Influence of Footshock Number and Intensity on the Behavioral and Endocrine Response to Fear Conditioning and Cognitive Fear Generalization in Male Rats

Nuria Daviu[a,f] #, Patricia Molina[a,b,g] #, Roser Nadal[a,c,d,e], Xavier Belda[a,b], Sara Serrano[a,b], Antonio Armario[a,b,d,e] *

[a] Institut de Neurociències, Universitat Autònoma de Barcelona, Spain

[b] Animal Physiology Unit, Faculty of Biosciences, Department of Cellular Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Spain

[c] Deparment of Psychobiology and Methodology of Health Sciences, Faculty of Psychology, Universitat Autònoma de Barcelona, Spain

[d] Unitat de Neurociència Traslacional, Parc Taulí Hospital Universitari, Institut d'Investigació i Innovació Parc Taulí I3PT, Universitat Autònoma de Barcelona, Spain

[e] CIBERSAM, Instituto de Salud Carlos III, Spain

[f] Present address: Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

[g] Present address: Department of Fundamental Neuroscience, University of Lausanne, 1005 Lausanne, Switzerland

 # co-first authors
*** corresponding author:** antonio.armario@uab.es

ABSTRACT

Foot-shock paradigms have provided valuable insights into the neurobiology of stress and fear conditioning. An extensive body of literature indicates that shock exposure can elicit both conditioned and unconditioned effects, although delineating between the two is a challenging task. This distinction holds crucial implications not only for the theoretical interpretation of fear conditioning, but also for properly evaluating putative preclinical models of post-traumatic stress disorder (PTSD) involving shock exposure. The characteristics of shocks (intensity and number) affect the strength of learning, but how these characteristics interact to influence conditioned and unconditioned consequences of shocks are poorly known. In this study, we aimed to investigate in adult male rats the impact of varying shock number and intensity on the endocrine and behavioral response to contextual fear conditioning and fear generalization to a novel environment markedly distinct from the shock context (i.e., fear generalization). Classical biological markers of stress (i.e., ACTH, corticosterone, and prolactin) were sensitive to manipulations of shock parameters, whereas these parameters had a limited effect on contextual fear conditioning (evaluated by freezing and distance traveled). In contrast, behavior in different novel contexts (fear generalization) was specifically sensitive to shock intensity. Notably, altered behavior in novel contexts markedly improved, but not completely normalized after fear extinction, hypoactivity apparently being the result of both conditioned and unconditioned effects of foot-shock exposure. The present results will contribute to a better understanding of shock exposure as a putative animal model of PTSD.

Keywords

Hypothalamic-Pituitary-Adrenal axis; Footshock Intensity and Number; Fear Conditioning; Fear Generalization; PTSD

1. Introduction

Electric foot-shock is an aversive stimulus that has been traditionally employed to study emotional learning and memory, particularly classical Pavlovian fear conditioning. In this paradigm, pairing an originally neutral stimulus (conditioned stimulus), such as a particular context or specific cues, with electric shocks (unconditioned stimulus) results in the development of a conditioned response (e.g., freezing behavior) to the mere presentation of the initially neutral stimulus. However, under specific conditions, shock exposure can be a severe stressor, and it has been used as a putative animal model for PTSD (Stam, 2007; Armario et al., 2008; Deslauriers et al., 2018; Török et al., 2019).

Notably, shock exposure can elicit not only conditioned responses but also unconditioned effects, both being relevant for PTSD. Pivotal experiments by Wotjak and colleagues showed that C57BL/6N mice exposed to a single intense foot-shock exhibited not only freezing in the shock context (conditioned effects), but also a heightened freezing response to a neutral tone in a novel environment (i.e., hyperarousal), which reflects a non-associative phenomenon (sensitization) (Siegmund and Wotjak, 2007a, 2007b). In addition, Fanselow and collaborators have shown that prior exposure to a single session of severe foot-shocks in a particular context induced a long-term enhancement of contextual and tone fear conditioning in a different context, which was still observed after fear extinction in the initial context, thus providing further evidence for long-lasting stress-induced sensitization (Rau et al., 2005; Rau and Fanselow, 2009; Perusini et al., 2016; Gonzalez et al., 2021).

One of the most consistent effects of acute or repeated exposure to shocks in rats has been long-lasting (days to weeks) hypoactivity in environments completely different from the shock context (e.g., an open field) and enhanced immobility after sudden changes in the environment occur (e.g., Levine et al., 1973; van Dijken et al., 1992a; 1992b; van den Berg et al., 1998; Pijlman & Van Ree, 2002). These effects were considered to be due to shock-induced sensitization and, therefore, a non-associative process. However, other studies in both rats and mice have shown that hypoactivity does not occur when fear conditioning acquisition is prevented (Radulovic et al., 1998; Daviu et al., 2010; Sauerhöfer et al., 2012), suggesting that it is, in fact, an associative phenomenon. Importantly, this hypoactivity can be observed in the absence of freezing (Daviu et al., 2010).

It has been classically considered that freezing observed in a different context after fear conditioning could be explained by a fear generalization phenomenon, due to presence of some common perceptual features between the shock context and the novel one (O'Reilly & Rudy, 2001; Fanselow, 2010). However, it is improbable that hypoactivity in apparatuses like a circular open field or a hole-board can be explained by the animals not distinguishing

between the shock chamber and those contexts. Therefore, we have proposed the term conditioned cognitive fear generalization (Daviu et al., 2014; Fuentes et al 2018) to remark that after experiencing shocks in a particular environment animals develop low levels of fear to any unknown environment despite the major dissimilarities from the shock context. Regardless of whether this new term is relevant to explain the phenomenon, this generalization is not dependent on an overall increase in anxiety, as evaluated by classical tests such as the elevated plus-maze (Radulovic et al., 1998; Daviu et al., 2010; 2014; Viviani et al., 2012). However, it might interfere with animal behavior in a variety of tests, potentially leading to some misinterpretations.

It is reasonable to consider that both the strength of conditioned fear learning and the development of conditioned fear generalization might be related to the parameters of the shock procedure (i.e., number and intensity). However, a comprehensive understanding of how these parameters influence the long-term behavioral consequences and stress response following fear conditioning remains elusive. Previous studies have mainly focused on the impact of shock parameters on the magnitude of fear conditioning. Whereas most reports support a positive relationship between shock intensity and context or cued fear conditioning (e.g., Cordero et al., 1998; Milanovic et al., 1998; Merino et al., 2000; Baldi et al., 2004; Santos et al., 2005; Wöhr et al., 2005; Quinn et al., 2008; Poulos et al., 2016; dos Santos Correa et al., 2019), others do not (Luyten et al., 2011a; Archbold et al., 2010). Regarding shock number, the available data consistently indicates reduced freezing levels after a single versus multiple shock exposures, with minimal or negligible influence when more than 3 shocks were administered (Laxmi et al., 2003; Quinn et al., 2008; Luyten et al., 2011b; Lattal and Maughan, 2012; Poulos et al., 2016). To our knowledge, only one study has investigated the impact of shock intensity on long-lasting hypoactivity in novel environments, wherein rats subjected to higher shock intensities displayed increased hypoactivity levels in a novel environment 5 days after 5 daily sessions of foot-shock exposure (Pijlman et al., 2002).

In the present study, we aimed to investigate the influence of foot-shock number and intensity on contextual fear conditioning and conditioned fear generalization. In addition, to assess whether distinct shock conditions induce a differential physiological stress response, we measured plasma levels of hypothalamic-pituitary-adrenal (HPA) hormones (adrenocorticotropic hormone, ACTH, and corticosterone) and prolactin, all of them well-characterized endocrine markers of stress intensity (Armario et al., 2020). PTSD involves both associative and non-associative consequences of trauma. Therefore, studying how foot-shock characteristics influence its endocrine and behavioral consequences (associative and non-associative) will contribute to a better characterization of this stressor as a putative animal model of PTSD, as this pathology involves both.

2. MATERIALS AND METHODS

2.1. Animals and general procedure

Sixty eight-week-old male Sprague–Dawley rats from the breeding center of the Universitat Autònoma de Barcelona were used. The animals were housed in pairs and maintained under standard conditions of temperature (21 ± 1 °C) and in a 12 h light-dark cycle (lights on at 8:00 am). Food and water were available *ad libitum*. The experimental protocol was approved by the Ethics Committee at the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, and it was carried out in accordance with the European Council Directive (2010/63/UE) and Spanish legislation (RD 53/2013).

All experimental procedures were conducted during the light period. Five days after their arrival, all animals were handled for 2 minutes per day for 3 days before the start of the experiments. Blood samples were taken by the tail-nick procedure, which allows obtaining true resting levels of hormones (Belda et al., 2004). A blood sample was collected under resting conditions 4 days before fear conditioning training to habituate the rats to the procedure. In a small subset of rats (n = 10), hormone levels were analyzed to obtain reference basal levels. Later, all rats were blood sampled after fear conditioning training and after all the behavioral tests. Cage-mates were always sampled simultaneously. Blood (300 µl) was collected within 2 min into ice-cold EDTA capillary tubes (Sarsted) and centrifuged at 4 °C, and the plasma was frozen at -20 °C until analysis. Plasma ACTH, corticosterone, and prolactin levels were determined by well-established double-antibody radioimmunoassays (RIAs) as described previously (Márquez et al., 2006).

2.2. Experimental design and procedures

Rats were assigned at random to six experimental groups (n = 10/group). Four groups were formed based on the number of 3-second duration shocks (2 or 15) and their intensity (low 0.4 or high 1.5 mA), thus resulting in the L2, L15, H2, and H15 groups. An additional H15 group was included that was not exposed to fear extinction (H15-NE). Finally, the control group was exposed to the shock chamber without receiving shocks. Shocks were generated by a LETICA LI 2700 instrument.

The detailed experimental protocol is illustrated in Fig. 1. On day 1 (fear training), after 3 min of habituation to the shock chamber (Context A), the animals received the corresponding number and intensity of shocks. Rats from the L2 and H2 groups received 2 shocks with an intertrial interval (ITI) of 7 min, whereas rats from the L15, H15 and H15-NE groups received 15 shocks with an ITI of 30 seconds. Animals were removed from the shock chamber 3 min after the last shock. The control group was exposed to the shock chamber for the same amount of time but without receiving shocks. On day 3 (48 h after contextual fear training), all animals

were exposed to a novel environment for 15 min (Context B). The animals were tested in Context B before being re-exposed to Context A to prevent possible interference from contextual fear extinction with the development of shock-induced hypoactivity. On days 4, 5, and 6, all animals (except those of the H15-NE group) were re-exposed for 15 min to the conditioned context A to undergo an extinction procedure (extinction sessions 1, 2, and 3). On day 7, all groups, including the H15 - NE group, were tested in another novel environment (Context C) for 15 min. Behavior was recorded during all sessions with a video camera for later analysis. Immediately after the tests, blood samples were taken after the shock sessions and after exposure to context A and context B.

The three contexts were Plexiglas cages of the same size (57 x 41 x 70 cm) with differential characteristics, although the frontal wall was always transparent to allow frontal recording of behavior. Notably, this context is bigger than those typically used for fear conditioning in rats, which have dimensions of approximately 20 cm (e.g., Fanselow 1985; Bevins et al., 1997) or 30 cm (Cordero et al., 1998) per side. This decision was based on the fact that fear conditioning apparatuses for mice, in which behaviors other than freezing have been assessed (Radulovic et al., 1998; Laxmi et al., 2003), are proportionally larger than the ones used for rats. The relatively limited space available to rats could limit the display of exploratory behaviors as an alternative to freezing. Thus, in the present study, apart from time spent freezing we also assessed the total distance traveled as a measure of activity.

Context A consisted of white lateral and back walls, with a grid floor of 44 stainless steel rods (Panlab, Harvard, Barcelona), located in a room with white walls and fluorescent light. The apparatus was cleaned between animals with ethanol (5% v/v). Animals were transported from the vivarium to the experimental room in a small white plastic box (29 x 27 x 14 cm) without bedding covered with a piece of cloth. Context B consisted of black and rough lateral and back walls, with a black floor containing four equidistant holes (4.5 cm diameter) to imitate a hole-board apparatus, placed in a room with black walls and red light. The apparatus was cleaned with a water solution containing soap. Animals were transported to the experimental room in their home cages. Context C consisted of black and smooth lateral and back walls (a little red rough piece of plastic in the middle of the walls), with a black and smooth floor, and it was located in a room with black walls and white light (25 W). The apparatus was cleaned with a water solution containing soap (dishwasher Mistol®, 1ml/1L of water). Animals were transported to the experimental room in their home cages.

2.3. Behavioral measures

Freezing was manually scored by an experimenter blind to the experimental condition. Freezing involved the absence of all movements except for respiration. The reliability of

freezing measurements was demonstrated by the high interrater correlation (r=0.99). The distance traveled was assessed by video tracking analysis using the center of gravity of the animal (Smart version 2.5.21, Panlab Harvard, Barcelona, Spain). Except for the training day, distance traveled and freezing were quantified in 3 blocks of 3 min, corresponding to 0-3 min, 6-9 min, and 12-15 min of the tests.

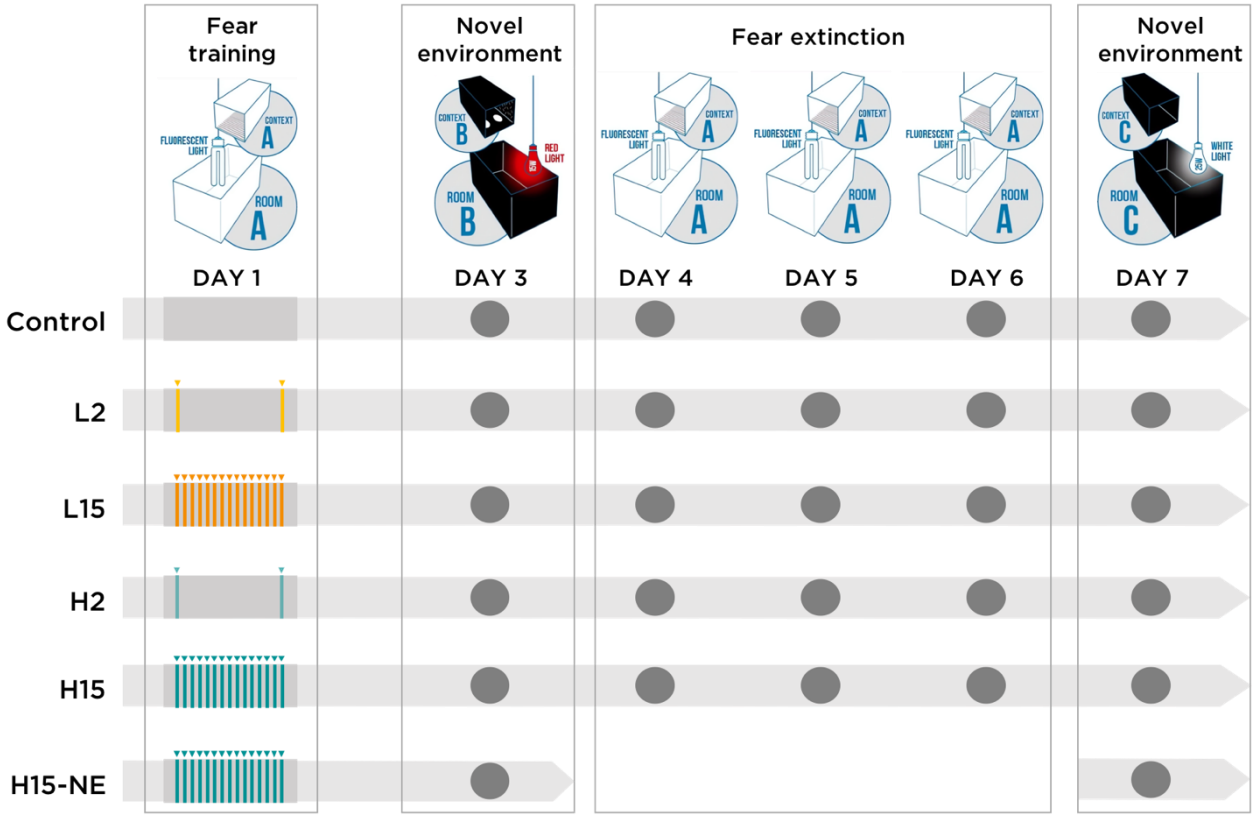

Figure 1. Schematic representation of the experimental design. Rats were randomly assigned to six experimental groups (n = 10/group) and underwent fear conditioning training on day 1 (context A). Group L2 and L15 were exposed to 2 and 15 0.4 mA shocks, respectively. Groups H2 and H15/H15-NE were exposed to 2 and 15 1.5 mA shocks, respectively. The control group was exposed to the context without receiving shocks. On day 3, all animals were exposed to a novel environment (context B). On days 4, 5, and 6 all groups, except the H15-NE group, underwent fear extinction in context A. On day 7, all groups were exposed to a novel environment (context C). The grey circles represent the behavioral paradigms to which the rats were exposed after fear training. Blood sampling was conducted after fear training and after all tests in all subjects.

2.4. Statistical analysis

Statistical analysis was conducted with the Statistical Package for Social Sciences (SPSS) version 24 for Windows. Generalized linear model (GzLM; McCulloch and Searle, 2001) were used when only between-subjects factors were included. Generalized estimating equations model (GEE; Hardin & Hilbe, 2003) were used when within-subjects factors were also included (e.g., behavior at different times within a particular session or between sessions). These

procedures were used instead of conventional ANOVA because they do not require normality or homogeneity of variances. The significance of the effects was determined by the Wald chi-square statistic (χ^2). Log x+1 for freezing in all tests and log of prolactin values during acquisition were used to improve the homogeneity of variances. The criterion for significance was set at $p < 0.05$.

A major purpose of the present study was to assess the behavioral impact of both number and intensity of shocks. Therefore, the main analysis excluded both the control group and the non-extinguished group (H15-NE) to specifically address the effects of the between-subjects factors number (2 levels) and intensity (2 levels) of shocks. In addition, the within-subjects factor time (3 levels) were included when analyzing behavior in context B and the within-subjects factor time (3 levels) and extinction session (3 levels) when analyzing behavior in the shock context A. If interactions between factors were found, further decomposition of interactions were done when they involved number or intensity of shocks.

For the analysis of the hormonal response, we used a partially different approach. A first preliminary GzLM analysis that included one single factor ("shock condition") with five levels (each of the four shocked groups and the control group) aimed to demonstrate which of the shocked groups differed from the group merely exposed to the shock chamber. In this case, if the factor was significant, planned post-hoc comparisons of each shocked group with the control group were done, without corrections. Then, in a second analysis, the specific contribution of the factors number and intensity of shocks (2 x 2 design) was studied. If an interaction was found, post-hoc comparisons were done using sequential Bonferroni correction.

Because all the analysis detailed above excluded the non-extinguished group (H15-NE), for the specific analysis of the contribution of extinction, we first used a t-test to compare this group with the H15 group before extinction and then a GzLM with the factor "group" (three levels: controls, H15 and H15-NE) to study the specific contribution of extinction. Post-hoc comparisons were performed when appropriate using sequential Bonferroni.

3. Results

3.1. Behavioral and endocrine response to contextual fear conditioning training

We evaluated the behavior of rats in the 3 min after the last shock during the fear training session. The analysis of the immediate post-acquisition behavior revealed a significant effect of the number of shocks for the distance traveled ($\chi^2(1) = 19.5$, $p < 0.001$; Fig. 2A) and time spent freezing ($\chi^2(1) = 69.3$, $p < 0.001$; Fig. 2B), but not of intensity.

Regarding the endocrine response, basal levels of hormones were measured in a subset of rats 4 days before fear training to have a reference for the magnitude of changes in response to training and testing. The values (mean and S.E.M., n=10) were: 67±7 pg/ml for ACTH, 9.9±2.7 ng/ml for corticosterone and 2.1±0.3 ng/ml for prolactin. During the training for fear conditioning in context A, a significant effect of "shock condition" was found for ACTH ($\chi^2(4) = 76.5$, $p < 0.001$), corticosterone ($\chi^2(4) = 20.6$, $p < 0.001$), and prolactin ($\chi^2(4) = 110.6$, $p < 0.001$). Planned post-hoc comparisons showed that ACTH (Fig. 2C) and prolactin (Fig. 2E) levels were significantly higher in all shocked groups compared to controls, whereas corticosterone (Fig. 2D) only differed from controls in the high-intensity shock groups (p at least < 0.05).

The analysis of the specific effects of shock number and intensity on ACTH (Fig. 2C) indicated significant effects of number ($\chi^2(1) = 16.9$, $p < 0.001$), intensity ($\chi^2(1) = 16.5$, $p < 0.001$) and number x intensity interaction ($\chi^2(1) = 4.417$, $p = 0.036$). Post-hoc comparisons with sequential Bonferroni corrections revealed that ACTH levels in the H15 group were significantly higher than in the other three groups ($p < 0.001$ in all cases). The analysis of corticosterone (Fig. 2D) showed only a significant effect of foot-shock intensity ($\chi^2(1) = 15.8$, $p < 0.001$). Finally, the analysis of prolactin levels (Fig. 2E) revealed significant effects of shock number ($\chi^2(1) = 14.7$, $p < 0.001$) and intensity ($\chi^2(1) = 10.5$, $p = 0.001$), without significant number x intensity interaction. Thus, these results highlight that the neuroendocrine response to contextual fear conditioning training is sensitive to shock number and intensity, albeit with subtle differences among the three hormones.

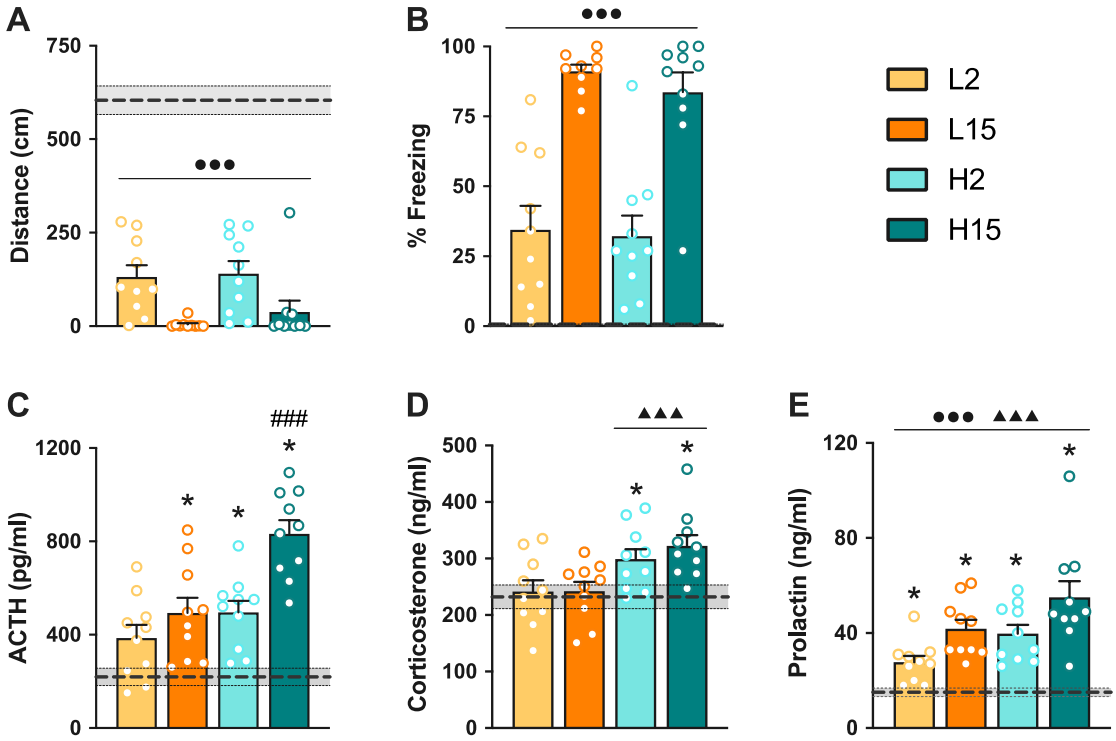

Figure 2. Behavioral and endocrine response to contextual fear conditioning training (context A). Data shown as mean + S.E.M. Distance traveled (A) and time spent freezing (B) immediately after the last shock of the fear training session and plasma levels of ACTH (C), corticosterone (D) and prolactin (E) immediately the session. The experiment includes a control non-shocked group, exposed to the shock chamber, and four shocked groups: L2 and L15, exposed to 2 or 15 shocks of low intensity (0.4 mA); and H2 and H15, exposed to 2 or 15 shocks of high intensity (1.5 mA), respectively. Dashed lines and shadowed area represent the average values and S.E.M., respectively, of the control group. * indicates significant differences with respect to the control group (p at least < 0.05). # indicates significant differences compared with the other three shock groups, ▲ significant overall effect of shock intensity, ● significant overall effect of shock number; in all cases, one symbol indicates p< 0.05, two symbols p< 0.01 and three symbols p< 0.001.

3.2. Behavioral and endocrine response to a novel context B before test/extinction

We exposed control and shocked rats to a novel environment (hole-board-like, context B) two days after fear conditioning and evaluated their behavioral and endocrine response.

The analysis of the distance traveled (Fig. 3A) revealed a marginally significant effect of intensity ($\chi^2(1) = 3.6$, $p = 0.059$), a significant effect of time ($\chi^2(2) = 32.1$, $p < 0.001$) and marginally significant interactions number x time ($\chi^2(2) = 5.4$, $p = 0.067$) and intensity x time ($\chi^2(2) = 5.3$, $p = 0.072$). Decomposition of the interactions did not reveal any significant effect of number at any time, but revealed that high intensity shocks resulted in lower activity at the two first time blocks ($p = 0.012$ and $p = 0.023$, respectively).

The analysis of freezing levels (Fig. 3B) revealed significant effects of intensity ($\chi^2(1) = 7.7$, $p = 0.006$), time ($\chi^2(2) = 34.1$, $p < 0.001$) and the interaction intensity x time ($\chi^2(2) = 11.1$, $p = 0.002$). Decomposition of the interaction indicated that intensity increased freezing at time block 1 ($p = 0.018$) and 2 ($p < 0.001$), but not at time block 3.

Regarding the endocrine response to context B, we found a significant effect of "shock condition" on ACTH ($\chi^2(4) = 22.0$, $p < 0.001$; Fig. 3C) and corticosterone ($\chi^2(4) = 22.3$, $p < 0.001$; Fig. 3D), but not on prolactin (Fig. 3E). Planned post-hoc comparisons of ACTH response revealed significantly higher levels in all shocked groups compared to controls (p at least < 0.05). Post-hoc comparisons of corticosterone levels indicated significantly higher levels in all shock groups (p at least < 0.05) except the L2 group.

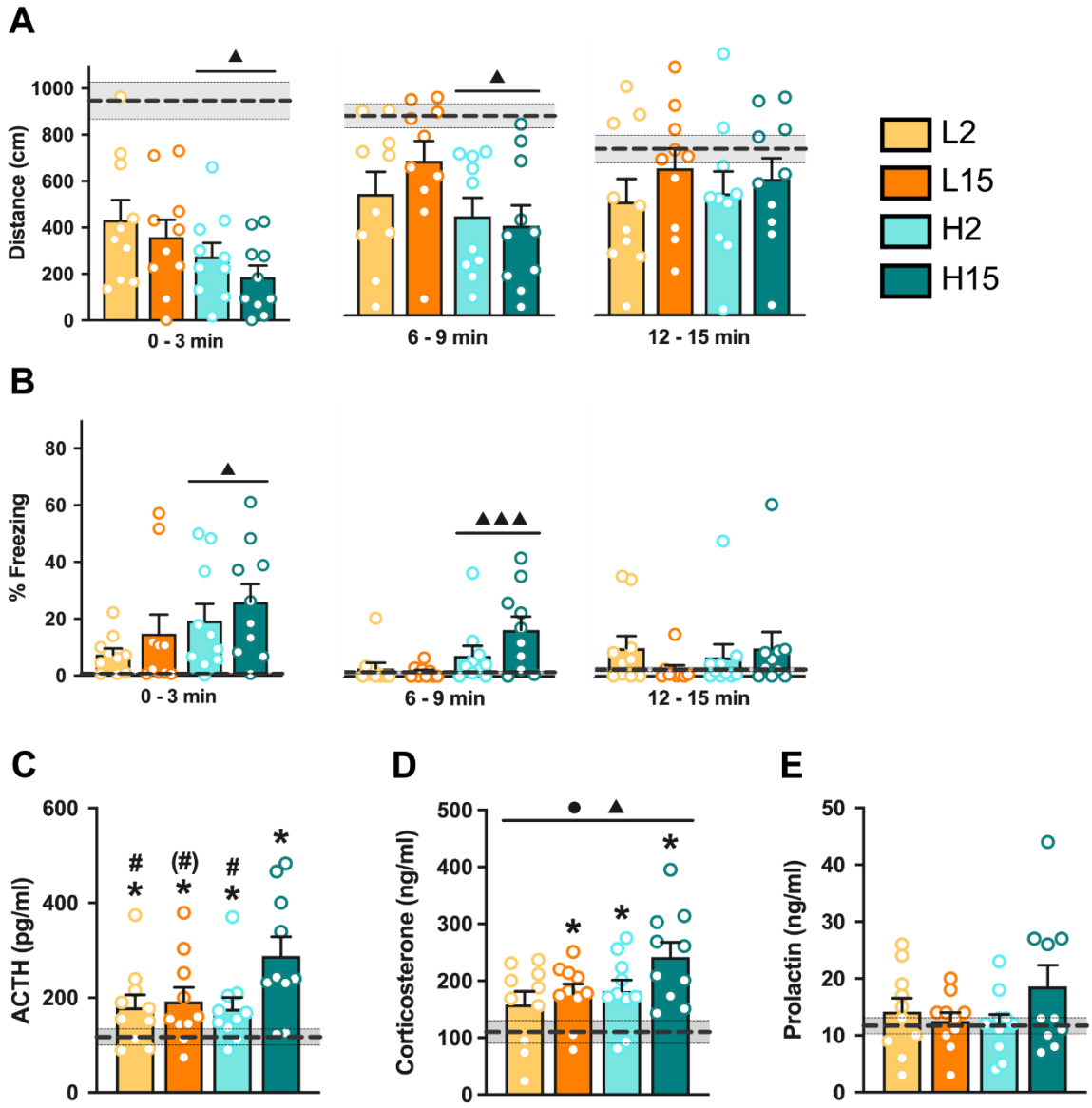

Figure 3. Behavioral and endocrine response of control and shocked rats to a novel context (context B, hole-board). Data shown as mean + S.E.M. Total distance traveled (A) and percentage of time freezing (B) in three blocks of 3 min each. Plasma levels of ACTH (C), corticosterone (D) and prolactin (E) immediately after 15 min exposure to context B. Dashed lines and shadowed area from C to G represent the average values and the S.E.M., respectively, of the control group (note that time freezing was near cero in the control group). * indicates significant differences with respect to the control group for each particular block; # indicates significant difference and (#) marginally significant difference with respect to the H15 group; ▲ indicates overall effect of shock intensity and ● indicates overall effect of shock number. Note that significant effect of time was typically found but not indicated. In all cases, one symbol indicates $p< 0.05$, two symbols $p< 0.01$ and three symbols $p< 0.001$.

These results point towards a marked hypoactivity in novel environments following contextual fear conditioning that is notably influenced by shock intensity but not by shock number. Moreover, ACTH and corticosterone response is generally higher in shocked rats, with a modest contribution of number or intensity of shocks.

3.3. Conditioned behavioral and endocrine response to context A

We analyzed behavioral response over time and sessions, using GEE. The analysis of the distance traveled (Fig. 4A) revealed significant effects of time ($\chi^2(2) = 12.3$, $p = 0.002$), session ($\chi^2(2) = 77.5$, $p = 0.022$) and the interactions intensity x time ($\chi^2(2) = 7.1$, $p = 0.028$), time x session ($\chi^2(4) = 37.4$, $p < 0.001$) and number x time x session ($\chi^2(4) = 15.4$, $p = 0.004$). Decomposition of the interactions intensity x time did not reveal any effect of intensity at a specific time. Decomposition of the number x time x session interaction indicated that rats exposed to 15 shocks showed less activity than those exposed to 2 shocks in the first time block of extinction 2 session ($p = 0.046$). No other relevant effect was found.

The analysis of freezing (Fig. 4B) only revealed significant effects of time ($\chi^2(2) = 8.9$, $p = 0.012$), session ($\chi^2(2) = 61.5$, $p < 0.001$) and the interaction time x session ($\chi^2(4) = 44.2$, $p < 0.001$). As expected freezing decreased over time within each session and between sessions.

Regarding hormones, during re-exposure to context A for the first extinction session, no significant effect of “shock condition” was found for ACTH (Fig. 4C) or prolactin (Fig. 4E), but it was for corticosterone ($\chi^2(4) = 11.9$, $p = 0.018$) (Fig. 4D). Post-hoc comparisons showed that corticosterone levels were higher than in controls only in the H15 group ($p <0.05$). Further analysis with shock number and intensity as factors revealed an effect of shock intensity on corticosterone levels ($\chi^2(1) = 6.9$, $p = 0.009$), without significant effects on ACTH and prolactin. After the second and third extinction session no significant effect of “shock condition” was found for ACTH or corticosterone (data not shown). Prolactin was not measured because of low availability of iodinated hormone and the lack of effects in context B and the first extinction session.

Together, these results suggest that neither the number nor the intensity of shocks have a clear influence in the development of contextual fear conditioning, although a higher number of shocks appears to modestly slow down the extinction of the hypoactivity. In addition, we observed a low sensitivity of hormones to fear conditioning.

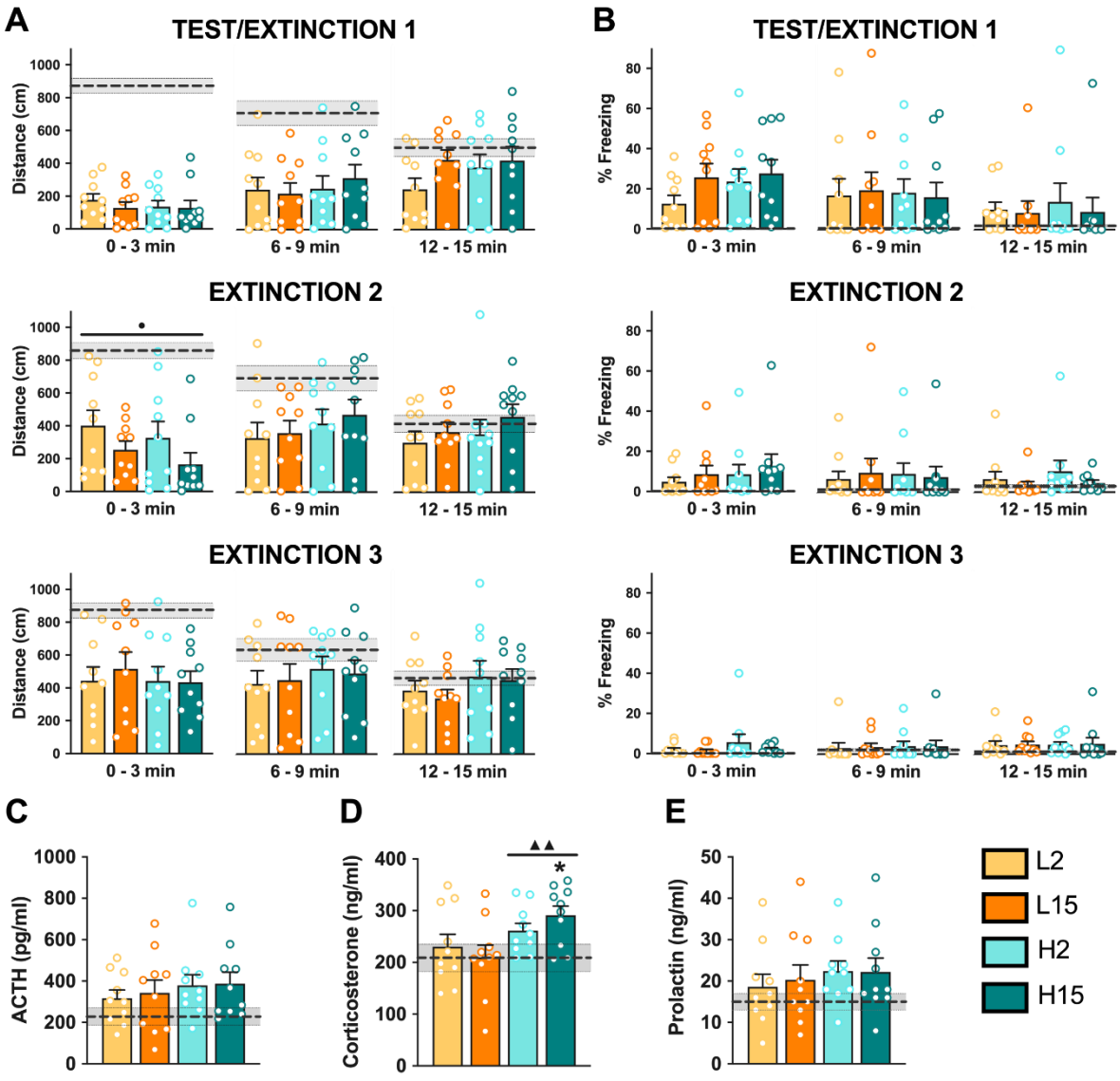

Figure 4. Behavioral and endocrine response to extinction sessions in the conditioned context A in control and shocked rats. Data shown as mean + S.E.M. Total distance traveled (A) and percentage of time freezing (B) in three blocks of 3 min each for each of the three extinction sessions (extinction 1-3) are indicated. Plasma levels of ACTH (C), corticosterone (D) and prolactin (E) immediately after the first extinction session. In the subsequent extinction sessions, no significant endocrine changes were found for ACTH or corticosterone (data not shown) and prolactin was not measured. Dashed lines and shadowed area from C to G represent the average values and the S.E.M., respectively, of the control group (please not that freezing levels of controls were near cero). * indicates significant differences with respect to the control group for each particular block or over all blocks; ● indicates overall effect of shock number at this particular session. ▲ indicates overall effect of shock intensity. Note that significant effect of time and session were typically found but not indicated in the figure. In all cases, one symbol indicates p< 0.05, two symbols p< 0.01 and three symbols p< 0.001.

3.4. Behavioral and endocrine response to a novel context C after fear extinction

Rats were exposed to a novel context (context C) after fear extinction and the behavioral and hormonal responses were analyzed following the same procedure as in context B.

The GEE analysis of distance traveled (Fig. 5A) revealed only a significant effect of time, with activity decreasing throughout the session. The GEE analysis of freezing (Fig. 5B) revealed significant effects of time ($\chi^2(2) = 46.5$, $p < 0.001$) and the interaction number x time ($\chi^2(2) = 6.2$, $p < 0.045$), but decomposition of the interaction revealed no specific effect shock number at any time. In addition, no significant effect of "shock condition" was found for ACTH or corticosterone response to context C (Fig. 5C, D). Prolactin was not measured, as explained previously.

Thus, these findings indicate that extinction following contextual fear conditioning markedly reduces freezing behavior in a novel context and slightly decreases hypoactivity, and this process seems to be independent on the number or intensity of shocks used during fear conditioning.

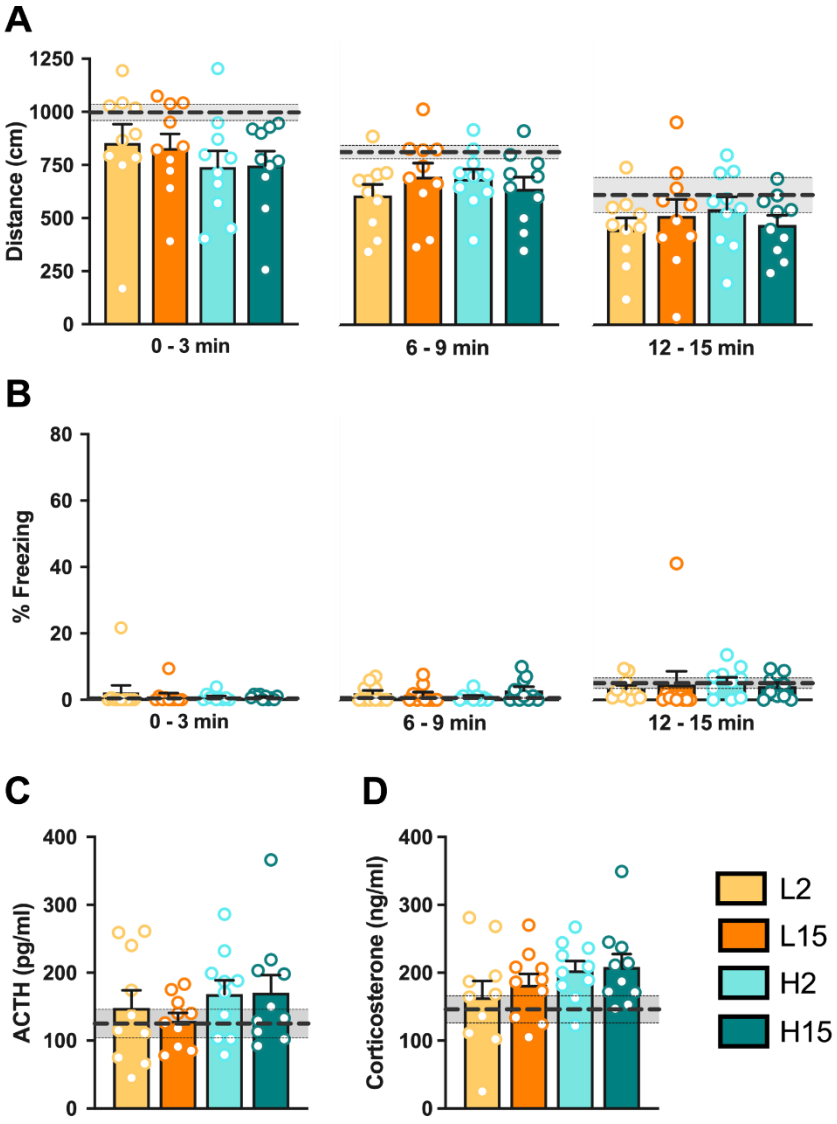

Figure 5. Behavioral response of control and shocked rats in a novel context (context C) after 3 extinction sessions in context A. Data shown as mean + S.E.M. Total distance traveled (A) and percent time spent freezing (B) in control and shocked groups throughout three time blocks of 3 min in context C. Plasma levels of ACTH (C) and corticosterone (D) immediately after exposure to context C. Prolactin was not measured. Dashed lines and shadowed area from D-F represent the average values and the S.E.M., respectively, of the control group (freezing levels of controls were near cero and are not shown). Note that significant effect of time was found but not indicated.

3.5. Comparison of the behavioral response to a novel context C in no-extinction and extinction groups

We first aimed to confirm that the H15 and H15 - NE groups did not differ in any of the parameters evaluated before extinction. T-test analysis indicated that the two groups did not differ in the endocrine response (ACTH, corticosterone and prolactin) to fear conditioning training or the levels of activity and freezing in context B (Fig. 6A-E). Then, we compared the two groups together with the control group using a GzLM with the factor “group” (three levels: controls, H15 and H15-NE), followed by sequential Bonferroni corrections for post-hoc comparisons.

The analysis of the distance traveled during the 3 first minutes in context C (Fig. 6F) revealed a significant “group” effect ($\chi^2(2) = 39.4$, $p < 0.001$) with both H15 and H15 - NE groups showing reduced distance traveled compared to the control group ($p = 0.01$ and $p < 0.001$, respectively). Notably, the activity of the H15 - NE group was much lower than that of the H15 group ($p < 0.001$). Analysis of the percent time spent freezing (Fig. 6G) showed a significant effect of “group” ($\chi^2(2) = 34.9$, $p < 0.001$), but in this case the H15 - NE group displayed significantly higher freezing levels than the control and H15 groups ($p < 0.001$ for both), while no differences were observed between the H15 group and the control. Together, these findings suggest that fear extinction mitigates hypoactivity in a subsequent exposure to a novel context, while it reduces freezing behavior to levels comparable to controls.

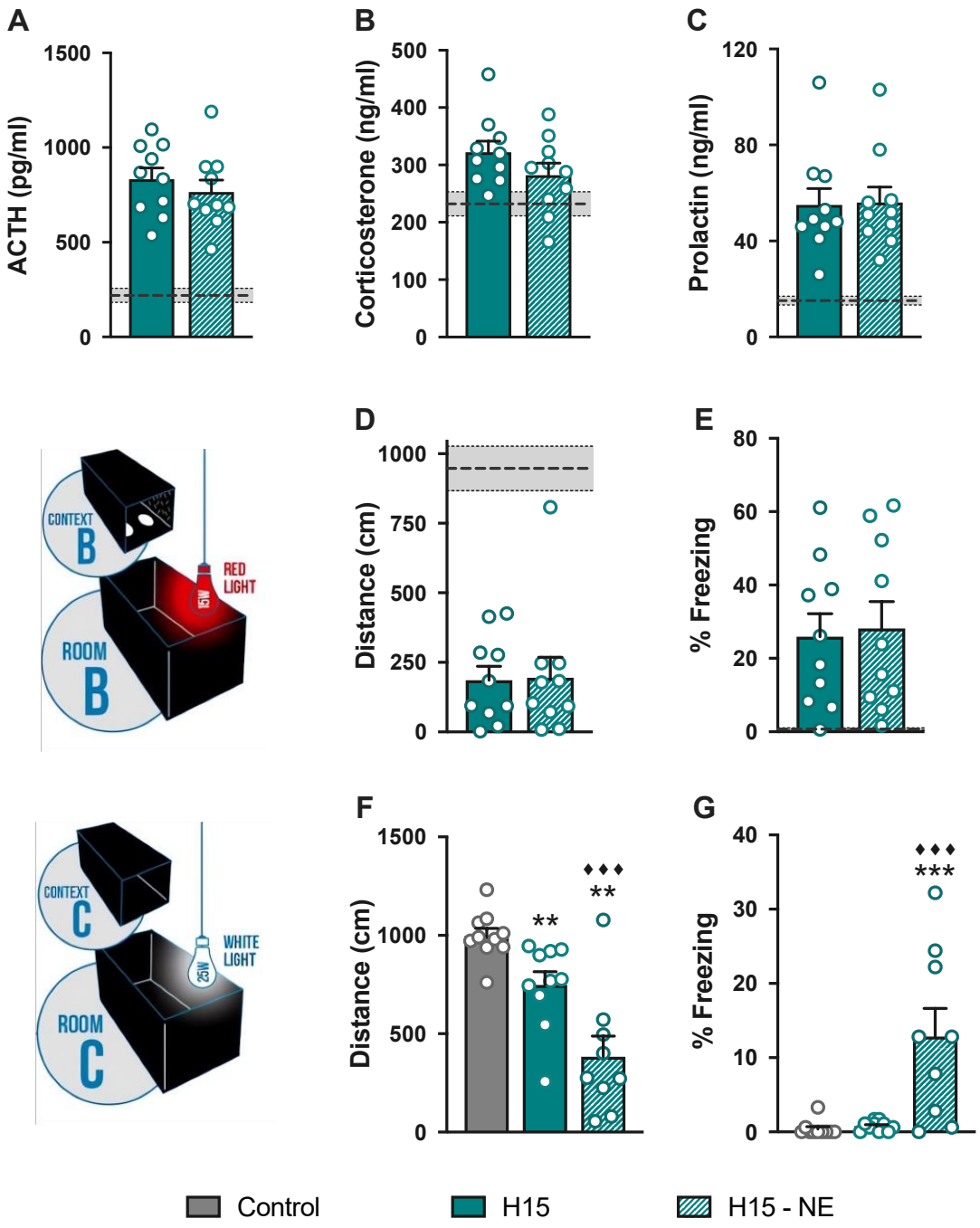

Figure 6. Prior fear extinction affects the behavioral response to a novel context C. Data shown as mean + S.E.M. One H15 group was exposed to fear extinction and another H15 group was not (H15-NE). Note that ACTH (A), corticosterone (B) and prolactin (C) responses to shock exposure during training as well as the that total distance traveled (D) and the percent time spent freezing (E) in the first 3 minutes of exposure to context B were similar in the H15 and H15-NE groups. Dashed lines and shadowed area from A to E represent the average values and the S.E.M., respectively, of the control group (please not that freezing levels of controls were near cero). After extinction (H15 group), total distance traveled (F) and percent time spent freezing (G) in the first 3 minutes of exposure to context C were measured in the control, H15 and H15-NE groups. * indicates differences versus control and ◆ differences versus H15. In all cases, two symbols $p < 0.01$ and three symbols $p < 0.001$.

3.6. Correlation between hormones and behavior

Pearson correlations between behavior (freezing time and distance traveled) and ACTH, corticosterone and prolactin levels were calculated after the training session, context B (generalization) and context A (test/first extinction session) considering the four groups exposed to shocks (n = 40). We found significant correlations of freezing time with ACTH and prolactin levels after training ($r = 0.39$, $p = 0.014$; $r = 0.42$, $p = 0.008$, respectively), significant correlation between freezing time and corticosterone response to context B ($r = 0.33$, $p = 0.039$) and between freezing time and prolactin levels during the first session in context A ($r = 0.34$, $p = 0.033$).

4. Discussion

In the present study, we assessed the influence of the number and intensity of shocks during contextual fear conditioning training on the response of well-characterized biological markers of stress, the conditioned behavior in the shock context, and the generalization of fear to different novel environments. Our results indicated that the number and intensity of shocks during training had an impact on different biological markers of stress, with both factors impacting ACTH and prolactin levels. However, the behavioral response followed a different pattern: (i) post-acquisition freezing was influenced by the number of shocks but not their intensity, (ii) fear memory showed minimal sensitivity to variations in shock parameters, and (iii) fear generalization to novel environments was dependent on the severity of the shocks. Fear generalization (i.e., hypoactivity) was only partially prevented by fear extinction, suggesting a dual contribution of associative and non-associative components.

4.1. Endocrine consequences of fear conditioning

To investigate the impact of the number and/or the intensity of shocks on the stress levels experienced by rats during fear training (context A), we relied on plasma levels of ACTH, corticosterone, and prolactin. ACTH and corticosterone are commonly used markers of stressor intensity that reflect activation of the HPA axis, although with certain limitations in the case of corticosterone (see Armario et al., 2020). We incorporated prolactin as an additional marker of stress intensity independent of the HPA axis, thereby strengthening the robustness of our findings. The overall picture confirmed that the neuroendocrine response was sensitive to variations in shock number and intensity, in accordance with previous findings (e.g., Kant et al., 1983; Cordero et al., 1998; Merino et al., 2000). Notably, subtle distinctions emerged among the three markers: ACTH was particularly sensitive to the combination of high intensity and high shock number, corticosterone primarily to intensity, and prolactin to both parameters. While interpreting these subtle differences remains speculative due to the scarcity of studies

including the three hormones, this topic holds potential interest for further investigation and highlights the importance of simultaneously measuring different stress-related hormones.

Two days after fear conditioning training, rats were exposed to a novel environment (context B) that markedly differed from the shock context to study fear generalization. Whereas prolactin levels were similar in all groups, ACTH and corticosterone levels showed group differences similar to those observed during training. However, during re-exposure to shock context A the following day, only corticosterone exhibited group differences, with higher levels in the two groups exposed to the highest shock intensities (Fig. 2B). As hypoactivity and freezing during exposure to contexts B and A showed low sensitivity to shock number or intensity (see next section), the changes observed in HPA hormones do not appear to reflect fear conditioning. Instead, they could potentially be explained by sensitization (non-associative) of the HPA axis caused by shock exposure during training, as the magnitude and duration of sensitization has been found to be proportional to the intensity of the stressors (Belda et al., 2015; 2016). Importantly, this phenomenon is most prominent within the first 48 hours after stress exposure, progressively diminishing thereafter. Given the transient nature of HPA sensitization, this was not observed regarding ACTH during exposure to the shock context the following day, although it was still noted with corticosterone in the high shock intensity groups. These results align with previous data indicating that corticosterone sensitization is more robust and long-lasting than ACTH sensitization, probably reflecting an impact on adrenal sensitivity to ACTH (Belda et al., 2015; 2020).

In the present study, ACTH levels were not sensitive to fear conditioning during the testing session, whereas corticosterone levels appeared to be sensitive only in the groups exposed to high-intensity shocks. The effects observed in corticosterone could be attributed to a sensitization mechanism rather than to fear conditioning itself because the increased levels were not present in the groups exposed to low-intensity shocks, which showed a clear contextual conditioning at the behavioral level. These results contrast with previous studies from our laboratory, where both hormones reliably reflected conditioning (Daviu et al., 2010; 2014). It is plausible that subtle differences, such as the stressful properties of the chamber itself, whose size was greater than usual in the present study, may have masked the specific contribution of conditioning on the endocrine response. Nevertheless, our results suggest that behavior might be more sensitive to the consequences of fear conditioning than the endocrine response.

We performed correlations between the behavioral and endocrine responses to fear training in context A, fear generalization in context B and fear testing in context A. After fear training, low but significant positive correlations were found between freezing time and levels of ACTH and prolactin, but not corticosterone. In contrast, no correlation between hormones and

distance traveled was observed. This suggests that despite the apparent lack of relationship between hormones and freezing when considering the means of the groups, the response of these two hormones may partially reflect freezing of individual subjects. In the other conditions, significant correlations between freezing and corticosterone in context B and freezing and prolactin during context A testing were observed. It is possible that correlation in context B reflects a common contribution of sensitization to both corticosterone response and freezing, whereas correlation in context A points to a greater sensitivity of prolactin versus ACTH to reflect conditioning. Although both hormones can potentially reflect fear conditioning (van der Kar et al., 1999), differential sensitivity has not been explored yet.

4.2. Impact of shock intensity and number on contextual fear conditioning and extinction

Freezing behavior has proven to be a reliable index of fear conditioning in rodents across different experimental conditions. Nonetheless, some studies in mice have shown that other parameters, such as exploratory activity and risk assessment, might add relevant information regarding conditioned fear (Stiedl et al., 1999; Laxmi et al., 2003). For this reason, alongside freezing, we opted for measuring activity (i.e., distance traveled) as a complementary measure of fear. Remarkably, to favor the display of behaviors beyond freezing, we employed a shock context larger in size than what is typically used for contextual fear conditioning in rats.

In this study, shocked rats, regardless of the number and intensity of shocks, displayed a typical behavioral pattern during the three sessions of exposure to the shock context A (Fig. 4). There was a progressive reduction in freezing levels within and between sessions, indicative of successful fear extinction. Activity followed roughly an opposite trend, but shocked rats still displayed lower levels of activity than controls during the third extinction session, whereas freezing levels did not differ. These findings suggest that hypoactivity may reflect a lower level of fear than freezing and that it could serve as a valuable complementary measure of fear. When examining the specific contribution of the number and intensity of shocks to fear expression and extinction, hypoactivity showed a modest sensitivity to shock number during the second extinction while freezing was unaffected by these parameters, at least under the conditions of the present study.

Overall, the present results suggest that fear conditioning is not highly sensitive to manipulations of shock number and intensity, although we observed that freezing measured immediately after shocks was influenced by shock number but not intensity. We are not aware of any study about contextual fear conditioning reporting such immediate impact of shock number. Nevertheless, the lack of effect of shock intensity and number on long-term contextual fear memory is in accordance with some previous studies (e.g. Bevins et al., 1997; Baldi et al.,

2004 for shock intensity, Quinn et al., 2008 for shock number). In contrast, other authors have reported that freezing levels are positively related to shock intensity (e.g., Merino et al., 2000; Wöhr et al., 2005; Luyten et al., 2011a; dos Santos Correa et al., 2019). Discrepancies may be partially attributed to the specific range of shock intensities used in the previous studies, as differences are clearly observed when the range of shock intensities include very low levels (0.1-0.2 mA), but not when intensities are higher than 0.6 mA, pointing towards a ceiling effect on the expression of conditioned fear with relatively high shock intensities. The number of shocks could also be relevant when using a few number (Landeira-Fernandez et al., 2006). Some procedural variations such as shock duration and the specific sensitivities of the species/strains used in the experiments might contribute to these inconsistencies. Studies using auditory fear conditioning (Kamprath and Wotjak, 2004) have suggested that the differential impact of shock intensity might be primarily explained by sensitization caused by high intensity shocks, rather than by actual differences in fear memory, although more studies are needed on this topic.

4.3. Impact of shock intensity and number on fear generalization and the influence of fear extinction

One of the main aims of this study was to assess whether shock intensity and number during contextual fear conditioning training could influence cognitive generalization of fear. Hence, animals were tested two days after training in a novel context (context B) with several critical features strongly differing from the shock context (e.g., color and texture of the walls and floor, odor). We observed increased freezing time and reduced activity in all animals previously exposed to shock. Hypoactivity is in full agreement with earlier studies (e.g., Van Dijken et al., 1992b; Radulovic et al., 1998; Van den Berg et al., 1998; Daviu et al., 2010; 2014). Although neither the intensity nor the number of shocks affected the magnitude of the conditioned fear response during subsequent extinction in context A, higher shock intensity was associated with increased freezing levels and hypoactivity in context B (Fig. 3A,B). These findings support the hypotheses that the intensity of the aversive stimulus is important for the generalization of fear to other contexts (Laxmi et al., 2003; Baldi et al., 2004), a critical aspect to consider for the development of animal models of PTSD.

Some studies have found that hypoactivity in novel environments was not observed when contextual fear memory was prevented by administering a single shock immediately after exposure to the context (Radulovic et al., 1998; Daviu et al., 2010), a condition, named immediate shock deficit, which disrupts the proper association between the context and the shock (Fanselow, 1990). Consequently, we hypothesized that fear extinction could reverse this cognitive generalization of fear when tested in a different novel environment (context C). Accordingly, seven days after fear conditioning, animals from the H15 group that underwent

fear extinction to context A exhibited minimal freezing levels in context C, not differing from control animals. Meanwhile, the H15-NE (non-extinguished) group displayed higher freezing levels (Fig. 6G). Importantly, extinction substantially reduced hypoactivity in context C in the H15 group compared to the H15-NE group, but a certain level of hypoactivity still persisted compared to the control group.

These results suggest that hypoactivity in novel environments is more resistant to extinction than freezing. Moreover, the residual hypoactivity still observed after extinction suggests a non-associative process, probably reflecting shock-induced emotional sensitization. In line with this, Siegmund and Wotjak (2007b) showed in mice enhanced acoustic startle response after shock exposure that was independent of fear conditioning and probably dependent on shock-induced sensitization. Additionally, this sensitization phenomenon could also explain the long-lasting potentiation of fear conditioning induced by prior exposure to inescapable foot-shocks, extensively characterized by Fanselow and colleagues (Rau et al., 2005; Perusini et al., 2016).

Freezing and/or hypoactivity observed in contexts differing from the original shock context are commonly attributed to fear generalization due to the incapability of the animals to distinguish between the original shock context and novel environments. While this might be the case when discrete configurational changes are made in the original context, it is difficult to accept this explanation when dramatic changes are introduced. We then consider that the best explanation is that animals that experience shock in a novel apparatus and learn this association will consider any novel environment as potentially dangerous (generalization of fear). However, in parallel to this associative phenomenon, under certain shock conditions, additional non-associative processes can develop. In the present study, this non-associative component was unveiled through fear extinction and reflected by this residual hypoactivity, whereas in other studies it has been reflected in the potentiation of further fear conditioning. We consider that hypoactivity in novel environments might reflect low levels of fear, based on the results obtained after extinction in the present study, although the characterization of the precise factors underlying hypoactivity requires further investigation. Considering that many behavioral tests involve exposure to novel environments, both fear generalization and sensitization-like processes could influence those paradigms to varying extents, potentially leading to misinterpretations. Therefore, it is crucial to dissect the specific contribution of these two components in future studies.

5. Conclusions and limitations

Clinical symptoms of exposure to traumatic situations in humans include both associative and non-associative aspects (Lissek & Van Meurs 2015; Liberzon & Abelson 2016). Our findings

suggest that the development of associative and non-associative behavioral changes after exposure to high-intensity foot-shocks might be a distinctive feature of this model compared with other animal models, placing inescapable shocks as one of the best animal models of PTSD. However, the present study has limitations that warrant further investigation. First, our results are limited to males, and it is crucial to evaluate in further studies the influence of sex on fear learning, fear extinction and fear generalization, especially because fear-related disorders, such as PTSD, are more prevalent in women (Olff et al., 2007). In addition, the present results highlight the need for studies addressing the contribution of distinct shock parameters, such as number and particularly intensity, to the associative and non-associative long-lasting consequences of shock exposure. Further follow-up studies should employ experimental designs that introduce more than two levels of shock intensity or use a group with daily exposure to a single shock in conditions that still maintain the immediate shock deficit but can potentially enhance sensitization due to repeated shock experience (Landeira et al., 2006). These investigations are crucial to better characterize the validity of foot-shock as a putative animal model of PTSD.

Fundings

This work was supported by Spanish grants (SAF2017-83430-R and PID2020-118844RB-I00) and the Generalitat de Catalunya (SGR21-00158) to AA and RN. Our group belongs to REIS (Red Temática Española de Investigación en Estrés), Ministerio de Ciencia e Innovación, REF. RED2022-134191-T. PM was recipient of a scholarship from Ministerio de Educación, Cultura y Deporte (FPU16/ 05416).

Ethical statements

The experimental protocol was approved by the Ethics Committee at the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, and it was carried out in accordance with the European Council Directive (2010/63/UE) and Spanish legislation (RD 53/2013).

Credit authorship contribution statement

Nuria Daviu: conceptualization, formal analysis, investigation, methodology, validation, review and editing; Patricia Molina: investigation, data curation, formal analysis, validation, visualization, writing original draft, review and editing; Roser Nadal: conceptualization, supervision, funding, project administration, resources, review and editing. Xavier Belda: investigation; Sara Serrano: data curation; formal analysis, validation, visualization; Antonio Armario: conceptualization, data curation, funding, project administration, resources, supervision, validation, writing original draft, review and editing.

Conflicts of interest

The authors declare no competing financial interest or potential conflict of interest.

5. BIBLIOGRAPHY

Archbold, G. E. B., Bouton, M. E., & Nader, K. (2010). Evidence for the persistence of contextual fear memories following immediate extinction. *European Journal of Neuroscience*, 31(7), 1303–1311. https://doi.org/10.1111/j.1460-9568.2010.07161.x

Armario, A., Escorihuela, R. M., & Nadal, R. (2008). Long-term neuroendocrine and behavioural effects of a single exposure to stress in adult animals. *Neuroscience and biobehavioral reviews*, *32*(6), 1121–1135. https://doi.org/10.1016/j.neubiorev.2008.04.003

Armario, A., Labad, J., & Nadal, R. (2020). Focusing attention on biological markers of acute stressor intensity: Empirical evidence and limitations. *Neuroscience and biobehavioral reviews*, *111*, 95–103. https://doi.org/10.1016/j.neubiorev.2020.01.013

Baldi, E., Lorenzini, C. A., & Bucherelli, C. (2004). Footshock intensity and generalization in contextual and auditory-cued fear conditioning in the rat. *Neurobiology of learning and memory*, 81(3), 162–166. https://doi.org/10.1016/j.nlm.2004.02.004

Belda, X., Fuentes, S., Daviu, N., Nadal, R., & Armario, A. (2015). Stress-induced sensitization: the hypothalamic-pituitary-adrenal axis and beyond. *Stress (Amsterdam, Netherlands),* 18(3), 269–279. https://doi.org/10.3109/10253890.2015.1067678

Belda, X., Fuentes, S., Labad, J., Nadal, R., & Armario, A. (2020). Acute exposure of rats to a severe stressor alters the circadian pattern of corticosterone and sensitizes to a novel stressor: Relationship to pre-stress individual differences in resting corticosterone levels. *Hormones and behavior*, *126*, 104865. https://doi.org/10.1016/j.yhbeh.2020.104865

Belda, X., Márquez, C., & Armario, A. (2004). Long-term effects of a single exposure to stress in adult rats on behavior and hypothalamic-pituitary-adrenal responsiveness: comparison of two outbred rat strains. *Behavioural brain research*, *154*(2), 399–408. https://doi.org/10.1016/j.bbr.2004.03.011

Belda, X., Nadal, R., & Armario, A. (2016). Critical features of acute stress-induced cross-sensitization identified through the hypothalamic-pituitary-adrenal axis output. *Scientific reports*, *6*, 31244. https://doi.org/10.1038/srep31244

Bevins, R. A., McPhee, J. E., Rauhut, A. S., & Ayres, J. J. (1997). Converging evidence for one-trial context fear conditioning with an immediate shock: importance of shock potency. *Journal of experimental psychology. Animal behavior processes*, *23*(3), 312–324. https://doi.org/10.1037//0097-7403.23.3.312

Cordero, M. I., Merino, J. J., & Sandi, C. (1998). Correlational relationship between shock intensity and corticosterone secretion on the establishment and subsequent expression of contextual fear conditioning. *Behavioral neuroscience*, *112*(4), 885–891. https://doi.org/10.1037//0735-7044.112.4.885

Daviu, N., Andero, R., Armario, A., & Nadal, R. (2014). Sex differences in the behavioural and hypothalamic-pituitary-adrenal response to contextual fear conditioning in rats. *Hormones and behavior*, *66*(5), 713–723. https://doi.org/10.1016/j.yhbeh.2014.09.015

Daviu, N., Fuentes, S., Nadal, R., & Armario, A. (2010). A single footshock causes long-lasting hypoactivity in unknown environments that is dependent on the development of contextual fear conditioning. *Neurobiology of learning and memory*, *94*(2), 183–190. https://doi.org/10.1016/j.nlm.2010.05.005

Deslauriers, J., Toth, M., Der-Avakian, A., & Risbrough, V. B. (2018). Current Status of Animal Models of Posttraumatic Stress Disorder: Behavioral and Biological Phenotypes, and Future Challenges in Improving Translation. *Biological psychiatry*, *83*(10), 895–907. https://doi.org/10.1016/j.biopsych.2017.11.019

Dos Santos Corrêa, M., Vaz, B. D. S., Grisanti, G. D. V., de Paiva, J. P. Q., Tiba, P. A., & Fornari, R. V. (2019). Relationship between footshock intensity, post-training corticosterone release and contextual fear memory specificity over time. *Psychoneuroendocrinology*, *110*, 104447. https://doi.org/10.1016/j.psyneuen.2019.104447

Fanselow, M. S. (1985). Odors released by stressed rats produce opioid analgesia in unstressed rats. *Behavioral Neuroscience, 99*(3), 589–592. https://doi.org/10.1037//0735-7044.99.3.589

Fanselow, M. S. (1990). Factors governing one-trial contextual conditioning. *Animal Learning & Behavior, 18*(3), 264–270. https://doi.org/10.3758/BF03205285

Fanselow M. S. (2010). From contextual fear to a dynamic view of memory systems. *Trends in cognitive sciences, 14*(1), 7–15. https://doi.org/10.1016/j.tics.2009.10.008

Fuentes, S., Daviu, N., Gagliano, H., Belda, X., Armario, A., & Nadal, R. (2018). Early life stress in rats sex-dependently affects remote endocrine rather than behavioral consequences of adult exposure to contextual fear conditioning. *Hormones and behavior, 103*, 7–18. https://doi.org/10.1016/j.yhbeh.2018.05.017

Gonzalez, S. T., Marty, V., Spigelman, I., Reise, S. P., & Fanselow, M. S. (2021). Impact of stress resilience and susceptibility on fear learning, anxiety, and alcohol intake. *Neurobiology of Stress, 15*, 100335. https://doi.org/10.1016/j.ynstr.2021.100335

Hardin, J. W., & Hilbe, J. M. (2002). Generalized estimating equations. In Generalized Estimating Equations.

Jean Kant, G., Mougey, E. H., Pennington, L. L., & Meyerhoff, J. L. (1983). Graded footshock stress elevates pituitary cyclic AMP and plasma beta-endorphin, beta-LPH corticosterone and prolactin. *Life Sciences, 33*(26), 2657–2663. https://doi.org/10.1016/0024-3205(83)90350-8

Kamprath, K., & Wotjak, C. T. (2004). Nonassociative learning processes determine expression and extinction of conditioned fear in mice. *Learning & Memory (Cold Spring Harbor, N.Y.), 11*(6), 770–786. https://doi.org/10.1101/LM.86104

Landeira-Fernandez, J., DeCola, J. P., Kim, J. J., & Fanselow, M. S. (2006). Immediate shock deficit in fear conditioning: effects of shock manipulations. *Behavioral neuroscience, 120*(4), 873–879. https://doi.org/10.1037/0735-7044.120.4.873

Lattal, K. M., & Maughan, D. A. K. (2012). A parametric analysis of factors affecting acquisition and extinction of contextual fear in C57BL/6 and DBA/2 mice. *Behavioural Processes, 90*(1), 49–57. https://doi.org/10.1016/j.beproc.2012.03.008

Laxmi, T. R., Stork, O., & Pape, H. C. (2003). Generalisation of conditioned fear and its behavioural expression in mice. *Behavioural brain research*, *145*(1-2), 89–98. https://doi.org/10.1016/s0166-4328(03)00101-3

Levine, S., Madden IV, J., Conner, R. L., Moskal, J. R., & Chris Anderson, D. (1973). Physiological and behavioral effects of prior aversive stimulation (preshock) in the rat. *Physiology & Behavior, 10*(3), 467–471. https://doi.org/10.1016/0031-9384(73)90207-2

Liberzon, I., & Abelson, J. L. (2016). Context Processing and the Neurobiology of Post-Traumatic Stress Disorder. *Neuron, 92*(1), 14–30. https://doi.org/10.1016/j.neuron.2016.09.039

Lissek, S., & van Meurs, B. (2015). Learning models of PTSD: Theoretical accounts and psychobiological evidence. *International Journal of Psychophysiology, 98*(3), 594–605. https://doi.org/10.1016/j.ijpsycho.2014.11.006

Luyten, L., Vansteenwegen, D., van Kuyck, K., Deckers, D., & Nuttin, B. (2011). Optimization of a contextual conditioning protocol for rats using combined measurements of startle amplitude and freezing: the effects of shock intensity and different types of conditioning. *Journal of neuroscience methods*, *194*(2), 305–311. https://doi.org/10.1016/j.jneumeth.2010.11.005

Luyten, L., Vansteenwegen, D., Kuyck, K. V., & Nuttin, B. (2011). Towards chronic contextual conditioning in rats: the effects of different numbers of unpaired tone-shock presentations on freezing time and startle. *Acta neurobiologiae experimentalis, 71*(3), 331–338. https://doi.org/10.55782/ane-2011-1855

Márquez, C., Nadal, R., & Armario, A. (2006). Influence of reactivity to novelty and anxiety on hypothalamic-pituitary-adrenal and prolactin responses to two different novel environments in adult male rats. *Behavioural brain research*, *168*(1), 13–22. https://doi.org/10.1016/j.bbr.2005.10.004

McCulloch, C., & Searle, S. (2001). Generalized, Linear, and Mixed Models Generalized, Linear, and Mixed Models. In Wiley-Interscience (p. 325). https://doi.org/10.1198/tech.2003.s13

Merino, J. J., Cordero, M. I., & Sandi, C. (2000). Regulation of hippocampal cell adhesion molecules NCAM and L1 by contextual fear conditioning is dependent upon time and stressor intensity. *The European journal of neuroscience*, *12*(9), 3283–3290. https://doi.org/10.1046/j.1460-9568.2000.00191.x

Milanovic, S., Radulovic, J., Laban, O., Stiedl, O., Henn, F., & Spiess, J. (1998). Production of the Fos protein after contextual fear conditioning of C57BL/6N mice. *Brain research*, *784*(1-2), 37–47. https://doi.org/10.1016/s0006-8993(97)01266-3

Olff, M., Langeland, W., Draijer, N. & Gersons, B. P. R. (2007). Gender differences in posttraumatic stress disorder. *Psychological Bull. 133*, 183–204. https://doi: 10.1037/0033-2909.133.2.183

O'Reilly, R. C., & Rudy, J. W. (2001). Conjunctive representations in learning and memory: principles of cortical and hippocampal function. *Psychological review, 108*(2), 311–345. https://doi.org/10.1037/0033-295x.108.2.311

Perusini, J. N., Meyer, E. M., Long, V. A., Rau, V., Nocera, N., Avershal, J., Maksymetz, J., Spigelman, I., & Fanselow, M. S. (2016). Induction and Expression of Fear Sensitization Caused by Acute Traumatic Stress. *Neuropsychopharmacology, 41*(1), 45–57. https://doi.org/10.1038/npp.2015.224

Pijlman, F. T., & van Ree, J. M. (2002). Physical but not emotional stress induces a delay in behavioural coping responses in rats. *Behavioural brain research*, *136*(2), 365–373. https://doi.org/10.1016/s0166-4328(02)00128-6

Pijlman, F. T., Wolterink, G., & van Ree, J. M. (2002). Cueing unavoidable physical but not emotional stress increases long-term behavioural effects in rats. *Behavioural brain research*, *134*(1-2), 393–401. https://doi.org/10.1016/s0166-4328(02)00053-0

Poulos, A. M., Mehta, N., Lu, B., Amir, D., Livingston, B., Santarelli, A., Zhuravka, I., & Fanselow, M. S. (2016). Conditioning- and time-dependent increases in context fear and generalization. *Learning & memory (Cold Spring Harbor, N.Y.)*, *23*(7), 379–385. https://doi.org/10.1101/lm.041400.115

Quinn, J. J., Wied, H. M., Ma, Q. D., Tinsley, M. R., & Fanselow, M. S. (2008). Dorsal hippocampus involvement in delay fear conditioning depends upon the strength of the tone-footshock association. *Hippocampus*, *18*(7), 640–654. https://doi.org/10.1002/hipo.20424

Radulovic, J., Kammermeier, J., & Spiess, J. (1998). Generalization of fear responses in C57BL/6N mice subjected to one-trial foreground contextual fear conditioning. *Behavioural brain research*, *95*(2), 179–189. https://doi.org/10.1016/s0166-4328(98)00039-4

Rau, V., DeCola, J. P., & Fanselow, M. S. (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neuroscience and biobehavioral reviews*, *29*(8), 1207–1223. https://doi.org/10.1016/j.neubiorev.2005.04.010

Rau, V., & Fanselow, M. S. (2009). Exposure to a stressor produces a long lasting enhancement of fear learning in rats. *Stress (Amsterdam, Netherlands)*, *12*(2), 125–133. https://doi.org/10.1080/10253890802137320

Rudy, J. W., & Pugh, C. R. (1996). A comparison of contextual and generalized auditory-cue fear conditioning: Evidence for similar memory processes. *Behavioral Neuroscience, 110*(6), 1299–1308. https://doi.org/10.1037/0735-7044.110.6.1299

Santos, J. M., Gárgaro, A. C., Oliveira, A. R., Masson, S., & Brandão, M. L. (2005). Pharmacological dissociation of moderate and high contextual fear as assessed by freezing behavior and fear-potentiated startle. *European neuropsychopharmacology, 15*(2), 239–246. https://doi.org/10.1016/j.euroneuro.2004.10.004

Sauerhöfer, E., Pamplona, F. A., Bedenk, B., Moll, G. H., Dawirs, R. R., von Hörsten, S., Wotjak, C. T., & Golub, Y. (2012). Generalization of contextual fear depends on associative rather than non-associative memory components. *Behavioural brain research, 233*(2), 483–493. https://doi.org/10.1016/j.bbr.2012.05.016

Siegmund, A., & Wotjak, C. T. (2007a). A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. *Journal of psychiatric research*, *41*(10), 848–860. https://doi.org/10.1016/j.jpsychires.2006.07.017

Siegmund, A., & Wotjak, C. T. (2007b). Hyperarousal does not depend on trauma-related contextual memory in an animal model of Posttraumatic Stress Disorder*. Physiology & Behavior, 90*(1), 103–107. https://doi.org/10.1016/J.PHYSBEH.2006.08.032

Stam R. (2007). PTSD and stress sensitisation: a tale of brain and body Part 2: animal models. *Neuroscience and biobehavioral reviews*, *31*(4), 558–584. https://doi.org/10.1016/j.neubiorev.2007.01.001

Stiedl, O., Radulovic, J., Lohmann, R., Birkenfeld, K., Palve, M., Kammermeier, J., Sananbenesi, F., & Spiess, J. (1999). Strain and substrain differences in context- and tone-dependent fear conditioning of inbred mice. *Behavioural Brain Research, 104*(1–2), 1–12. https://doi.org/10.1016/S0166-4328(99)00047-9

Török, B., Sipos, E., Pivac, N., & Zelena, D. (2019). Modelling posttraumatic stress disorders in animals. *Progress in neuro-psychopharmacology & biological psychiatry*, *90*, 117–133. https://doi.org/10.1016/j.pnpbp.2018.11.013

Van den Berg, C. L., Lamberts, R. R., Wolterink, G., Wiegant, V. M., & Van Ree, J. M. (1998). Emotional and footshock stimuli induce differential long-lasting behavioural effects in rats; involvement of opioids. *Brain research*, *799*(1), 6–15. https://doi.org/10.1016/s0006-8993(98)00397-7

Van Dijken, H. H., Mos, J., van der Heyden, J. A. M., & Tilders, F. J. H. (1992). Characterization of stress-induced long-term behavioural changes in rats: Evidence in favor of anxiety. *Physiology & Behavior, 52*(5), 945–951. https://doi.org/10.1016/0031-9384(92)90375-C

Van Dijken, H. H., Van Der Heyden, J. A. M., Mos, J., & Tilders, F. J. H. (1992). Inescapable footshocks induce progressive and long-lasting behavioural changes in male rats. *Physiology & Behavior, 51(*4), 787–794. https://doi.org/10.1016/0031-9384(92)90117-K

Viviani, D., Haegler, P., Strasser, D. S., & Steiner, M. A. (2012). Sex comparison on long-lasting behavioral and physiological disturbances induced by single shock experience in rats. *Physiology & behavior, 107*(2), 243–251. https://doi.org/10.1016/j.physbeh.2012.06.018

Wöhr, M., Borta, A., & Schwarting, R. K. (2005). Overt behavior and ultrasonic vocalization in a fear conditioning paradigm: a dose-response study in the rat. *Neurobiology of learning and memory*, *84*(3), 228–240. https://doi.org/10.1016/j.nlm.2005.07.004