Repeated ethanol exposure following avoidance conditioning impairs avoidance extinction and modifies conditioning-associated prefrontal dendritic changes in a mouse model of post-traumatic stress disorder

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Reply to Reviewer # 2

Reviewer # 2 has raised 5 points. Please find below our corresponding replies.

POINT 1: The authors provided an explanation and rationale for the behavioral tests (original point 3#) in the response to the review. Please incorporate some of this text into the manuscript to help the reader understand the interpretations of, and relationships between, behaviors in the CPP and passive avoidance procedures.

REPLY TO POINT 1: The requested change has been made in the revised manuscript in the Introduction section (page 4).

POINT 2: In the response to point #4, the authors describe the biased CPP training as "positive reinforcement." It can also be described as negative reinforcement (or both could be going on) because you are alleviating the aversion to the non-preferred place. Please clearly state in the manuscript that the CPP procedure is a "biased" procedure. Please also mention an alternative interpretation of the CPP data with biased (vs. unbiased) and negative reinforcement in mind and provide a citation from the literature.

REPLY TO POINT 2: The requested changes have been made. Firstly, in the Material and Methods section, we have stated that the CPP procedure is a "biased" procedure (page 5). Secondly, in the Discussion section, we have discussed the "positive reinforcement" and the negative reinforcement of our CPP procedure (pages 20-21) and biased and unbiased procedures for CPP (page 21).

POINT 3: Also in the response to point #4, the authors state that they don't think that their data would change with the addition of unpaired control groups receiving footshock stress and ethanol separately from the cues. ("…we can guess that the results would have been like those

expressed by our EtOH + groups.”). This response is not sufficient and not supported by any data from the published literature. At a minimum, the authors need to state that a limitation of the study designs using Pavlovian conditioning procedures is that they do not include control groups that have received equal exposures to the unconditioned (and conditioned) stimuli in an unpaired fashion. Otherwise, they cannot rule out that behavior changes were due to exposures to these stimuli. Please review Rescorla (https://uk.sagepub.com/sites/default/files/upm-binaries/23600_Ch_1.pdf) and mention the lack of these control groups as a limitation of the study design.

REPLY TO POINT 3: We have now stated this limitation in the conclusion of the Discussion section (page 28).

POINT 4: The CPP data are not given enough attention in the discussion so that the results are interpreted in the context of the original hypothesis/rationale for CPP testing. There is no mention in the conclusion of the significant finding that trauma exposed mice showed stronger ethanol-induced CPP. Instead, the conclusion line only refers to the passive avoidance results. According to the authors the CPP data were integral to the study design and rationale, yet, the discussion does not tie these findings together. Please discuss the findings in the context of how changes in sensitivity to rewarding effects of alcohol (assessed by CPP) relate to fear sensitization.

REPLY TO POINT 4: We have now added the CPP finding to the conclusion (page 28). We have also discussed the link between the sensitivity of the rewarding effects of ethanol and susceptibility status (page 20-21).

POINT 5: Please put the Ns per group in the figure legends.

REPLY TO POINT 5: The total number of animals per group has been added to figure legends.

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Abstract

Treatment of post-traumatic stress disorder is complicated by the presence of alcohol use disorder comorbidity. Little is known about the underlying brain mechanisms. We have recently shown, in mice, that the post-traumatic stress disorder-like phenotype is characterized by the increase and decrease in total dendritic number and length in the prelimbic and infralimbic areas of the medial prefrontal cortex, respectively. Here, we examined whether repeated ethanol exposure would exacerbate these changes, and whether this would be associated with difficulty to extinguish passive avoidance behavior, as an indicator of treatment resistance. We also analyzed whether other known trauma-associated changes, like increased or decreased corticosterone and decreased brain-derived neurotrophic factor levels, would also be exacerbated. Male mice underwent trauma exposure (1.5-mA footshock), followed, 8 days later, by a conditioned place preference training with ethanol. Tests for fear sensitization, passive avoidance, anxiety-like behavior, extinction acquisition and relapse susceptibility were used to assess behavior changes. Plasma corticosterone and brain-derived neurotrophic factor levels and prefrontal dendritic changes were subsequently measured. Trauma-susceptible mice exposed to ethanol acquired a strong place preference and behaved differently from those not exposed to ethanol, with delayed avoidance extinction and higher avoidance relapse vulnerability. Ethanol potentiated trauma-associated dendritic changes in the prelimbic area and suppressed trauma-associated dendritic changes in the infralimbic area. However, ethanol had no effect on trauma-induced increased corticosterone and decreased brain-derived neurotrophic factor levels. These data suggest that the modification of prefrontal trauma-related changes, due to alcohol use, can characterize, and probably support, treatment-resistant post-traumatic stress disorder.

1. INTRODUCTION

Post-traumatic stress disorder (PTSD) is a mental illness that may arise after an individual experiences or witnesses a life-threatening event. Although most people with PTSD recover by one year **(Freedman *et al.,* 1999)**, the remaining patients form a chronic subgroup that never fully remits even after many years **(Zlotnick *et al.,* 1999; Galatzer-Levy *et al.,* 2013)**. One of the conditions contributing to the chronic aspect of the disorder is the comorbidity with alcohol use disorder **(McFarlane, 1988; Breslau and Davis, 1991; Bremner *et al.*, 1996)**. According to one of the theoretical models addressing this comorbidity, PTSD patients often turn to alcohol to alleviate their symptoms, but this ultimately results in the maintenance of their symptoms **(Zlotnick *et al.*, 1999; Kaysen *et al.*, 2011; Hawn *et al.,* 2020)**. Although the understanding of the risk factor of alcohol for PTSD maintenance has enabled clinicians to identify patients who need continued care, little is known about the brain changes associated with alcohol-induced PTSD maintenance. This can be, in part, addressed with studies based on fear extinction.

During the last decades, our understanding of the development and maintenance of PTSD has improved by the means of advances in the analysis of learning mechanisms related to fear conditioning and extinction **(Maeng & Milad, 2017)**. Consistent with conditioning theories, PTSD patients often express fear responses, such as avoidance, when exposed to trauma-related cues. In addition, maintenance of PTSD symptoms is hypothesized to involve disruption of fear extinction processes **(Wessa & Flor, 2007)**. It is therefore possible that alcohol use may contribute to treatment-resistant PTSD through the disruption of fear extinction processes.

Basic rodent studies have shown that increased activity in the prelimbic (PrL) area of the medial prefrontal cortex (mPFC) is implicated in extinction disruption **(Burgos-robles *et al.*, 2009; Herry *et al.*, 2010; Bravo-Rivera *et al.*, 2015)**. However, studies exploring the potential link between changes in the PrL area and ethanol-induced extinction disruption are still missing.

Regarding ethanol administration, several methods have been successfully used in rodents. For instance, animals can be allowed to consume ethanol using the choice between ethanol and water with

the two-bottle choice paradigm (e.g., **Huynh *et al.,* 2019)**. They can also be allowed to self-administer ethanol while having access to a natural reward like a sweet solution (e.g., **Augier *et al.,* 2018).** The other method is to submit animals to the conditioned place preference (CPP) task in which they learn to associate one compartment of a two- or three-compartment apparatus with ethanol injections (e.g., **Cunningham and Prather, 1992)**; this latter procedure also allows studying drug related preference and motivation behavior **(Tzschentke, 2007)**. Interestingly, our recent rat study with cocaine, based on a self-administration procedure **(Lguensat *et al.,* 2021)**, and our preliminary mouse study with ethanol, based on the CPP procedure, revealed that both procedures similarly disrupt extinction. Given another of our recent rodent studies showing a positive correlation between passive avoidance behavior and the increase in total dendritic number and length in the PrL area (**Lguensat *et al.,* 2019)**, we hypothesized that the disruption of passive avoidance extinction with the CPP procedure would be associated with the exacerbation of the PrL dendritic changes. To test this hypothesis, mice were exposed to a high intensity (1.5-mA) footshock to acquire passive avoidance behavior and, 8 days later, to ethanol injections for CPP acquisition. Following behavioral testing (including tests for fear sensitization, passive avoidance, anxiety-like behavior, extinction acquisition and relapse vulnerability), dendritic changes in the mPFC were measured using the Golgi-cox method. We also investigated changes in plasma concentrations of corticosterone (CORT) and brain-derived neurotrophic factor (BDNF), knowing their dysregulation under the conditions of PTSD with rodent models (increased CORT: e.g., **Sillivan *et al.*, 2017**; decreased BDNF: e.g., **Burstein *et al.*, 2018**).

2. METHODS AND MATERIALS

2.1. Animals

Eighty outbred adult male Swiss mice aged between 2 and 3 months at the start of the experiment and bred in the central animal facility of the Faculty of Science Semlalia, Marrakech, Morocco, were used in this study. The animals were group housed and maintained under constant conditions of temperature

(22 ±2°C) and under a 12h light/12h dark cycle (ZT0=7h), with food and water available *Ad libitum*. All animal procedures were in strict accordance with the guidelines of European Council Directive (EU2010/63). All efforts were made to minimize animal suffering.

2.2. Behavioral apparatus and protocols

The apparatus and behavioral procedures used, for passive avoidance training, classical conditioning, passive avoidance test, sensitization test, anxiety test using an elevated plus maze (EPM) as well as passive avoidance extinction and post-extinction priming exposure, have been described in detail in our previous studies **(Bentefour *et al.*, 2015, 2016)**.

The CPP apparatus consisted of three compartments of different appearances, arranged to have a neutral compartment (15.5 x 14 x 13 cm), which served as a start compartment with transparent walls without any pattern on them. The start compartment had access to two other compartments (13 x 17 x 13 cm each) through a sliding door. Both compartments had a visual (dotted *vs*. stripped walls) and tactile (smooth *vs*. rough floors) contextual differences. This unit was cleaned with 20% ethanol solution and the mouse was placed in the apparatus 5 minutes later to avoid any olfactory cue that could interfere with the CPP training.

The experimental timeline is shown in **Figure 1**. On Day 1, the PTSD-like symptoms were induced using a passive avoidance apparatus (context A), where mice were put in the light compartment of the apparatus and were left to freely enter the dark compartment, where, when locked in, they received two foot-shocks of 2 seconds at 1.5-mA each, separated by 6 seconds. The apparatus was cleaned using 70% ethanol after each animal. Following an incubation period of 7 days in the animal facility, mice were subjected to a biased procedure of ethanol induced CPP, which was adapted from **Li *et al.*, (2015)** and lasted for 6 days. This biased procedure consisted in associating reward to the least preferred compartment **(Prus *et al.*, 2009)**. On Day 8 of the protocol, the CPP procedure started (preconditioning phase) by placing mice in the start compartment and allowing the animals to freely explore the apparatus for 30 minutes. Mouse behavior was recorded, and the time spent in each compartment and the number

of entries were quantified. From Day 9 to Day 12 (conditioning phase), mice were subjected to the conditioning procedure, where the least preferred compartment (the one where the animal spent the lesser time during the preconditioning phase) was associated with ethanol injections, while the most preferred compartment was associated with saline injections. Ethanol (1.6g/kg) was dissolved in saline solution (0.9%) and injected intraperitoneally at a volume of 10ml/kg while saline solution was injected with an equal volume. On Days 9 and 11, mice trained for CPP (the EtOH+ groups) received ethanol in the morning and saline solution in the afternoon. This order was alternated on Days 10 and 12 with mice in the EtOH+ groups receiving four ethanol injections in total. The other mice (the EtOH- groups) were subjected to the same regimen, except that ethanol was replaced by saline. After receiving each injection, mice were locked in the appropriate compartment for 25 minutes. The morning and afternoon sessions were separated by at least 5 h. On Day 13 (post-conditioning: testing phase), mice were exposed to the CPP apparatus as on Day 8.

On the following day (Day 14 of the experiment), all mice underwent classical fear conditioning in a different context (context B) using 3 footshocks of 1 second at a lower intensity (0.4-mA) and separated by 70 seconds. On Day 15, in the morning, passive avoidance behavior was tested by putting mice back in the light compartment of the passive avoidance apparatus (context A) and measuring step-through latency time to enter the dark compartment using a stopwatch. A maximum time of 480 seconds (8 minutes) was allowed for the animal to enter the dark compartment and in case of no entrance, the mouse was removed from the apparatus. In the afternoon, they were placed back in the classical conditioning chamber (context B) to assess fear sensitization. On Day 16, anxiety-like behavior was assessed in the EPM. The mouse was placed in the center of the maze for free exploration of all arms of the maze for 5 min. From Day 18 to Day 22, mice were exposed daily to the passive avoidance apparatus (context A); where they were left to freely enter the dark compartment. Each day (Days 18-22), once in the dark compartment, they were locked there for 10 minutes, with no exposure to footshock to induce passive avoidance extinction. Starting from Day 19, if an animal spends 480 seconds without going to the dark compartment, it was forced to enter and was locked there for 10 minutes. On Day 23, animals were subjected to trauma re-exposure in context A as on Day 1, but with a very low intensity footshock

(0.15-mA instead of 1.5-mA: passive avoidance priming exposure). The following day (Day 24), passive avoidance relapse was assessed in the same apparatus (context A).

2.3. Behavioral scoring and analysis

The behavior of each mouse was videotaped with a JVC camera for later analysis. Behavioral data from the CPP and EPM tests were quantified using Ethovision XT Noldus 8.5 video-tracking program (Noldus Information Technology b.v., Wageningen, The Netherlands). For passive avoidance behavior, step-through latency (from the light compartment to the dark compartment) was scored using a stopwatch during each exposure to the shuttle box apparatus. For the CPP paradigm (preconditioning and post conditioning), the time spent in each compartment was measured and used to calculate the preference score following the formula used by **Montanari *et al.*, (2018)**:

Preference score = Time spent on the drug paired side in post conditioning day -Time spent in the same compartment on the preconditioning day.

For fear sensitization, the amount of time spent freezing was measured using a 1-s time sampling technique. A mouse was considered to freeze when it adopted a motionless posture, refraining from all but respiratory movements **(Fanselow *et al.*, 1994)**. For anxiety-like behavior, the anxiety index was calculated as follows:

Anxiety index = 1 – [(open-arm time/total time) + (open-arm entries/total entries)]/2.

For passive avoidance relapse correlation analysis, we calculated a relapse score based on the time spent in the trauma-associated chamber, as follows:

Relapse score = Time spent in the footshock-associated chamber on day 24 -Time spent in the same compartment on day 23

Immediately following the incubation period in the animal facility, mice from shocked and control groups were divided into EtOH- (with no ethanol exposure) and EtOH+ (with ethanol exposure). To discriminate the susceptible and resilient mice, we followed the same method that we used in our previous study **(Lguensat *et al.*, 2019)**. Briefly, after examining the step-through latency time recorded

during the passive avoidance test, shocked mice expressed a heterogeneous response and displayed a bimodal distribution **(Figure 2B)**. Therefore, animals in the top and bottom tertiles of step-through latency time were separated into high-avoiders (HA: trauma-susceptible) and low-avoiders (LA: stress-resilient) groups **(Table 1).** Mice that expressed intermediate behavior were eliminated from the study.

2.4. Golgi-Cox staining

After the end of the behavioral experiment, mice (n=31) were deeply anesthetized with an intraperitoneal injection of a lethal dose of urethane (>40mg/kg) and transcardially perfused with 0.9% saline followed by 4% of paraformaldehyde. Brains were then extracted, post-fixed overnight in 4% paraformaldehyde. The Golgi-Cox staining method consisted of immersing the brains in 3% solution of potassium dichromate for 15 days. The brains were then transferred in 2% silver nitrate solution for 3 days. After this delay, they were cut with a vibratom (Leica VT 1200 S), into 100µm sections, which were immediately mounted onto gelatin coated slides and dehydrated in graded ethanol starting from 50%, then xylene, before cover slipping the slide.

2.5. Image acquisition and morphometric analysis

Slides were analyzed using Olympus BH-2 microscope equipped with an Olympus DP71 camera. The regions of interest were identified using the atlas of **Paxinos and Franklin (2007)**. Pyramidal neurons in these regions were recognized according to their triangular soma shape and apical and basilar dendrites. To select neurons with less bias, we considered the same criteria as in our previous study **(Lguensat *et al.*, 2019)**. The images were taken using a 40x objective and the number and length of apical and basilar dendrites were quantified for each neuron. These parameters are the most used to assess the effect of stress on dendritic plasticity. We used image j software (NIH) to process images of neurons and for stack construction following the same method as in our previous study **(Lguensat *et***

***al.*, 2019).** We also used Adobe Photoshop CS2 for neuron image processing. Dendritic measurements were performed blind to the treatment conditions.

2.6. Blood collection method and CORT and BDNF measurements

After the completion of the behavioral tests, on Day 17, blood was collected using the restrained tail snip. We chose this method because it causes minimal stress-related changes in plasma CORT levels due to handling **(Kim *et al.*, 2018).**

Blood collection was performed between 11:30 am and 2 pm to avoid potential effects of circadian variations on plasma CORT concentrations. Each mouse (n=33) was placed in a restriction tube and a small puncture at the level of the lateral tail vein was made using a sterile blade. Blood samples were collected into EDTA tubes, which were stored on ice until centrifugation. To obtain plasma, blood samples were centrifuged for 15 minutes at 1600g at 4°C. The supernatant (20-80µl) was collected and stored at -80 °C until analysis.

Plasma CORT analysis was performed on all the mice (n=33) while BDNF measurements were done on 31 mice. Due to outliers, we discarded the results of one mouse from BDNF measurements. We performed these measures using enzyme-linked immunosorbent assay (ELISA) kits: Enzyme-linked immunosorbent Assay kit (My BioSource, San Diego, California, USA) and BDNF Emax ® ImmunoAssay (Promega, Madison, Wisconsin, USA) in accordance with the recommendations of the manufacturer.

2.7. Statistical analysis

Data analysis and graphs plotting were performed using SigmaPlot v.11 (SigmaPlot, San Jose, California, USA) and GraphPad Prism v7 (GraphPad software, California, USA) software. AICs (Akaike information criterion) were calculated using XLSTAT and Akaike weights (w) were determined as following: exp (-0.5*ΔAIC) divided by the sum of these values across all models. **(Burnham and**

Anderson, 2003). Shapiro Wilk's normality or lognormality test was run on data sets, and parametric tests were used whenever the test passed. When the test failed, nonparametric tests were used. Thus, the Mann-Whitney U test was used to measure the difference between the control and shocked mice regarding their passive avoidance behavior and Two-way ANOVA were used to assess the difference between the groups regarding their anxiety index, plasma CORT and BDNF levels. CPP preference score and sensitization test were analyzed using Kruskal-Wallis analysis of variance followed by Dunn's *post-hoc* for multiple comparisons. Data on passive avoidance extinction and dendritic changes from the PrL and IL areas were analyzed by three-way ANOVA followed by two-way ANOVA of data of each day separately (passive avoidance extinction and relapse) and from each region separately (dendritic parameters). The Holm-Sidak *post-hoc* was used for multiple comparisons. Values are reported as mean ±SEM and the significance was set at $p<0.05$.

CPP data were analyzed after the separation of mice into trauma-susceptible and trauma-resilient subgroups. The preference score was considered during the analysis. Correlation analysis of relapse score data was performed according to data distribution (Spearman's correlation for non-normally distributed data).

3. RESULTS

3.1. Distinction of trauma-susceptible and stress-resilient mice

Passive avoidance testing that followed avoidance training showed that most shocked mice acquired avoidance behavior **(Figure 2A)**. The Mann-Whitney test revealed a significant difference between the Control and Shocked groups (U=231, p=0.032). The normality test showed that the Control group expressed a normal distribution (Shapiro-Wilk's normality test passed: p=0.345). However, passive avoidance data from shocked mice failed the normality test (Shapiro-Wilk's normality test not passed: p<0.001), implying that they manifested a multimodal distribution. Further analysis showed that frequency distribution of the shocked group follows a bimodal distribution with two peaks matching the

bin centers of 50 and 500 seconds **(Figure 2B)**. In fact, the bimodal Gaussian function (function 1) provided the best fit relative to unimodal Gaussian function (function 2) [(a low AIC indicates the model that fits the data better: Δ_1(AIC)=1.51 and Δ_2(AIC)=45.81, Akaike weight for these models: w_1= 0.997 and w_2=0.00024)]. Thus, we considered animals in the top and bottom tertiles of the distribution and segregated them into trauma-resilient (or low avoiders: LA) and trauma-susceptible (or high avoiders: HA) subgroups within the ethanol exposed group (EtOH+/LA and EtOH+/HA groups). The other half, mice not exposed to ethanol (EtOH- group), received saline injections (EtOH-/LA and EtOH-/HA groups) and served as controls.

3.2. Ethanol-induced CPP in trauma-susceptible and stress-resilient mice

Data demonstrate that all mice exposed to ethanol [the non-shocked (EtOH+/Ctrl) and the shocked (EtOH+/LA, EtOH+/HA) groups] acquired a conditioned preference towards the ethanol-paired side of the apparatus **(Figure 2C)**. Given that preference score data were not normally distributed (Shapiro-Wilk's normality test not passed: p=0.002), we used a non-parametric test for the analysis. The Kruskal-Wallis one-way analysis of variance of preference score data indicated a highly significant difference between the groups (H (5) =39.28, p<0.001). The Dunn's multiple comparison test revealed significant increases of preference score for the groups exposed to ethanol (EtOH+/Ctrl, EtOH+/LA and EtOH+/HA) compared to the EtOH-/Ctrl group (q=3.032, p<0.034; q=4.589, p<0.001 and q=5.902, p<0.001; respectively). The preference score expressed by the EtOH+/HA group was significantly higher compared with the EtOH+/Ctrl group (q=3.074, p=0.043), but was not significant compared with the EtOH+/LA group (q=1.623, p=0.997). In addition, the EtOH+/HA group showed significant increases of the preference score when compared with the EtOH-/LA and EtOH-/HA groups (q=3.544, p=0.008 and q=3.351, p=0.015, respectively).

Regarding the EtOH- groups, we found that none of the associated subgroups (EtOH-/LA and EtOH-/HA) manifested a significant elevation of their preference score when compared to the EtOH-

/Ctrl group (Kruskal Wallis test followed by Dunn's multiple comparison test, EtOH-/LA *vs.* EtOH-/Ctrl: q=2.529, p=0.159 and EtOH-/HA *vs.* EtOH-/Ctrl: q=2.736, p=0.096).

3.3. PTSD-like behaviors across the groups

3.3.1. Fear sensitization based on a fear-conditioning test

Mice in the EtOH+/HA group displayed a marked freezing behavior in the new context in comparison with the other groups **(Figure 3A)**. A Kruskal-Wallis one-way analysis of variance (data non-normally distributed: Shapiro-Wilk's normality test not passed: p<0.001) of freezing time showed a difference between the groups (H (5) =14.07, p=0.015). The Dunn's multiple comparison test demonstrated that within the EtOH+ group, the EtOH+/HA subgroup expressed significant high levels of freezing as compared to the EtOH-/Ctrl subgroup (q=2.937, p=0.023). Moreover, even if the freezing expressed by EtOH+/ HA group was high, this increase was not significant compared with the EtOH-/ HA group (q=0.864, p=0.990). In addition, we did not find any significant increases in freezing for the EtOH+/LA group compared to the EtOH-/LA group (q=1.458, p=0.992), nor did we obtained significant increases in freezing for the EtOH+/Ctrl group in comparison with the EtOH-/Ctrl group (q=1.224, p=0.999).

3.3.2. Anxiety-like behavior based on an EPM test

Results demonstrated that shocked mice tended to express high anxiety index **(Figure 3B)**. A two-way ANOVA on anxiety-like behavior data (treatment and post-footshock avoidance) did not reveal any effect of treatment ($F_{(1, 72)}$ =0.242, p=0.624), but a significant effect of post-footshock avoidance was found ($F_{(2, 71)}$ =7.449, p=0.001). The interaction between these factors was not significant ($F_{(2, 72)}$ =1.269, p=0.287). However, the Holm-Sidak *post-hoc* test showed that, within the groups that were not exposed to ethanol, anxiety index was significantly higher in the EtOH-/HA group as compared to the EtOH-/Ctrl and EtOH-/LA groups (t=3.363, p=0.003 and t=3.036, p=0.006; respectively). No significant

difference was recorded between the EtOH+/HA and EtOH-/HA groups (t=0.806, p=0.666), the EtOH+/LA and EtOH-/LA groups (t=1.467, p=0.379) nor between the EtOH-/Ctrl and EtOH+/Ctrl groups (t=0.271, p=0.786).

3.3.3. Passive avoidance extinction and relapse

All trauma (1.5mA footshock) exposed mice exhibited progressive decreases in step-through latency during extinction training **(Figure 3C).** A three-way ANOVA (session, treatment and post-footshock avoidance) revealed significant effect of the three factors ($F_{(7;\ 68)}$ =35.21, p<0.001, $F_{(1;\ 72)}$ =44.38, p<0.001 and $F_{(2;\ 71)}$ =180.94, p<0.001, respectively). The interactions between session and post-footshock avoidance and between treatment and post-footshock avoidance were significant ($F_{(14;\ 71)}$ =11.83, p<0.001 and $F_{(2;\ 72)}$ =18.44; p<0.001, respectively), while the interaction between session and treatment was not significant ($F_{(2;\ 72)}$ =1.750, p=0.095). The Holm-Sidak *post-hoc* test (following a two-way ANOVA analysis performed on each day separately) showed that on Day 15 (avoidance test) and Day 18 (extinction session 1), values of step-through latency were significantly higher in HA mice from the EtOH+ group, compared with Ctrl and LA mice from both the EtOH- and EtOH+ groups, while the difference with the EtOH-/HA group was not significant**.** From Day 19 (extinction session 2) to Day 23 (pre-footshock priming), the EtOH+/HA group continued to display significant higher step-through latency time compared with each of the other groups, including the EtOH-/HA group **(Table 2).** On Day 24 (post-footshock priming), the EtOH+/HA group expressed elevated step-through latency time that was significantly higer compared with the remaining groups, including the EtOH-/HA group (**Table 2**), indicating that passive avoidance behavior returned only in HA mice treated with ethanol.

3.4. Quantification of Golgi-Cox material

Branching complexity of the apical and basilar dendrites, dendritic length, and branch order **(Figure 4)** were quantified in two areas of the mPFC: PrL and IL. The analysis included 182 PrL neurons (with a

number ranging from 24 to 45 neurons/group) and 147 IL neurons (ranging from 20 to 29 neurons/group) from 31 mice. We considered the individual as a unit of analysis.

3.4.1. Number of dendrites

Apical dendrites: The total number of apical dendrites was increased only in the PrL area of trauma-susceptible mice exposed to ethanol (**Figure 5A)**. A three-way ANOVA (mPFC area, treatment and post-footshock avoidance) on these data showed a significant effect of treatment ($F_{(1, 28)}$ =19.462, p<0.001), but the effects of the two other factors were not significant (mPFC area: $F_{(1, 28)}$ =0.071, p=0.790; post-footshock avoidance: $F_{(2, 27)}$ =1.050, p=0.358). The interaction between area and post-footshock avoidance was significant ($F_{(2, 28)}$ =11.160, p<0.001), as well as the interaction between treatment and area ($F_{(1, 28)}$ =4.267, p=0.044). However, the remaining interaction (i.e., between treatment and post-footshock avoidance) was not significant ($F_{(2, 28)}$ =1.711, p=0.191).

Thereafter, we used a two-factor ANOVA to assess the effect of treatment and post-footstock on the dendrites within each area separately. For PrL data, this analysis indicated significant effects of both factors (treatment: $F_{(1, 28)}$ =19.356, p<0.001; post-footshock avoidance: $F_{(2, 27)}$ =6.305, p=0.006). In fact, the Holm-Sidak *post-hoc* test showed that the EtOH+/HA group expressed a significant elevation of the total number of dendrites compared with the EtOH-/HA group (t=3.500, p=0.002). The same group (EtOH+/HA) also expressed a significant elevation compared with the EtOH-/LA group (t=2.749, p=0.032) (**Figure 5A:** PrL).

Similarly, for IL data, a two-way ANOVA analysis, with treatment and post-footstock avoidance as factors, showed a significant effect of post-footshock avoidance ($F_{(2, 27)}$ =5.878, p=0.008) but no effect was observed for treatment ($F_{(1, 28)}$ =3.003, p=0.095). The Holm-Sidak *post-hoc* test showed that the EtOH-/HA group had a significant diminution of the total number of dendrites compared to the EtOH-/LA group (t=3.873, p=0.002). The EtOH+/HA group also expressed higher dendritic number compared with the EtOH-/HA group (t=2.151, p=0.041) (**Figure 5A:** IL).

Basilar dendrites: Data indicated that the total number of basilar dendrites increased in the PrL of EtOH+/HA animals (**Figure 5B)**. A three-way ANOVA on these data (mPFC area, treatment and post-footshock avoidance) revealed main effects of treatment and post-footshock avoidance ($F_{(1, 28)}$ =5.584, p=0.022 and $F_{(2, 27)}$ =3.202, p=0.049, respectively), but no significant effect of area was found ($F_{(1, 28)}$ =0.512, p=0.477). The interactions between area and treatment and between area and post-footshock avoidance were significant ($F_{(2, 28)}$ =4.226, p=0.045 and $F_{(1, 28)}$ =13.517, p<0.001, respectively). However, the interaction between treatment and post-footshock avoidance was not significant ($F_{(2, 28)}$ =2.593, p=0.084).

A more detailed analysis of PrL data revealed a significant effect of treatment but no effect of post-footshock avoidance was found [two-way ANOVA: treatment ($F_{(1, 28)}$ =12.501, p=0.002); post-footshock avoidance: ($F_{(1, 28)}$ =2.103, p=0.143)]. The Holm-Sidak *post-hoc* test showed a significant elevation of the total number of basilar dendrites in the EtOH+/HA group compared with the EtOH-/HA group (t=2.957, p=0.007) (**Figure 5B:** PrL).

Regarding data from the IL area, two-way ANOVA did not reveal any effect of treatment ($F_{1, 28)}$ =0.000003, p=1.000). However, the factor post-footshock avoidance was highly significant ($F_{(1, 28)}$ =15.021, p<0.001). The Holm-Sidak *post-hoc* test indicated that the EtOH-/HA group had a significant reduction of the overall number of dendrites when compared to the EtOH-/LA and EtOH+/Ctrl groups (t=6.219, p<0.001 and t=2.584, p=0.016). However, the EtOH-/LA group manifested a significant increase of the number of dendrites when compared with the EtOH-/Ctrl group (t=4.398, p<0.001) (**Figure 5B:** IL).

3.4.2. Dendrite branch length

Apical dendrites: The total length of apical dendrites was markedly increased only in the PrL area of HA mice from the EtOH+ group (**Figure 5C)**. A three-way ANOVA on these data (mPFC area, treatment and post-footshock avoidance) did not reveal any significant effect of area, neither any effect of post-footshock avoidance ($F_{(1,28)}$ = 0.655,p=0.422 and $F_{(2,27)}$ = 0.852,p=0.433), but a significant

treatment effect was found ($F_{(1,28)} = 6.815$, $p=0.012$). The interactions between area and treatment and between area and post-footshock avoidance were also significant ($F_{(2, 28)} = 5.950$, $p=0.018$ and $F_{(1, 28)} = 15.063$, $p<0.001$; respectively). However, the interaction between treatment and post-footshock avoidance was not significant ($F_{(2, 28)} = 2.516$, $p=0.123$).

A detailed analysis of PrL data indicated a significant effect of treatment and post-footshock avoidance (two-way ANOVA: treatment ($F_{(1, 28)} = 15.316$, $p<0.001$); post-footshock avoidance: ($F_{(1, 28)} = 9.500$, $p<0.001$)). The Holm-Sidak *post-hoc* test showed that the EtOH+/HA group expressed a significant elevation of the total length of dendrites compared to the EtOH+/LA and EtOH-/HA groups ($t=3.059$, $p=0.016$ and $t=2.699$, $p=0.012$, respectively) (**Figure 5B:** PrL).

The same analysis on data from the IL area showed a significant effect of post-footshock avoidance ($F_{(1, 28)} = 10.390$, $p<0.001$) but no effect of treatment was found ($F_{(1, 28)} = 0.093$, $p=0.763$). The Holm-Sidak *post-hoc* test revealed that EtOH-/HA group expressed a significant reduction of the total length compared with both the EtOH-/Ctrl and EtOH-/LA groups ($t=2.512$; $p=0.019$ and $t=5.634$, $p<0.001$, respectively). The EtOH-/LA group also expressed a significantly higher elevation as compared with the EtOH-/Ctrl group ($t=2.989$, $p=0.012$). No such differences were observed within the EtOH+ groups (**Figure 5C:** IL).

Basilar dendrites: The total length of basilar dendrites was markedly increased only in the PrL area of HA mice from the EtOH+ groups (**Figure 5D).** A three-way ANOVA on the total branch length (mPFC area, treatment and post-footshock avoidance) did not indicate any significant effect of area ($F_{(1,28)} = 0.897$, $p=0.348$), or post-footshock avoidance ($F_{(2,28)} = 1.839$, $p=0.169$), but a main effect of treatment was found ($F_{(1,28)} = 10.288$, $p=0.002$). The interactions between area and treatment and between area and post-footshock avoidance were significant ($F_{(2, 28)} = 5.040$, $p=0.029$ and $F_{(1, 28)} = 22.869$, $p<0.001$; respectively). However, the interaction between treatment and post-footshock avoidance was not significant ($F_{(2, 28)} = 0.233$, $p=0.793$).

Regarding PrL data, both treatment and post-footshock avoidance had significant effects [two-way ANOVA: treatment ($F_{(1, 28)} = 13.903$, $p<0.001$) and post-footshock avoidance ($F_{(2, 28)} = 6.399$, $p=0.006$)]. The Holm-Sidak *post-hoc* test revealed that the EtOH+/HA group had a significant increase in the overall length of basilar dendrites as compared with the EtOH+/Ctrl and EtOH+/HA groups

(t=2.893, p=0.023 and t=2.728, p=0.011; respectively). Within the EtOH- groups, EtOH-/HA mice demonstrated an increased branch length compared with EtOH-/LA mice (t=2.650, p=0.041) (**Figure 5D:** PrL).

When considering the IL area, the analysis showed that the factor post-footshock avoidance was significant ($F_{(2, 28)} = 9.454$, $p<0.001$), while there was no effect of the factor treatment ($F_{(1, 28)} = 1.237$, p=0.277). The EtOH+/HA group did not differ from each of the two groups who received ethanol (EtOH+/Ctrl and EtOH+/LA; t=1.184, p=0.434 and t=1.421, p=0.423, respectively). However, the EtOH-/HA group showed a significant reduction of the length of basilar dendrites in comparison with the EtOH-/LA group (t=4.803, $p<0.001$), while the EtOH-/LA group displayed a significant elevation compared with the EtOH-/Ctrl group (2.831, p=0.018) (**Figure 5D:** IL).

3.5. Correlation between behavior and dendritic morphology in the PrL

Spearman correlation analysis on relapse score data and the total number of PrL dendrites revealed a positive correlation between the two parameters (r = 0.653, p=0.040), thus supporting the existence of a strong link between high dendrite number in the PrL and the increase of susceptibility to relapse **(Figure 6).**

3.6. CORT and BDNF measurements across the groups

3.6.1. CORT levels using ELISA assay

Higher levels of CORT were seen in HA mice, regardless of their EtOH treatment condition **(Figure 7A)**. A two-way ANOVA analysis revealed a significant effect of post-footshock avoidance ($F_{(2, 28)} = 19.80$, $p<0.001$) with no effect seen for treatment ($F_{(2, 28)} = 0.035$, p=0.853). The interaction between these two factors was not significant ($F_{(4, 28)} = 0.518$, p=0.601). The Holm-Sidak multiple comparisons showed significant elevation in the circulating CORT levels of the EtOH-/HA group, compared with the EtOH-/Ctrl and EtOH-/LA groups (t=3.536; p=0.014 and t=3.069; p=0.038, respectively). The levels

expressed by the EtOH+/HA group were also significantly elevated compared with the EtOH+/Ctrl and EtOH+/LA groups (t=4.175; p=0.003 and t=4.635; p=0.001; respectively).

3.6.2. BDNF levels using ELISA assay

Significantly lower levels of BDNF were found in HA, but not LA, mice in both the EtOH- and EtOH+ groups (**Figure 7B).** A two-way ANOVA performed on BDNF data (with treatment and post-footshock avoidance) revealed a main effect of post-footshock avoidance ($F_{(2, 27)}$ =16.350, p<0.001). There was no significant effect of treatment ($F_{(2, 27)}$ =0.380, p=0.543). Moreover, the interaction between treatment and post-footshock avoidance was not significant ($F_{(4, 27)}$ =0.625, p=0.543). The Holm-Sidak *post-hoc* test showed that each of the HA groups had significantly lower levels of BDNF as compared to the Ctrl groups within each treatment (EtOH-/HA vs. EtOH-/Ctrl: t=4.666, p<0.001 and EtOH+/HA *vs.*EtOH+/Ctrl: t=3.401, p=0.007). The two trauma-susceptible groups (EtOH-/HA and EtOH+/HA) did not differ from each other, showing that ethanol exposure did not modulate BDNF concentrations in plasma.

4. DISCUSSION

In the present work, we analyzed the effects of repeated ethanol injections on trauma-related behavioral and biological changes (using a high intensity, 1.5-mA, footshock for trauma). Regarding the trauma-related behavioral changes, we assessed passive avoidance towards the trauma chamber, sensitization of post-trauma contextual fear (using a low intensity, 0.4-mA, footshock in a different context), anxiety-like behavior (using the elevated plus maze test), extinction of passive avoidance towards the trauma chamber and the susceptibility to relapse (using a very low intensity, 0.15-mA, footshock in the trauma chamber). Regarding the trauma-related biological changes, on the one hand, we examined plasma concentrations of CORT and BDNF. On the other hand, using the Golgi-Cox method, we investigated changes in total dendritic number and length in the mPFC. We found that repeated exposure of trauma-susceptible mice to ethanol altered their extinction behavior, by delaying extinction acquisition and

increasing the susceptibility to relapse. These behavioral changes were associated with higher CPP acquisition. We also found higher increases in total dendritic number and length in the PrL area. Interestingly, the relapse score correlated with changes in dendritic density in the PrL area. Finally, there were no effects of ethanol on trauma-induced changes in plasma concentrations of CORT and BDNF.

4.1. Acquisition of ethanol-induced CPP in trauma-susceptible and stress-resilient mice

Our data showed that all trauma-susceptible and trauma-resilient mice subjected to the ethanol CPP procedure developed a preference for the ethanol-paired side. However, trauma-susceptible mice exhibited a significantly higher preference when compared with the non-stressed controls. This mouse finding is in accordance with other recent rodent data, showing that pronounced effects of ethanol characterize animals expressing maladaptive behavioral responses to stress. These effects were observed either with the CPP paradigm (**Bahi and Dreyer, 2014; Yu *et al.*, 2016; Macedo *et al.*, 2018; Bahi and Dreyer, 2020)**, with the two-bottle free choice paradigm **(Meyer *et al.,* 2013; Manjoch *et al.,* 2016; Cannady *et al.,* 2021),** or with the self-administration paradigm **(Edwards *et al.,* 2013; Barchiesi *et al.,* 2021; Makhijani *et al.,* 2021).** Nevertheless, studies with other drugs (such as cocaine and methamphetamine) have shown that stress does not necessarily lead to potentiate the effect of the drug or increase its consumption (e.g., cocaine: **Enman *et al.,* 2015; Hadad *et al.,* 2016; Lguensat *et al.,* 2021;** methamphetamine: **Eagle and Perrine, 2013**).

When considering the effect of stress on ethanol consumption, it seems that this effect depends on the type of stress applied **(Spanagel *et al.,* 2014; Weera and Gilpin, 2019)**. For instance, footshock stress and forced swim stress, but not restraint stress, have been found to enhance ethanol intake **(Noori *et al.,* 2014).** Another factor playing a key role is the degree of sensitivity to stress. Studies investigating the interaction between stress susceptibility and ethanol intake have generally reported increased consumption in rodents most affected by stress **(Edwards *et al.,* 2013; Meyer *et al.,* 2013; Manjoch *et al.,* 2016; Weera *et al.,* 2020; Barchiesi *et al.,* 2021).** In addition, the degree of ethanol preference

before stress may also play a key role **(Darnaudéry *et al.,* 2007),** as animals with a high preference for ethanol show increased consumption after stress **(Vengeliene *et al*, 2003; Chester *et al.,* 2004).** Finally, another factor to be considered is the method used to measure ethanol intake **(Noori *et al*, 2014)**. According to these authors, stress does not typically result in enhanced ethanol consumption in operant self-administration paradigms but leads to increased home cage drinking. This may, in part, explain our present finding obtained with mice subjected to the 1.5-mA footshock before the CPP procedure. However, ethanol was not self-administered, but injected by the experimenter. Moreover, the CPP procedure only enabled us to assess preference and motivation towards ethanol in trauma-susceptible and trauma-resilient mice. To reliably assess the effect of stress-susceptibility on ethanol consumption and intake, different ethanol administration methods need to be tested.

4.2. Changes in sensitivity to reward associated with trauma susceptibility

In humans, following a traumatic event, susceptible people who develop PTSD show highlighted vulnerability to develop drug abuse (**Chilcoat and Breslau, 1998**). Several animal studies managed to mimic this feature have showed that sensitivity to the rewarding properties of drugs change as a function of stress susceptibility (**Krishnan *et al.*, 2007; Breit and Chester, 2016; Brodnik *et al.,* 2017**). However, determining stress susceptibility status in laboratory animals requires using methods based on behavioral classifications. These classifications consider either two or more trauma-related symptoms **(Cohen *et al.,* 2004; Toledano and Gisquet-Verrier, 2014)** or one major symptom **(Dopfel *et al.,* 2019; Lguensat *et al.,* 2019).** We based our classification on avoidance measures **(Lguensat *et al.,* 2019)** as the major symptom with our mouse model. Moreover, our trauma-susceptible (high avoiders) mice expressed higher place preference, which joins other reports**,** showing that the motivation to consume the drug **(e.g., Brodnik *et al.,* 2017)** and preference for the drug measured using CPP **(e.g., Krishnan *et al.*, 2007)** are increased in stress-susceptible animals. Changes in drug preference as a function of stress susceptibility could be driven by the sensitization of the dopamine system (**Krishnan *et al.*, 2007; Brodnik *et al.,* 2017)**, knowing its role in motivation. Furthermore, the widespread view is that CPP is

mainly mediated by the rewarding properties of the drug **(McKendrick and Graziane, 2020)** and thus by the drug's positive reinforcement properties. This view is supported both by human **(e.g., Childs and De Wit, 2016)** and rodent **(e.g., Tzschentke, 2007)** data. However, CPP can also result from the ability of the drug to remove or reduce an aversive state **(e.g., Koob and Le Moal, 2008)** and therefore it can result from negative reinforcement. In fact, it is largely admitted that ethanol exerts both positive and negative reinforcing effects **(Pautassi *et al.,* 2010).** It is therefore possible that our CPP findings could be largely due to ethanol's negative reinforcement effects given that susceptible animals showed greater preference for the ethanol-paired side compared to the non-stressed group.

4.3. Procedural and practical considerations for interpreting CPP data

When it comes to interpreting CPP data regarding CPP procedure design (biased/unbiased), an important distinction needs to be made. According to **Cunningham and colleagues, (2003)**, the biased/unbiased description is often used to refer to either the typical response of untrained animals to the apparatus or to describe the experimental procedure for assigning the drug-paired conditioned stimulus. In the present study, we used this description to refer to the fact that ethanol injections were associated with the least preferred side of the apparatus (biased design). Several reports have indicated that adopting this method allows a better development of the conditioned preference compared to associating the drug with the preferred side or cue **(Schenk *et al.*, 1985; Nomikos and Spyraki 1988; Cunningham *et al.,* 2003)**. Here, using the biased design, the conditioned preference was obtained, but we did not test the unbiased design for a better comparison. However, other studies **(e.g., Cunningham *et al.,* 2006; Cunningham and Shields, 2018)** have demonstrated that ethanol can also induce reliable CPP with the unbiased design. Nevertheless, we also considered the fact that associating ethanol with a chamber where the animal spent, for example, 20% of its time during the preconditioning session may give more room for detecting differences than starting with an equal preference of the two chambers in an unbiased design **(Roma and Riley, 2005)**.

4.4. Effects of post-trauma repeated ethanol exposure on trauma-associated behaviors and passive avoidance extinction

4.4.1. Fear sensitization and anxiety-like behavior

Fear sensitization is a form of non-associative learning and a hallmark of PTSD **(Siegmund & Wotjak, 2007)**. It can be assessed in rats or mice, either by measuring acoustic startle response **(Cohen *et al.,* 2004)**, by exposing them to a neutral tone in a neutral chamber **(Siegmund & Wotjak, 2007),** or by conditioning animals using a low intensity footshock, and re-exposing them, at least 24 hours later, to the conditioning context **(Bentefour *et al.,* 2015)**. Under these different conditions, during tests, it has been often reported that the stressed group displays more fear (fear sensitization) compared to the non-stressed group (**Lewis and Gould, 2003; Owen *et al*., 2003; Bentefour *et al*., 2015, 2016, 2018**).

Our data show that ethanol (1.6g/kg) had no effect on the fear sensitization phenomenon, joining the findings of **Lewis and Gould, (2003)** and **Owens *et al.,* (2003).** These studies reported that a proximate dose of ethanol (1.5g/kg) had no effect on fear sensitization, while the effect was only detected at either lower (0.5g/kg: **Lewis and Gould, 2003**) or higher (2.25g/kg: **Owens *et al.,* 2003**) doses. In these studies, as in the present one, ethanol was administered via IP injections. However, these studies evaluated the effects of ethanol acutely following one injection. Together with our present study, one can suggest that the effect of ethanol on fear sensitization is not related to the number of injections, but rather to the dose. Using a different approach, **Albrechet-Souza and colleagues (2020)** have reported that a history of chronic ethanol consumption can provoke an accentuation of fear sensitization (measured by the means of acoustic startle response) in a sex-specific manner (stress-susceptible males only). Altogether, one can hypothesize that ethanol may enhance fear sensitization in stress sensitive individuals as a function of some factors, such as the method and the chronicity of its administration.

Like fear sensitization, anxiety is often reported in PTSD patients **(Olatunji *et al.,* 2018).** Interestingly, it is also often considered in studies with rodent models of PTSD, with increased anxiety-like behavior characterizing trauma-susceptible animals **(Lguensat *et al.*, 2019)**.

Our finding on anxiety-like behavior did not reveal any effect of ethanol (1.6g/kg). These data are in accordance with those of **Varlinskaya and Spear, (2012)**, in which ethanol injections (1g/kg) did not have any effect in adult stressed animals. Moreover, it seems that the anxiolytic effects of ethanol are only noticeable in less anxious animals **(Langen *et al.,* 2002)**; ethanol-preferring animals **(Stewart *et al.,* 1993)** and defeated animals with a history of ethanol intake **(Kudryavtseva *et al.,* 2006)**. We therefore expected to detect a potential effect of anxiety on one of two trauma-exposed groups (trauma-susceptible or trauma-resilient mice). In our study, even though anxiety levels displayed by the trauma-susceptible group was slightly attenuated (anxiety index close to the one expressed by the control group), the lack of any significant difference with the trauma-resilient group discarded our initial hypothesis.

4.4.2. Passive avoidance extinction

Impairment of extinction of conditioned fear is considered as a hallmark of PTSD **(Milad *et al.,* 2006, 2008)**. This is, in part, supported by studies showing, in PTSD patients, either impaired extinction learning **(e.g., Blechert *et al.*, 2007)** or normal extinction learning, but impaired extinction memory **(e.g., Milad *et al.*, 2008)**. Studies using a single prolonged stress paradigm, as an animal model of PTSD, have also demonstrated impaired extinction learning **(Eskandarian *et al.,* 2013; Matsumoto *et al.,* 2013).** However, in those human and rodent studies, the authors tested the impairment in the ability to extinguish conditioned fear that was acquired after trauma (intense footshock in rodents). Here, as in our previous studies **(Bentefour *et al.,* 2018; Lguensat *et al.,* 2019)**, we focused on extinction of conditioned fear of trauma reminders. Indeed, exposure therapy, one of the gold-standard treatments for PTSD **(Graham & Milad, 2011)**, is based on extinction of conditioned fear of trauma reminders. We found that ethanol injections impaired passive avoidance extinction learning and facilitated the return of the extinguished fear. These data agree with our cocaine study in rats **(Lguensat *et al.,* 2021)**, showing impaired fear extinction and increased susceptibility to relapse. In mice, a single injection of 1.5 g/kg of ethanol 5–10 min prior to extinction session has also been found to delay extinction learning **(Lattal, 2007)**. In humans, alcohol has been shown to alter extinction behavior **(Bisby *et al.,* 2015).**

We have previously shown, in mice, that passive avoidance extinction training suppresses not only avoidance towards trauma-related context, but also the other PTSD-like symptoms (including non-conditioned symptoms, such as fear sensitization and anxiety-like behavior; **Bentefour *et al.*, 2015, 2016)**. Similarly, provocation of passive avoidance relapse, with a priming footshock applied in the trauma-related context, has been found to induce the return of all PTSD-like symptoms **(Bentefour *et al.*, 2015, 2016)**. One can hypothesize that impairment of avoidance extinction may support resistance to exposure therapy reported in PTSD patients **(Schottenbauer *et al.*, 2008)**, indicating PTSD chronicization. Indeed, patients who respond to exposure therapy for PTSD display suppression of all symptoms (including non-conditioned symptoms; **Yehuda *et al.*, 2009**). Therefore, given the strong association between factors such as substance abuse and the course of chronic PTSD **(Zlotnick *et al.*, 1999)**, our current finding suggests that post-stress ethanol exposure can provoke chronicization of PTSD-like symptoms. Nevertheless, one of the limitations of our study is the use of a cut-off for passive avoidance at 480s. Since values above 480s were not measured, one cannot know whether exposure to ethanol potentiated avoidance (values above 480s). In this case, the normal rate of extinction would appear as extinction learning impairment, with avoidance values of the ethanol group remaining high, when extinction would be almost achieved with the group not exposed to ethanol. However, this potential effect of ethanol does not support the fact that relapse was facilitated.

4.5. Effects of post-stress repeated ethanol exposure on stress-associated dendritic changes in the mPFC

Several neuroimaging studies have investigated brain structural and functional alteration in PTSD. These studies have shown reduced volumes and changes in activities in various regions, including the mPFC **(e.g., Bremner, 1999; De Bellis *et al.*, 2000; Rauch *et al.*, 2003; Shin *et al.*, 2004)**. In our previous rodent study, we have also found that the PTSD-like phenotype is associated with changes in the mPFC, corresponding to decreases in total number and length of apical and basilar dendrites in the IL area **(Lguensat *et al.*, 2019)**. Overall, in the PrL area, we have observed increases in the total number

and length of apical dendrites. Here, these increases were not observed clearly in trauma-susceptible mice, but they significantly emerged in trauma-susceptible mice exposed to ethanol. It seems that ethanol by itself did not induce significant dendritic changes in the IL area. However, in the IL area, we noted that changes in the dendritic parameters of trauma-susceptible mice treated with ethanol (EtOH+/HA) no longer differ from those of trauma-resilient mice that also received ethanol (EtOH+/LA). This suggests that ethanol could have influenced both the PrL and IL areas (**Makhijani *et al.,* 2021; Cannady *et al.,* 2021)**. As mentioned above, ethanol exposure impaired fear extinction. Interestingly, in a study in which mice were exposed to chronic intermittent ethanol vapor, followed by auditory fear conditioning, an impaired fear extinction associated with an elevated length of the apical dendrites was found in the PrL area, with no change in the IL area **(Holmes *et al*., 2012)**. Moreover, the dendritic expansion in the PrL area was more accentuated far from the soma of neurons. A fine structured analysis of PrL neurons considering the distance from soma would have given us more detailed information about the distribution and the extent of dendritic expansion. Similarly, in other rodent studies, repeated **(Quiñones-Laracuente *et al.,* 2015)** and chronic **(Cannady *et al.,* 2021)** exposure to ethanol was found to enhance activity in the PrL area, which was associated with impaired extinction memory **(Quiñones-Laracuente *et al.,* 2015)**.

Several studies have shown that the PrL and IL areas have opposite influences on conditioned fear expression **(Gilmartin & McEchron, 2005; Vidal-Gonzalez *et al.,* 2006).** Contrary to the IL area, the PrL area is, indeed, implicated in the production and retrieval of conditioned fear **(Milad & Quirk, 2002; Sierra-Mercado *et al.,* 2011)**. The persistence of PrL activity after extinction training has also been associated with failure to express extinction memory **(Burgos-robles *et al.,* 2009).** Consistent with our present finding, it has also been reported that persistent increased PrL activity is associated with persistent active avoidance **(Bravo-Rivera *et al.,* 2015)**. This PrL morphological change may interact with avoidance extinction probably through direct projection of the PrL to the ventral striatum and/or to the basal amygdala **(Bravo-Rivera *et al.,* 2015).**

4.6. Effects of post-stress repeated ethanol exposure on plasma CORT and

BDNF concentrations in trauma-susceptible mice

4.6.1. CORT

Glucocorticoids are the end-product of the activation of the hypothalamo-pituitary-adrenal axis. Various dysfunctions of this axis have been repeatedly reported in patients with PTSD, including low levels of cortisol in urine and plasma, enhanced suppression of cortisol in response to administration of low dose of dexamethasone (a synthetic glucocorticoid), and glucocorticoid receptor abnormalities **(Yehuda *et al.,* 2001)**. Low levels of plasma CORT have also been reported, in rats, with the single prolonged stress model of PTSD **(Wang *et al.,* 2018).** However, there are also studies indicating, on the contrary, increased plasma CORT levels in rats subjected to the single prolonged stress model **(Qiu *et al.,* 2018)** and after exposure to synthetic predator odor **(Makhijani *et al.,* 2021)**. In humans, bedtime salivary cortisol can be higher in some patients, while the same patients were found to display low levels of urinary cortisol **(Yehuda *et al.,* 2014).** Further studies are therefore necessary to better understand changes in the levels of cortisol in PTSD.

Here, we found that trauma exposure induced high levels of plasma CORT in all trauma-susceptible mice, regardless of ethanol exposure. Surprisingly, although administration of ethanol, like many other drugs of abuse, is known to activate the hypothalamo-pituitary-adrenal axis **(Thiagarajan *et al.*, 1989)**, we here found that the levels of CORT were not affected by ethanol exposure. Of note, we obtained this finding with male mice, which seems to agree with studies showing that females, but not males, respond to drug with higher CORT secretion **(Peričić and Pivac, 1995; Willey *et al.*, 2012; Albrechet-Souza *et al.,* 2020)**.

Since we observed that ethanol exposure was associated with extinction impairment, it is possible that this impairment was not related to the increases in CORT levels in trauma-susceptible mice. On the contrary, increased levels of glucocorticoids have been reported to enhance fear extinction consolidation both in humans **(Brueckner *et al.*, 2019)** and in rodents **(Cai *et al.*, 2006).**

4.6.2. BDNF

BDNF is involved in synaptic plasticity, neuronal differentiation, and survival of neurons **(Klein *et al.,* 2011).** Although plasma BDNF levels have been shown to correlate with cortical integrity **(Lang *et al.,* 2007)**, we acknowledge that changes in plasma BDNF levels remains a kind of "surrogate marker", which does not necessarily reflect intracerebral dynamics in BDNF secretion. In a study investigating the clinical correlates of plasma BDNF concentrations in a clinical population showing PTSD symptomatology, the authors found that the subjects with full PTSD showed lower levels of BDNF than subjects with partial PTSD and controls **(Stratta *et al.,* 2016).** Using the single prolonged stress paradigm in rats, it has also been reported that this procedure decreases the expression of BDNF mRNA in the hippocampus **(Lee *et al.,* 2016)**. In the present study, we found that trauma-susceptible mice that were not exposed to ethanol and those exposed to this treatment expressed identical decreases in the levels of plasma BDNF as compared to controls, indicating no effect of ethanol on BDNF levels. This result can be, in part, explained by the low dose of ethanol that we used. Indeed, effect of ethanol on BDNF protein expression has been reported with much higher doses, yielding blood ethanol levels near 50–70 mg per 100 ml **(Stragier *et al.,* 2015)**.

Although BDNF blockade interferes with extinction memory **(Radiske *et al.,* 2015),** it is possible that the observed impairment of fear extinction in the present study was not associated with any effect of ethanol exposure on BDNF.

4.7. Conclusion

Results of the present study provide evidence for altered passive avoidance extinction in trauma-susceptible mice exposed to repeated ethanol injections. This suggests that exposure to ethanol induced persistent PTSD-like symptoms in the most vulnerable mice. Interestingly, the treatment of PTSD has been shown to be less efficient in people with a PTSD diagnosis who also drink alcohol **(McCauley *et al.,* 2012).** Moreover, trauma-susceptible mice showed stronger ethanol-induced CPP indicating that

these mice were more sensitive to ethanol effects, which potentially resulted from their high avoidance status.

Our data also show, with trauma-susceptible mice exposed to ethanol, a positive correlation between passive avoidance relapse and dendritic changes in the PrL. However, it is worth mentioning that our study was only done on male mice, which constitutes one of its major limitations. For our data to gain more translational value, the same experimental procedures need to be applied on female mice as well. Another limitation of the present study is that the designs for Pavlovian conditioning procedures did not include control groups receiving equal exposures to the unconditioned (and conditioned) stimuli in an unpaired fashion.

Together, these findings suggest that PTSD patients who self-medicate their symptoms for relief seeking **(Baker *et al.,* 2004; Read *et al.,* 2014)**, expose themselves to difficulty to recover from the disorder **(Zlotnick *et al.,* 1999; Kaysen *et al.,* 2011)** via drug-induced neuroadaptive mechanisms **(Holmes *et al.*, 2012; Quiñones-Laracuente *et al.,* 2015)**, which may include changes in the PrL dendritic plasticity.

CONFLICT OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

A.L, R.G., M.B and S.B. conceived and planned the experiment. A.L. and Z.B. collected the data. A.L., Z.B. and S.B. performed statistical analyses. A.L., S.B. and R.G. wrote the manuscript. Z.B, E.G., C.B. revised the manuscript. S.B. and R.G. supervised the research.

DATA AVAILABILITY STATEMENT

To access the data, please visit the following Figshare link:

https://figshare.com/articles/dataset/Donn_es_brutes_EJN_18092021_xlsx/16692031

List of abbreviations:

AIC: Akaike Information Criterion

BDNF: Brain Derived Neurotrophic Factor

CORT Corticosterone

CPP: Conditioned Place Preference

Ctrl: Control

EtOH: Ethanol

HA: High avoider

IL: infralimbic cortex

LA: Low avoider

m-A: Milliamp

mPFC: Medial Prefrontal Cortex

PrL : Prelimbic cortex

PTSD: Post-Traumatic Stress Disorder

w: Akaike weight

ZT: Zitgeber

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FIGURE LEGENDS

Figure 1. Timeline of the behavioral experiment.

Figure 2. Fear response to the 1.5-mA footshock and Preference score. (A) Step-through latency as expressed by non-shocked (Control group) and shocked mice during passive avoidance test performed 15 days after training (footshock administration) (Mann-Whitney's test: * $p<0.05$ as significant differences compared to the Control group). (B) Frequency distribution of step-through latency time in the Control and Shocked groups including Low avoiders (LA) and High avoiders (HA). (C) Preference score expressed by mice that were not exposed (EtOH-) and that were exposed (EtOH+) to ethanol (Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons: $^{*}p<0.05$ and $^{***}p<0.001$ indicate significant differences). EtOH-/Ctrl: n=13, EtOH-/LA: n=13, EtOH-/HA: n=13, EtOH+/Ctrl: n=13, EtOH+/LA: n=13, EtOH+/HA: n=10.

Figure 3. Stress-related behavioral testing and passive avoidance extinction testing. (A) Freezing behavior (as fear sensitization indicator) relative to a weak contextual fear conditioning performed two weeks after passive avoidance acquisition (Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons: $^{*}p<0.05$ as significant differences compared to the EtOH-/Ctrl group). (B) Anxiety index recorded during the elevated plus maze test (two-way ANOVA followed by Holm-Sidak *post-hoc* test: ** $p<0.01$ indicate significant differences). (C) Step-through latency recorded during Day 15 (passive avoidance test), Days 18-22 (extinction sessions) and Days 23-24 (pre and post footshock priming) (two-way ANOVA for each day followed by Holm-Sidak *post-hoc* test: $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ significant differences compared to the EtOH-/Ctrl group; $^{d}p<0.05$, $^{e}p<0.01$ and $^{f}p<0.001$ represent significant differences compared to the EtOH+/Ctrl group; $^{g}p<0.05$, $^{h}p<0.01$ and $^{i}p<0.001$ represent significant differences compared to the EtOH-/LA group; $^{j}p<0.05$, $^{k}p<0.01$ and $^{l}p<0.001$ represent significant differences compared to the EtOH-/HA group ; $^{m}p<0.05$, $^{n}p<0.01$ and $^{o}p<0.001$ represent significant differences compared to the EtOH+/LA group). Data are presented as mean ± SEM. EtOH-/Ctrl: n=13, EtOH-/LA: n=13, EtOH-/HA: n=13, EtOH+/Ctrl: n=13, EtOH+/LA: n=13, EtOH+/HA: n=10.

Figure 4. Photomicrographs showing representative neurons in the PrL and IL areas in a stress-susceptible mouse and a stress-resilient mouse exposed to ethanol (EtOH+/HA and EtOH+/LA; respectively). Scale bar= 20µm.

Figure 5. Dendritic morphological changes in the mPFC. (A) Total number of apical dendrites in the PrL and IL areas. (B) Total number of basilar dendrite changes in the PrL and IL areas. (C) Total apical dendrites branch length changes in the PrL and IL areas. (D) Total basilar branch length changes in the PrL and IL areas. Data in panels A-D are shown as mean (±SEM). Two-way ANOVA within each region, followed by Holm-Sidak *post-hoc*: *p<0.05, **p<0.01 and ***p<0.01 indicate significant differences. EtOH-/Ctrl: n=5, EtOH-/LA: n=5, EtOH-/HA: n=6, EtOH+/Ctrl: n=5, EtOH+/LA: n=5, EtOH+/HA: n=5.

Figure 6: The relationship between passive avoidance return (relapse) and the total number of dendrites in the PrL. Spearman correlation between relapse score and the total number of dendrites in the PrL. Dark magenta dots : EtOH+/HA mice (n=5). Grey dots : EtOH+/LA mice (n=5).

Figure 7. Changes in basal plasma CORT (A) and BDNF (B) levels across the groups. Two-way ANOVA followed by Holm-Sidak *post-hoc* test: *p<0.05, **p<0.01 and ***p<0.001 indicate significant differences. CORT: EtOH-/Ctrl: n=5, EtOH-/LA: n=5, EtOH-/HA: n=6, EtOH+/Ctrl: n=5, EtOH+/LA: n=7, EtOH+/HA: n=5. BDNF: EtOH-/Ctrl: n=5, EtOH-/LA: n=5, EtOH-/HA: n=5, EtOH+/Ctrl: n=5, EtOH+/LA: n=5, EtOH+/HA: n=5.

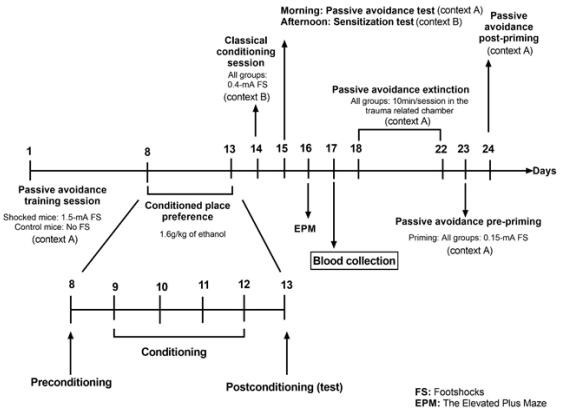

Figure 1. Timeline of the behavioral experiment.

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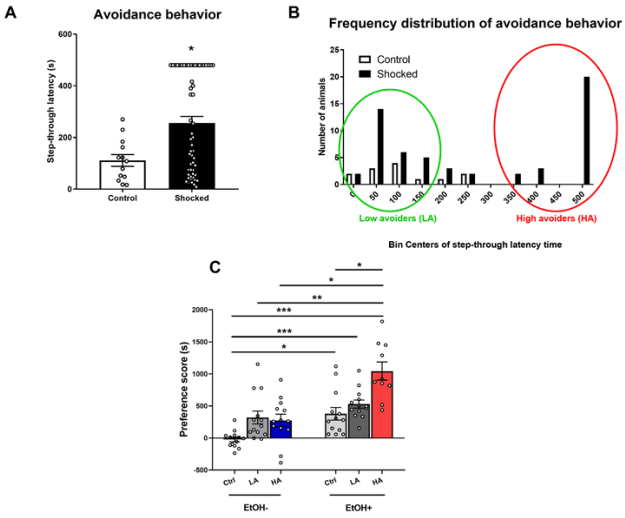

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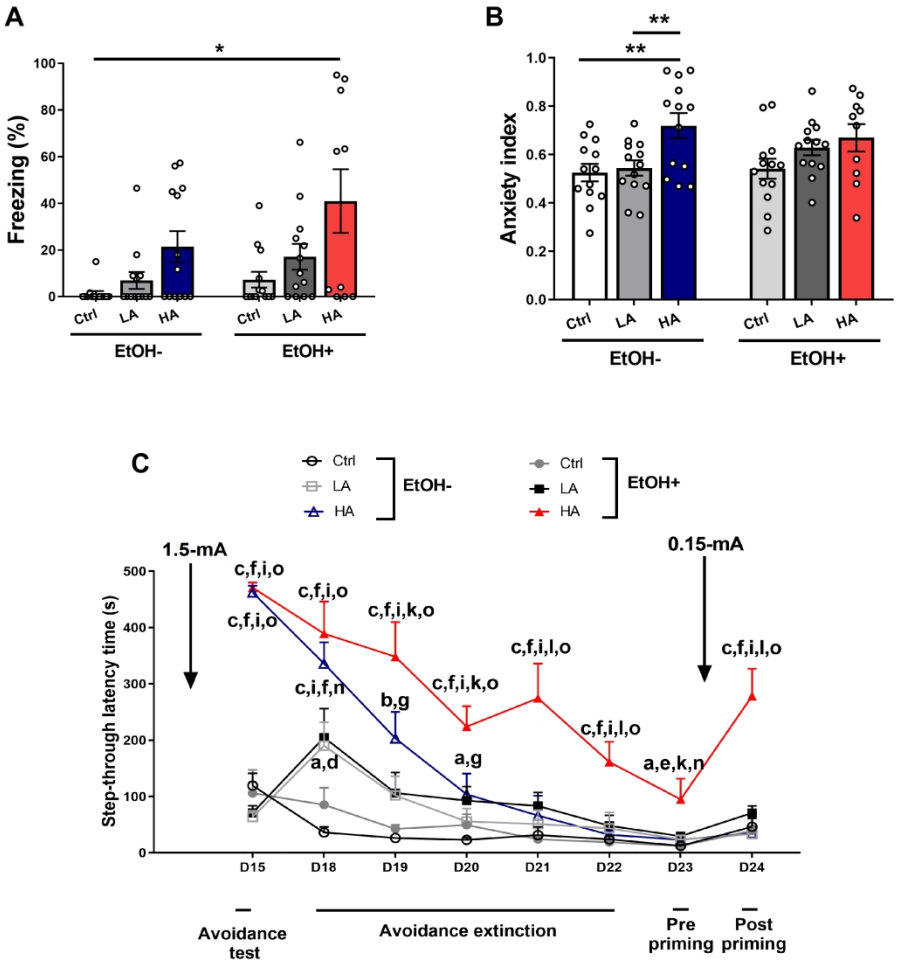

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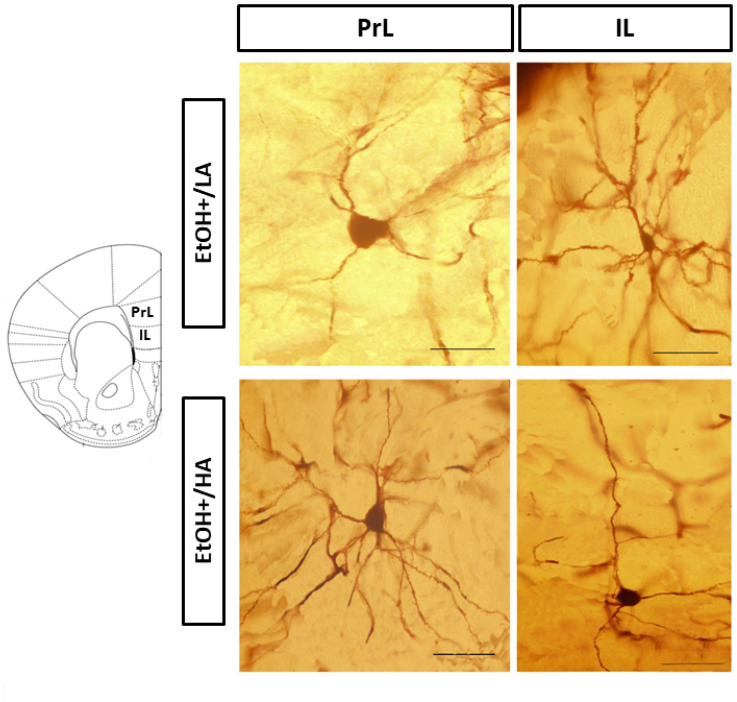

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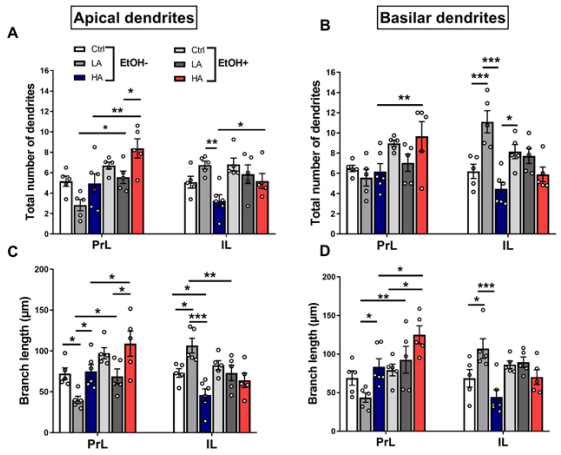

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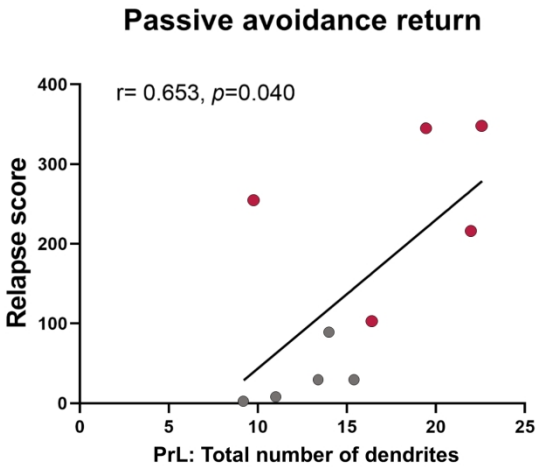

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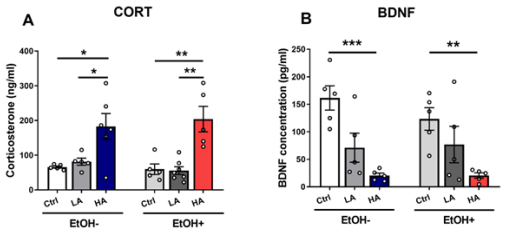

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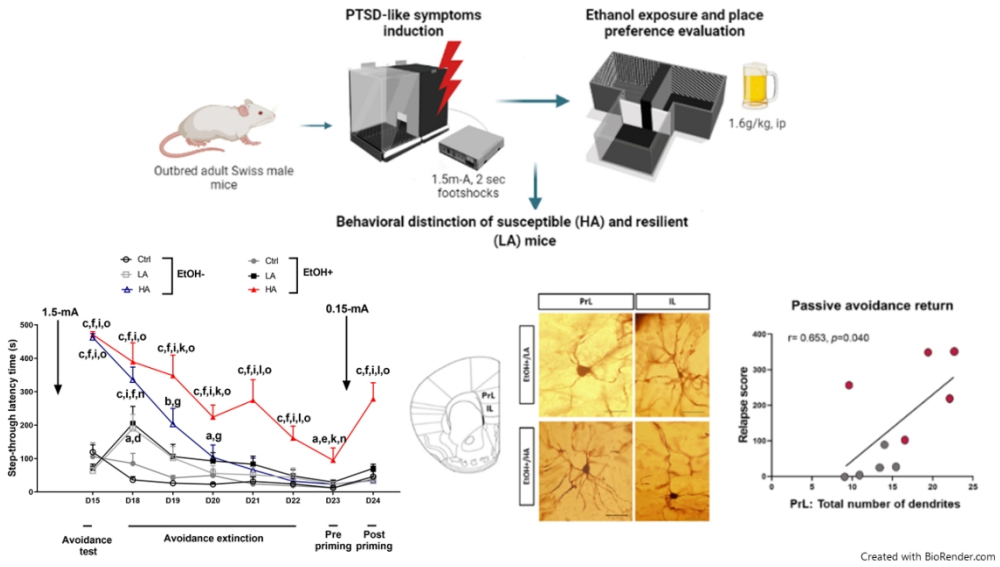
Table 1: Groups' distribution

Initial groups	**EtOH treatment**	**Trauma-related condition**	**Final groups' nomination**
Control (n=26)	EtOH- (n=13)	-	EtOH-/Ctrl
	EtOH+ (n=13)	-	EtOH+/Ctrl
Shocked (n=54) (Including 5 intermediate mice)	EtOH- (n=26)	EtOH-/LA (n=13)	EtOH-/LA
		EtOH-/HA (n=13)	EtOH-/HA
	EtOH+ (n=23)	EtOH+/LA (n=13)	EtOH+/LA
		EtOH+/HA (n=10)	EtOH+/HA

Table 2: Different comparisons of step-through latency time recorded for the EtOH+/HA group during Day 15 (passive avoidance test) and Days 18-22 (passive extinction sessions) and Days 23-24 (pre and post footshock priming). [a]$p<0.05$, [b]$p<0.01$ and [c]$p<0.001$ represent significant differences compared to the EtOH-/Ctrl group; [d]$p<0.05$, [e]$p<0.01$ and [f]$p<0.001$ significant differences compared to the EtOH+/Ctrl group; [g]$p<0.05$, [h]$p<0.01$ and [i]$p<0.001$ significant differences compared to the EtOH-/LA group; [j]$p<0.05$, [k]$p<0.01$ and [l]$p<0.001$ significant differences compared to the EtOH-/HA group ; [m]$p<0.05$, [n]$p<0.01$ and [o]$p<0.001$ significant differences compared to the EtOH+/LA group.

	Session	**EtOH-/Ctrl (mean±SEM)**	**EtOH-/LA (mean±SEM)**	**EtOH-/HA (mean±SEM)**	**EtOH+/Ctrl (mean±SEM)**	**EtOH+/LA (mean±SEM)**	**EtOH+/HA (mean±SEM)**	*Post-hoc* **EtOH+/HA** vs. **EtOH-/Ctrl**	*Post-hoc* **EtOH+/HA** vs. **EtOH-/LA**	*Post-hoc* **EtOH+/HA vs. EtOH-/HA**	*Post-hoc* **EtOH+/HA** vs. **EtOH+/Ctrl**	*Post-hoc* **EtOH+/HA** vs. **EtOH+/LA**
Avoidance test	**Day 15**	119.23 ±21.75	63.36±11.28	462.46±11.87	105.81±41.51	70.92±12.49	471.00±9.00	**c**	**i**	ns	**f**	**o**
Extinction	**Day 18**	35.94±9.93	190.23±41.89	335.87±37.90	85.15±30.15	204.87±51.09	389.30±56.89	**c**	**i**	ns	**f**	**o**
	Day 19	26.21±4.48	101.61±34.15	203.04±47.01	42.23±7.23	106.11±36.51	348.00±61.57	**c**	**i**	**k**	**f**	**o**
	Day 20	22.80±3.82	55.38±23.11	103.91±36.47	49.22±19.23	92.70±24.90	224.11±35.98	**c**	**i**	**k**	**f**	**o**
	Day 21	31.2±14.94	51.00±24.48	66.04±35.31	23.98±5.98	83.09±24.18	274.11±61.44	**c**	**i**	**l**	**f**	**o**
	Day 22	23.88±5.54	43.30±28.52	31.83±7.60	18.80±3.57	48.03±18.64	161.26±35.88	**c**	**i**	**l**	**f**	**o**
Pre-priming	**Day 23**	12.16±2.41	23.52±7.11	23.02±7.20	12.05±2.57	29.24±5.86	94.67±37.10	**a**	ns	**k**	**e**	**n**
Post-priming	**Day 24**	46.15±15.04	33.22±9.62	35.10±10.19	39.20±8.80	70.81±12.20	278.28±48.61	**c**	**i**	**l**	**f**	**o**

Ethanol exposure (ip injections at the dose of 1.6g/kg, during a conditioned place preference training that was performed 8 days following trauma with a high intensity, 1.5 mA, footshock), altered avoidance extinction and increased susceptibility to avoidance relapse. It also provoked an increase in total dendritic number and length in the prelimbic area of the medial prefrontal cortex. A positive correlation was found between susceptibility to avoidance relapse and changes in both dentritic parameters in the prelimbic area.



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Repeated ethanol exposure following avoidance conditioning impairs avoidance extinction and modifies conditioning-associated prefrontal dendritic changes in a mouse model of post-traumatic stress disorder

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Keywords: PTSD-like phenotype. Mice. Ethanol exposure. Avoidance extinction impairment

Running title: Ethanol exposure and avoidance extinction impairment

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Abstract

Treatment of post-traumatic stress disorder is complicated by the presence of alcohol use disorder comorbidity. Little is known about the underlying brain mechanisms. We have recently shown, in mice, that the post-traumatic stress disorder-like phenotype is characterized by the increase and decrease in total dendritic number and length in the prelimbic and infralimbic areas of the medial prefrontal cortex, respectively. Here, we examined whether repeated ethanol exposure would exacerbate these changes, and whether this would be associated with difficulty to extinguish passive avoidance behavior, as an indicator of treatment resistance. We also analyzed whether other known trauma-associated changes, like increased or decreased corticosterone and decreased brain-derived neurotrophic factor levels, would also be exacerbated. Male mice underwent trauma exposure (1.5-mA footshock), followed, 8 days later, by a conditioned place preference training with ethanol. Tests for fear sensitization, passive avoidance, anxiety-like behavior, extinction acquisition and relapse susceptibility were used to assess behavior changes. Plasma corticosterone and brain-derived neurotrophic factor levels and prefrontal dendritic changes were subsequently measured. Trauma-susceptible mice exposed to ethanol acquired a strong place preference and behaved differently from those not exposed to ethanol, with delayed avoidance extinction and higher avoidance relapse vulnerability. Ethanol potentiated trauma-associated dendritic changes in the prelimbic area and suppressed trauma-associated dendritic changes in the infralimbic area. However, ethanol had no effect on trauma-induced increased corticosterone and decreased brain-derived neurotrophic factor levels. These data suggest that the modification of prefrontal trauma-related changes, due to alcohol use, can characterize, and probably support, treatment-resistant post-traumatic stress disorder.

1. INTRODUCTION

Post-traumatic stress disorder (PTSD) is a mental illness that may arise after an individual experiences or witnesses a life-threatening event. Although most people with PTSD recover by one year **(Freedman *et al.,* 1999)**, the remaining patients form a chronic subgroup that never fully remits even after many years **(Zlotnick *et al.,* 1999; Galatzer-Levy *et al.,* 2013)**. One of the conditions contributing to the chronic aspect of the disorder is the comorbidity with alcohol use disorder **(McFarlane, 1988; Breslau and Davis, 1991; Bremner *et al.*, 1996)**. According to one of the theoretical models addressing this comorbidity, PTSD patients often turn to alcohol to alleviate their symptoms, but this ultimately results in the maintenance of their symptoms **(Zlotnick *et al.*, 1999; Kaysen *et al.*, 2011; Hawn *et al.,* 2020)**. Although the understanding of the risk factor of alcohol for PTSD maintenance has enabled clinicians to identify patients who need continued care, little is known about the brain changes associated with alcohol-induced PTSD maintenance. This can be, in part, addressed with studies based on fear extinction.

During the last decades, our understanding of the development and maintenance of PTSD has improved by the means of advances in the analysis of learning mechanisms related to fear conditioning and extinction **(Maeng & Milad, 2017)**. Consistent with conditioning theories, PTSD patients often express fear responses, such as avoidance, when exposed to trauma-related cues. In addition, maintenance of PTSD symptoms is hypothesized to involve disruption of fear extinction processes **(Wessa & Flor, 2007)**. It is therefore possible that alcohol use may contribute to treatment-resistant PTSD through the disruption of fear extinction processes.

Basic rodent studies have shown that increased activity in the prelimbic (PrL) area of the medial prefrontal cortex (mPFC) is implicated in extinction disruption **(Burgos-robles *et al.*, 2009; Herry *et al.*, 2010; Bravo-Rivera *et al.*, 2015)**. However, studies exploring the potential link between changes in the PrL area and ethanol-induced extinction disruption are still missing.

Regarding ethanol administration, several methods have been successfully used in rodents. For instance, animals can be allowed to consume ethanol using the choice between ethanol and water with

the two-bottle choice paradigm (e.g., **Huynh *et al.,* 2019)**. They can also be allowed to self-administer ethanol while having access to a natural reward like a sweet solution (e.g., **Augier *et al.,* 2018).** The other method is to submit animals to the conditioned place preference (CPP) task in which they learn to associate one compartment of a two- or three-compartment apparatus with ethanol injections (e.g., **Cunningham and Prather, 1992)**; this latter procedure also allows studying drug related preference and motivation behavior **(Tzschentke, 2007)**. Interestingly, our recent rat study with cocaine, based on a self-administration procedure **(Lguensat *et al.*, 2021)**, and our preliminary mouse study with ethanol, based on the CPP procedure, revealed that both procedures similarly disrupt extinction. Given another of our recent rodent studies showing a positive correlation between passive avoidance behavior and the increase in total dendritic number and length in the PrL area (**Lguensat *et al.*, 2019)**, we hypothesized that the disruption of passive avoidance extinction with the CPP procedure would be associated with the exacerbation of the PrL dendritic changes. To test this hypothesis, mice were exposed to a high intensity (1.5-mA) footshock to acquire passive avoidance behavior and, 8 days later, to ethanol injections for CPP acquisition. Following behavioral testing (including tests for fear sensitization, passive avoidance, anxiety-like behavior, extinction acquisition and relapse vulnerability), dendritic changes in the mPFC were measured using the Golgi-cox method. We also investigated changes in plasma concentrations of corticosterone (CORT) and brain-derived neurotrophic factor (BDNF), knowing their dysregulation under the conditions of PTSD with rodent models (increased CORT: e.g., **Sillivan *et al.*, 2017**; decreased BDNF: e.g., **Burstein *et al.*, 2018**).

2. METHODS AND MATERIALS

2.1. Animals

Eighty outbred adult male Swiss mice aged between 2 and 3 months at the start of the experiment and bred in the central animal facility of the Faculty of Science Semlalia, Marrakech, Morocco, were used in this study. The animals were group housed and maintained under constant conditions of temperature

(22 ±2°C) and under a 12h light/12h dark cycle (ZT0=7h), with food and water available *Ad libitum*. All animal procedures were in strict accordance with the guidelines of European Council Directive (EU2010/63). All efforts were made to minimize animal suffering.

2.2. Behavioral apparatus and protocols

The apparatus and behavioral procedures used, for passive avoidance training, classical conditioning, passive avoidance test, sensitization test, anxiety test using an elevated plus maze (EPM) as well as passive avoidance extinction and post-extinction priming exposure, have been described in detail in our previous studies **(Bentefour *et al.*, 2015, 2016)**.

The CPP apparatus consisted of three compartments of different appearances, arranged to have a neutral compartment (15.5 x 14 x 13 cm), which served as a start compartment with transparent walls without any pattern on them. The start compartment had access to two other compartments (13 x 17 x 13 cm each) through a sliding door. Both compartments had a visual (dotted *vs*. stripped walls) and tactile (smooth *vs*. rough floors) contextual differences. This unit was cleaned with 20% ethanol solution and the mouse was placed in the apparatus 5 minutes later to avoid any olfactory cue that could interfere with the CPP training.

The experimental timeline is shown in **Figure 1**. On Day 1, the PTSD-like symptoms were induced using a passive avoidance apparatus (context A), where mice were put in the light compartment of the apparatus and were left to freely enter the dark compartment, where, when locked in, they received two foot-shocks of 2 seconds at 1.5-mA each, separated by 6 seconds. The apparatus was cleaned using 70% ethanol after each animal. Following an incubation period of 7 days in the animal facility, mice were subjected to a biased procedure of ethanol induced CPP, which was adapted from **Li *et al.*, (2015)** and lasted for 6 days. This biased procedure consisted in associating reward to the least preferred compartment **(Prus *et al.*, 2009)**. On Day 8 of the protocol, the CPP procedure started (preconditioning phase) by placing mice in the start compartment and allowing the animals to freely explore the apparatus for 30 minutes. Mouse behavior was recorded, and the time spent in each compartment and the number

of entries were quantified. From Day 9 to Day 12 (conditioning phase), mice were subjected to the conditioning procedure, where the least preferred compartment (the one where the animal spent the lesser time during the preconditioning phase) was associated with ethanol injections, while the most preferred compartment was associated with saline injections. Ethanol (1.6g/kg) was dissolved in saline solution (0.9%) and injected intraperitoneally at a volume of 10ml/kg while saline solution was injected with an equal volume. On Days 9 and 11, mice trained for CPP (the EtOH+ groups) received ethanol in the morning and saline solution in the afternoon. This order was alternated on Days 10 and 12 with mice in the EtOH+ groups receiving four ethanol injections in total. The other mice (the EtOH- groups) were subjected to the same regimen, except that ethanol was replaced by saline. After receiving each injection, mice were locked in the appropriate compartment for 25 minutes. The morning and afternoon sessions were separated by at least 5 h. On Day 13 (post-conditioning: testing phase), mice were exposed to the CPP apparatus as on Day 8.

On the following day (Day 14 of the experiment), all mice underwent classical fear conditioning in a different context (context B) using 3 footshocks of 1 second at a lower intensity (0.4-mA) and separated by 70 seconds. On Day 15, in the morning, passive avoidance behavior was tested by putting mice back in the light compartment of the passive avoidance apparatus (context A) and measuring step-through latency time to enter the dark compartment using a stopwatch. A maximum time of 480 seconds (8 minutes) was allowed for the animal to enter the dark compartment and in case of no entrance, the mouse was removed from the apparatus. In the afternoon, they were placed back in the classical conditioning chamber (context B) to assess fear sensitization. On Day 16, anxiety-like behavior was assessed in the EPM. The mouse was placed in the center of the maze for free exploration of all arms of the maze for 5 min. From Day 18 to Day 22, mice were exposed daily to the passive avoidance apparatus (context A); where they were left to freely enter the dark compartment. Each day (Days 18-22), once in the dark compartment, they were locked there for 10 minutes, with no exposure to footshock to induce passive avoidance extinction. Starting from Day 19, if an animal spends 480 seconds without going to the dark compartment, it was forced to enter and was locked there for 10 minutes. On Day 23, animals were subjected to trauma re-exposure in context A as on Day 1, but with a very low intensity footshock

(0.15-mA instead of 1.5-mA: passive avoidance priming exposure). The following day (Day 24), passive avoidance relapse was assessed in the same apparatus (context A).

2.3. Behavioral scoring and analysis

The behavior of each mouse was videotaped with a JVC camera for later analysis. Behavioral data from the CPP and EPM tests were quantified using Ethovision XT Noldus 8.5 video-tracking program (Noldus Information Technology b.v., Wageningen, The Netherlands). For passive avoidance behavior, step-through latency (from the light compartment to the dark compartment) was scored using a stopwatch during each exposure to the shuttle box apparatus. For the CPP paradigm (preconditioning and post conditioning), the time spent in each compartment was measured and used to calculate the preference score following the formula used by **Montanari *et al.*, (2018)**:

Preference score = Time spent on the drug paired side in post conditioning day -Time spent in the same compartment on the preconditioning day.

For fear sensitization, the amount of time spent freezing was measured using a 1-s time sampling technique. A mouse was considered to freeze when it adopted a motionless posture, refraining from all but respiratory movements **(Fanselow *et al.*, 1994)**. For anxiety-like behavior, the anxiety index was calculated as follows:

Anxiety index = 1 – [(open-arm time/total time) + (open-arm entries/total entries)]/2.

For passive avoidance relapse correlation analysis, we calculated a relapse score based on the time spent in the trauma-associated chamber, as follows:

Relapse score = Time spent in the footshock-associated chamber on day 24 -Time spent in the same compartment on day 23

Immediately following the incubation period in the animal facility, mice from shocked and control groups were divided into EtOH- (with no ethanol exposure) and EtOH+ (with ethanol exposure). To discriminate the susceptible and resilient mice, we followed the same method that we used in our previous study **(Lguensat *et al.*, 2019)**. Briefly, after examining the step-through latency time recorded

during the passive avoidance test, shocked mice expressed a heterogeneous response and displayed a bimodal distribution **(Figure 2B)**. Therefore, animals in the top and bottom tertiles of step-through latency time were separated into high-avoiders (HA: trauma-susceptible) and low-avoiders (LA: stress-resilient) groups **(Table 1).** Mice that expressed intermediate behavior were eliminated from the study.

2.4. Golgi-Cox staining

After the end of the behavioral experiment, mice (n=31) were deeply anesthetized with an intraperitoneal injection of a lethal dose of urethane (>40mg/kg) and transcardially perfused with 0.9% saline followed by 4% of paraformaldehyde. Brains were then extracted, post-fixed overnight in 4% paraformaldehyde. The Golgi-Cox staining method consisted of immersing the brains in 3% solution of potassium dichromate for 15 days. The brains were then transferred in 2% silver nitrate solution for 3 days. After this delay, they were cut with a vibratom (Leica VT 1200 S), into 100µm sections, which were immediately mounted onto gelatin coated slides and dehydrated in graded ethanol starting from 50%, then xylene, before cover slipping the slide.

2.5. Image acquisition and morphometric analysis

Slides were analyzed using Olympus BH-2 microscope equipped with an Olympus DP71 camera. The regions of interest were identified using the atlas of **Paxinos and Franklin (2007)**. Pyramidal neurons in these regions were recognized according to their triangular soma shape and apical and basilar dendrites. To select neurons with less bias, we considered the same criteria as in our previous study **(Lguensat *et al.*, 2019)**. The images were taken using a 40x objective and the number and length of apical and basilar dendrites were quantified for each neuron. These parameters are the most used to assess the effect of stress on dendritic plasticity. We used image j software (NIH) to process images of neurons and for stack construction following the same method as in our previous study **(Lguensat *et***

***al.*, 2019).** We also used Adobe Photoshop CS2 for neuron image processing. Dendritic measurements were performed blind to the treatment conditions.

2.6. Blood collection method and CORT and BDNF measurements

After the completion of the behavioral tests, on Day 17, blood was collected using the restrained tail snip. We chose this method because it causes minimal stress-related changes in plasma CORT levels due to handling **(Kim *et al.*, 2018).**

Blood collection was performed between 11:30 am and 2 pm to avoid potential effects of circadian variations on plasma CORT concentrations. Each mouse (n=33) was placed in a restriction tube and a small puncture at the level of the lateral tail vein was made using a sterile blade. Blood samples were collected into EDTA tubes, which were stored on ice until centrifugation. To obtain plasma, blood samples were centrifuged for 15 minutes at 1600g at 4°C. The supernatant (20-80µl) was collected and stored at -80 °C until analysis.

Plasma CORT analysis was performed on all the mice (n=33) while BDNF measurements were done on 31 mice. Due to outliers, we discarded the results of one mouse from BDNF measurements. We performed these measures using enzyme-linked immunosorbent assay (ELISA) kits: Enzyme-linked immunosorbent Assay kit (My BioSource, San Diego, California, USA) and BDNF Emax ® ImmunoAssay (Promega, Madison, Wisconsin, USA) in accordance with the recommendations of the manufacturer.

2.7. Statistical analysis

Data analysis and graphs plotting were performed using SigmaPlot v.11 (SigmaPlot, San Jose, California, USA) and GraphPad Prism v7 (GraphPad software, California, USA) software. AICs (Akaike information criterion) were calculated using XLSTAT and Akaike weights (w) were determined as following: exp (-0.5*ΔAIC) divided by the sum of these values across all models. **(Burnham and**

Anderson, 2003). Shapiro Wilk's normality or lognormality test was run on data sets, and parametric tests were used whenever the test passed. When the test failed, nonparametric tests were used. Thus, the Mann-Whitney U test was used to measure the difference between the control and shocked mice regarding their passive avoidance behavior and Two-way ANOVA were used to assess the difference between the groups regarding their anxiety index, plasma CORT and BDNF levels. CPP preference score and sensitization test were analyzed using Kruskal-Wallis analysis of variance followed by Dunn's *post-hoc* for multiple comparisons. Data on passive avoidance extinction and dendritic changes from the PrL and IL areas were analyzed by three-way ANOVA followed by two-way ANOVA of data of each day separately (passive avoidance extinction and relapse) and from each region separately (dendritic parameters). The Holm-Sidak *post-hoc* was used for multiple comparisons. Values are reported as mean ±SEM and the significance was set at $p<0.05$.

CPP data were analyzed after the separation of mice into trauma-susceptible and trauma-resilient subgroups. The preference score was considered during the analysis. Correlation analysis of relapse score data was performed according to data distribution (Spearman's correlation for non-normally distributed data).

3. RESULTS

3.1. Distinction of trauma-susceptible and stress-resilient mice

Passive avoidance testing that followed avoidance training showed that most shocked mice acquired avoidance behavior **(Figure 2A)**. The Mann-Whitney test revealed a significant difference between the Control and Shocked groups (U=231, p=0.032). The normality test showed that the Control group expressed a normal distribution (Shapiro-Wilk's normality test passed: p=0.345). However, passive avoidance data from shocked mice failed the normality test (Shapiro-Wilk's normality test not passed: p<0.001), implying that they manifested a multimodal distribution. Further analysis showed that frequency distribution of the shocked group follows a bimodal distribution with two peaks matching the

bin centers of 50 and 500 seconds **(Figure 2B)**. In fact, the bimodal Gaussian function (function 1) provided the best fit relative to unimodal Gaussian function (function 2) [(a low AIC indicates the model that fits the data better: Δ_1(AIC)=1.51 and Δ_2(AIC)=45.81, Akaike weight for these models: w_1= 0.997 and w_2=0.00024)]. Thus, we considered animals in the top and bottom tertiles of the distribution and segregated them into trauma-resilient (or low avoiders: LA) and trauma-susceptible (or high avoiders: HA) subgroups within the ethanol exposed group (EtOH+/LA and EtOH+/HA groups). The other half, mice not exposed to ethanol (EtOH- group), received saline injections (EtOH-/LA and EtOH-/HA groups) and served as controls.

3.2. Ethanol-induced CPP in trauma-susceptible and stress-resilient mice

Data demonstrate that all mice exposed to ethanol [the non-shocked (EtOH+/Ctrl) and the shocked (EtOH+/LA, EtOH+/HA) groups] acquired a conditioned preference towards the ethanol-paired side of the apparatus **(Figure 2C)**. Given that preference score data were not normally distributed (Shapiro-Wilk's normality test not passed: p=0.002), we used a non-parametric test for the analysis. The Kruskal-Wallis one-way analysis of variance of preference score data indicated a highly significant difference between the groups (H (5) =39.28, p<0.001). The Dunn's multiple comparison test revealed significant increases of preference score for the groups exposed to ethanol (EtOH+/Ctrl, EtOH+/LA and EtOH+/HA) compared to the EtOH-/Ctrl group (q=3.032, p<0.034; q=4.589, p<0.001 and q=5.902, p<0.001; respectively). The preference score expressed by the EtOH+/HA group was significantly higher compared with the EtOH+/Ctrl group (q=3.074, p=0.043), but was not significant compared with the EtOH+/LA group (q=1.623, p=0.997). In addition, the EtOH+/HA group showed significant increases of the preference score when compared with the EtOH-/LA and EtOH-/HA groups (q=3.544, p=0.008 and q=3.351, p=0.015, respectively).

Regarding the EtOH- groups, we found that none of the associated subgroups (EtOH-/LA and EtOH-/HA) manifested a significant elevation of their preference score when compared to the EtOH-

/Ctrl group (Kruskal Wallis test followed by Dunn's multiple comparison test, EtOH-/LA *vs.* EtOH-/Ctrl: q=2.529, p=0.159 and EtOH-/HA *vs.* EtOH-/Ctrl: q=2.736, p=0.096).

3.3. PTSD-like behaviors across the groups

3.3.1. Fear sensitization based on a fear-conditioning test

Mice in the EtOH+/HA group displayed a marked freezing behavior in the new context in comparison with the other groups **(Figure 3A)**. A Kruskal-Wallis one-way analysis of variance (data non-normally distributed: Shapiro-Wilk's normality test not passed: p<0.001) of freezing time showed a difference between the groups (H (5) =14.07, p=0.015). The Dunn's multiple comparison test demonstrated that within the EtOH+ group, the EtOH+/HA subgroup expressed significant high levels of freezing as compared to the EtOH-/Ctrl subgroup (q=2.937, p=0.023). Moreover, even if the freezing expressed by EtOH+/ HA group was high, this increase was not significant compared with the EtOH-/ HA group (q=0.864, p=0.990). In addition, we did not find any significant increases in freezing for the EtOH+/LA group compared to the EtOH-/LA group (q=1.458, p=0.992), nor did we obtained significant increases in freezing for the EtOH+/Ctrl group in comparison with the EtOH-/Ctrl group (q=1.224, p=0.999).

3.3.2. Anxiety-like behavior based on an EPM test

Results demonstrated that shocked mice tended to express high anxiety index **(Figure 3B)**. A two-way ANOVA on anxiety-like behavior data (treatment and post-footshock avoidance) did not reveal any effect of treatment ($F_{(1, 72)}$ =0.242, p=0.624), but a significant effect of post-footshock avoidance was found ($F_{(2, 71)}$ =7.449, p=0.001). The interaction between these factors was not significant ($F_{(2, 72)}$ =1.269, p=0.287). However, the Holm-Sidak *post-hoc* test showed that, within the groups that were not exposed to ethanol, anxiety index was significantly higher in the EtOH-/HA group as compared to the EtOH-/Ctrl and EtOH-/LA groups (t=3.363, p=0.003 and t=3.036, p=0.006; respectively). No significant

difference was recorded between the EtOH+/HA and EtOH-/HA groups (t=0.806, p=0.666), the EtOH+/LA and EtOH-/LA groups (t=1.467, p=0.379) nor between the EtOH-/Ctrl and EtOH+/Ctrl groups (t=0.271, p=0.786).

3.3.3. Passive avoidance extinction and relapse

All trauma (1.5mA footshock) exposed mice exhibited progressive decreases in step-through latency during extinction training **(Figure 3C).** A three-way ANOVA (session, treatment and post-footshock avoidance) revealed significant effect of the three factors ($F_{(7;\ 68)}$ =35.21, p<0.001, $F_{(1;\ 72)}$ =44.38, p<0.001 and $F_{(2;\ 71)}$ =180.94, p<0.001, respectively). The interactions between session and post-footshock avoidance and between treatment and post-footshock avoidance were significant ($F_{(14;\ 71)}$ =11.83, p<0.001 and $F_{(2;\ 72)}$ =18.44; p<0.001, respectively), while the interaction between session and treatment was not significant ($F_{(2;\ 72)}$ =1.750, p=0.095). The Holm-Sidak *post-hoc* test (following a two-way ANOVA analysis performed on each day separately) showed that on Day 15 (avoidance test) and Day 18 (extinction session 1), values of step-through latency were significantly higher in HA mice from the EtOH+ group, compared with Ctrl and LA mice from both the EtOH- and EtOH+ groups, while the difference with the EtOH-/HA group was not significant**.** From Day 19 (extinction session 2) to Day 23 (pre-footshock priming), the EtOH+/HA group continued to display significant higher step-through latency time compared with each of the other groups, including the EtOH-/HA group **(Table 2).** On Day 24 (post-footshock priming), the EtOH+/HA group expressed elevated step-through latency time that was significantly higer compared with the remaining groups, including the EtOH-/HA group (**Table 2**), indicating that passive avoidance behavior returned only in HA mice treated with ethanol.

3.4. Quantification of Golgi-Cox material

Branching complexity of the apical and basilar dendrites, dendritic length, and branch order **(Figure 4)** were quantified in two areas of the mPFC: PrL and IL. The analysis included 182 PrL neurons (with a

number ranging from 24 to 45 neurons/group) and 147 IL neurons (ranging from 20 to 29 neurons/group) from 31 mice. We considered the individual as a unit of analysis.

3.4.1. Number of dendrites

Apical dendrites: The total number of apical dendrites was increased only in the PrL area of trauma-susceptible mice exposed to ethanol (**Figure 5A)**. A three-way ANOVA (mPFC area, treatment and post-footshock avoidance) on these data showed a significant effect of treatment ($F_{(1, 28)}$ =19.462, p<0.001), but the effects of the two other factors were not significant (mPFC area: $F_{(1, 28)}$ =0.071, p=0.790; post-footshock avoidance: $F_{(2, 27)}$ =1.050, p=0.358). The interaction between area and post-footshock avoidance was significant ($F_{(2, 28)}$ =11.160, p<0.001), as well as the interaction between treatment and area ($F_{(1, 28)}$ =4.267, p=0.044). However, the remaining interaction (i.e., between treatment and post-footshock avoidance) was not significant ($F_{(2, 28)}$ =1.711, p=0.191).

Thereafter, we used a two-factor ANOVA to assess the effect of treatment and post-footstock on the dendrites within each area separately. For PrL data, this analysis indicated significant effects of both factors (treatment: $F_{(1, 28)}$ =19.356, p<0.001; post-footshock avoidance: $F_{(2, 27)}$ =6.305, p=0.006). In fact, the Holm-Sidak *post-hoc* test showed that the EtOH+/HA group expressed a significant elevation of the total number of dendrites compared with the EtOH-/HA group (t=3.500, p=0.002). The same group (EtOH+/HA) also expressed a significant elevation compared with the EtOH-/LA group (t=2.749, p=0.032) (**Figure 5A:** PrL).

Similarly, for IL data, a two-way ANOVA analysis, with treatment and post-footstock avoidance as factors, showed a significant effect of post-footshock avoidance ($F_{(2, 27)}$ =5.878, p=0.008) but no effect was observed for treatment ($F_{(1, 28)}$ =3.003, p=0.095). The Holm-Sidak *post-hoc* test showed that the EtOH-/HA group had a significant diminution of the total number of dendrites compared to the EtOH-/LA group (t=3.873, p=0.002). The EtOH+/HA group also expressed higher dendritic number compared with the EtOH-/HA group (t=2.151, p=0.041) (**Figure 5A:** IL).

Basilar dendrites: Data indicated that the total number of basilar dendrites increased in the PrL of EtOH+/HA animals (**Figure 5B)**. A three-way ANOVA on these data (mPFC area, treatment and post-footshock avoidance) revealed main effects of treatment and post-footshock avoidance ($F_{(1, 28)}$ =5.584, p=0.022 and $F_{(2, 27)}$ =3.202, p=0.049, respectively), but no significant effect of area was found ($F_{(1, 28)}$ =0.512, p=0.477). The interactions between area and treatment and between area and post-footshock avoidance were significant ($F_{(2, 28)}$ =4.226, p=0.045 and $F_{(1, 28)}$ =13.517, p<0.001, respectively). However, the interaction between treatment and post-footshock avoidance was not significant ($F_{(2, 28)}$ =2.593, p=0.084).

A more detailed analysis of PrL data revealed a significant effect of treatment but no effect of post-footshock avoidance was found [two-way ANOVA: treatment ($F_{(1, 28)}$ =12.501, p=0.002); post-footshock avoidance: ($F_{(1, 28)}$ =2.103, p=0.143)]. The Holm-Sidak *post-hoc* test showed a significant elevation of the total number of basilar dendrites in the EtOH+/HA group compared with the EtOH-/HA group (t=2.957, p=0.007) (**Figure 5B:** PrL).

Regarding data from the IL area, two-way ANOVA did not reveal any effect of treatment ($F_{1, 28)}$ =0.000003, p=1.000). However, the factor post-footshock avoidance was highly significant ($F_{(1, 28)}$ =15.021, p<0.001). The Holm-Sidak *post-hoc* test indicated that the EtOH-/HA group had a significant reduction of the overall number of dendrites when compared to the EtOH-/LA and EtOH+/Ctrl groups (t=6.219, p<0.001 and t=2.584, p=0.016). However, the EtOH-/LA group manifested a significant increase of the number of dendrites when compared with the EtOH-/Ctrl group (t=4.398, p<0.001) (**Figure 5B:** IL).

3.4.2. Dendrite branch length

Apical dendrites: The total length of apical dendrites was markedly increased only in the PrL area of HA mice from the EtOH+ group (**Figure 5C)**. A three-way ANOVA on these data (mPFC area, treatment and post-footshock avoidance) did not reveal any significant effect of area, neither any effect of post-footshock avoidance ($F_{(1,28)}$ = 0.655,p=0.422 and $F_{(2,27)}$ = 0.852,p=0.433), but a significant

treatment effect was found ($F_{(1,28)} = 6.815$, $p=0.012$). The interactions between area and treatment and between area and post-footshock avoidance were also significant ($F_{(2, 28)} = 5.950$, $p=0.018$ and $F_{(1, 28)} = 15.063$, $p<0.001$; respectively). However, the interaction between treatment and post-footshock avoidance was not significant ($F_{(2, 28)} = 2.516$, $p=0.123$).

A detailed analysis of PrL data indicated a significant effect of treatment and post-footshock avoidance (two-way ANOVA: treatment ($F_{(1, 28)} = 15.316$, $p<0.001$); post-footshock avoidance: ($F_{(1, 28)} = 9.500$, $p<0.001$)). The Holm-Sidak *post-hoc* test showed that the EtOH+/HA group expressed a significant elevation of the total length of dendrites compared to the EtOH+/LA and EtOH-/HA groups ($t=3.059$, $p=0.016$ and $t=2.699$, $p=0.012$, respectively) (**Figure 5B:** PrL).

The same analysis on data from the IL area showed a significant effect of post-footshock avoidance ($F_{(1, 28)} = 10.390$, $p<0.001$) but no effect of treatment was found ($F_{(1, 28)} = 0.093$, $p=0.763$). The Holm-Sidak *post-hoc* test revealed that EtOH-/HA group expressed a significant reduction of the total length compared with both the EtOH-/Ctrl and EtOH-/LA groups ($t=2.512$; $p=0.019$ and $t=5.634$, $p<0.001$, respectively). The EtOH-/LA group also expressed a significantly higher elevation as compared with the EtOH-/Ctrl group ($t=2.989$, $p=0.012$). No such differences were observed within the EtOH+ groups (**Figure 5C:** IL).

Basilar dendrites: The total length of basilar dendrites was markedly increased only in the PrL area of HA mice from the EtOH+ groups (**Figure 5D).** A three-way ANOVA on the total branch length (mPFC area, treatment and post-footshock avoidance) did not indicate any significant effect of area ($F_{(1,28)} = 0.897$, $p=0.348$), or post-footshock avoidance ($F_{(2,28)} = 1.839$, $p=0.169$), but a main effect of treatment was found ($F_{(1,28)} = 10.288$, $p=0.002$). The interactions between area and treatment and between area and post-footshock avoidance were significant ($F_{(2, 28)} = 5.040$, $p=0.029$ and $F_{(1, 28)} = 22.869$, $p<0.001$; respectively). However, the interaction between treatment and post-footshock avoidance was not significant ($F_{(2, 28)} = 0.233$, $p=0.793$).

Regarding PrL data, both treatment and post-footshock avoidance had significant effects [two-way ANOVA: treatment ($F_{(1, 28)} = 13.903$, $p<0.001$) and post-footshock avoidance ($F_{(2, 28)} = 6.399$, $p=0.006$)]. The Holm-Sidak *post-hoc* test revealed that the EtOH+/HA group had a significant increase in the overall length of basilar dendrites as compared with the EtOH+/Ctrl and EtOH+/HA groups

(t=2.893, p=0.023 and t=2.728, p=0.011; respectively). Within the EtOH- groups, EtOH-/HA mice demonstrated an increased branch length compared with EtOH-/LA mice (t=2.650, p=0.041) (**Figure 5D:** PrL).

When considering the IL area, the analysis showed that the factor post-footshock avoidance was significant ($F_{(2, 28)} = 9.454$, $p<0.001$), while there was no effect of the factor treatment ($F_{(1, 28)} = 1.237$, p=0.277). The EtOH+/HA group did not differ from each of the two groups who received ethanol (EtOH+/Ctrl and EtOH+/LA; t=1.184, p=0.434 and t=1.421, p=0.423, respectively). However, the EtOH-/HA group showed a significant reduction of the length of basilar dendrites in comparison with the EtOH-/LA group (t=4.803, $p<0.001$), while the EtOH-/LA group displayed a significant elevation compared with the EtOH-/Ctrl group (2.831, p=0.018) (**Figure 5D:** IL).

3.5. Correlation between behavior and dendritic morphology in the PrL

Spearman correlation analysis on relapse score data and the total number of PrL dendrites revealed a positive correlation between the two parameters (r = 0.653, p=0.040), thus supporting the existence of a strong link between high dendrite number in the PrL and the increase of susceptibility to relapse **(Figure 6).**

3.6. CORT and BDNF measurements across the groups

3.6.1. CORT levels using ELISA assay

Higher levels of CORT were seen in HA mice, regardless of their EtOH treatment condition **(Figure 7A)**. A two-way ANOVA analysis revealed a significant effect of post-footshock avoidance ($F_{(2, 28)} = 19.80$, $p<0.001$) with no effect seen for treatment ($F_{(2, 28)} = 0.035$, p=0.853). The interaction between these two factors was not significant ($F_{(4, 28)} = 0.518$, p=0.601). The Holm-Sidak multiple comparisons showed significant elevation in the circulating CORT levels of the EtOH-/HA group, compared with the EtOH-/Ctrl and EtOH-/LA groups (t=3.536; p=0.014 and t=3.069; p=0.038, respectively). The levels

expressed by the EtOH+/HA group were also significantly elevated compared with the EtOH+/Ctrl and EtOH+/LA groups (t=4.175; p=0.003 and t=4.635; p=0.001; respectively).

3.6.2. BDNF levels using ELISA assay

Significantly lower levels of BDNF were found in HA, but not LA, mice in both the EtOH- and EtOH+ groups (**Figure 7B).** A two-way ANOVA performed on BDNF data (with treatment and post-footshock avoidance) revealed a main effect of post-footshock avoidance ($F_{(2, 27)}$ =16.350, p<0.001). There was no significant effect of treatment ($F_{(2, 27)}$ =0.380, p=0.543). Moreover, the interaction between treatment and post-footshock avoidance was not significant ($F_{(4, 27)}$ =0.625, p=0.543). The Holm-Sidak *post-hoc* test showed that each of the HA groups had significantly lower levels of BDNF as compared to the Ctrl groups within each treatment (EtOH-/HA vs. EtOH-/Ctrl: t=4.666, p<0.001 and EtOH+/HA *vs.*EtOH+/Ctrl: t=3.401, p=0.007). The two trauma-susceptible groups (EtOH-/HA and EtOH+/HA) did not differ from each other, showing that ethanol exposure did not modulate BDNF concentrations in plasma.

4. DISCUSSION

In the present work, we analyzed the effects of repeated ethanol injections on trauma-related behavioral and biological changes (using a high intensity, 1.5-mA, footshock for trauma). Regarding the trauma-related behavioral changes, we assessed passive avoidance towards the trauma chamber, sensitization of post-trauma contextual fear (using a low intensity, 0.4-mA, footshock in a different context), anxiety-like behavior (using the elevated plus maze test), extinction of passive avoidance towards the trauma chamber and the susceptibility to relapse (using a very low intensity, 0.15-mA, footshock in the trauma chamber). Regarding the trauma-related biological changes, on the one hand, we examined plasma concentrations of CORT and BDNF. On the other hand, using the Golgi-Cox method, we investigated changes in total dendritic number and length in the mPFC. We found that repeated exposure of trauma-susceptible mice to ethanol altered their extinction behavior, by delaying extinction acquisition and

increasing the susceptibility to relapse. These behavioral changes were associated with higher CPP acquisition. We also found higher increases in total dendritic number and length in the PrL area. Interestingly, the relapse score correlated with changes in dendritic density in the PrL area. Finally, there were no effects of ethanol on trauma-induced changes in plasma concentrations of CORT and BDNF.

4.1. Acquisition of ethanol-induced CPP in trauma-susceptible and stress-resilient mice

Our data showed that all trauma-susceptible and trauma-resilient mice subjected to the ethanol CPP procedure developed a preference for the ethanol-paired side. However, trauma-susceptible mice exhibited a significantly higher preference when compared with the non-stressed controls. This mouse finding is in accordance with other recent rodent data, showing that pronounced effects of ethanol characterize animals expressing maladaptive behavioral responses to stress. These effects were observed either with the CPP paradigm (**Bahi and Dreyer, 2014; Yu *et al.*, 2016; Macedo *et al.*, 2018; Bahi and Dreyer, 2020)**, with the two-bottle free choice paradigm **(Meyer *et al.,* 2013; Manjoch *et al.,* 2016; Cannady *et al.,* 2021),** or with the self-administration paradigm **(Edwards *et al.,* 2013; Barchiesi *et al.,* 2021; Makhijani *et al.,* 2021).** Nevertheless, studies with other drugs (such as cocaine and methamphetamine) have shown that stress does not necessarily lead to potentiate the effect of the drug or increase its consumption (e.g., cocaine: **Enman *et al.,* 2015; Hadad *et al.,* 2016; Lguensat *et al.,* 2021;** methamphetamine: **Eagle and Perrine, 2013**).

When considering the effect of stress on ethanol consumption, it seems that this effect depends on the type of stress applied **(Spanagel *et al.,* 2014; Weera and Gilpin, 2019)**. For instance, footshock stress and forced swim stress, but not restraint stress, have been found to enhance ethanol intake **(Noori *et al.,* 2014).** Another factor playing a key role is the degree of sensitivity to stress. Studies investigating the interaction between stress susceptibility and ethanol intake have generally reported increased consumption in rodents most affected by stress **(Edwards *et al.,* 2013; Meyer *et al.,* 2013; Manjoch *et al.,* 2016; Weera *et al.,* 2020; Barchiesi *et al.,* 2021).** In addition, the degree of ethanol preference

before stress may also play a key role **(Darnaudéry *et al.*, 2007),** as animals with a high preference for ethanol show increased consumption after stress **(Vengeliene *et al*, 2003; Chester *et al.*, 2004).** Finally, another factor to be considered is the method used to measure ethanol intake **(Noori *et al*, 2014)**. According to these authors, stress does not typically result in enhanced ethanol consumption in operant self-administration paradigms but leads to increased home cage drinking. This may, in part, explain our present finding obtained with mice subjected to the 1.5-mA footshock before the CPP procedure. However, ethanol was not self-administered, but injected by the experimenter. Moreover, the CPP procedure only enabled us to assess preference and motivation towards ethanol in trauma-susceptible and trauma-resilient mice. To reliably assess the effect of stress-susceptibility on ethanol consumption and intake, different ethanol administration methods need to be tested.

4.2. Changes in sensitivity to reward associated with trauma susceptibility

In humans, following a traumatic event, susceptible people who develop PTSD show highlighted vulnerability to develop drug abuse (**Chilcoat and Breslau, 1998**). Several animal studies managed to mimic this feature have showed that sensitivity to the rewarding properties of drugs change as a function of stress susceptibility (**Krishnan *et al.*, 2007; Breit and Chester, 2016; Brodnik *et al.*, 2017**). However, determining stress susceptibility status in laboratory animals requires using methods based on behavioral classifications. These classifications consider either two or more trauma-related symptoms **(Cohen *et al.*, 2004; Toledano and Gisquet-Verrier, 2014)** or one major symptom **(Dopfel *et al.*, 2019; Lguensat *et al.*, 2019).** We based our classification on avoidance measures **(Lguensat *et al.*, 2019)** as the major symptom with our mouse model. Moreover, our trauma-susceptible (high avoiders) mice expressed higher place preference, which joins other reports**,** showing that the motivation to consume the drug **(e.g., Brodnik *et al.*, 2017)** and preference for the drug measured using CPP **(e.g., Krishnan *et al.*, 2007)** are increased in stress-susceptible animals. Changes in drug preference as a function of stress susceptibility could be driven by the sensitization of the dopamine system (**Krishnan *et al.*, 2007; Brodnik *et al.*, 2017**), knowing its role in motivation. Furthermore, the widespread view is that CPP is

mainly mediated by the rewarding properties of the drug **(McKendrick and Graziane, 2020)** and thus by the drug's positive reinforcement properties. This view is supported both by human **(e.g., Childs and De Wit, 2016)** and rodent **(e.g., Tzschentke, 2007)** data. However, CPP can also result from the ability of the drug to remove or reduce an aversive state **(e.g., Koob and Le Moal, 2008)** and therefore it can result from negative reinforcement. In fact, it is largely admitted that ethanol exerts both positive and negative reinforcing effects **(Pautassi *et al.*, 2010).** It is therefore possible that our CPP findings could be largely due to ethanol's negative reinforcement effects given that susceptible animals showed greater preference for the ethanol-paired side compared to the non-stressed group.

4.3. Procedural and practical considerations for interpreting CPP data

When it comes to interpreting CPP data regarding CPP procedure design (biased/unbiased), an important distinction needs to be made. According to **Cunningham and colleagues, (2003)**, the biased/unbiased description is often used to refer to either the typical response of untrained animals to the apparatus or to describe the experimental procedure for assigning the drug-paired conditioned stimulus. In the present study, we used this description to refer to the fact that ethanol injections were associated with the least preferred side of the apparatus (biased design). Several reports have indicated that adopting this method allows a better development of the conditioned preference compared to associating the drug with the preferred side or cue **(Schenk *et al.*, 1985; Nomikos and Spyraki 1988; Cunningham *et al.*, 2003)**. Here, using the biased design, the conditioned preference was obtained, but we did not test the unbiased design for a better comparison. However, other studies **(e.g., Cunningham *et al.*, 2006; Cunningham and Shields, 2018)** have demonstrated that ethanol can also induce reliable CPP with the unbiased design. Nevertheless, we also considered the fact that associating ethanol with a chamber where the animal spent, for example, 20% of its time during the preconditioning session may give more room for detecting differences than starting with an equal preference of the two chambers in an unbiased design **(Roma and Riley, 2005)**.

4.4. Effects of post-trauma repeated ethanol exposure on trauma-associated behaviors and passive avoidance extinction

4.4.1. Fear sensitization and anxiety-like behavior

Fear sensitization is a form of non-associative learning and a hallmark of PTSD **(Siegmund & Wotjak, 2007)**. It can be assessed in rats or mice, either by measuring acoustic startle response **(Cohen *et al.,* 2004)**, by exposing them to a neutral tone in a neutral chamber **(Siegmund & Wotjak, 2007),** or by conditioning animals using a low intensity footshock, and re-exposing them, at least 24 hours later, to the conditioning context **(Bentefour *et al.,* 2015)**. Under these different conditions, during tests, it has been often reported that the stressed group displays more fear (fear sensitization) compared to the non-stressed group (**Lewis and Gould, 2003; Owen *et al*., 2003; Bentefour *et al*., 2015, 2016, 2018**).

Our data show that ethanol (1.6g/kg) had no effect on the fear sensitization phenomenon, joining the findings of **Lewis and Gould, (2003)** and **Owens *et al.,* (2003).** These studies reported that a proximate dose of ethanol (1.5g/kg) had no effect on fear sensitization, while the effect was only detected at either lower (0.5g/kg: **Lewis and Gould, 2003**) or higher (2.25g/kg: **Owens *et al.,* 2003**) doses. In these studies, as in the present one, ethanol was administered via IP injections. However, these studies evaluated the effects of ethanol acutely following one injection. Together with our present study, one can suggest that the effect of ethanol on fear sensitization is not related to the number of injections, but rather to the dose. Using a different approach, **Albrechet-Souza and colleagues (2020)** have reported that a history of chronic ethanol consumption can provoke an accentuation of fear sensitization (measured by the means of acoustic startle response) in a sex-specific manner (stress-susceptible males only). Altogether, one can hypothesize that ethanol may enhance fear sensitization in stress sensitive individuals as a function of some factors, such as the method and the chronicity of its administration.

Like fear sensitization, anxiety is often reported in PTSD patients **(Olatunji *et al.,* 2018).** Interestingly, it is also often considered in studies with rodent models of PTSD, with increased anxiety-like behavior characterizing trauma-susceptible animals **(Lguensat *et al.*, 2019)**.

Our finding on anxiety-like behavior did not reveal any effect of ethanol (1.6g/kg). These data are in accordance with those of **Varlinskaya and Spear, (2012)**, in which ethanol injections (1g/kg) did not have any effect in adult stressed animals. Moreover, it seems that the anxiolytic effects of ethanol are only noticeable in less anxious animals **(Langen *et al.,* 2002)**; ethanol-preferring animals **(Stewart *et al.,* 1993)** and defeated animals with a history of ethanol intake **(Kudryavtseva *et al.,* 2006)**. We therefore expected to detect a potential effect of anxiety on one of two trauma-exposed groups (trauma-susceptible or trauma-resilient mice). In our study, even though anxiety levels displayed by the trauma-susceptible group was slightly attenuated (anxiety index close to the one expressed by the control group), the lack of any significant difference with the trauma-resilient group discarded our initial hypothesis.

4.4.2. Passive avoidance extinction

Impairment of extinction of conditioned fear is considered as a hallmark of PTSD **(Milad *et al.,* 2006, 2008)**. This is, in part, supported by studies showing, in PTSD patients, either impaired extinction learning **(e.g., Blechert *et al.*, 2007)** or normal extinction learning, but impaired extinction memory **(e.g., Milad *et al.*, 2008)**. Studies using a single prolonged stress paradigm, as an animal model of PTSD, have also demonstrated impaired extinction learning **(Eskandarian *et al.,* 2013; Matsumoto *et al.,* 2013).** However, in those human and rodent studies, the authors tested the impairment in the ability to extinguish conditioned fear that was acquired after trauma (intense footshock in rodents). Here, as in our previous studies **(Bentefour *et al.,* 2018; Lguensat *et al.,* 2019)**, we focused on extinction of conditioned fear of trauma reminders. Indeed, exposure therapy, one of the gold-standard treatments for PTSD **(Graham & Milad, 2011)**, is based on extinction of conditioned fear of trauma reminders. We found that ethanol injections impaired passive avoidance extinction learning and facilitated the return of the extinguished fear. These data agree with our cocaine study in rats **(Lguensat *et al.,* 2021)**, showing impaired fear extinction and increased susceptibility to relapse. In mice, a single injection of 1.5 g/kg of ethanol 5–10 min prior to extinction session has also been found to delay extinction learning **(Lattal, 2007)**. In humans, alcohol has been shown to alter extinction behavior **(Bisby *et al.,* 2015).**

We have previously shown, in mice, that passive avoidance extinction training suppresses not only avoidance towards trauma-related context, but also the other PTSD-like symptoms (including non-conditioned symptoms, such as fear sensitization and anxiety-like behavior; **Bentefour *et al.*, 2015, 2016)**. Similarly, provocation of passive avoidance relapse, with a priming footshock applied in the trauma-related context, has been found to induce the return of all PTSD-like symptoms **(Bentefour *et al.*, 2015, 2016)**. One can hypothesize that impairment of avoidance extinction may support resistance to exposure therapy reported in PTSD patients **(Schottenbauer *et al.*, 2008)**, indicating PTSD chronicization. Indeed, patients who respond to exposure therapy for PTSD display suppression of all symptoms (including non-conditioned symptoms; **Yehuda *et al.*, 2009**). Therefore, given the strong association between factors such as substance abuse and the course of chronic PTSD **(Zlotnick *et al.*, 1999)**, our current finding suggests that post-stress ethanol exposure can provoke chronicization of PTSD-like symptoms. Nevertheless, one of the limitations of our study is the use of a cut-off for passive avoidance at 480s. Since values above 480s were not measured, one cannot know whether exposure to ethanol potentiated avoidance (values above 480s). In this case, the normal rate of extinction would appear as extinction learning impairment, with avoidance values of the ethanol group remaining high, when extinction would be almost achieved with the group not exposed to ethanol. However, this potential effect of ethanol does not support the fact that relapse was facilitated.

4.5. Effects of post-stress repeated ethanol exposure on stress-associated dendritic changes in the mPFC

Several neuroimaging studies have investigated brain structural and functional alteration in PTSD. These studies have shown reduced volumes and changes in activities in various regions, including the mPFC **(e.g., Bremner, 1999; De Bellis *et al.*, 2000; Rauch *et al.*, 2003; Shin *et al.*, 2004)**. In our previous rodent study, we have also found that the PTSD-like phenotype is associated with changes in the mPFC, corresponding to decreases in total number and length of apical and basilar dendrites in the IL area **(Lguensat *et al.*, 2019)**. Overall, in the PrL area, we have observed increases in the total number

and length of apical dendrites. Here, these increases were not observed clearly in trauma-susceptible mice, but they significantly emerged in trauma-susceptible mice exposed to ethanol. It seems that ethanol by itself did not induce significant dendritic changes in the IL area. However, in the IL area, we noted that changes in the dendritic parameters of trauma-susceptible mice treated with ethanol (EtOH+/HA) no longer differ from those of trauma-resilient mice that also received ethanol (EtOH+/LA). This suggests that ethanol could have influenced both the PrL and IL areas (**Makhijani *et al.,* 2021; Cannady *et al.,* 2021)**. As mentioned above, ethanol exposure impaired fear extinction. Interestingly, in a study in which mice were exposed to chronic intermittent ethanol vapor, followed by auditory fear conditioning, an impaired fear extinction associated with an elevated length of the apical dendrites was found in the PrL area, with no change in the IL area **(Holmes *et al*., 2012)**. Moreover, the dendritic expansion in the PrL area was more accentuated far from the soma of neurons. A fine structured analysis of PrL neurons considering the distance from soma would have given us more detailed information about the distribution and the extent of dendritic expansion. Similarly, in other rodent studies, repeated **(Quiñones-Laracuente *et al.,* 2015)** and chronic **(Cannady *et al.,* 2021)** exposure to ethanol was found to enhance activity in the PrL area, which was associated with impaired extinction memory **(Quiñones-Laracuente *et al.,* 2015)**.

Several studies have shown that the PrL and IL areas have opposite influences on conditioned fear expression **(Gilmartin & McEchron, 2005; Vidal-Gonzalez *et al.,* 2006).** Contrary to the IL area, the PrL area is, indeed, implicated in the production and retrieval of conditioned fear **(Milad & Quirk, 2002; Sierra-Mercado *et al.,* 2011)**. The persistence of PrL activity after extinction training has also been associated with failure to express extinction memory **(Burgos-robles *et al.,* 2009).** Consistent with our present finding, it has also been reported that persistent increased PrL activity is associated with persistent active avoidance **(Bravo-Rivera *et al.,* 2015)**. This PrL morphological change may interact with avoidance extinction probably through direct projection of the PrL to the ventral striatum and/or to the basal amygdala **(Bravo-Rivera *et al.,* 2015).**

4.6. Effects of post-stress repeated ethanol exposure on plasma CORT and

BDNF concentrations in trauma-susceptible mice

4.6.1. CORT

Glucocorticoids are the end-product of the activation of the hypothalamo-pituitary-adrenal axis. Various dysfunctions of this axis have been repeatedly reported in patients with PTSD, including low levels of cortisol in urine and plasma, enhanced suppression of cortisol in response to administration of low dose of dexamethasone (a synthetic glucocorticoid), and glucocorticoid receptor abnormalities **(Yehuda *et al.,* 2001)**. Low levels of plasma CORT have also been reported, in rats, with the single prolonged stress model of PTSD **(Wang *et al.,* 2018).** However, there are also studies indicating, on the contrary, increased plasma CORT levels in rats subjected to the single prolonged stress model **(Qiu *et al.,* 2018)** and after exposure to synthetic predator odor **(Makhijani *et al.,* 2021)**. In humans, bedtime salivary cortisol can be higher in some patients, while the same patients were found to display low levels of urinary cortisol **(Yehuda *et al.,* 2014).** Further studies are therefore necessary to better understand changes in the levels of cortisol in PTSD.

Here, we found that trauma exposure induced high levels of plasma CORT in all trauma-susceptible mice, regardless of ethanol exposure. Surprisingly, although administration of ethanol, like many other drugs of abuse, is known to activate the hypothalamo-pituitary-adrenal axis **(Thiagarajan *et al.*, 1989)**, we here found that the levels of CORT were not affected by ethanol exposure. Of note, we obtained this finding with male mice, which seems to agree with studies showing that females, but not males, respond to drug with higher CORT secretion **(Peričić and Pivac, 1995; Willey *et al.*, 2012; Albrechet-Souza *et al.,* 2020)**.

Since we observed that ethanol exposure was associated with extinction impairment, it is possible that this impairment was not related to the increases in CORT levels in trauma-susceptible mice. On the contrary, increased levels of glucocorticoids have been reported to enhance fear extinction consolidation both in humans **(Brueckner *et al.*, 2019)** and in rodents **(Cai *et al.*, 2006).**

4.6.2. BDNF

BDNF is involved in synaptic plasticity, neuronal differentiation, and survival of neurons **(Klein *et al.,* 2011).** Although plasma BDNF levels have been shown to correlate with cortical integrity **(Lang *et al.,* 2007)**, we acknowledge that changes in plasma BDNF levels remains a kind of "surrogate marker", which does not necessarily reflect intracerebral dynamics in BDNF secretion. In a study investigating the clinical correlates of plasma BDNF concentrations in a clinical population showing PTSD symptomatology, the authors found that the subjects with full PTSD showed lower levels of BDNF than subjects with partial PTSD and controls **(Stratta *et al.,* 2016).** Using the single prolonged stress paradigm in rats, it has also been reported that this procedure decreases the expression of BDNF mRNA in the hippocampus **(Lee *et al.,* 2016)**. In the present study, we found that trauma-susceptible mice that were not exposed to ethanol and those exposed to this treatment expressed identical decreases in the levels of plasma BDNF as compared to controls, indicating no effect of ethanol on BDNF levels. This result can be, in part, explained by the low dose of ethanol that we used. Indeed, effect of ethanol on BDNF protein expression has been reported with much higher doses, yielding blood ethanol levels near 50–70 mg per 100 ml **(Stragier *et al.,* 2015)**.

Although BDNF blockade interferes with extinction memory **(Radiske *et al.,* 2015),** it is possible that the observed impairment of fear extinction in the present study was not associated with any effect of ethanol exposure on BDNF.

4.7. Conclusion

Results of the present study provide evidence for altered passive avoidance extinction in trauma-susceptible mice exposed to repeated ethanol injections. This suggests that exposure to ethanol induced persistent PTSD-like symptoms in the most vulnerable mice. Interestingly, the treatment of PTSD has been shown to be less efficient in people with a PTSD diagnosis who also drink alcohol **(McCauley *et al.,* 2012).** Moreover, trauma-susceptible mice showed stronger ethanol-induced CPP indicating that

these mice were more sensitive to ethanol effects, which potentially resulted from their high avoidance status.

Our data also show, with trauma-susceptible mice exposed to ethanol, a positive correlation between passive avoidance relapse and dendritic changes in the PrL. However, it is worth mentioning that our study was only done on male mice, which constitutes one of its major limitations. For our data to gain more translational value, the same experimental procedures need to be applied on female mice as well. Another limitation of the present study is that the designs for Pavlovian conditioning procedures did not include control groups receiving equal exposures to the unconditioned (and conditioned) stimuli in an unpaired fashion.

Together, these findings suggest that PTSD patients who self-medicate their symptoms for relief seeking **(Baker *et al.,* 2004; Read *et al.,* 2014)**, expose themselves to difficulty to recover from the disorder **(Zlotnick *et al.,* 1999; Kaysen *et al.,* 2011)** via drug-induced neuroadaptive mechanisms **(Holmes *et al.*, 2012; Quiñones-Laracuente *et al.,* 2015)**, which may include changes in the PrL dendritic plasticity.

CONFLICT OF INTEREST

The authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

A.L, R.G., M.B and S.B. conceived and planned the experiment. A.L. and Z.B. collected the data. A.L., Z.B. and S.B. performed statistical analyses. A.L., S.B. and R.G. wrote the manuscript. Z.B, E.G., C.B. revised the manuscript. S.B. and R.G. supervised the research.

DATA AVAILABILITY STATEMENT

To access the data, please visit the following Figshare link:

https://figshare.com/articles/dataset/Donn_es_brutes_EJN_18092021_xlsx/16692031

List of abbreviations:

AIC: Akaike Information Criterion

BDNF: Brain Derived Neurotrophic Factor

CORT Corticosterone

CPP: Conditioned Place Preference

Ctrl: Control

EtOH: Ethanol

HA: High avoider

IL: infralimbic cortex

LA: Low avoider

m-A: Milliamp

mPFC: Medial Prefrontal Cortex

PrL : Prelimbic cortex

PTSD: Post-Traumatic Stress Disorder

w: Akaike weight

ZT: Zitgeber

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FIGURE LEGENDS

Figure 1. Timeline of the behavioral experiment.

Figure 2. Fear response to the 1.5-mA footshock and Preference score. (A) Step-through latency as expressed by non-shocked (Control group) and shocked mice during passive avoidance test performed 15 days after training (footshock administration) (Mann-Whitney's test: * $p<0.05$ as significant differences compared to the Control group). (B) Frequency distribution of step-through latency time in the Control and Shocked groups including Low avoiders (LA) and High avoiders (HA). (C) Preference score expressed by mice that were not exposed (EtOH-) and that were exposed (EtOH+) to ethanol (Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons: $^{*}p<0.05$ and $^{***}p<0.001$ indicate significant differences). EtOH-/Ctrl: n=13, EtOH-/LA: n=13, EtOH-/HA: n=13, EtOH+/Ctrl: n=13, EtOH+/LA: n=13, EtOH+/HA: n=10.

Figure 3. Stress-related behavioral testing and passive avoidance extinction testing. (A) Freezing behavior (as fear sensitization indicator) relative to a weak contextual fear conditioning performed two weeks after passive avoidance acquisition (Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons: $^{*}p<0.05$ as significant differences compared to the EtOH-/Ctrl group). (B) Anxiety index recorded during the elevated plus maze test (two-way ANOVA followed by Holm-Sidak *post-hoc* test: ** $p<0.01$ indicate significant differences). (C) Step-through latency recorded during Day 15 (passive avoidance test), Days 18-22 (extinction sessions) and Days 23-24 (pre and post footshock priming) (two-way ANOVA for each day followed by Holm-Sidak *post-hoc* test: $^{a}p<0.05$, $^{b}p<0.01$ and $^{c}p<0.001$ significant differences compared to the EtOH-/Ctrl group; $^{d}p<0.05$, $^{e}p<0.01$ and $^{f}p<0.001$ represent significant differences compared to the EtOH+/Ctrl group; $^{g}p<0.05$, $^{h}p<0.01$ and $^{i}p<0.001$ represent significant differences compared to the EtOH-/LA group; $^{j}p<0.05$, $^{k}p<0.01$ and $^{l}p<0.001$ represent significant differences compared to the EtOH-/HA group ; $^{m}p<0.05$, $^{n}p<0.01$ and $^{o}p<0.001$ represent significant differences compared to the EtOH+/LA group). Data are presented as mean ± SEM. EtOH-/Ctrl: n=13, EtOH-/LA: n=13, EtOH-/HA: n=13, EtOH+/Ctrl: n=13, EtOH+/LA: n=13, EtOH+/HA: n=10.

Figure 4. Photomicrographs showing representative neurons in the PrL and IL areas in a stress-susceptible mouse and a stress-resilient mouse exposed to ethanol (EtOH+/HA and EtOH+/LA; respectively). Scale bar= 20µm.

Figure 5. Dendritic morphological changes in the mPFC. (A) Total number of apical dendrites in the PrL and IL areas. (B) Total number of basilar dendrite changes in the PrL and IL areas. (C) Total apical dendrites branch length changes in the PrL and IL areas. (D) Total basilar branch length changes in the PrL and IL areas. Data in panels A-D are shown as mean (±SEM). Two-way ANOVA within each region, followed by Holm-Sidak *post-hoc*: **p*<0.05, ***p*<0.01 and ****p*<0.01 indicate significant differences. EtOH-/Ctrl: n=5, EtOH-/LA: n=5, EtOH-/HA: n=6, EtOH+/Ctrl: n=5, EtOH+/LA: n=5, EtOH+/HA: n=5.

Figure 6: The relationship between passive avoidance return (relapse) and the total number of dendrites in the PrL. Spearman correlation between relapse score and the total number of dendrites in the PrL. Dark magenta dots : EtOH+/HA mice (n=5). Grey dots : EtOH+/LA mice (n=5).

Figure 7. Changes in basal plasma CORT (A) and BDNF (B) levels across the groups. Two-way ANOVA followed by Holm-Sidak *post-hoc* test: **p*<0.05, ***p*<0.01 and ****p*<0.001 indicate significant differences. CORT: EtOH-/Ctrl: n=5, EtOH-/LA: n=5, EtOH-/HA: n=6, EtOH+/Ctrl: n=5, EtOH+/LA: n=7, EtOH+/HA: n=5. BDNF: EtOH-/Ctrl: n=5, EtOH-/LA: n=5, EtOH-/HA: n=5, EtOH+/Ctrl: n=5, EtOH+/LA: n=5, EtOH+/HA: n=5.