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Abstract

Exposure to electric foot-shocks can induce in rodents contextual fear conditioning, generalization of fear to other contexts and sensitization of the hypothalamic-pituitary-adrenal (HPA) axis to further stressors. All these aspects are relevant for the study of post-traumatic stress disorder. In the present work we evaluated in rats the sex differences and the role of early life stress (ELS) in fear memories, generalization and sensitization. During the first postnatal days subjects were exposed to restriction of nesting material along with exposure to a “substitute” mother. In the adulthood they were exposed to (i) a contextual fear conditioning to evaluate long-term memory and extinction and (ii) to a novel environment to study cognitive fear generalization and HPA axis heterotypic sensitization. ELS did not alter acquisition, expression or extinction of context fear conditioned behavior (freezing) in either sex, but reduced activity in novel environments only in males. Fear conditioning associated hypoactivity in novel environments (cognitive generalization) was greater in males than females but was not specifically affected by ELS. Although overally females showed greater basal and stress-induced levels of ACTH and corticosterone, an interaction between ELS, shock exposure and sex was found regarding HPA hormones. In males, ELS did not affect ACTH response in any situation, whereas in females, ELS reduced both shock-induced sensitization of ACTH and its conditioned response to the shock context. Also, shock-induced sensitization of corticosterone was only observed in males and ELS specifically reduced corticosterone response to stressors in males but not females. In conclusion, ELS seems to have only a minor impact on shock-induced behavioral conditioning, while affecting the unconditioned and conditioned responses of HPA hormones in a sex-dependent manner.

Keywords	Fear memory, Fear Generalization, Sex Differences, ACTH, corticosterone, PTSD models

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Early life stress in rats sex-dependently affects remote endocrine rather than behavioral consequences of adult exposure to contextual fear conditioning

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Summary

Exposure to electric foot-shocks can induce in rodents contextual fear conditioning, generalization of fear to other contexts and sensitization of the hypothalamic-pituitary-adrenal (HPA) axis to further stressors. All these aspects are relevant for the study of post-traumatic stress disorder. In the present work we evaluated in rats the sex differences and the role of early life stress (ELS) in fear memories, generalization and sensitization. During the first postnatal days subjects were exposed to restriction of nesting material along with exposure to a "substitute" mother. In the adulthood they were exposed to (i) a contextual fear conditioning to evaluate long-term memory and extinction and (ii) to a novel environment to study cognitive fear generalization and HPA axis heterotypic sensitization. ELS did not alter acquisition, expression or extinction of context fear conditioned behavior (freezing) in either sex, but reduced activity in novel environments only in males. Fear conditioning associated hypoactivity in novel environments (cognitive generalization) was greater in males than females but was not specifically affected by ELS. Although overall females showed greater basal and stress-induced levels of ACTH and corticosterone, an interaction between ELS, shock exposure and sex was found regarding HPA hormones. In males, ELS did not affect ACTH response in any situation, whereas in females, ELS reduced both shock-induced sensitization of ACTH and its conditioned response to the shock context. Also, shock-induced sensitization of corticosterone was only observed in males and ELS specifically reduced corticosterone response to stressors in males but not females. In conclusion, ELS seems to have only a minor impact on shock-induced behavioral conditioning, while affecting the unconditioned and conditioned responses of HPA hormones in a sex-dependent manner.

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Introduction

The development of fear to contexts associated with danger is a biological adaptive process. However, excessive or permanent contextual fear memories may lead to psychopathology (Maren et al., 2013), particularly to fear and anxiety disorders that have an estimated lifetime prevalence close to 29% of the population (Kessler et al., 2005). In rodents, fear memories can be studied by means of the Pavlovian contextual fear conditioning paradigm where an unconditioned stimulus or US (usually a shock) that elicits fear (unconditioned response), is paired with a particular context (conditioned stimulus, CS) (Fanselow and Poulos, 2005; Maren et al., 2013; LeDoux, 2014). When animals are later exposed to thecontext without shock, freezing is measured as an index of fear. Generally, the test is done 24-48 h afterthe acquisition of conditioning aiming to evaluating "recent" fear memories. However,.recent and remote contextual fear memories may engage different brain structures and biochemical mechanisms (Frankland et al., 2006; Restivo et al., 2009; Xu et al., 2012; Tayler et al., 2013; Gräff et al., 2014; Einarsson et al., 2015).

After Pavlovian contextual fear conditioning, animals not only fear the shock-paired context, but other contexts that have partial resemblance with the original one. This phenomenon is called fear generalization and interestingly remote fear memories are more prone to generalization (Houston et al., 1999; Balogh and Wehner, 2003; Biedenkapp and Ruddy, 2007; Wiltgen and Silva, 2007; Wiltgen et al., 2010; Poulos et al., 2016). Importantly, context fear conditioning causes hypoactivity in novel environments that have no resemblance at all with the context. This hypoactivity is dependent on the development of contextual fear learning rather than the mere exposure to shocks (Radulovic et al., 1998; Daviu et al., 2010). We have termed this phenomenon cognitive generalization as it does not appear to involve perceptual processes (Daviu et al., 2014). The study of fear generalization in animal models is particularly useful for translational research because patients with post-traumatic stress disorder (PTSD) show overgeneralization of fear and an inability to inhibit fear in front of safe cues (Jovanovic et al., 2012; Briscione et al., 2014). In addition, in animals, exposure to shocks can induce sensitization of the hypothalamic-pituitary-adrenal (HPA) axis (Johnson et al., 2002; Belda et al., 2016), the prototypical stress system in all vertebrates, suggesting that prior experience with shocks can induce both behavioral and endocrine sensitization.

Considering that behavioral consequences of exposure to shock can model PTSD, it is of note that different PTSD symptoms have been reported in men *versus* women (Carmassi et al., 2014; Carragher et al., 2016), and preliminary data suggest that increased generalization may be an important correlate of PTSD symptoms in females but less so in males (Radell et al., 2017). In animal models, sex differences in fear conditioning have been previously described (see Dalla and Shors, 2009 for a review), but the direction of the differences are not always concordant with those observed in humans. Males have stronger contextual recent fear memory, as measured by freezing (e.g. Barker and Galea, 2010; Daviu et al., 2014), but the conditioned HPA axis response is stronger in females (Daviu et al., 2014). Moreover, cognitive generalization of fear was higher in males than females (Daviu et al., 2014). All these data together suggest that fear is a multidimensional construct and thus, not all the indexes of fear may always develop in the same direction.

Early life experiences have a long-lasting impact on central nervous system functioning, behavior and cognition (Heim and Nemeroff, 2001; Shea et al., 2005; Morgan and Fisher, 2007). More specifically, fear memories are profoundly modulated by early life experiences and childhood trauma has been reported to increase vulnerability to PTSD in adulthood (Bremner et al., 1993; Breslau et al., 1999; Cougle et al., 2010). In animal models, early life postnatal stressful experiences (ELS) have also a long-term effect in emotional behavior. One of the most extensively studied models is maternal separation/deprivation that has been found to potentiate in adulthood the formation of recent contextual and/or auditory fear memories (Oomen et al., 2010; Diehl et al., 2014; Sampath et al., 2014; Toda et al., 2014), but opposite results have been found in other studies (Guijarro et al., 2007; Wang et

al., 2011). Moreover, maternal separation increases at adulthood fear generalization to other contexts and cues –e.g. a tone not paired with shocks in a paradigm of auditory fear conditioning- (Sampath et al., 2014). In contrast, consistent decreases in fear memory have been reported after early exposure to "protective" treatments such as postnatal handling (Meerlo et al., 1999; Claessens et al., 2012) or environmental enrichment (Barbelivien et al., 2006). Most of these studies have been performed in male rats and only in few cases both sexes were included (Wang et al., 2011; Diehl et al., 2014). On the other hand, one of the aspects that have deserved scarce attention is the impact of ELS on remote fear memories and generalization. In PTSD patients, the probability to interact again with the same contextual cues than the ones paired to the trauma is lower than the probability to be exposed to only partially similar cues. Therefore, the study in animal models of how ELS modulates fear generalization to different contexts and cues deserves more attention.

Given all the above, the general aim in the present study was to characterize how a model of ELS in rats is affecting at adulthood in a sex-dependent manner: (i) contextual fear acquisition and remote memory and extinction. To asses that, freezing, as a behavioral measure, and HPA axis functioning (measured by adrenocorticotrophic hormone (ACTH) and corticosterone plasma levels), as an endocrine measure, were used. (ii) generalization of fear to a novel environment, using hypoactivity as a behavioral measure,and (iii) HPA axis response to the novel environment that acted as a heterotypic stressor. As a model ofELS we used, as previously (Fuentes et al., 2014), a combination of restriction of nesting material (Moletet al., 2014) and exposure to a substitute mother (Roth et al., 2009). This ELS procedure has been shownto induce, at adulthood, a mixture of detrimental and adaptive effects in several behaviors and in the functioning of the HPA axis. In our hands, this model induces sex-dependent cognitive and emotional changes in adulthood and increases maternal care provided by the biological mother, which may "buffer" some of the "negative" effects of stress (Fuentes et al., 2014).

Material and methods

Subjects

Long-Evans (RjOrl:LE) outbred rats were housed in Makrolon transparent polycarbonate wire-topped cages with solid bottom (26.5 x 42.5 x 18.5 cm, Ref. 1291 Eurostandard Type III H) containing sawdust bedding (Lignocel 3/4, Harlan) in a climate-controlled environment at 20-21°C on a 12-hour light-dark cycle (lights on at 8:00 am). Behavioral studies were carried out during the light cycle. Food (SAFE-diet A04, Panlab S.L.U., Barcelona, Spain) and filtered tap-water were available ad libitum. All animal protocols were in accordance with the European Communities Council Directive 2010-63-EU and the Spanish legislation (BOE53-2013) and approved by the Ethics Committee for Human and Animal Research of the Universitat Autònoma de Barcelona and by the Generalitat de Catalunya. No specific environmental enrichment program was used in the animal facility. A maximal effort was done to minimize the number and suffering of animals. A detailed timeline of the study is provided in Figure 1.

Early life treatment

The pups of the present study represent a particular cohort of offspring coming from the same dams used in Fuentes et al. (2014), being the offspring a different cohort of rats. As mentioned (Fuentes et al.,2014), 30 mated pregnant dams arrived from Janvier (France) at GD 15. Each dam (approximately 8 weeks old and primiparous) was paired with a different male (1 male/1 female). In this specific experiment 28 different dams were used. All of the dams were at the same vivarium, special care was taken to restrict access to the room and the day of delivery was termed postnatal day (PND) 0. The nextday, the pups were weighed, counted, sexed and those litters higher than 12 were culled to 12(maintaining, if possible, a sex ratio between 0.4 and 0.6) and weighed. Cross-fostering was never performed. At PND 1, the dams were assigned to control (CTR) or ELS conditions. Final litter size and sexratio was not different between groups [litter size CTR: 9.3 ± 0.5; litter size ELS: 9.6 ± 0.5; sex ratio (males

to females) CTR: 0.59 ± 0.04; sex ratio ELS: 0.52 ± 0.03]. The bedding was not changed until PND 8 when the pups were again weighed. As mentioned (Fuentes et al., 2014), prior to treatment (PND 1) body weight was not affected by group and at PND 8 ELS decreased weight in, both, males and females.The ELS consisted of a combination of 2 different treatments (i) restriction of nesting material and (ii) exposure for 1 h/day to a "substitute" mother (see Fuentes et al., 2014 for more details). The ELS treatment lasted 7 days (PND 1-7), and after that all dams were returned to control conditions. Control dams remained undisturbed with the litter during all the lactation period, until weaning at PND 21. Maternal behavior was measured between PND 1 and 7, and again at PND 13 and PND 18. As it can beseen in Fuentes et al. (2014), ELS decreased the time dams spent off the nest and increased arched- back behavior during PND 1-7.

After weaning, pups were housed by sex in groups of 4 (each one from a different mother) and kept undisturbed until PND 60. In each one of the 4 groups (ELS and controls, males and females), only 1-2 pups from the same mother were used. Before initiating adult testing rats were handled at least for 3 days.

Apparatus

Fear conditioning boxes.

The fear conditioning experiments were conducted in 8 standard Skinner boxes (Ref LE1005, Panlab-Harvard, Barcelona, Spain). Each chamber (25 cm x 25 cm x 25 cm) had a clear Plexiglas door, a black stainless steel back wall and two aluminum sidewalls. The floor, composed of 19 stainless steel rods (3 mm in diameter), spaced 1 cm centre to centre, was wired to a shock generator and scrambler. A house light (4 cm diameter 2.4-W, 24-V) was placed in the right wall at 22 cm to the floor. The software (Packwin 2.00.2, Panlab-Harvard, Barcelona, Spain) controlled the administration of the different stimuli. The chambers were inside a sound attenuating box (67 cm x 53 cm x 55 cm) provided with a fan that helped to mitigate strange sounds. The room was dimly illuminated and the boxes were carefully cleaned between animals with a solution with ethanol in tap water (5% v/v).

Novel environment

To study fear generalization and endocrine sensitization to novel environments a modified conditioned place-preference box was used. The box had two compartments (36.5 cm x 42 cm x 34.5 cm) with distinct visual and tactile cues: one compartment had stripped black and white walls with the floor wood-made, black and smooth, and the other compartment had white walls with black dots and the floor was white, rough and glass-made, with guillotine doors that separated if needed the different compartments. Room was illuminated by white fluorescent light. Behavior was recorded by a camera (Sony SSC-M388 CE, BW) situated 150 cm above the centre of the apparatus. A digital video recorder (JVC VR-716) sampled the position of the rat (8.3 samples/s) and it was used to transfer the videos to a computer for video tracking analysis using the centre of gravity of the animal (Smart version 2.5.21, Panlab-Harvard, Barcelona, Spain).

Blood sampling and radioimmunoassay

During handling, rats were subjected once to the tail-nick procedure to habituate them to this blood sampling procedure. The tail-nick consisted of gently wrapping the animals with a cloth, making a 2 mm incision at the end of one of the tail veins and then massaging the tail while collecting, within 2 min, 300 µl of blood into ice-cold EDTA capillary tubes (Sarsted, Granollers, Spain). After centrifugation at 4°C, plasma was stored at -20º C. Cage-mates were processed simultaneously, including blood sampling (two experimenters were sampling at the same time and a third was gently holding the two rats). The tail-nick procedure is extensively used in our laboratory because low resting levels of hormones are obtained (i.e.

Belda et al., 2004; Vahl et al., 2005). Animals were always tested in a room different from the animal room and blood sampling room. The interventions were always done in the morning.

Plasma ACTH and corticosterone levels were determined by double-antibody radioimmunoassay (RIA) following our general procedures (Muñoz-Abellán et al., 2011). In brief, ACTH RIA used ^{125}I-ACTH (PerkinElmer Life Science, Boston, USA) as the tracer, rat synthetic ACTH 1-39 (Sigma, Barcelona, Spain) as the standard and an antibody raised against rat ACTH (rb7) kindly provided by Dr. W.C. Engeland (Department of Surgery, University of Minnesota, Minneapolis, USA). The characteristics of the antibody have been described previously (Engeland et al., 1989) and we followed a non-equilibrium procedure. Corticosterone RIA used ^{125}I-corticosterone-carboximethyloxime-tyrosine-methylester (ICN-Biolink 2000, Barcelona, Spain), synthetic corticosterone (Sigma, Barcelona, Spain) as the standard and an antibody raised in rabbits against corticosterone–carboximethyloxime-BSA kindly provided by Dr. G. Makara (Institute of Experimental Medicine, Budapest, Hungary). The characteristics of the antibody and the basic RIA procedure have been described previously (Zelena et al., 2003) and we followed an equilibrium procedure. All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was 5.1% for ACTH and 7.6 % for corticosterone. The sensitivity of the assays was 25 pg/ml for ACTH and 2 ng/ml for corticosterone.

General procedure at adulthood

After the ELS procedure, males and females were distributed at random among shock and non-shock groups. Thus, the experimental groups were: males non-ELS, non-shock (n=12, from 10 different mothers), males ELS, non-shock (n=12, from 10 different mothers), males non-ELS, shock (n=12, from 11 different mothers), males ELS, shock (n=13, from 12 different mothers), females non-ELS, non-shock (n=6, from 5 different mothers), females ELS, non-shock (n=12, from 10 different mothers), females non-ELS, shock (n=7, from 6 different mothers) and females ELS, shock (n=15, from 12 different mothers). All animals were exposed: (a) at PND 91-93 to an acquisition session of contextual fear conditioning (or exposed to the box in the non-shocked groups), (b) at PND 122-124 to a novel environment, (c) at PND 123-125 to a contextual fear memory test, and (d) at PND 125-127 to a extinction training session of contextual fear memory. After each session a blood sample was obtained by tail-nick to evaluate the reactivity of the HPA axis. Except for the acquisition session, the duration of the other tests was of 15 min because with durations of 5 min a conditioned HPA response cannot be detected (Armario et al., 2012). The order of the experimental groups tested was randomized across the session.

The estrous cycle was not monitored for several reasons. Some studies indicate that rats present equivalent basal levels of plasmatic ACTH across the different stages of the estrous cycle (Atkinson and Waddell, 1997) and there is no consistent evidence for a different HPA response to stress during the estrous cyclewhen samples are taken in the morning (Babb et al., 2013a; Iwasaki-Sekino et al., 2009; Viau and Meaney, 1991). Moreover, monitoring the estrous cycle may add a confounding factor when comparing both sexes because it has been described that may induce both some degree of stress in other rats (Sharp et al., 2003) and even conditioned place preference (Walker et al., 2002).

In the acquisition phase shocked animals were allowed to explore a chamber for 3 min and then received 3 foot-shocks (1.5 mA, 3 s of duration, ITI 60 s, non-continue squared current, frequency: 20 Hz, each pulse duration 8.3 ms, polarity: monophase, pulse current: effective) and after the last foot-shock, animals remained for 3 more min in the chamber. The non-shocked group was placed in the chamberfor the same amount of time without receiving shock. Behaviour was videotaped from the front by a camera situated inside the chamber. An experimenter blind to the treatment measured freezing behaviour with the Observer software (Noldus, Netherlands, version XT 11). Freezing is defined as the absence of all movement except for respiratory related movements (Blanchard and Blanchard, 1969).

To study the possible development of long-term generalization of fear to a context different from the training one, animals were exposed, 31 days later, to a novel environment. The animal was always placed into the activity box facing the wall. The procedure lasted 15 min and blood samples were taken immediately after the end of the test. Special care was paid to change putative proximal (size, shape, type of floor, odor, color, texture and lighting conditions of the novel environment) and distal (experimenter, transport, testing room) cues with respect to the acquisition session (Chang et al., 2009). A black curtain surrounded the apparatus and a fluorescent light was placed above the center. A soap solution was used to clean the box after the session. The test was conducted in a room different from the one used in the shock session. The experimenter, the box used to transport the rats from the vivarium to the room (white plastic box with litter 29 x 27 x 14 cm), and the route of transportation (the animals were moved down the corridor twice) were also changed. The behaviour was videotaped from the top and an experimenter blind to the treatment measured distance travelled by video tracking, to study hypoactivity.

Next day, 32 days after the acquisition session, all animals were re-exposed to the context paired with the shock (without receiving shock) for 15 min to test remote contextual fear memory and a blood sample was taken at the end of the test. An additional session of extinction training (equal to the previously described memory session) was given 2 days after. Freezing was measured during all those 2 sessions (memory test and extinction). Finally, to study the possible long-term impact of ELS in basal levels of ACTH and corticosterone, non-shocked animals were sampled by tail-nick 5 days after the extinction session.

Statistical analysis

Data were analyzed by means of the Statistical Program for Social Sciences (SPSS-IBM) (version 24). A repeated-measures analysis of variance (general linear model: GLM) was used with three between-subjects factors: SEX (two levels, male and female), early treatment (two levels, control and ELS) and SHOCK (two levels, shocked and non-shocked). A within-subject factor was included only when comparing ACTH levels after the novel environment and the fear conditioning test (see Results). To achieve homogeneity of variances log-transformations were made, if needed (see Results). To further analyze the interactions between factors separate analysis were made by sex or by shock. Partial eta squared indexes were included for each factor as a measure of the proportion of the total variance explained taking into account the effects of the other factors (Richardson, 2011).

Results

No body weight differences were found between controls and ELS at the beginning of the experimental procedure in adulthood (day before the acquisition session), being the only difference, as expected, due to SEX (data not shown, ELS, SHOCK, ELS x SEX, ELS x SHOCK, SEX x SHOCK, ELS x SEX s SHOCK: all NS, SEX: $F(1,81)=483.02$, $p<0.001$, partial $\eta^2=0.856$). Three days before the exposure to the novel environment animals were again weighed and no differences emerged due to ELS or to SHOCK [data not shown, ELS, SHOCK, ELS x SEX, ELS x SHOCK, SEX x SHOCK, ELS x SEX s SHOCK: all NS, SEX: $F(1,81)=781.49$, $p<0.001$, partial $\eta^2=0.906$].

In non-shocked animals, basal levels of ACTH and corticosterone (log-transformed) were not affected by ELS and the only statistically significant factor was SEX [ACTH: $F(1,38)=14.66$, $p<0.001$, partial $\eta^2=0.278$; Corticosterone: $F(1,38)=15.82$, $p<0.001$, partial $\eta^2=0.294$], being NS the interaction ELS x SEX. Both ACTH and corticosterone basal levels were higher in females than males [ACTH: in males 61.6 $\pm$ 6.6 in controls and 57.8 $\pm$ 6.3 in ELS animals, in females 99.3 $\pm$ 13.2 in controls and 99.4 $\pm$ 13.1 pg/ml in ELS; Corticosterone: in males 48.3 $\pm$ 24.3 in controls and 33 $\pm$ 25 in ELS animals, in females 212.5 $\pm$ 77.8 in controls and 127.1 $\pm$ 47.2 ng/ml in ELS].

Context fear conditioning training

In the non-shocked groups freezing was low across all sessions and only the shocked groups were further analyzed. Freezing measured in the acquisition session, before the presentation of the shock, during the 3 min of exploration of the context was near zero in all groups (Table 1). After the 3 shocks, freezing was again measured during the additional 3 min period and no statistical differences between groups were detected [SEX, ELS, SEX x ELS: all NS]. No between groups differences were observed in the memory test or in the extinction session (Table 1). When comparing freezing during the memory test with the extinction session the only statistically significant factor was TIME, being all the other factors and interactions NS. The levels of freezing during the extinction training were lower than during the memory test.

HPA response to shocks during acquisition session is shown in Figures 2A and 2B. Regardless of sex, animals that have been exposed to shock presented higher plasma levels of ACTH than their respective non-shocked group [SHOCK: $F(1,81)=63.12$, $p<0.001$, partial $\eta^2=0.438$], but females showed higher levels of ACTH than males [SEX: $F(1,81)=10.29$, $p<0.01$, partial $\eta^2=0.113$], The other factors and interactions were statistically NS. Regarding corticosterone (log-transformed), the statistical analysis revealed significant effects of SHOCK [$F(1,81)=14.76$, $p<0.001$, partial $\eta^2=0.154$], SEX [$F(1,81)=347.27$, $p<0.001$, partial $\eta^2=0.818$] and the interaction SEX x SHOCK [$F(1,81)=10.10$, $p<0.01$, partial $\eta^2=0.111$]. To further analyze the interaction SEX x SHOCK, separate analysis were done for males and females, and for shocked and non-shocked animals. In males, shock exposure increased corticosterone levels with respect to non-shocked animals [SHOCK, $F(1,45)=20.16$, $p<0.001$, partial $\eta^2=0.309$], regardless of ELS condition. In females, no influence of shock exposure or ELS condition was found, but females showed higher corticosterone levels than males in both non-shocked [SEX, $F(1,38)=216.00$, $p<0.001$, partial $\eta^2=0.850$] and shocked [SEX, $F(1,38)=131.62$, $p<0.001$, partial $\eta^2=0.754$] animals.

Long-term effects of context fear conditioning on behavioral and HPA response to novel environments

Thirty-one days after the acquisition session all animals were exposed to a novel environment to evaluate cognitive generalization of fear by measuring activity. The statistical analysis of distance travelled (log-transformed) showed (Figure 3) significant effects of SHOCK [$F(1,77)=96.40$, $p<0.001$, partial $\eta^2=0.556$], SEX [$F(1,77)=17.23$, $p<0.001$, partial $\eta^2=0.183$] and SEX x SHOCK [$F(1,77)=6.47$, $p<0.05$, partial $\eta^2=0.077$], whereas the ELS factor approached the significance [$F(1,77)=3.95$, $p=0.051$, partial $\eta^2=0.049$]. All other interactions were NS. Due to the interaction SEX x SHOCK, separate analysis by sex were made. In males, effects of ELS [$F(1,43)=6.25$, $p<0.05$, partial $\eta^2=0.127$] and SHOCK [$F(1,43)=85.26$, $p<0.001$, partial $\eta^2=0.665$] were statistically significant. Thus, males previously exposed to any of the two treatments (ELS and SHOCK) presented hypoactivity in the novel environment,being the shock-induced hypoactivity stronger. In females shock exposure significantly reduced activity [$F(1,34)=25.35$, $p<0.001$, partial $\eta^2=0.427$], whereas ELS had no effect. Sex differences only emerged in shock-exposed animals [SEX factor in shock group: $F(1,39)=14.38$, $p=0.001$, partial $\eta^2=0.269$], presenting shocked females less hypoactivity than shocked males.

HPA response to the novel environment was measured to evaluate the possible development ofendocrine sensitization to a heterotypic mild stressor. Regarding ACTH (Figures 4A), statistical analysisshowed significant effects of SHOCK [$F(1,77)=11.59$, $p=0.001$, partial $\eta^2=0.131$], SEX [$F(1,77)=11.11$, $p=0.001$, partial $\eta^2=0.126$] and the interactions SEX x SHOCK [$F(1,77)=6.19$, $p<0.05$, partial $\eta^2=0.074$] and ELS x SHOCK x SEX [$F(1,77)=4.52$, $p<0.05$, partial $\eta^2=0.055$]. All other interactions were NS. The decomposition of the triple interaction indicated that in males the ELS factor only approached significance [$F(1,43)=3.61$, $p=0.064$, partial $\eta^2=0.077$], being SHOCK and SEX x SHOCK both NS. In females, significant effects were found for SHOCK [$F(1,34)=10.82$, $p<0.01$, partial $\eta^2=0.241$] and ELS x SHOCK [$F(1,34)=4.38$, $p<0.05$, partial $\eta^2=0.114$]. Additional decompositions of the interaction ELS x

SHOCK in females showed that: (i) in controls previous exposure to shock increased ACTH response in comparison to non-shocked animals ($p<0.01$), but such effect was not observed in ELS females; and (ii) ELS rats showed lower ACTH levels after the novel environment only in rats previously exposed to the shock ($p<0.05$). Regarding sex differences, females presented higher ACTH levels than males in all groups except in controls not previously exposed to shock (between $p<0.05$ and $p<0.01$).

Results concerning corticosterone (Figures 4B), indicated significant effects of SHOCK [$F(1,77)=6.25$, $p<0.05$, partial $\eta^2=0.0756$], SEX [$F(1,77)=498.40$, $p<0.001$, partial $\eta^2=0.866$] and ELS x SEX [$F(1,77)=4.50$, $p<0.05$, partial $\eta^2=0.055$], being the other factors and interactions NS, although the ELS factor approached significance [$F(1,77)=3.87$, $p=0.053$, partial $\eta^2=0.048$]. To further analyze the interaction ELS x SEX, separate analysis were done for each sex. In males, ELS [$F(1,43)=19.44$, $p<0.001$, partial $\eta^2=0.311$] and SHOCK [$F(1,43)=4.09$, $p<0.05$, partial $\eta^2=0.087$] factors were statistically significant, whereas in females all the factors and interactions were NS. Thus, only in males, previous shock exposure increased corticosterone response, whereas ELS by itself reduced it. Sex differences were observed in both non-shocked and shocked rats, having females always higher levels of corticosterone ($p<0.001$ in both cases).

Behavioral and HPA response to context fear conditioning testing and extinction

ACTH and corticosterone levels were measured after fear memory testing 32 days after the acquisition session (Figures 5A and B). Statistical analysis of ACTH revealed significant effect of ELS [$F(1,81)=4.99$, $p<0.05$, partial $\eta^2=0.058$], SHOCK [$F(1,81)=62.82$, $p<0.001$, partial $\eta^2=0.437$], SEX [$F(1,81)=4.97$, $p<0.05$, partial $\eta^2=0.058$] and the interaction SEX x SHOCK [$F(1,81)=8.80$, $p<0.01$, partial $\eta^2=0.098$], being the other interactions NS. Although the interaction ELS x SHOCK x SEX was not statistically significant, additional decompositions of this triple interaction were done to better compare the results with those observed in response to the novel environment (see above). Previous shock exposure significantly increased ACTH response to the conditioned context in both sexes regardless of ELS treatment [males non-ELS: $F(1,22)=6.62$, $p<0.05$, partial $\eta^2=0.231$; males ELS: $F(1,23)=16.45$, $p<0.001$, partial $\eta^2=0.417$; females non-ELS: $F(1,11)=17.18$, $p<0.01$, partial $\eta^2=0.610$; females ELS: $F(1,25)=18.33$, $p<0.001$, partial $\eta^2=0.423$]. The ELS factor was only statistically significant in shocked females: ELS animals showed lower ACTH response to the fear context than non-ELS [$F(1,20)=5.73$, $p<0.05$, partial $\eta^2=0.223$]. Sex differences only appeared in shocked animals that did not receive ELS, having females higher ACTH levels than males [SEX: $F(1,17)=11.03$, $p<0.01$, partial $\eta^2=0.394$]. Regarding corticosterone levels (log-transformed), significant effects of SHOCK [$F(1,81)=14.08$, $p<0.001$, partial $\eta^2=0.148$], SEX [$F(1,81)=411.66$, $p<0.001$, partial $\eta^2=0.836$], ELS x SEX [$F(1,81)=7.10$, $p<0.01$, partial $\eta^2=0.081$] and SEX x SHOCK [$F(1,81)=6.57$, $p<0.05$, partial $\eta^2=0.075$]
were found. Separate analyses for males and females showed that in males, ELS [$F(1,45)=8.03$, $p<0.01$, partial $\eta^2=0.151$] and SHOCK [$F(1,45)=18.32$, $p<0.001$, partial $\eta^2=0.289$], but not the interaction ELS x SHOCK were statistically significant. ELS reduced corticosterone response after context exposure both in shocked and non-shocked males. But, regardless the ELS effect, shocked males presented higher corticosterone levels than non-shocked animals. In females, all the factors and interactions were NS. Focusing on sex differences, females presented higher corticosterone levels than males in both non-shocked ($p<0.001$) and shock-exposed animals ($p<0.001$).

In order to distinguish between the ACTH response to a novel environment (probably reflecting non-specific sensitization of the HPA axis) and the specific response of ACTH to contextual fear conditioning, we directly compared, in a separate within-subject analysis, ACTH levels of shocked animals after the novel environment and after the memory test, in both males and females. The levels were higher in the memory test ($p<0.001$ in both cases), supporting a specific response to conditioning.

After exposure to the extinction session, ACTH response (Figures 6A) was still higher in animals previously exposed to shock [SHOCK: $F(1,81)=27.92$, $p<0.001$, partial $\eta^2=0.256$] and higher in females

than in males [SEX: $F(1,81)=6.48$, $p<0.05$, partial $\eta^2=0.074$], being all the other factors and interactions NS. Thus, in agreement with behavioral data, from an endocrine point of view the fear response was not yet totally extinguished. Regarding corticosterone (log-transformed), the results (Figures 6B) followed the same pattern as in the memory test: significant effect of SHOCK [$F(1,81)=8.18$, $p<0.01$, partial $\eta^2=0.092$], SEX [$F(1,81)=596.29$, $p<0.001$, partial $\eta^2=0.880$], ELS x SEX [$F(1,81)=6.11$, $p<0.05$, partial $\eta^2=0.070$] and SEX x SHOCK [$F(1,81)=4.85$, $p<0.05$, partial $\eta^2=0.057$] were found, being the other factors and interactions NS. To further analyze the interaction SEX x SHOCK, separate analysis were made by sex and by shock. Separate analysis by sex indicated that: (i) in males, shock exposure increased [$F(1,45)=11.07$, $p<0.01$, partial $\eta^2=0.197$] whereas ELS decreased [$F(1,45)=6.65$, $p<0.05$, partial $\eta^2=0.129$] corticosterone levels; (ii) in females, all the factors and interactions were NS. Direct sex comparisons indicated that females always presented higher corticosterone levels than males both in shocked ($p<0.001$) and non-shocked ($p<0.001$) animals and also in non-ELS ($p<0.001$) and ELS ($p<0.001$) animals.

Discussion

The present results strongly support the importance of considering sex differences in the long-term response to stress. We showed that, after being exposed to a model of ELS, the behavioral and endocrine effects of contextual fear conditioning at adulthood affected differentially male and female rats. The contextual fear conditioning procedure was chosen considering the relevant literature demonstrating that early life negative experiences in humans increased the susceptibility to develop PTSD, probably by sensitizing fear conditioning.

Behavioral consequences of early life stress and exposure to contextual fear conditioning

ELS reduced activity in novel environments regardless of prior shock exposure in males but not females, suggesting that in our experimental conditions males are more sensitive to ELS than females. The ultimate reasons for this differential susceptibility is unclear. Since dams devoted more time to take care of males than females (Moore et al., 1997; Claessens et al., 2011), a differential impact of stress on maternal behavior towards the two sexes cannot be disregarded. Unfortunately with the current protocols of analysis of maternal behavior it is not possible to specifically study this subject. This decrease of adult exploration of novel environments is consistent with previous data using other ELS models (i.e. Avital et al., 2006; Tsoory and Richter-Levin, 2006; Tsoory et al., 2007, 2008; Fuentes et al., 2014). This effect of ELS may be related to a lack of interest/motivation for novel environments as the reduction of activity/exploration in novel environments has been interpreted as an anhedonic-like behavior (Fukushiro et al., 2012).

Freezing in the acquisition session or during exposure to the shock context (memory test and extinction sessions) were not affected by ELS either in males or females. In the acquisition session, freezing greatly increased immediately after the shocks in comparison to the pre-shock levels, but no ELS-induced differences were observed either before or after shock exposure. When animals were exposed again to the conditioned context (without shock) 32 days later to evaluate long-term contextual fear memory, they still remembered the context, as indicated by freezing, but this long-term contextual fear memory was not affected by ELS or sex. In a posterior extinction session two days later, freezing was still higher in previously shocked animals, regardless of ELS or sex. Although several studies have reported that male rats presented more conditioned freezing than females (see Dalla and Shors, 2009 for a review), usually recent rather than remote fear memories were studied. Also, we cannot rule out that sex-dependent differences could be greater in some strains of rats or mice (Bolivar et al., 2001, March et al., 2014). Our data showed no effect of ELS in fear memories evaluated by freezing. Although some studies support our findings (Guijarro et al., 2007; Wang et al., 2011), other data about ELS and fear memories (as evaluated by freezing) report an increase of freezing behavior (Oomen et al., 2010; Diehl et al., 2014; Sampath et al., 2014; Toda et al., 2014). In some of the above reports only male rats were used (Guijarro et al., 2007;

Sampath et al., 2014; Toda et al., 2014), but in other studies both sexes were evaluated an sex differences in the long-term impact of ELS on freezing behavior were not found (Wang et al., 2011; Diehlet al., 2014). Future studies are needed to ascertain whether different type of ELS procedures are the reasons of those discrepancies.

The study of fear generalization may contribute to understanding emotional disorders and basic long-term memory function (Jasnow et al., 2017). Fear generalization may develop towards contexts partially similar to the training one (that we will name "conditioned perceptual generalization") or towards other environments perceptually very different. In the latter scenario, animals typically display hypoactivity (Radulovic et al., 1998, Daviu et al., 2010, 2014, Girardi et al., 2013; Berardi et al., 2014). We have termed this later phenomenon as "conditioned cognitive generalization" of fear (see Daviu et al., 2014) as it is dependent on the establishment of contextual fear conditioning (Radulovic et al., 1998; Daviu et al., 2010; Sauerhöfer et al., 2012). Because in humans fear generalization goes beyond "perceptual" generalization to high-order cognitive processes (Dunsmoor and Murphy, 2015), it would be important to study in rodents cognitive generalization. In the present study, when rats were exposed to a completely different novel environment hypoactivity emerged in shock-conditioned rats as compared with non-shocked rats, suggesting that this phenomenon is extremely long-lasting. The precise time-course of the expression of this cognitive generalization is unclear. In the study of Radulovic et al. (1998) in mice already appeared 24 h post-shock, whereas in rats have been found in the interval of 6-22 days (Daviu et al., 2010, 2014; Girardi et al., 2013). In contrast, Berardi et al. (2014) were unable to detect hypoactivity at 1 or 7 days post-shock, but they did observe hypoactivity at 14 days. However, in the latter study the novel environment used was the elevated plus-maze, where overall activity is more difficult to separate from other behaviors.

The development of conditioned hypoactivity in novel environments does not seem to be related to an increase in anxiety. There is a dissociation between increased anxiety as measured by classical tests (elevated plus maze, light and dark, among others) and the presence of hypoactivity in novel environments after fear conditioning procedures (Radulovik et al., 1998; Kamprath and Wotjak, 2004; Daviu et al., 2010). Moreover, classical anxiolytics only partially blocked this hypoactivity (Van Dijken et al., 1992; Bruijnzeel et al., 2001).

In the present study cognitive generalization was stronger in males than females, despite similar levels of contextual fear conditioning memory. In our previous work in rats hypoactivity was only evident in males (Daviu et al., 2014), supporting a greater impact in males and perhaps the existence of sex and strain interactions. Barker and Galea (2010) described in gonadectomized rats that males presented more fear than females to the original context and more generalized fear to a novel context described asvery different in terms of proximal and distal cues (differences that were not affected by estradiol administration). However, the apparently lower cognitive generalization of fear in females contrasts to other recent data that can be included within the framework of perceptual generalization. Day et al. (2016) reported that females generalized between a CS+ (tone predicting shock) and a CS- (predicting safety). When using two "similar" contexts (in both cases a grid floor, which is a very salient stimulus) in a passive avoidance procedure, context generalization appeared in females (but not in males) at 5 days(but not before) after shock exposure (Lynch et al., 2013). Also, Keiser et al. (2017) observed in mice more generalization of fear to a similar context in females. In fact, pattern separation in spatial tasks is better in males than females (Yagi et al., 2016). It is therefore possible that females present more perceptual but less cognitive generalization than males, a hypothesis that merits to be further tested.

The functional implications of the sex differences in conditioned cognitive generalization are not clear. The adaptive meaning of generalization versus discrimination in fear conditioning depends on the circumstances that surround the subject. Under some stressful or uncertain environments, generalization may be "protective", increasing the chances of survival, but in other situations would be more "beneficial" to restrict defensive behavior to specific contexts/cues and promoting novelty-

seeking or risk-taking behaviors. Thus, it may be an optimal degree of generalization depending on the situation, and excessive or too little generalization may led to psychopathology.

Overall sex differences in HPA function

In the present study basal levels of ACTH and corticosterone in non-shocked animals were measured at the end of the experiment (5 days after the extinction session). Females showed higher levels of both hormones than males in resting conditions. There is a high consistency in the literature regarding the higher basal corticosterone levels of adult females but these differences have not always been detected in ACTH (e.g. Aloisi et al., 1994; Atkinson and Waddell, 1997; Babb et al., 2013b; Gagliano et al., 2014). In response to the stressful conditions tested (exposure to novel environments, exposure to shocks or context fear conditioning), we observed consistently higher ACTH response in females that was associated with more pronounced differences in corticosterone. The clearly greater corticosterone response to stress in female than male rats is a consistent finding in the literature, but less consistent results have been reported regarding ACTH response (e.g. Erskine et al., 1975; Aloisi et al., 1994; Rivier, 1999; Babb et al., 2013a; Iwasaki-Sekino et al., 2009; Peña et al., 2009). Thus, there are discrepancies in the literature to whether or not female rats respond to stress more than males in terms of ACTH, probably because the response might depend on the type of stressor (e.g. Spinedi et al., 1994; Babb et al., 2013b). It is unclear at present which are the characteristics of stressors that determine whether or not sex differences will appear, but the higher ACTH response observed in the present work is consistent with those previous studies using shock as the stressor (Daviu et al., 2014; Erskine et al., 1975; Rivier, 1999). The influence of the estrous cycle in the ACTH response to stress is unlikely given that most reports showed no clear differences (Viau and Meaney, 1991; Iwasaki-Sekino et al., 2009; Babb et al., 2013a). Despite higher plasmatic levels of corticosterone in females, it seems that brain corticosterone levels are similar in both sexes (Droste et al., 2009), probably because levels of corticosterone binding globulin are higher in females (Gala and Westphal, 1965), thus producing similar free circulating corticosterone levels in both sexes.

It is of note that plasma corticosterone in females was markedly responsive to exposure to a mild stressor such as a novel environment, but quite insensitive to a higher intensity stressful conditions (e.g. shock exposure, conditioned fear). This situation is probably reflecting a prompt saturation of glucocorticoid synthesis at the adrenal cortex with modest levels of ACTH. This saturation has been well-characterized in male rats unless the area under the curve of the corticosterone response is followed until it reaches again resting levels (Keller-Wood et al., 1983). This saturation is likely to occur even before in females considering the already high resting levels, but unfortunately there are no studies in females. This fact would make corticosterone much less useful than ACTH to reflect HPA activity in females than males.

Endocrine consequences of early life stress upon contextual fear conditioning

Our model of ELS decreased transiently body weight gain (Fuentes et al., 2014), supporting that the procedure had an impact on the pups, although did not alter the behavioral expression of fear conditioning or had any effects on body weight and resting levels of ACTH and corticosterone at adulthood. Nevertheless, prior ELS did influence HPA response to fear conditioning in a sex-dependent manner.

In the acquisition session, the mere exposure of the animals to the shock chambers increased both ACTH and corticosterone, but the increase in ACTH was greater after exposure to shock in both sexes. In contrast, as discussed previously, plasma corticosterone levels were higher after shock exposure than shock chamber exposure only in males, whereas in females no additional shock-induced increase in plasma corticosterone was observed. In this case, the response of HPA hormones was not affected by prior ELS exposure.

In response to a novel environment (31 days after the acquisition of the fear conditioning), ACTH response was not affected by ELS or prior shocks in males. However, corticosterone response was affected by both factors revealing that prior shock exposure caused a modest sensitization to the novel environment (in both control and ELS animals), but ELS reduced the response to both the shock chamber and the shocks. A different pattern was observed in females: prior shock caused sensitization of the ACTH response to the novel environment in control but not in ELS rats, whereas no effect of ELS or prior shocks was found in corticosterone levels. Overall, the data indicate that shock-induced sensitization is restricted to corticosterone in males, whereas in females sensitization is restricted to ACTH and not observed in ELS females. HPA sensitization caused by exposure to severe predominantly emotional stressors (e.g. tail- or foot-shocks, immobilization) has been previously demonstrated in both male and female rats (Johnson et al., 2002; Belda et al., 2008, 2016; Gagliano et al., 2014). This HPA sensitization is mainly dependent on the intensity of the triggering stressor, although duration of exposure is important to determine how long-lasting sensitization is (Belda et al., 2016). Given that rats were only exposed to 3 high intensity shocks and the long interval of time between exposure to shocks and the novel environment (one month), it is surprising that sensitization of the response to the novel environment, although modest and restricted to some groups, was still evident. In general, HPA sensitization has been studied from 1 day to more than one month after the triggering stressor (Belda et al., 2016), but the effect is more persistent (28 *versus* 14 days) after immune (physical) than after emotional stressors (van Dijken et al., 1993; Schmidt et al., 1995, 2003; Hayley et al., 1999; Johnson et al., 2002; Belda et al., 2008). Since no previous study used Long-Evans rats, this strain might be particularly sensitive to sensitization. The finding that in males shock-induced sensitization was observed in corticosterone but not ACTH suggests that sensitization can also affect responsiveness of the adrenal cortex to circulating ACTH, these adrenal changes persisting far more than ACTH sensitization. Regardless of the putative mechanisms involved, these results suggest caution when interpreting sensitization observed only measuring glucocorticoids as reflecting central sensitization of the HPA axis.

In opposite direction to sensitization, prior ELS appears to reduce, in males but not females, the adrenal responsiveness to ACTH as observed in response to the novel environment but also in response to the conditioned context. There are several possibilities to explain the altered responsiveness of the adrenal cortex to ACTH after ELS or adult shock exposure, including alterations in ACTH (melacortin type 2) receptors, sympathetic signals to the adrenal or the presence in blood of cytokines or other factors modulating adrenocortical secretion (Bornstein and Chrousos, 1999; Armario, 2006). The characterization of the precise mechanisms deserves further studies.

When firstly exposed to the shock context 32 days after conditioning, conditioned males showed greater ACTH and corticosterone responses than non-conditioned animals, in accordance with previous reports using shocks (Daviu et al., 2010, 2012) or cat odor (Muñoz-Abellán et al., 2009) as the aversive stimuli. The same pattern was observed in the following extinction session. ELS had no effect in males except for reduced corticosterone response as previously commented. In females, a conditioned ACTH but not corticosterone response was observed during the test session, but prior ELS reduced the conditioned (in the shocked animals) but not the unconditioned (in the non-shocked animals) ACTH during the memory test, the effect disappearing in the extinction session. These data, together with the reduced response of ELS females to shocks after the novel environment exposure and during the conditioning, suggest that prior ELS exerted a protective effect in female responsiveness to stressors during adulthood. Whether this reduced response was not observed during extinction due to the decline in ACTH response as compared with the other conditions or whether control females showed a greater sensitivity to extinction than ELS rats (despite no differences in freezing behavior) is unclear.

Although an important number of papers have reported that ELS in rats increased adult basal activity of the HPA axis and its reactivity to stress (Liu et al., 2000; Francis et al., 2002; Huot et al., 2002), there are also reports of decreased corticosterone response to stress in males (Greisen et al., 2005; Roman et al., 2006; Rüedi-Bettschen et al., 2006) and females (Rees et al., 2006). Our ELS treatment is able to

increase the maternal care received by the pups (Fuentes et al., 2014). This effect probably buffers some of the detrimental effects of the stress exposure, being one of the factors to take into account to explain the lower HPA response induced by ELS in the adulthood after the exposure to some stressors (Fuentes et al., 2014). In addition to maternal care (Macri et al., 2011), other factor seems involved in the "protective" versus "detrimental" long-term impact of ELS treatments, such as the intensity of the stressor, the type of adult context or the genetic background (Schmidt, 2011; Daskalakis et al., 2013). It may be possible that with other more severe models of ELS contextual fear conditioning and memory at the behavioral and endocrine level would be more affected.

Conclusions

The present results demonstrated that prior ELS can exert long-lasting sex-dependent effects onbehavior and HPA function, although the two types of effects are dissociated. ELS males are hypoactivein novel environments, but similarly sensitive as non-ELS males to behavioral and endocrine consequences of adult shock exposure. In addition, they showed reduced adrenocortical responsivenessto ACTH. In females, ELS does not affect behavior, but appears to reduce shock-induced ACTHsensitization and ACTH responsiveness to certain stressful conditions, with no evidence for altered adrenocortical function as compared with non-ELS females. The above data, together with the fact that cognitive generalization of fear was greater in males than females, suggest that the consideration of female rodents as more vulnerable to stress than males is not obvious and further studies are required to characterize the specific vulnerability of each sex.

Captions for Figures and Tables.

Table 1. Freezing behavior (%) during the acquisition session (pre and post-shock, 3 min in both cases), in the memory test (15 min) and during the extinction session (15 min), in male and female rats exposed or not to an early-life stress (CTRL or ELS). Mean and S.E.M. are shown.

Figure 1. General procedure.

Figure 2. Plasma levels of ACTH and corticosterone in males and females, for control (CTR) and early-life stress (ELS) rats after the acquisition of contextual fear conditioning. Mean and S.E.M. are shown. Shocked animals were allowed to explore during 3 min the context. After this habituation period 3 shocks of 1.5 mA were administered followed by a post-shock period of 3 min. Non-shocked animals were exposed to the conditioned context for the same period of time without receiving shock. +++ $p<0.001$ versus non-shocked animals; ΔΔ $p<0.01$, ΔΔΔ $p<0.001$ vs males.

Figure 3. Distance travelled during 15 min exposure to a novel environment. Mean and S.E.M. are shown. Thirty-one days after fear acquisition control (CTR) and ELS animals were exposed to a novel environment to study long-term cognitive fear generalization. * $p<0.05$ vs respective controls; +++ $p<0.001$ vs non-shocked and ΔΔΔ $p<0.001$ vs males.

Figure 4. Plasma levels of ACTH and corticosterone in males and females, for control (CTR) and early-life stress (ELS) rats in response to a novel environment 31 days after fear acquisition. Mean and S.E.M. are shown. * $p<0.05$, *** $p<0.001$ vs respective controls; + $p<0.05$, ++ $p<0.01$ vs respective non-shocked group; and ΔΔ $p<0.01$, ΔΔΔ $p<0.001$ vs respective males.

Figure 5. Plasma levels of ACTH and corticosterone in male and female control (CTR) and early-life stress (ELS) rats in response to the fear conditioned context (15 min), 32 days after the acquisition session. Mean and S.E.M. are shown. ** $p<0.01$ vs respective controls; + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$ vs respective non-shocked groups; and ΔΔΔ $p<0.001$ vs respective males.

Figure 6. Plasma levels of ACTH and corticosterone in males and females, for control (CTR) and early-life stress (ELS) rats after an extinction session (15 min), 48 h after the fear conditioning testing session. Mean and S.E.M. are shown. * $p<0.05$ vs respective controls; ++ $p<0.01$, +++ $p<0.001$ vs respective non-shocked groups; and Δ $p<0.05$, ΔΔΔ $p<0.001$ vs respective males.

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Table 1. Freezing behavior (%) during the acquisition session (pre and post-shock, 3 min in both cases), in the memory test (15 min) and during the extinction session (15 min), in male and female rats exposed or not to an early-life stress (CTR or ELS).

Early treatment	Males				Females			
	Acquisition Pre-shock	Acquisition Post-shock	Memory test	Extinction session	Acquisition Pre-shock	Acquisition Post-shock	Memory test	Extinction session
CTR	0.1 ± 0.07	72.7 ± 5.5	75.8 ± 4.1	59.5 ± 7.8	0.1 ± 0.08	82.1 ± 5.9	74.9 ± 7.3	57.0 ± 10.7
ELS	0.6 ± 0.28	84.2 ± 5.3	76.6 ± 4.4	77.4 ± 6.9	0.1 ± 0.03	85.3 ± 3.4	78.8 ± 2.5	59.1 ± 7.9

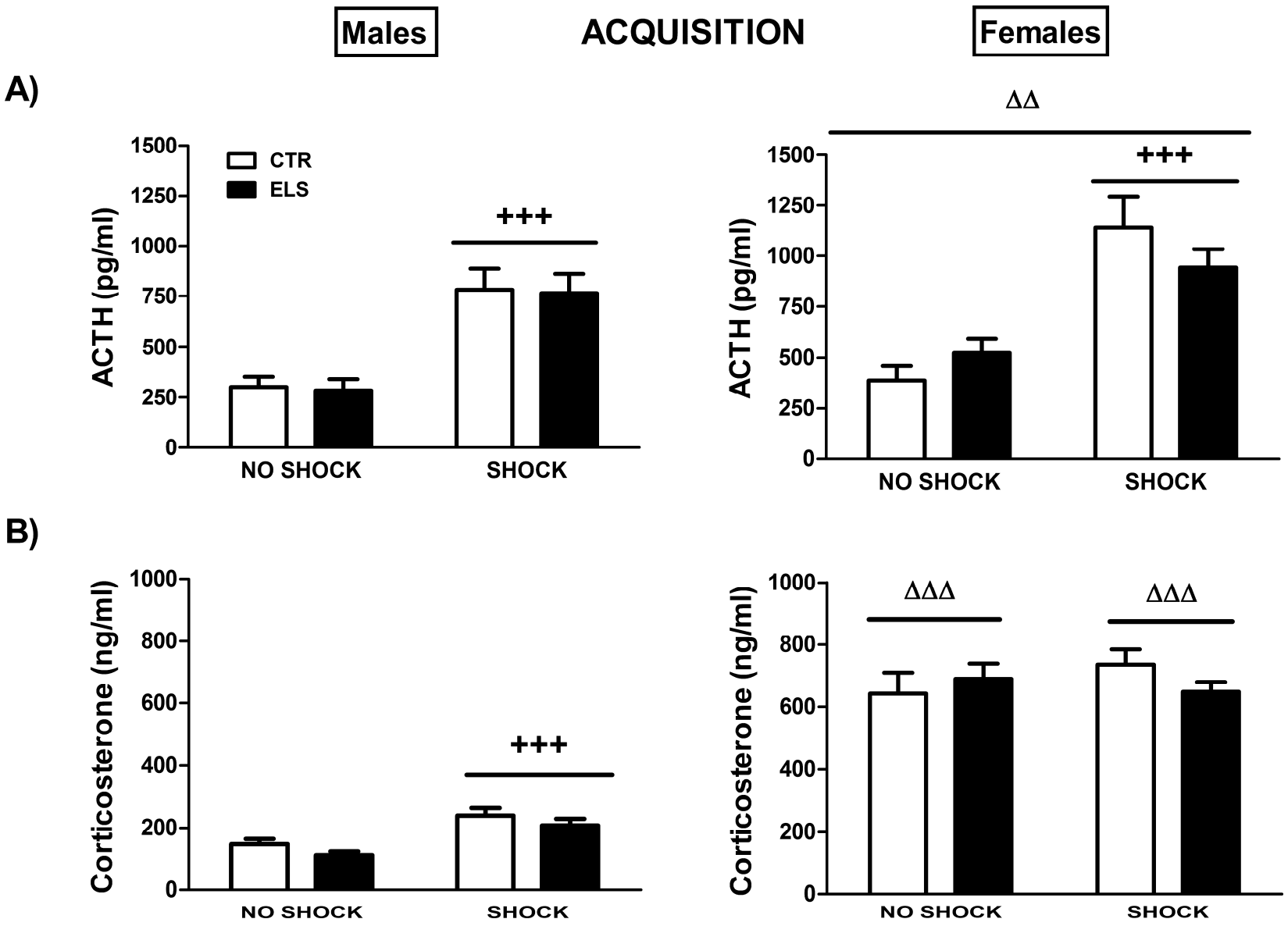
Means ± SEM are shown

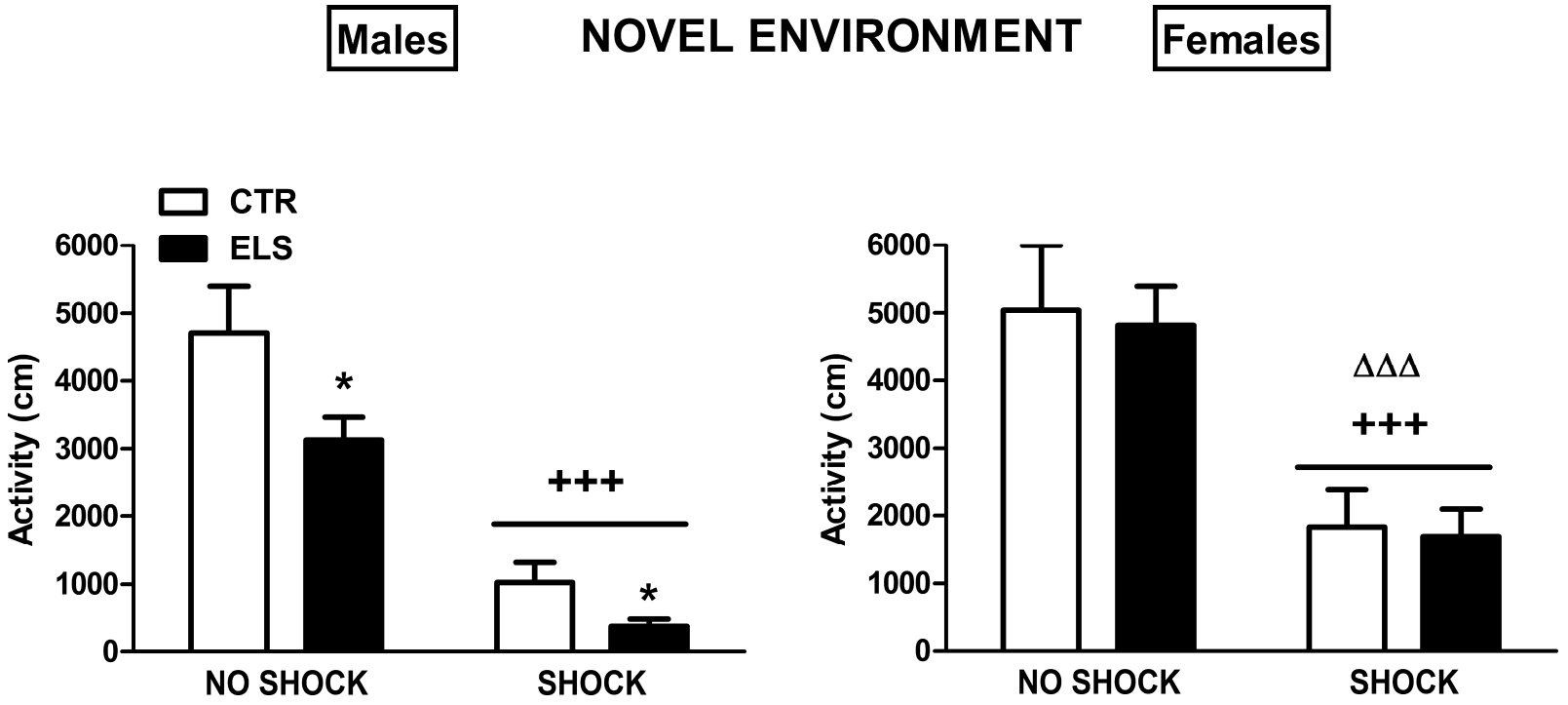
Figure 1. General procedure.

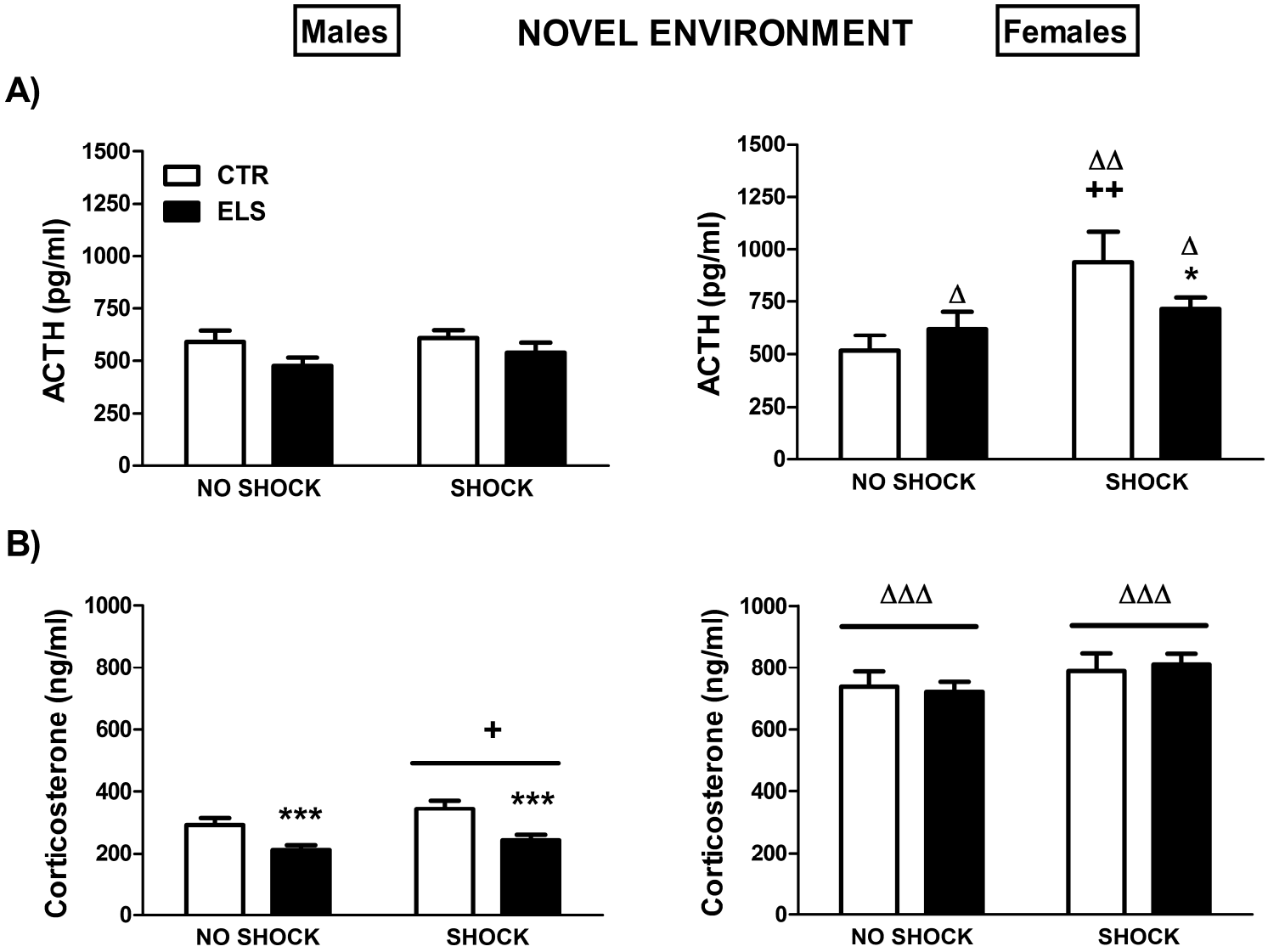
Day	GD 15	PND 1 to PND 7	PND 13	PND 18	PND 21	PND 27	PND 60-62
Intervention	Pregnant dams arrived	ELS: Restriction of nesting material (all day) + "Substitute mother" (1h/day) CTR: undisturbed Maternal behavior (biological and "substitute mother")	Maternal behavior (biological mother)	Maternal behavior (biological mother)	Weaning	Body weight	Body weight + Handling

Day	PND 62-64 / PND 65-67	PND 90-92	PND 91-93	PND 119-121	PND 122-124	PND 123-125	PND 125-127
Intervention	Handling	Body weight + Handling + Habituation to BS	Fear conditioning acquisition + BS	Body weight	Novel environment + BS	Memory test + BS	Extinction + BS

BS: blood sampling; GD: gestational day; PND: postnatal day; ELS: early-life stress; CTR: control.


Males
ACQUISITION
Females
A)
B)
CTR
ELS
ACTH (pg/ml)
Corticosterone (ng/ml)
NO SHOCK
SHOCK
+++
ΔΔ
ΔΔΔ
0
250
500
750
1000
1250
1500
200
400
600
800


Males
NOVEL ENVIRONMENT
Females
CTR
ELS
Activity (cm)
6000
5000
4000
3000
2000
1000
0
*
+++
*
NO SHOCK
SHOCK
ΔΔΔ
+++


Males
NOVEL ENVIRONMENT
Females
A)
ACTH (pg/ml)
1500
1250
1000
750
500
250
0
CTR
ELS
NO SHOCK
SHOCK
ΔΔ
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B)
Corticosterone (ng/ml)
1000
800
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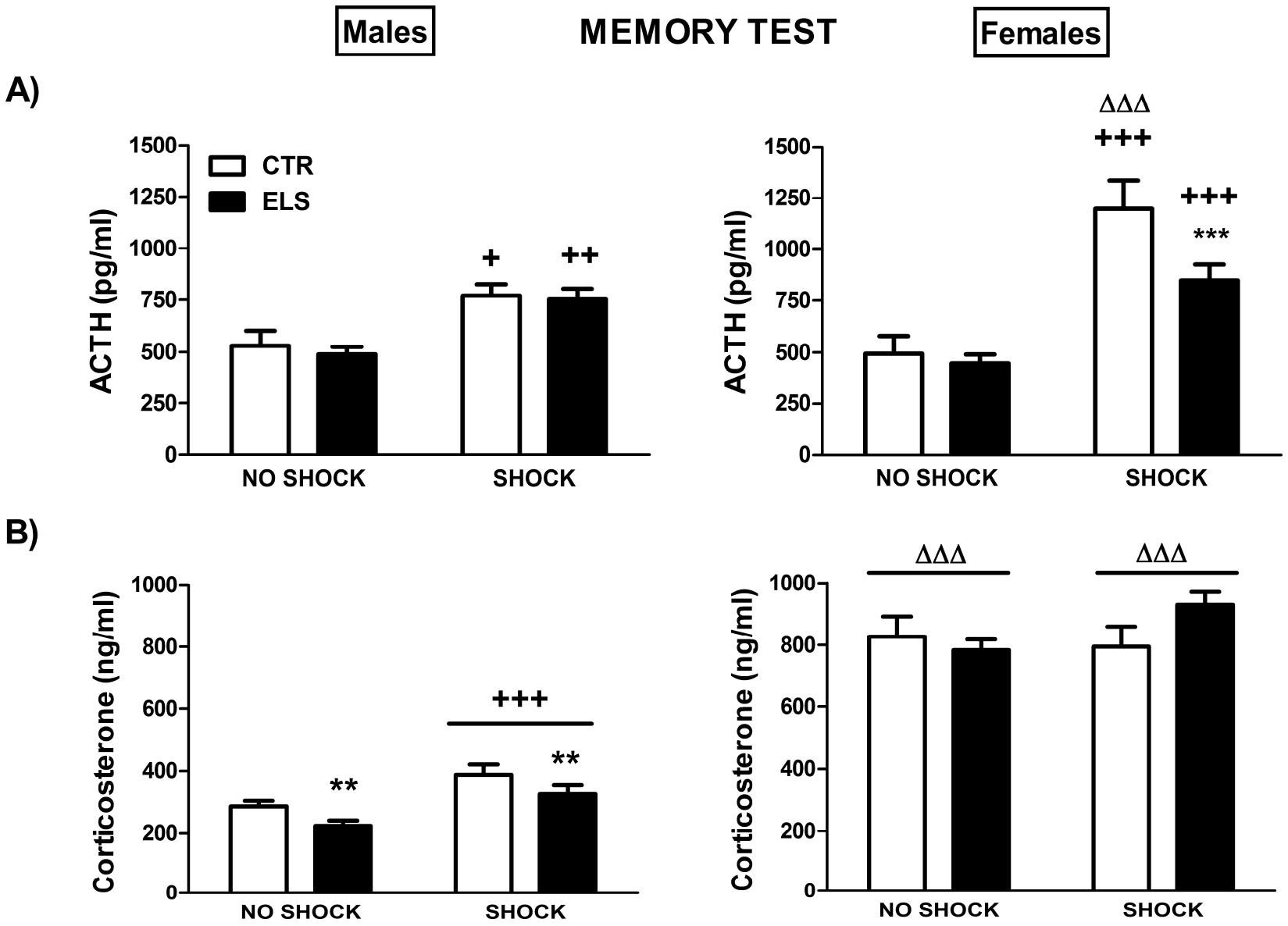
Males
MEMORY TEST
Females
A)
B)
CTR
ELS
ACTH (pg/ml)
Corticosterone (ng/ml)
NO SHOCK
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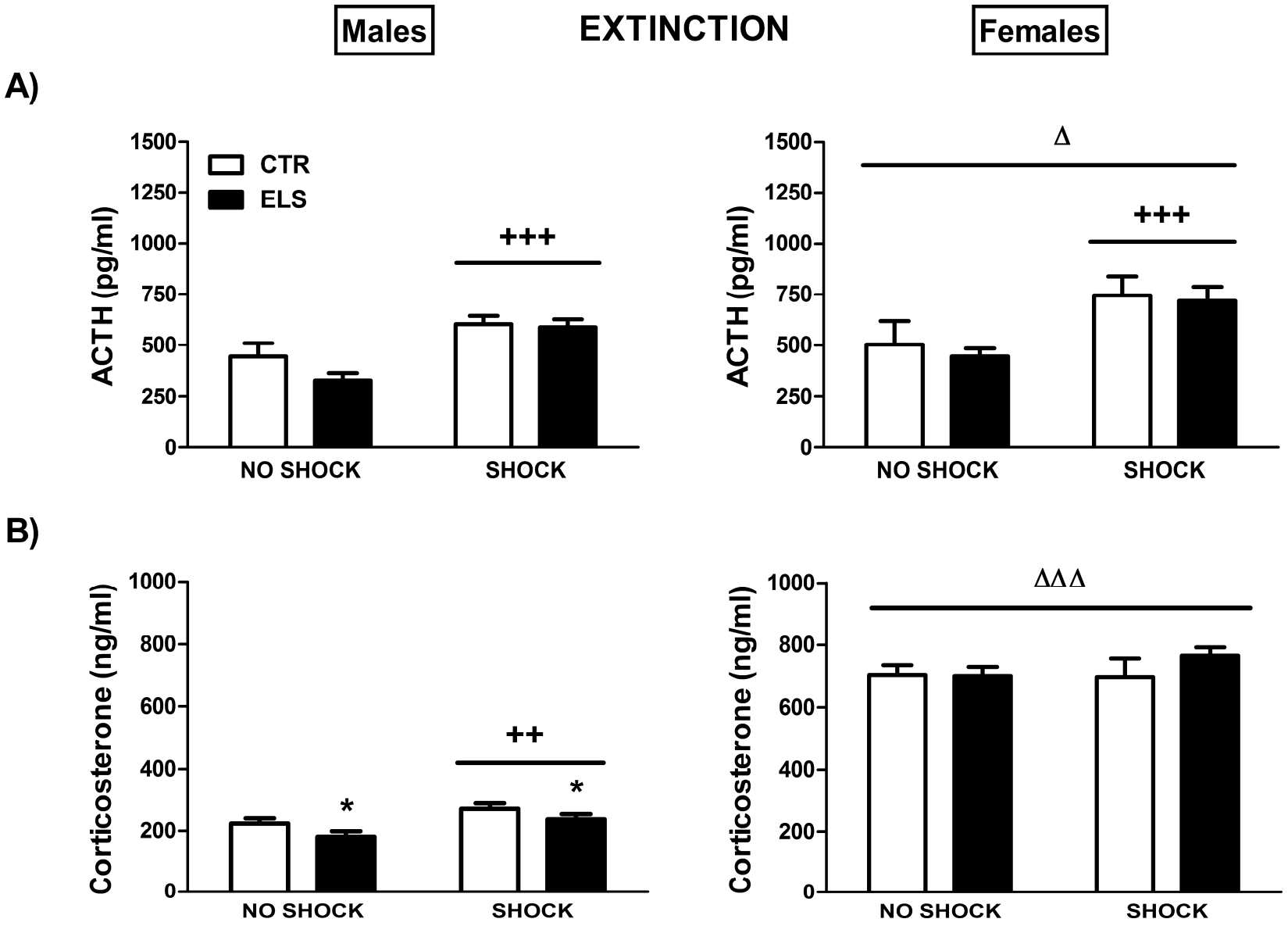
Males
EXTINCTION
Females
A)
B)
CTR
ELS
ACTH (pg/ml)
1500
1250
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NO SHOCK
SHOCK
Corticosterone (ng/ml)
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