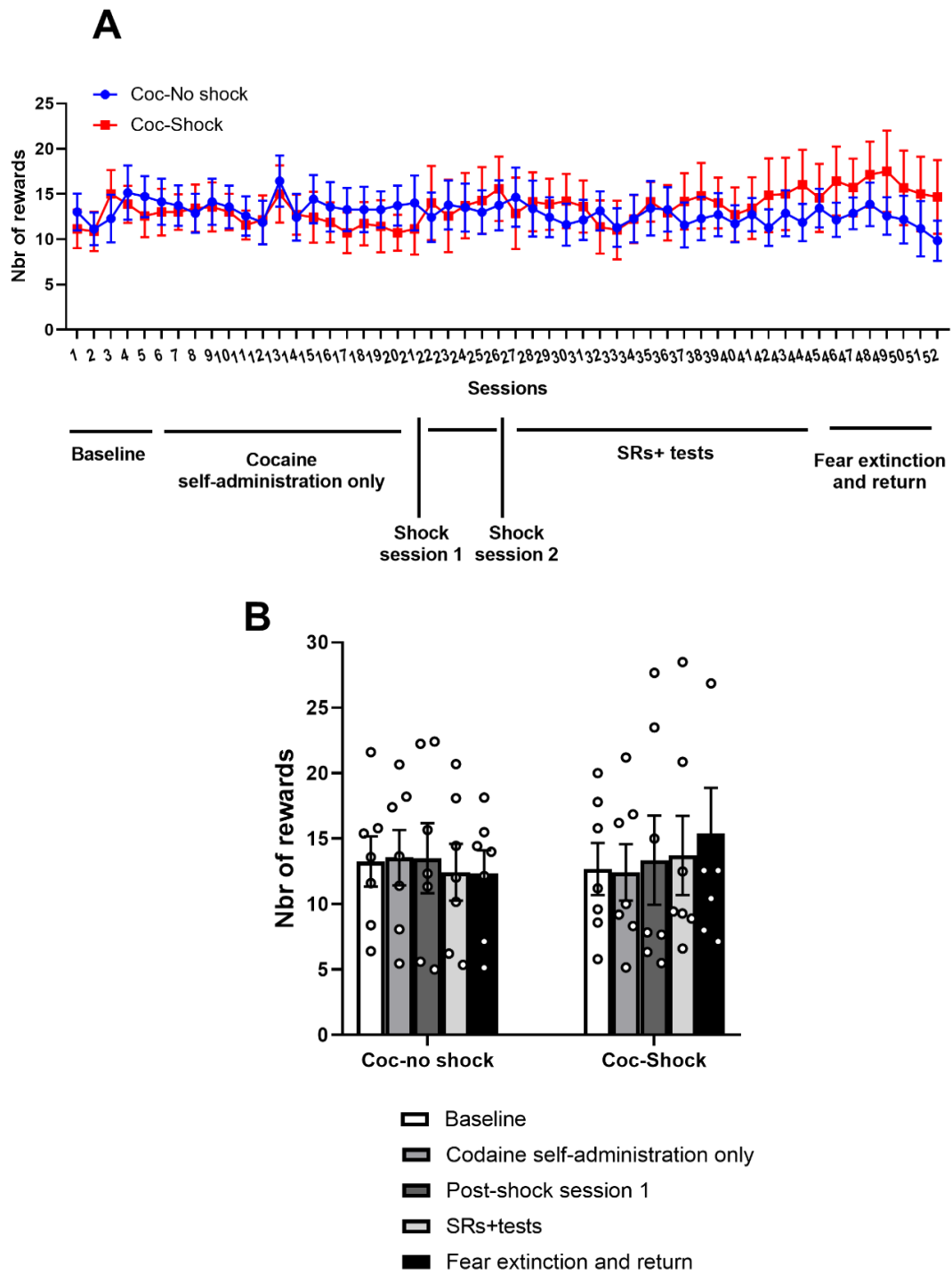


Figure 6:

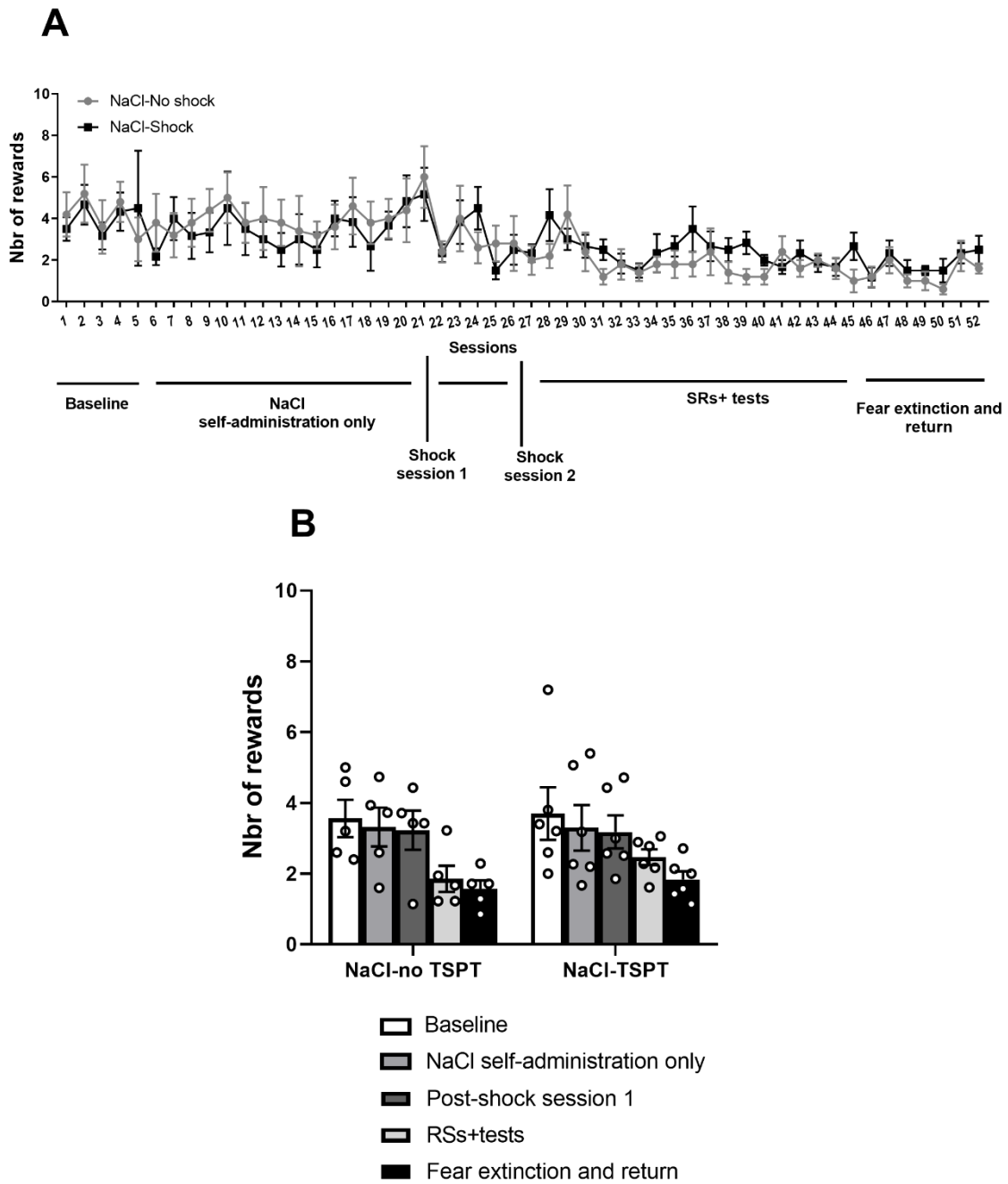
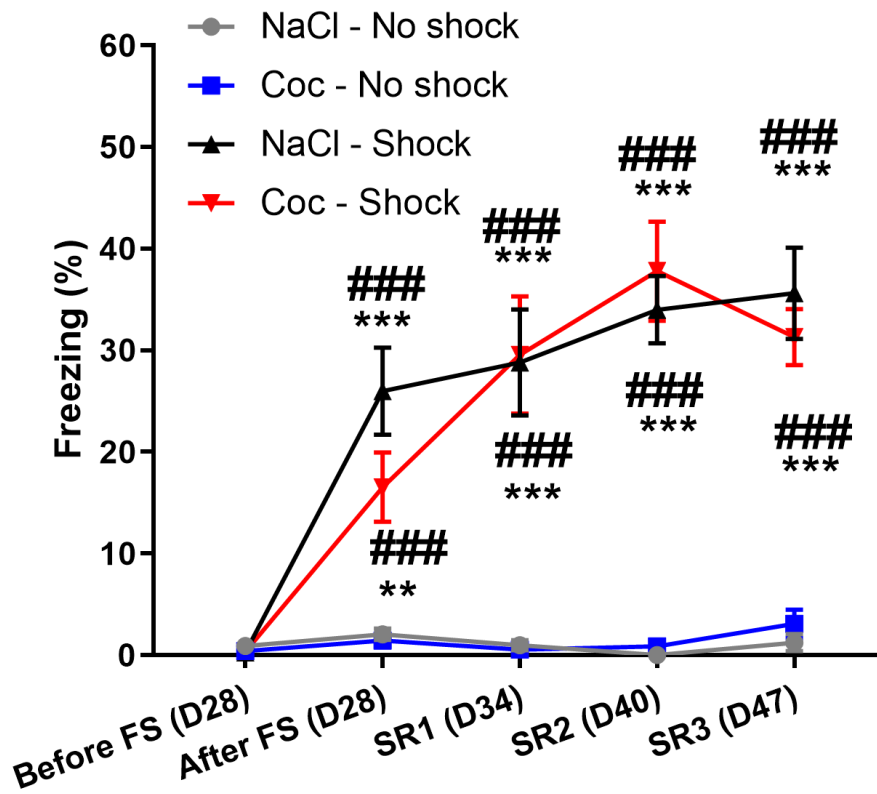


Figure 7:

Situational reminders



FS: Footshock
SR: Situational Reminder

Figure 8

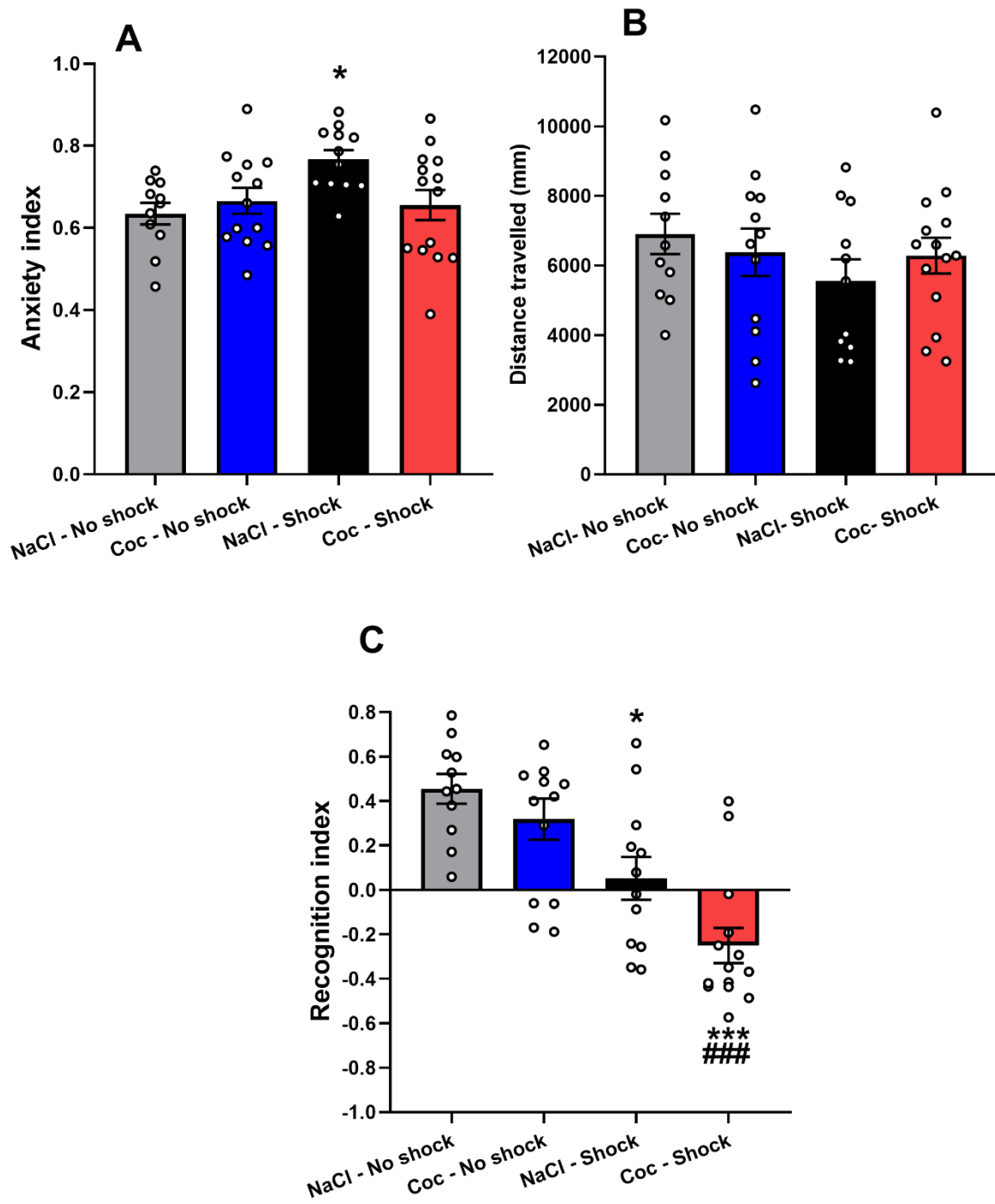
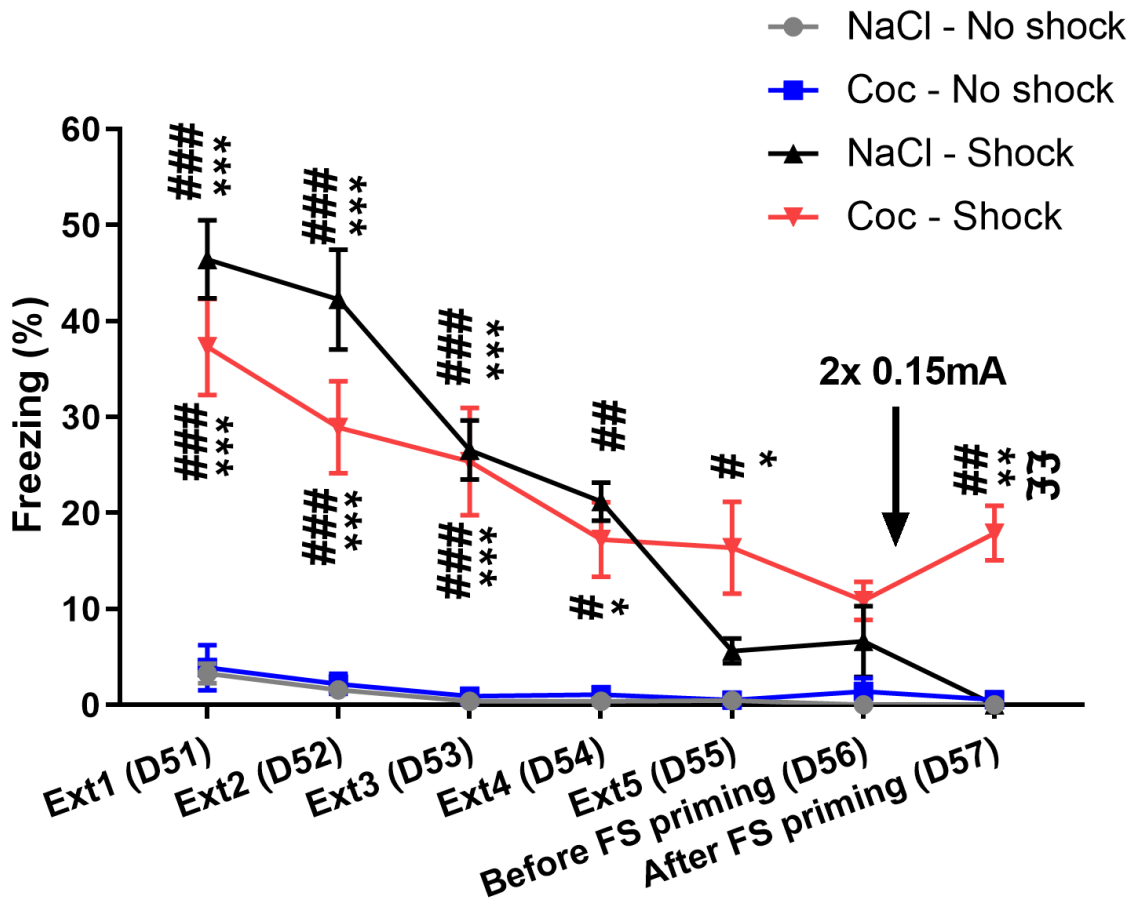


Figure 9

Fear extinction and return

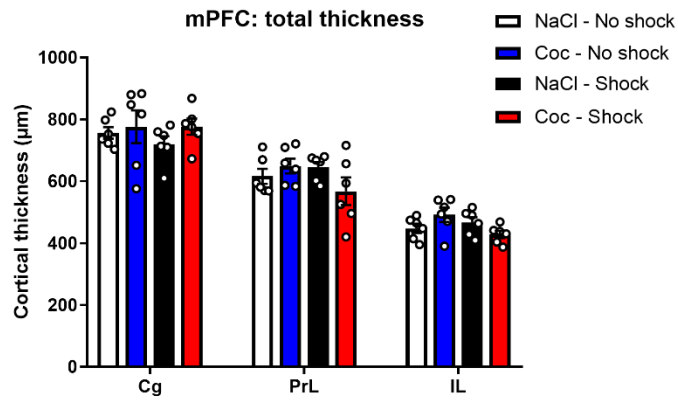


Ext: Extinction
FS: Footshock

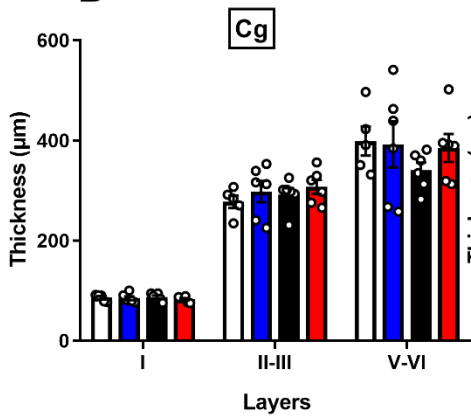
Figure 10

mPFC : layer thickness

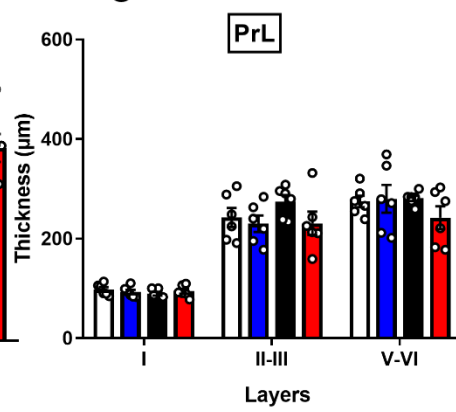
A



B



C



D

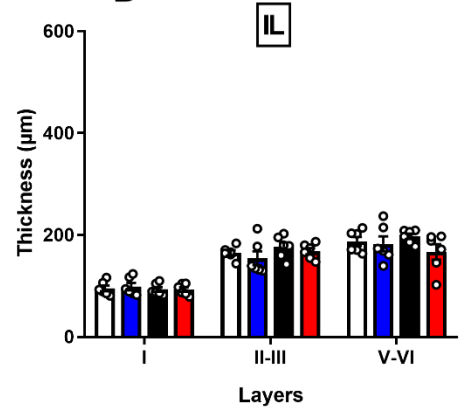


Figure 11

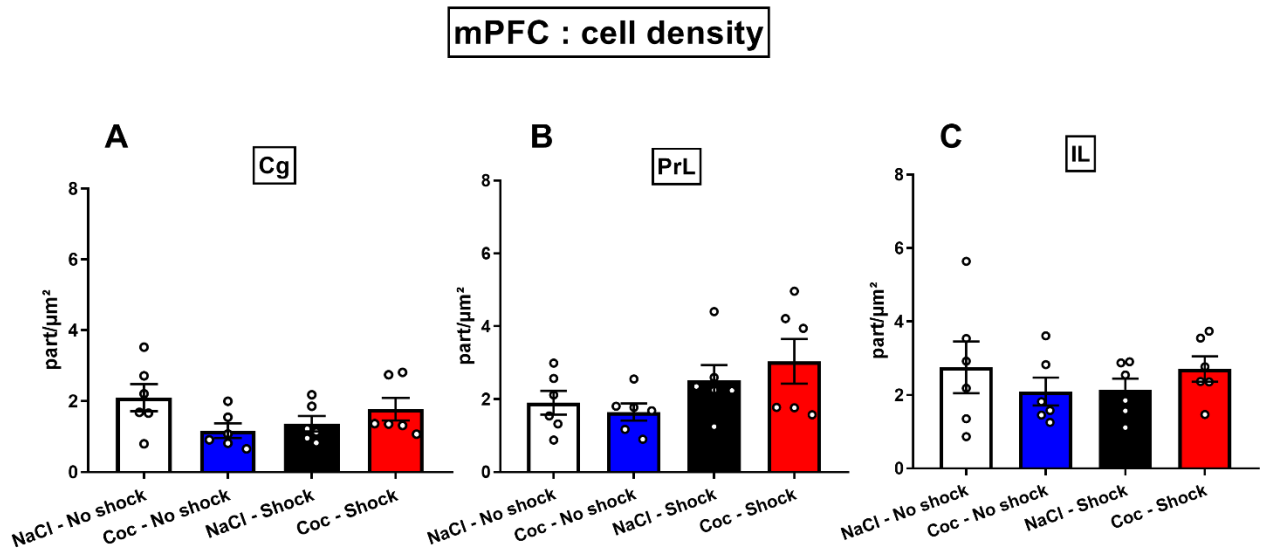


Figure 12

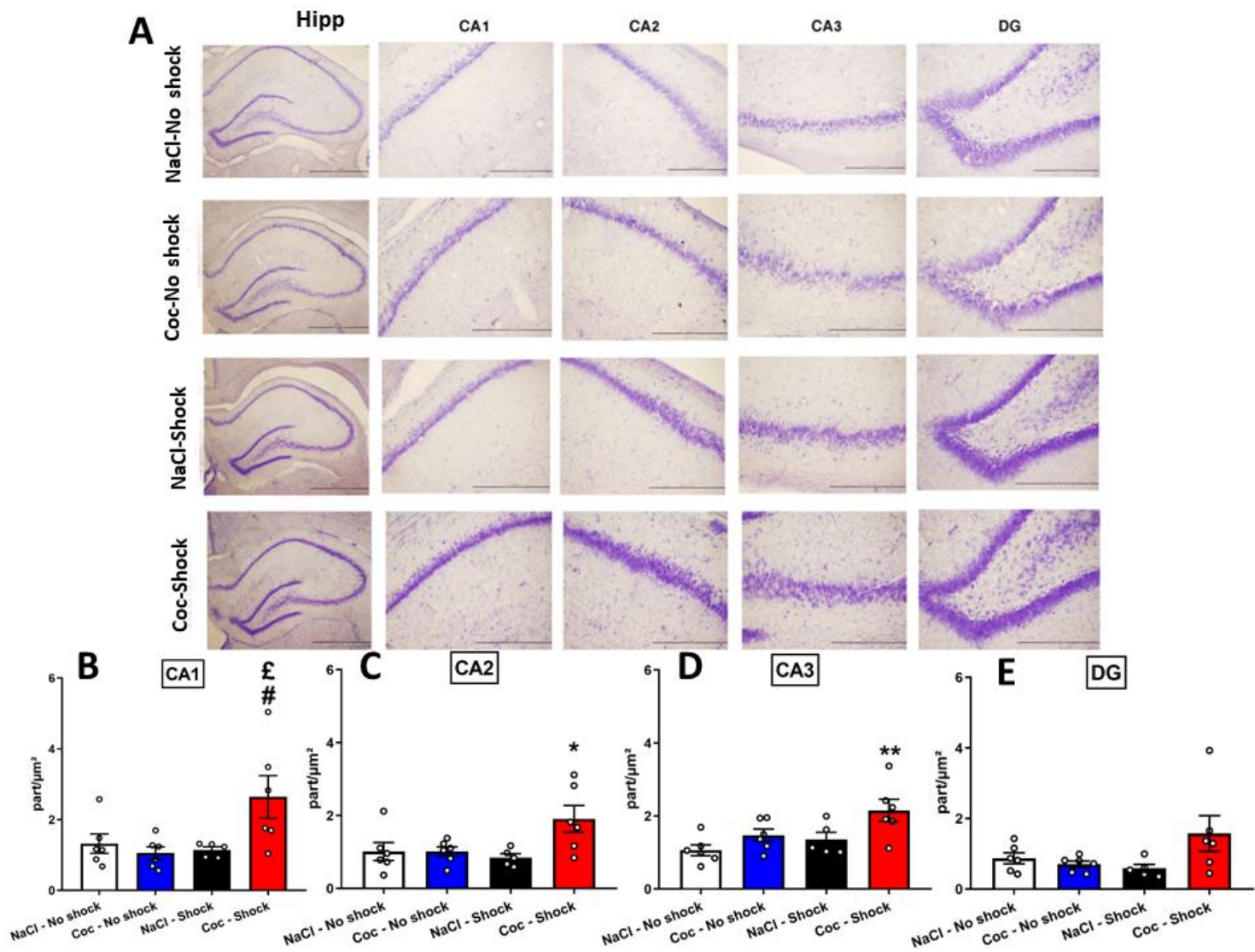


Figure 13

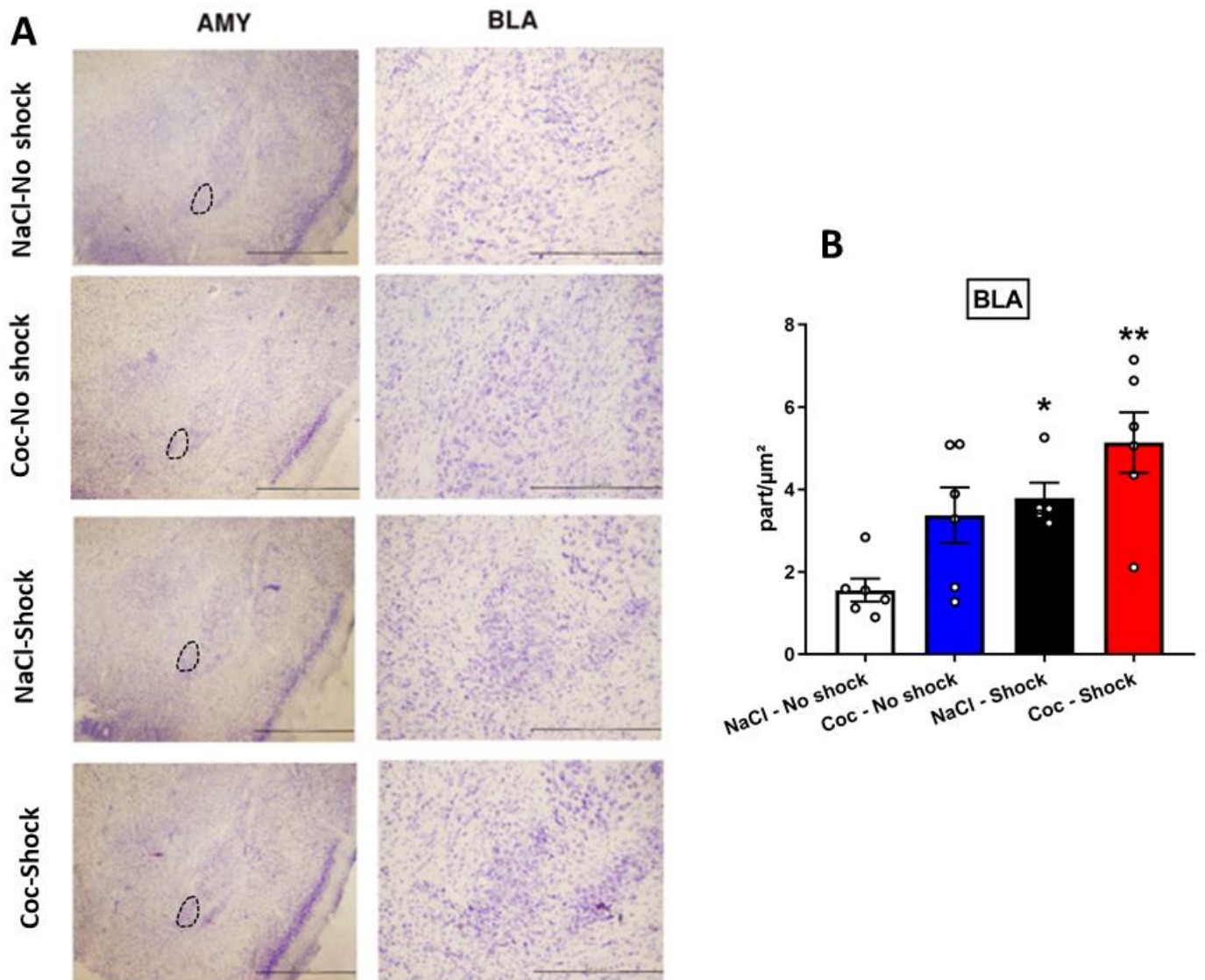


Table 1 : statistical values of the comparison between Cocaine and NaCl groups

Comparison : Cocaine vs. NaCl				
Session	NaCl (mean ±SEM)	Cocaine (mean ±SEM)	Post-hoc (NaCl vs. Cocaine)	
			t	p
1	3.13±0.39	11.25± 0.92	8.113	***
2	4.26±0.61	10.77± 0.92	8.457	***
3	3.51±0.48	12.14 ±1.08	7.454	***
4	3.34±0.47	12.88±1.11	8.172	***
5	3.78±0.23	12.22±1.01	9.172	***
6	3.13±0.41	12.22±1.03	8.822	***
7	4.21±0.82	12±0.89	7.754	***
8	3.17±0.54	12.51±1.02	6.791	***
9	4.86±1.32	12.37±1.11	6.201	**
10	4.78±0.85	12.37±0.92	8.249	***
11	3.65±0.61	11.18±0.82	7.257	***
12	3.56±0.55	11.14±1.00	8.254	***
13	3.26±0.50	13.66±1.26	5.217	***
14	3.21±0.50	12.22±0.99	6.575	***
15	3.52±0.55	12.33±1.13	7.531	***
16	3.60±0.68	12.37±0.96	8.567	***
17	3.82±0.69	11.81±0.99	7.263	***
18	2.95±0.51	11.96±1.02	7.600	***
19	4.13±0.67	11.74±1.00	6.319	***
20	3.95±0.65	11.77±0.93	6.172	***

Table 2 : Statistical data of the different comparisons made between groups during different situational reminder (SR) sessions.

Session	NaCl-Shock (mean±SEM)	Coc-Shock (mean±SEM)	<i>Post-hoc</i> (NaCl-Shock vs. NaCl-No shock)		<i>Post-hoc</i> (NaCl-Shock vs. Coc-No shock)		<i>Post-hoc</i> (Coc-Shock vs. NaCl-No shock)		<i>Post-hoc</i> (Coc-Shock vs. Coc-No shock)	
			t	p	t	p	t	p	t	p
Before footshocks (D28)	0.278±0.278	0.331±0.184	0.33	ns	0.26	ns	0.08	ns	0.15	ns
After footshocks (D28)	25.980±4.300	16.533±3.411	5.71	***	6.11	###	3.51	**	3.83	###
SR1 (D34)	28.817±5.212	29.538±5.753	6.65	***	7.03	###	6.95	***	7.36	###
SR2 (D40)	33.992±3.306	37.787±4.862	8.12	***	8.25	###	9.19	***	9.38	###
SR3 (D47)	35.612±4.493	31.305±2.755	8.21	***	8.10	###	7.31	***	7.17	###

Table 3: Different comparisons made during extinction and relapse sessions.

Session	NaCl-Shock (mean±SEM)	Coc-Shock (mean±SEM)	<i>Post-hoc</i> (NaCl-Shock vs. NaCl-No shock)		<i>Post-hoc</i> (NaCl-Shock vs. Coc-No Shock)		<i>Post-hoc</i> (Coc-Shock vs. NaCl-No Shock)		<i>Post-hoc</i> (Coc-Shock vs. Coc-No Shock)	
			t	p	t	p	t	p	t	p
Ext1 (D51)	46.43±4.05	37.32±5.00	10.19	***	8.33	###	8.18	***	10.14	###
Ext2 (D52)	42.25±5.21	28.93±4.80	9.27	***	7.27	###	7.01	***	9.83	###
Ext3 (D53)	26.56±3.07	25.37±5.58	9.82	***	8.03	###	6.82	***	7.92	###
Ext4 (D54)	21.18±1.96	17.28±3.88	1.82	ns	5.92	##	5.02	*	6.19	#
Ext5 (D55)	5.62±1.29	16.39±4.78	1.38	ns	2.01	ns	4.91	*	5.29	#
Before FS priming (D56)	6.62±3.69	10.85±1.97	1.02	ns	0.98	ns	1.81	ns	0.87	ns
After FS priming (D57)	0.00±0.00	17.91±2.855	2.11	ns	1.23	ns	0.23	**	0.79	##

Dr. Lee A. Shapiro
Associate Editor
Brain Structure and Function

Re: Ms. No.: BSAF-D-21-00049

Title: Repeated cocaine exposure prior to fear conditioning induces persistency of PTSD-like symptoms and enhancement of hippocampal and amygdala cell density in male rats.

Dear Doctor Shapiro,

My colleagues and I would like to thank the Reviewers for their useful comments, which we have fully considered (all changes are in red in the revised manuscript).

We would like to thank you for giving us this chance to improve our manuscript.

We hope that the manuscript is now suitable for publication in Brain Structure and Function.

We look forward to your decision.

Sincerely,

Prof. Rene Garcia