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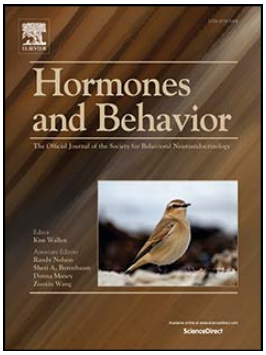

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Sex differences in the behavioural and hypothalamic-pituitary-adrenal response to contextual fear conditioning in rats

Núria Daviu[1,2], Raül Andero[1,2,#], Antonio Armario[1,2 *], Roser Nadal[1,3 *],

1 Institut de Neurociències and Red de Transtornos Adictivos (RTA), 2 Animal Physiology (School of Biosciences) and 3 Psychobiology (School of Psychology), Universitat Autònoma de Barcelona, Cerdanyola del Vallès 08193, Spain

Present address: Department of Psychiatry and Behavioural Sciences, Center for Behavioural Neuroscience, Yerkes National Primate Research Center, Emory University, Atlanta, GA, 30329, USA.

* Both authors contributed equally. Correspondence should be addressed to:
Dr. **Roser Nadal**
Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, SPAIN.
roser.nadal@uab.es
Phone 34-935868535
Fax 34-935812390
Dr. **Antonio Amario**
Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, SPAIN.
Antonio.armario@uab.es
Phone 34-935811840
Fax 34-935812390

Running head: Sex differences in contextual learning conditioning.

Summary

In recent years, special attention is being paid to sex differences in susceptibility to disease. In this regard, there is evidence that male rats present higher levels of both cued and contextual fear conditioning than females. However, little is known about the concomitant hypothalamic-pituitary-adrenal (HPA) axis response to those situations which is critical in emotional memories. Here, we studied the behavioural and HPA responses of male and female Wistar rats to context fear conditioning using electric footshock as the aversive stimulus. Fear-conditioned rats showed a much greater ACTH and corticosterone response than those merely exposed to the fear conditioning chamber without receiving shocks. Moreover, males presented higher levels of freezing whereas HPA axis response was greater in females. Accordingly, during the fear extinction tests, female rats consistently showed less freezing and higher extinction rate, but greater HPA activation than males. Exposure to an open-field resulted in lower activity/exploration in fear-conditioned males, but not females, suggesting greater conditioned cognitive generalization in males than females. It can be concluded that important sex differences in fear conditioning are observed in both freezing and HPA activation, but the two set of variables are affected in the opposite direction: enhanced behavioural impact in males, but enhanced HPA responsiveness in females. Thus, the role of sex differences on fear-related stimuli may depend on the variables chosen to evaluate it, the greater responsiveness of the HPA axis in females perhaps being an important factor to be further explored.

Keywords: Sex Differences, ACTH, Corticosterone, HPA Axis, Anxiety, Freezing, Fear Generalization.

Introduction

Exposure to electric footshock (FS) is the most extensively used experimental paradigm for the study of emotional memory and fear conditioning. One or a few FS exposures in a single session results in the development of long-lasting fear conditioning to the FS context (i.e. Fendt and Fanselow, 1999; Le Doux, 2003; Maren, 2008). More recently, FS exposure has gained interest as a putative animal model for post-traumatic stress disorder (PTSD) because it may induce long-lasting behavioural changes reminiscent of those typically observed in PTSD patients: avoidance of places and other cues associated to the traumatic event, generalization of fear/anxiety to context/cues having certain similarities with the original ones and sensitization to other putative stressful situations (Armario et al., 2008; Siegmund and Wotjak, 2007).

One typical consequence of exposure of rats to brief sessions of FS, widely described by several labs in the 90's, is a long-lasting (days to weeks) reduction of activity/exploration (herein hypoactivity) in novel environments (Bruijnzeel et al., 2001a, 2001b; Van den Berg et al., 1998; Van Dijken et al., 1992a, 1992b, 1992c). Although such hypoactivity could be at first sight interpreted in terms of FS-induced unconditioned increases in anxiety, the experimental evidence supporting this is scarce (Bruijnzeel et al., 2001a; Pijlman et al., 2003; Pijlman and Van Ree, 2002). In fact, mice previously exposed to FS showed hypoactivity in the elevated plus-maze (EPM), a classical test of anxiety, in accordance with earlier reports in rats. However, time spent in the open arms of the EPM was increased rather than reduced (Radulovic et al., 1998), arguing against enhanced anxiety. Importantly, in both mice and rats FS-induced hypoactivity in novel environments appears to be dependent on the development of fear to the original context because generalization did not develop when animals were shocked immediately after being placed into the chamber (Daviu et al., 2010, 2012; Radulovic et al., 1998), a procedure known to prevent contextual fear conditioning. It thus appears that hypoactivity is not explained by unconditioned (sensitization) and requires the development of contextual fear conditioning. This process has been termed conditioned generalization (Radulovic et al., 1998). Conditioned generalization has also been observed in mice after cued (tone) fear conditioning in that such enhanced response was not observed in the groups that received unpaired tones and FS during the training session (Laxmi et al., 2003).

Shock-induced hypoactivity in novel environments could reflect a weak degree of fear generalization, not intense enough to be detected with classical tests of anxiety. Activation of the hypothalamic-pituitary-adrenal (HPA) axis, a consistent biological marker of stress intensity (see Armario et al., 2012 for review), does not support that previous exposure to shock greatly enhances fear/anxiety in a novel environment. Thus, while exposure to the FS context results in high levels of freezing and greater HPA activation in prior shocked rats than controls, FS-induced hypoactivity in different novel environments is not associated with greater HPA response in male rats (Daviu et al., 2010). We interpreted those results as the development of a

long-lasting cautious behaviour associated to any unknown environment, which was not enough fear-provoking to induce activation of the HPA axis. We then propose the term of “conditioned cognitive generalization” to describe such a phenomenon to emphasize that this generalization is not based on configurational similarities with the original fear conditioning chamber.

The characterization of sex differences in responsiveness to stress and stress-related pathologies has gained considerable interest in the last years. There is general consensus that women are more vulnerable to develop anxiety disorders including PTSD and depression (Toufexis et al., 2006) but the neurobiological bases for this vulnerability remains elusive. Thus, the enhanced vulnerability is consonant with the higher sensitivity of females to emotional events and emotional memory (i.e. Canli et al., 2002), but fear conditioning appears to be sex-independent or greater in men than women (i.e Fredrikson et al., 1976; Milad et al., 2010; Schell et al., 1991). Similarly in animals, both contextual and cue fear conditioning, as evaluated by freezing, is higher in male than female rats and mice (see Dalla and Shors, 2009 for a review). These sex differences are more consistent with contextual fear conditioning than with cued fear conditioning (Anagnostaras et al., 1998). In addition, male rats also show longer retention of fear memory (Gresack et al., 2009) and present reduced extinction of contextual fear conditioning (Brunzell et al., 2002; Chang et al., 2009) than females.

The fact that fear conditioning is greater in males than females, measuring freezing, does not preclude that other consequences of exposure to fear conditioning or other fear-sensitive parameters follow the same pattern. In this regard, whether or not conditioned cognitive generalization differs in male and female is not known. Moreover, although there is an extensive literature on sex differences in the HPA axis functioning in rodents (Rhodes and Rubin, 1999), there is no study about sex differences in HPA response to fear conditioning. This may be of special relevance because it is widely known that the role of the HPA axis is crucial in emotional memories. Although most studies on stress focuses on plasma levels of corticosterone rather than ACTH, the latter hormone is a more direct reflection of stress-induced brain activation and has some advantages as a stress biomarker (Armario et al., 2012). Sex-differences in the endocrine response to fear conditioning are important for two main reasons. First, sex differences in ACTH response to stress in rats appear to be dependent on the particular type of stressor studied (Babb et al., 2013b; Spinedi et al., 1994; Watanobe, 2002). Second, characterization of ACTH response to fear-conditioning can contribute to determine whether or not females are actually less sensitive than males to fear conditioning. Thus, the aims of the present work were to compare male and female rats regarding: (i) the acquisition and the extinction of contextual fear conditioning as assessed by freezing and HPA axis activation, (ii) the degree of HPA sensitization and hypoactivity induced in a novel environment markedly different from the fear conditioning chamber as a consequence of previous exposure to shock.

Methods

Animals

We used 60 days old male and female Wistar rats, from Harlan Laboratories breeders (Sant Feliu de Codines, Spain). They were housed in pairs in a 1000 cm^3 plastic cages with sawdust bedding (Lignocel, Panlab S.L.U., Barcelona, Spain) in standard conditions of temperature (21 ± 1ºC) and in a 12-h light/12-h dark schedule (lights on at 08.00h). Males and females were housed in the same vivarium. Food (SAFE-diet A04, Panlab S.L.U., Barcelona, Spain) and water were available ad libitum. The experimental protocol was approved by the Committee of Ethics of the Universitat Autònoma de Barcelona and the Generalitat of Catalunya, followed the "Principles of laboratory animal care" and was carried out in accordance with the European Communities Council Directives (2010/63/EU) and the Spanish Legislation (RD 53/2013).

General procedure

The experimental procedures were always performed in the morning and handling started two days after being housed in pairs. Animals were handled for three consecutive days for approximately 2 min a day. The last day of handling, animals were subjected to a tail nick procedure to habituate animals to blood sampling procedure. The tail nick consisted of gently wrapping the animals with a cloth, making a 2 mm incision at the end of the tail veins and then massaging the tail while collecting, within 2 min, 300 µl of blood into ice-cold EDTA capillary tubes (Stardest, Granollers Spain). The cage-mates were sampled simultaneously (two experimenters were sampling at the same time and a third was gently holding the two rats). This procedure is extensively used in our lab and by others because low resting levels of hormones are obtained (e.g. Andero et al., 2012, Belda et al., 2004; Vahl et al., 2005).

Two days later, animals were randomly assigned to the experimental groups: Control Male (n = 8), Shock Male (n = 7), Control Female (n = 8) and Shock Female (n = 8). Wistar 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 cycle when samples are taken in the morning (Babb et al., 2013a; Isawaki-Sekino et al., 2009; Viau and Meaney, 1991). Moreover, monitoring the estrous cycle may have added some degree of stress which could interfere with the HPA axis measures of this study. For all these reasons, the estrous cycle was not monitored. The two animals of the same home-cage had the same treatment. On day 1 (fear acquisition), all animals were exposed to the fear conditioning chambers. Two identical fear conditioning chambers (Panlab S.L.U., Barcelona, Spain) were used, one for each cage-mate. Each chamber (25 cm X 25 cm X 25 cm) had a clear *Plexiglas* door and black aluminium 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 white house light (4 cm diameter) was placed in the right wall at 17.5 cm to the floor. The software (Freezing 1.3.0.1, Panlab S.L.U., Barcelona, Spain) controlled the house light and FS administration. The chambers were inside the sound attenuating box (67 cm X 53 cm X 55 cm, LE 111-118.8-116) provided with a fan that helps to mitigate strange sounds. The front door of this box allowed video camera recording and the image was transferred to a digital video recorder (JVC VR-716) and from there to a computer for manual analysis by stopwatch by a blind experimenter to the treatment. Freezing, a reliable measure of learned fear was assessed during the fear acquisition, context and extinction tests. Freezing involved the absence of all movement other than respiratory-related movements (Blanchard and Blanchard, 1969). The chambers were placed in a black painted room next to the vivarium which was illuminated by an indirect white 25 W bulb and the animals were transported to the test room into their home-cage. The chambers were cleaned carefully between animals with a tap water solution containing ethanol (5%, v/v). During the fear acquisition, the house light signalled the start of the session and at the end of a 3 min of habituation period shock groups received 3 FS, 3 s of duration, 1.5 mA of intensity, inter-trial interval (ITI) 1 min (non-continue squared current, frequency: 20 Hz, each pulse duration 8.3 ms, polarity: monophase, pulse current: effective), followed by 4 min of post-shock period. The control groups were exposed to the fear chamber the same time than the shock groups, without receiving shock (9 min). Immediately after the end of the session, blood samples were taken as mentioned to measure ACTH and corticosterone plasmatic levels.

Seven days after the fear conditioning acquisition all animals were placed in an open-field which acts as a novel environment for 15 min. The open-field was a grey rectangular plastic cage (56 cm X 36.5 cm X 31 cm) opened at the top and illuminated by a red 15 W bulb placed 150 cm above the centre of the cage. Behaviour was videotaped from the top by a video camera (Sony SSC-M388 CE, BW) situated 150 cm above the centre of the cage. The digital video recorder sampled the position of the rat (8.3 samples/s) and it was used to transfer the videos to a computer for subsequent video tracking analysis using the centre of gravity of the animal (Smart version 2.5.19, Panlab, S.L.U, Barcelona, Spain). The distance travelled in these tracks and the time spent immobile were analysed by an experimenter blind to the treatment. Immobility was defined as locomotor activity lower than 2 cm/s. Of note, freezing activity was found to be negligible. In addition, time spent in central areas was studied as a measure of anxiety. The apparatus was cleaned carefully between animals with a tap water solution containing ethanol (5% v/v). Immediately after the end of the session, blood samples were taken. To reduce putative cues associated with the fear conditioning apparatus, animals were tested in a different room, manipulated by a different experimenter and rats were transported from the vivarium to the room where the open-field was placed inside little plastic cages (27.5 cm X 17.5 cm X 11.5 cm). The cage-mates were tested simultaneously in the same room in two similar apparatuses.

Nine (context test/extinction 1), ten and eleven days (extinction tests) after the acquisition of the fear conditioning, all the animals were exposed to the fear conditioning chambers during 15 min without receiving shock to measure contextual fear retention and extinction by freezing. Although the reduced space of the chamber did not allow measuring horizontal activity the number of rearing episodes was measured as an index of exploration/activity. Each day of exposure to the chambers, blood samples were taken immediately after the end of the session.

In the present study, basal levels were taken three days after the end of the experiment (14 days after FS). Although it could be argued that by the time of exposure to the novel environment (7 days after FS) basal levels could have increased in shock groups (Albrecht et al., 2013), this is unlikely because unpublished data from our laboratory indicate that 7 days after exposure to a similar FS treatment and even much stronger stressors, HPA hormones are at basal levels.

Biochemical analysis

Plasma ACTH and corticosterone levels were determined by double-antibody radioimmunoassays (RIA). 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-methyl ester (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 previously described (Zelena et al., 2003). All samples to be statistically compared were run in the same assay to avoid inter-assay variability. The intra-assay coefficient of variation was less than 6 % for ACTH and corticosterone. The sensitivity was 12.5 pg/ml for ACTH and 1 ng/ml for corticosterone.

Statistical analysis

Data were analysed by the Statistical Program for Social Sciences (SPSS), VERSION 15. Log-transformation of data was applied when necessary to achieve or improve homogeneity of variances. Behavioural and hormonal response in a one single-point was analysed by means of a generalized linear model (GENLIN) (McCulloch and Searle, 2001) with two between-subjects factors (SHOCK: control and shocked, and SEX: male and female). A generalized estimated equation model (GEE) was used to analyse repeated-measures data (Hardin and Hilbe, 2003)

with one within-subjects factor (BLOCK: three periods of 5 min), and two between-subjects factors, SHOCK and SEX. In all cases, if a statistical significant interaction was found, additional pairwise comparisons were made. As a method of estimation, the maximum likelihood (ML) was used. Normality distribution and identity as a link function was always used. The significance of the effects was determined by the Wald chi-square statistic. The generalized liner model is a more flexible statistical tool than the standard general lineal model (GLM) because several types of distributions of the data and different covariance structures of the repeated-measures data could be chosen.

Results

Behavioural response in the fear conditioning procedure

Freezing response in the fear conditioning chamber is represented in Fig 1. In the post-shock period (fear acquisition, Fig 1A), the statistical analysis showed significant effects for SHOCK (Wald X^2 (1) = 195.49, $p < 0.001$), SEX (Wald X^2 (1) = 7.42, $p < 0.01$) and the interaction SHOCK x SEX (Wald X^2 (1) = 7.28, $p < 0.01$). Decomposition of this interaction showed that shock exposure increased freezing behaviour in both sexes ($p < 0.001$), but the increase was greater in males than females ($p < 0.001$). No sex differences were observed in controls.

Exposure to the conditioned context nine days later (Context test/Extinction 1, Fig 1B) showed significant effects for SHOCK (Wald X^2 (1) = 38.64, $p < 0.001$), SEX (Wald X^2 (1) = 9.94, $p < 0.01$), BLOCK (Wald X^2 (2) = 16.70, $p < 0.001$) and the interactions SHOCK x SEX (Wald X^2 (1) = 4.23, $p < 0.05$) and SHOCK x BLOCK (Wald X^2 (2) = 20.16, $p < 0.001$). The latter interaction reflects that the low levels of freezing in controls were maintained over the blocks, whereas in shocked rats progressively declined. The decomposition of the interaction SHOCK x SEX revealed that differences in freezing mainly emerged in shocked animals, showing shocked males higher levels of freezing than shocked females.

In the second extinction session (Fig 1C), the analysis revealed significant effects for SHOCK (Wald X^2 (1) = 7.65, $p < 0.01$), SEX (Wald X^2 (1) = 10.86, $p < 0.01$), BLOCK (Wald X^2 (2) = 20.06, $p < 0.001$), and the interactions SHOCK x BLOCK (Wald X^2 (2) = 50.40, $p < 0.001$); and SHOCK x SEX x BLOCK (Wald X^2 (2) = 22.31, $p < 0.001$). The decomposition of the interaction SHOCK x SEX x BLOCK revealed that differences between control and shocked males were statistically significant in the first block ($p < 0.001$). In contrast, no differences were observed in shocked and control females at any time, indicating that the freezing response was already extinguished. Moreover, lower levels of freezing were noted in control females as compared to control males in the last two blocks ($p< 0.05$, $p< 0.01$, respectively), whereas in shocked

animals, females showed lower levels of freezing than males in the three blocks ($p<0.01$, $p<0.05$ and $p<0.05$, respectively).

The analysis of the third extinction session (Fig 1D) only revealed significant effect for SEX (Wald X^2 (1) = 7.14, $p < 0.01$) in that females showed lower levels of freezing than males regardless of shock and block.

Regarding rearing activity (Fig 2), the analysis of the post-shock period in the fear conditioning chamber (Fig 2A) showed significant effects for SHOCK (Wald X^2 (1) = 97.39, $p < 0.001$) and SEX (Wald X^2 (1) = 12.36, $p < 0.001$). Exposure to shock resulted in a reduction of rearing activity in the two sexes and females showed higher levels of rearing than males, regardless of exposure to shock.

The analysis of day 9 (Fig 2B, context test/extinction 1) revealed significant effects for SHOCK (Wald X^2 (1) = 42.22, $p < 0.001$), SEX (Wald X^2 (1) = 27.92, $p < 0.001$), and the interactions SHOCK X SEX (Wald X^2 (1) = 5.49, $p < 0.05$) and SHOCK X BLOCK (Wald X^2 (1) = 7.14, $p < 0.01$). Further analysis revealed that while shocked males presented during all the session less rearing activity than control males ($p < 0.001$ in all time blocks), shocked females presented reduced rearing activity than controls only during the first block ($p < 0.01$). Overall, females presented more rearing activity than males, particularly in shocked animals (see Figure 2B for specific sex differences).

During the second extinction session (Fig 2C) statistical analysis showed significant effects for SEX (Wald X^2 (1) = 14.23, $p < 0.001$), BLOCK (Wald X^2 (2) = 94.40, $p < 0.001$) and the interactions SEX X BLOCK (Wald X^2 (2) = 11.09, $p < 0.01$), SHOCK X BLOCK (Wald X^2 (2) = 104.86, $p < 0.001$) and SHOCK X SEX X BLOCK (Wald X^2 (2) = 30.27, $p < 0.001$). The decomposition of the latter interaction revealed that shocked males showed less rearing activity than control males only during the first time block ($p < 0.01$), whereas no effect of shock was observed in females. Overall females showed higher levels of rearing activity than males, although differences emerged at specific times and conditions (see Figure 2C for specific sex differences).

The analysis of the third extinction session (Fig 2D) showed significant for SHOCK (Wald X^2 (1) = 3.96, $p < 0.05$), SEX (Wald X^2 (1) = 4.89, $p < 0.05$), BLOCK (Wald X^2 (2) = 69.73, $p < 0.001$) and the interactions SEX X BLOCK (Wald X^2 (2) = 6.65, $p < 0.05$) and SHOCK X BLOCK (Wald X^2 (2) = 8.25, $p < 0.05$). Further comparisons revealed less rearing activity in shocked than controls males during the first block ($p < 0.05$), and in shocked than control females during the second block ($p < 0.05$). Sex differences were attenuated in this session: only in the last block control females presented higher rearing activity than control males ($p < 0.05$).

Behavioural response to a novel environment

As shown in Fig 3A, during the exposure to the open-field previously shocked male rats showed reduced active with no evidence for enhanced anxiety. The factors SHOCK (Wald X^2 (1) = 9.295, $p < 0.01$); SEX (Wald X^2 (1) = 24.14, $p < 0.001$); BLOCK (Wald X^2 (2) = 14.91, $p < 0.001$); and the interactions SHOCK x SEX (Wald X^2 (1) = 6.53, $p < 0.05$); SHOCK x BLOCK (Wald X^2 (2) = 26.78, $p < 0.001$); SEX x BLOCK (Wald X^2 (2) = 29.92, $p < 0.001$); and SHOCK x SEX x BLOCK (Wald X^2 (2) = 8.71, $p < 0.05$) were statistically significant. Decomposition of the latter interaction showed that previous exposure to shock during the fear conditioning procedure produced a decrease in motor activity only in shocked males who showed hypoactivity in the first ($p < 0.001$) and the second block ($p < 0.01$) of time. Increased activity in females compared with males was observed in both control and shocked groups during the first part of the session ($p < 0.001$, blocks 1 and 2). By the end of the session (block 3), SEX and SHOCK effects disappeared. Time spent immobile was studied with results mirroring those of distance (not shown). The analysis of the percentage of time spent in central area (Fig 3B) revealed significant effects for SEX (Wald X^2 (1) = 6.86, $p < 0.01$), BLOCK (Wald X^2 (2) = 10.49, $p < 0.01$) and the interaction SEX X SHOCK X BLOCK (Wald X^2 (2) = 7.90, $p < 0.05$). Decomposition of the interaction revealed that shocked males spent more time in the central area than control males during the first time block ($p < 0.05$), whereas no effect of shocks were observed in females. Minor sex differences were observed (see Fig 3B), although females spent overall less time in the central area.

HPA activity

Resting levels

The analysis of basal levels taken 3 days after the end of the experiment (Table 1), reveals that only the SEX factor was statistically significant in both ACTH (Wald X^2 (1) = 31.90, $p < 0.001$) and corticosterone (Wald X^2 (1) = 81.89, $p < 0.001$). As expected, females showed higher levels of both hormones than males.

Response to fear conditioning

ACTH levels after exposure to shock (fear acquisition) are shown in Fig 4A. The analysis revealed significant effects for SHOCK (Wald X^2 (1) = 134.19, $p < 0.001$), SEX (Wald X^2 (1) = 31.35, $p < 0.001$) and the interaction SHOCK x SEX (Wald X^2 (1) = 195.49, $p < 0.001$). Decomposition of the interaction SHOCK x SEX showed that shock increased ACTH levels in both males and females in comparison to control groups ($p < 0.001$ in both cases), but females showed greater shock-induced ACTH levels than males ($p < 0.001$), in contrast to the freezing response. In controls, no sex differences were found.

The analysis of the ACTH levels after exposure to the conditioned context nine days later (Context test/Extinction 1, Fig 4B) showed significant effects for SHOCK (Wald X^2 (1) = 51.82, $p < 0.001$), SEX (Wald X^2 (1) = 4.91, $p < 0.05$) and the interaction SHOCK x SEX (Wald X^2 (1) = 3.83, $p < 0.05$). Further comparisons showed that although both males and females shocked animals showed higher levels of ACTH than control ($p < 0.001$ in both cases), shocked females presented higher levels of ACTH than shocked males ($p < 0.001$). In controls no sex differences were found. After the second and third extinction sessions (Fig 4C and Fig 4D), the analysis of ACTH response showed significant effects for SHOCK only (Wald X^2 (1) = 11.41, $p < 0.001$; Wald X^2 (1) = 6.79, $p < 0.01$; respectively), resulting in higher levels in shocked animals regardless of sex. Hence, in contrast to behavioural data, the endocrine response does not suggest a total extinction even after the third session.

The analysis of corticosterone during the conditioning session (fear acquisition, Fig 5A) showed significant effects for SHOCK (Wald X^2 (1) = 54.22, $p < 0.001$), SEX (Wald X^2 (1) = 308.39, $p < 0.001$) and the interaction SHOCK x SEX (Wald X^2 (1) = 9.10, $p < 0.01$). Decomposition of the interaction showed that: (i) shock exposure increased corticosterone levels in both males and females ($p < 0.001$ in the two cases), (ii) females showed higher levels of corticosterone than males in both control ($p < 0.01$) and shock conditions ($p < 0.001$).

The analysis of corticosterone response to the shock context nine days later (context test/extinction 1, Fig 5B) showed significant effects for SHOCK (Wald X^2 (1) = 37.19, $p < 0.001$), SEX (Wald X^2 (1) = 93.32, $p < 0.001$) and the interaction SHOCK x SEX (Wald X^2 (1) = 4.16, $p < 0.05$). Further comparisons showed higher levels of corticosterone in shocked than control animals in both males ($p < 0.01$) and females ($p < 0.001$), and higher levels in females than males both in control and shocked animals ($p < 0.001$ in both cases). Similar results were obtained during the second extinction test (extinction 2, Fig 5C), with significant effects for SHOCK (Wald X^2 (1) = 44.05, $p < 0.001$), SEX (Wald X^2 (1) = 226.91, $p < 0.001$), and SHOCK x SEX (Wald X^2 (1) = 6.27, $p < 0.05$). Further comparisons yielded the same results as during Context test/Extinction 1. During the last exposure to the shock context (extinction 3, Fig 5D) the analysis revealed significant effects for SHOCK (Wald X^2 (1) = 8.10, $p < 0.01$) and SEX (Wald X^2 (1) = 51.17, $p < 0.001$), with no interaction. These results indicated that the sex differences observed in the previous fear acquisition and extinction days were still observed in the fear extinction 3 in both control and shock groups. Moreover, the effect of having received shocks on the fear acquisition day was still noted regardless of sex. This also suggests that there is no extinction of the HPA axis response to the fear extinction 3 test.

HPA response to a novel environment

With regard to the ACTH response to the open-field (Fig 6A), SHOCK (Wald X^2 (1) = 14.91, $p < 0.001$) and SEX (Wald X^2 (1) = 4.74, $p < 0.05$) factors were statistically significant, but the

interaction SHOCK x SEX was not statistically significant. Thus, female rats had higher ACTH levels than males ($p < 0.05$) and the previous exposure to the shock increased the ACTH response to the open-field ($p < 0.001$). This analysis indicated that shock-induced sensitization was independent of sex.

Corticosterone levels after open-field exposure followed the same pattern as ACTH (Fig 6B): SHOCK (Wald X^2 (1) = 13.99, $p < 0.001$) and SEX (Wald X^2 (1) = 183.33, $p < 0.001$) factors were statistically significant, but not the interaction SHOCK x SEX. As in the case of ACTH, sensitization induced by previous shock was independent of sex.

Discussion

In the present work we have studied sex differences in behavioural and neuroendocrine consequences of shock-induced contextual fear conditioning and generalization. The results confirm previous reports of greater shock-induced contextual fear conditioning in male than female rats, as measured by freezing. In addition, we have extended those previous reports by demonstrating that: (i) shock-induced conditioned cognitive generalization to novel environments (e.g. an open-field), as measured by hypoactivity, was only observed in males, (ii) HPA response to shock, contextual fear conditioning and extinction were stronger in females.

Behavioural response

In response to FS both males and females developed contextual fear conditioning. However, freezing behaviour in the fear conditioning chamber was lower in females than males after shock and also in subsequent extinction tests. The opposite pattern was observed with rearing activity, thus supporting lower levels of fear conditioning in female rats, in accordance with previous studies (see for a review Dalla and Shors, 2009). Of note, there were no sex differences in freezing under control conditions, probably because of the very low levels, but rearing activity was higher in females both in control conditions and after shock. This suggests that the overall higher levels of activity in females (Armario and Nadal, 2013) might at least partially contribute to the reduced fear-conditioned freezing behaviour.

To study sex differences in extinction, the animals were repeatedly exposed to the fear conditioning chamber for two additional days and both behavioural and HPA responses were evaluated. Both male and female rats showed evidence for extinction of freezing behaviour, but this extinction was faster in females than males, confirming previous data (Brunzell et al., 2002; Chang et al., 2009). Although fear conditioning testing typically lasts for 5 min (i.e. Brunzell et al., 2002; Chang et al., 2009), exposure to the conditioned context (without FS) were longer (15 min) in our study to measure behavioural and endocrine changes (Armario et al., 2012). In those conditions, extinction already developed during the Context Test/Extinction 1 session in

females, whose freezing behaviour decreased across the 3 blocks of 5 min. In contrast, male freezing was more stable across time, suggesting less within-session extinction.

Rearing activity revealed additional interesting sex differences other than the overall higher levels of females. In control males, rearing activity increased over the sessions, particularly during the first 5 min, whereas in control females it was quite stable over the days. This is likely explained by a higher initial anxiety of males than females in the chamber that progressively diminished over the sessions. Regarding fear extinction, the results supported greater extinction rate in females, although some residual effect of conditioning was still observed in the two sexes in extinction 3, suggesting that not all measures are equally sensitive to extinction.

Although we did not measure pain sensitivity levels in our study, it has been found to be greater in females than males using different nociceptive stimuli, including FS (Barker and Galea, 2010; Basso et al., 2011; Beatty and Beatty, 1970; Cook and Moore, 2006; Li et al., 2009). In fact, females learn better than males an active avoidance task that uses FS as the aversive stimulus (Dalla and Shors, 2009). Therefore, neither differences in FS sensitivity between sexes seem to account for differences in fear conditioning, nor reduced fear conditioning in females is associated with impaired learning capabilities.

Animals exposed to a shock in a particular environment rapidly develop fear conditioning to the shock chamber, but also show freezing in another novel environment partially different from that where they were shocked, a phenomenon called contextual generalization. Contextual fear generalization, which is highly dependent on the hippocampus, is opposite to contextual discrimination (Frankland et al., 1998). Fear generalization increases with the intensity of shocks (Baldi et al., 2004), the resemblance of the fear conditioning context and a novel context (Gonzalez et al., 2003) and the time elapsed between training and testing (Houston et al., 1999; Wiltgen and Silva, 2007). Additionally, it is also dependent on the particular strain of mice studied (Radulovic et al., 1998). However, hypoactivity in novel environments appears to be a very special type of generalization that is not dependent on the configuration of the environment as there was not any resemblance with the fear conditioning chamber (Bruijnzeel et al., 2001a, 2001b; Daviu et al., 2010, 2012; Radulovic et al., 1998; Van den Berg et al., 1998; Van Dijken et al., 1992a, 1992b, 1992c). Moreover, hypoactivity is dependent on the development of contextual fear conditioning and not of shock exposure itself (Daviu et al., 2010; Radulovic et al., 1998), thus ruling out non-specific shock-induced sensitization. Of note, signals associated to the shock may be related not only to the particular context where shock occurred, but also to the experimenter that handled the animals, the way the animals are transported and the particular characteristics and the type of illumination of the room (distal context) (Kissinger and Riccio, 1995). In the present experiment, all these putative distal signals were maintained when the contextual fear conditioning was studied (fear acquisition, context test and extinction sessions), but all them were changed when the animals were exposed to the novel

environment. Despite elimination of all putative conditioned signals, when previously shocked rats were exposed to an open-field completely different from the fear conditioning chamber, hypoactivity was observed in males but not in females. In addition, according with previous results this hypoactivity was not associated with enhanced anxiety (Daviu et al., 2010, Radulovic et al., 1998). In fact, time spent in central area of the open-field was found to be higher in shocked than control males and not altered in shocked females. Therefore, it appears that some conditioned cognitive generalization of fear to any unknown environment, independent on its particular configuration, may underlie this behaviour. The present concept of conditioned cognitive generalization is reminiscent of the generalization of aversion to odours other than that associated to shock reported in mice (Pamplona et al., 2011).

To the best of our knowledge, this is the first report specifically addressing sex differences in conditioned cognitive generalization. Hypoactivity was observed in shocked males, but not females, which is compatible with the lower levels of shock-induced contextual fear conditioning in females since conditioned cognitive generalization is associated with the degree of contextual fear conditioning as explained above. It is unlikely that this lack of cognitive generalization of the females could be due to a better discrimination than males between the shock context and the other different environments for two main reasons. First, it is well-characterized that whereas lesions of the hippocampus before shock exposure do not greatly affect contextual fear conditioning, this brain area is critical for discrimination between the original context and other contexts sharing certain common features, although freezing in a completely different environment was not affected (e.g. Frankland et al., 1998). Second, the huge differences between the two environments make it difficult not to discriminate between them, but if females better discriminated between shock context and the novel environment than males, this would suggest an enhanced hippocampal functioning in females. This hypothesis is not supported by previous studies demonstrating that males perform similarly as or better than females in classical tests of hippocampal functioning such as spatial memory in a Morris water maze (i.e. Bucci et al., 1995; Carr et al., 2003; Perrot-Sinal et al., 1996; Roof and Stein, 1999; Silva-Gomez et al., 2003; Wagner et al., 2004; Wang et al., 2000).

The present data are compatible with another report showing that gonadectomized males presented more freezing than gonadectomized females in the shock context and in a different environment (Barker and Galea, 2010), suggesting greater conditioned cognitive generalization in males. Moreover, this pattern was not altered by estradiol administration. Thus, these sex differences do not appear to be dependent on the presence of sex hormones but rather on their early organizational effects. More recently, it has been suggested that the effects of estrogen in fear learning appear to be limited to certain processes. Endogenous estrogen influences extinction recall but neither fear conditioning nor fear extinction in both naturally cycling women and female rats (Lebron-Milad and Milad, 2012). Thus, it is unlikely that in our study

endogenous estrogen influenced the freezing levels of females during fear acquisition and fear context test.

Endocrine response

Basal levels of both ACTH and corticosterone were higher in female than male rats being these differences particularly evident in corticosterone levels. Our data are in line with previous studies from our lab (Fuentes et al., 2014; Peña et al., 2009) and with most previous reports measuring basal plasma corticosterone (e.g. Patchev et al., 1999; Rhodes et al., 2002; Seale et al., 2004a). Less consistent results have been obtained with resting ACTH levels likely because differences are usually small (e.g. Aloisi et al., 1994; Isawaki-Sekino et al., 2009; Peña et al.,2009; Rivier, 1999).

Regarding the response to stress, females showed greater ACTH and corticosterone response to both FS stress and fear extinction than males. In contrast, no differences were observed between males and females in the hormonal response to the low intensity stressor of being exposed to an unknown environment (control animal exposed to shock context without receiving shock), particularly when differences in resting levels were considered. Whereas a greater corticosterone response to stress in females than males is consistently observed across experiments (Aloisi et al., 1994; Armario et al., 1995; Galea et al., 1997; Peña et al., 2009; Patchev et al., 1999; Seale et al., 2004b), sex differences in ACTH responsiveness to stressors are not consistent (Aloisi et al., 1994; Erskine et al., 1975; Faraday et al., 2005; Isawaki-Sekino et al., 2009; Peña et al., 2009; Rivier, 1999; Spinedi et al., 1994). It is unlikely that discrepancies could be due to the phase of the estrous cycle as most results failed to find differences (Babb et al., 2013a; Isawaki-Sekino et al., 2009; Viau and Meaney, 1991). Interestingly, there is evidence to suggest that sex differences in ACTH response may be dependent on the particular type of stressor (Babb et al., 2013b; Spinedi et al., 1994), and this is supported by our present results demonstrating greater sex differences in the ACTH response to shock and conditioning than to novel environments. It is of note that brain corticosterone levels appears to be similar in the two sexes despite higher total levels of corticosterone in females (Droste et al., 2009), probably due to the higher levels of corticosteroid-binding globulin in females (i.e. Gala and Westphal, 1965), which result in similar free circulating levels of corticosterone in the two sexes.

When exposed to an open-field, those rats previously exposed to FS showed, regardless of sex, a greater HPA response than non-shocked controls. It is unlikely that this enhanced HPA response may be explained by enhanced fear/anxiety of previously-shocked rats in any unknown environment as endocrine sensitization was similarly observed in male and female rats, whereas hypoactivity was only observed in males. Alternatively, a long-lasting shock-induced HPA sensitization could better explain the results. There is ample evidence about long-lasting stress-induced sensitization of the HPA axis after previous exposure to tail-shock and

immobilization on boards (IMO). However, the magnitude of the effect is dependent on the severity and length of exposure to the stressors (see Armario et al., 2008 for a review). Results from our lab show that after an acute exposure to IMO a long-term endocrine and/or behavioural sensitization to novel environments can develop, but both phenomena appear to be dissociated (Belda et al., 2008, 2012; Gagliano et al., 2008; Muñoz-Abellán et al., 2008).

In previously shocked rats, the HPA response to the open-field was lower than that observed in the fear conditioning chamber, suggesting that exposure to the shock context caused activation of the HPA axis that can be specifically attributed to the development of contextual fear conditioning rather than to non-specific sensitization. Although there was earlier evidence for a specific activation of the HPA axis by the context previously associated to shock (e.g. Merino et al., 2000; Van de Kar et al., 1991), to our knowledge only recent studies from our lab have made a distinction between shock-induced contextual fear conditioning and sensitization in male rats (Daviu et al., 2010, 2012), with results similar to those reported here. Concordantly, the HPA axis also reflects contextual fear conditioning associated to predator odour exposure (Muñoz-Abellán et al., 2009).

The ACTH and corticosterone responses to the fear conditioning chamber were greater in females than males, in contrast to the behavioural data demonstrating lower levels of freezing in females. Therefore, the increased HPA response to the fear-conditioning context in females could reflect a general greater activation of the HPA response to stressors in females rather than sex differences in fear conditioning. Moreover, in contrast to the extinction of freezing observed under the present experimental conditions, a higher HPA axis responsiveness to the context was maintained during the second day of extinction in both females and males. Even after the third day of extinction, HPA activation was slightly higher in shocked animals, although during this last session we cannot rule out that this merely reflected a residual long-lasting HPA sensitization. Thus, the temporal course of extinction appears to be different from endocrine and behavioural perspectives, which points to the importance of measuring different dimensions of fear. All these data are particularly exciting because, to the best of our knowledge, this is the first study which simultaneously measures freezing behaviour and the HPA response during extinction.

Conclusions

The present data confirm that female rats showed lower levels of shock-induced contextual fear conditioning than males in terms of freezing and rearing activity. In addition, we demonstrate that the lower impact of shock in females is also reflected in the absence of hypoactivity in an unknown environment (open-field), which is typically observed in shocked males. Given the huge differences between the shock chamber and the open-field, sex differences in the

behaviour in the open-field is unlikely to be related to a deficit of females to establish a hippocampus-dependent representation of the context, but to higher levels of conditioned cognitive generalization in males. The present results also demonstrate that, in contrast to results obtained measuring freezing, females are more responsive to shock than males in terms of HPA hormones both immediately after shock and during fear conditioning testing and extinction. This differential HPA response to shock and shock-induced fear conditioning may be an intrinsic property of the HPA axis not related to a particular overall susceptibility to these stressful conditions. However, the enhanced HPA response of females to shock and shock-induced fear conditioning may be important. For instance, it has been suggested that high corticosterone levels might have anxiolytic properties in mice exposed to shock-induced fear conditioning (Albrecht et al., 2013), although the precise role of glucocorticoids in anxiety is a controversial topic that needs to be more systematically addressed. On the other hand, certain physiological consequences of stress linked to the activity of the HPA axis (i.e. immune suppression) might be stronger in females than males.

From the perspective of animal models of human psychopathologies, the present results fits with some, but not all, previous data reporting lower levels of fear conditioning in women (see Introduction). However some important caveats remain. First, there are no data on HPA responsiveness to fear conditioning in humans, but cortisol response to stress is in general lower in women (Kudielka and Kirschbaum, 2005), in contrast to the present results. Second, we have offered no evidence for a higher susceptibility of females to shock-induced behavioural alterations. Quite the opposite, female rats appears to be less vulnerable than male rats. Third, it is still unclear why women are more vulnerable to anxiety and depression, but sex differences are evolutionary linked to environmental challenges each species have encountered. So, it cannot be rule out that rodents are not the most appropriate species to model certain aspects of human psychopathologies.

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Captions for Figures

Fig.1. Percent time spent in freezing behaviour in the shock context (fear conditioning chamber) in male and female rats. Shock groups were exposed to 3 shocks (1.5 mA, 3 s, ITI 60 s). Means and S.E.M. are shown. (1A) Post-shock period, (1B) Context test, and two consecutive days of extinction (1C, 1D). Data correspond to 3 blocks of 5 min. *** $p<0.001$ vs respective control groups. +++ $p<0.001$ vs respective male groups.

Fig.2. Number of rearings in shock context (fear conditioning chamber) in male and female rats. (2A) Post-shock period, (2B) Context test, and two consecutive days of extinction (2C, 2D). Data corresponds to 3 blocks of 5 min. * $p<0.05$, ** $p<0.01$, *** $p<0.01$ vs respective control group. + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$ vs respective male groups.

Fig.3. (A) Total distance travelled and (B) percentage of time spent in central area (divided into 3 blocks of 5 min) during 15 min of exposure to an open-field, in male and female control rats and rats previously exposed to 3 shocks (1.5 mA, 3 s, ITI 60 s) 7 days before. Means and S.E.M. are shown. ** $p<0.01$, *** $p<0.001$ vs respective control groups. + $p<0.05$, +++ $p<0.001$ vs respective male groups.

Fig.4. Plasma ACTH levels in male and female control rats and in rats previously exposed to 3 shocks (1.5 mA, 3 s, ITI 60 s). Means and S.E.M. are shown. Hormone levels were measured immediately after exposure to shock (4A) or some days later after 15 min exposure to the shock context (2B) and two consecutive days of extinction (4C, 4D). Dotted lines correspond to basal ACTH levels obtained on a different day. ** $p<0.01$, *** $p<0.001$ vs respective control groups; +++ $p<0.001$ vs respective male groups.

Fig.5. Plasma corticosterone levels in male and female control rats and in rats previously exposed to 3 shocks (1.5 mA, 3 s, ITI 60 s). Means and S.E.M. are shown. Hormone levels were measured immediately after exposure to shock (5A) or some days later after 15 min exposure to the shock context (5B) and two consecutive days of extinction (5C, 5D). Dotted lines correspond to basal ACTH levels obtained on a different day. ** $p<0.01$, *** $p<0.001$ vs respective control groups; ++ $p<0.01$, +++ $p<0.001$ vs respective male groups.

Fig.6. Plasma ACTH levels (6A) and plasma corticosterone (6B) in males and females rats after 15 min of open-field exposure. Dotted lines correspond to basal ACTH or corticosterone levels obtained on a different day. ** $p<0.01$, *** $p<0.001$ vs respective control groups; + $p<0.05$, +++ $p<0.001$ vs respective male groups.

Table captions.

Table 1. Basal plasma ACTH and corticosterone levels in male and female rats 14 days after they had been exposed to shock or not (controls). Means ± SEM are shown.

	ACTH (pg/ml)	Corticosterone (ng/ml)
Control male	53 ± 14	10 ± 6
Shock male	57 ± 16	17 ± 16
Control female	90 ± 26 +++	226 ± 110 +++
Shock female	95 ± 20 +++	258 ± 98 +++

Mean ± S.E.M. are shown. +++ $p<0.001$ vs male

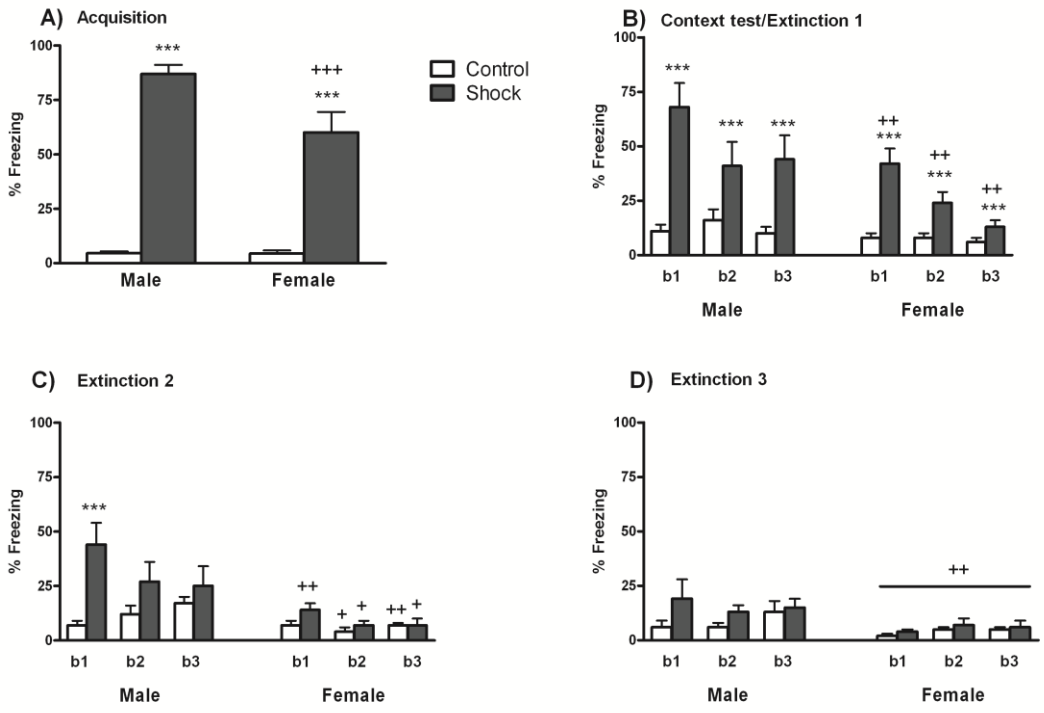

Figure 1

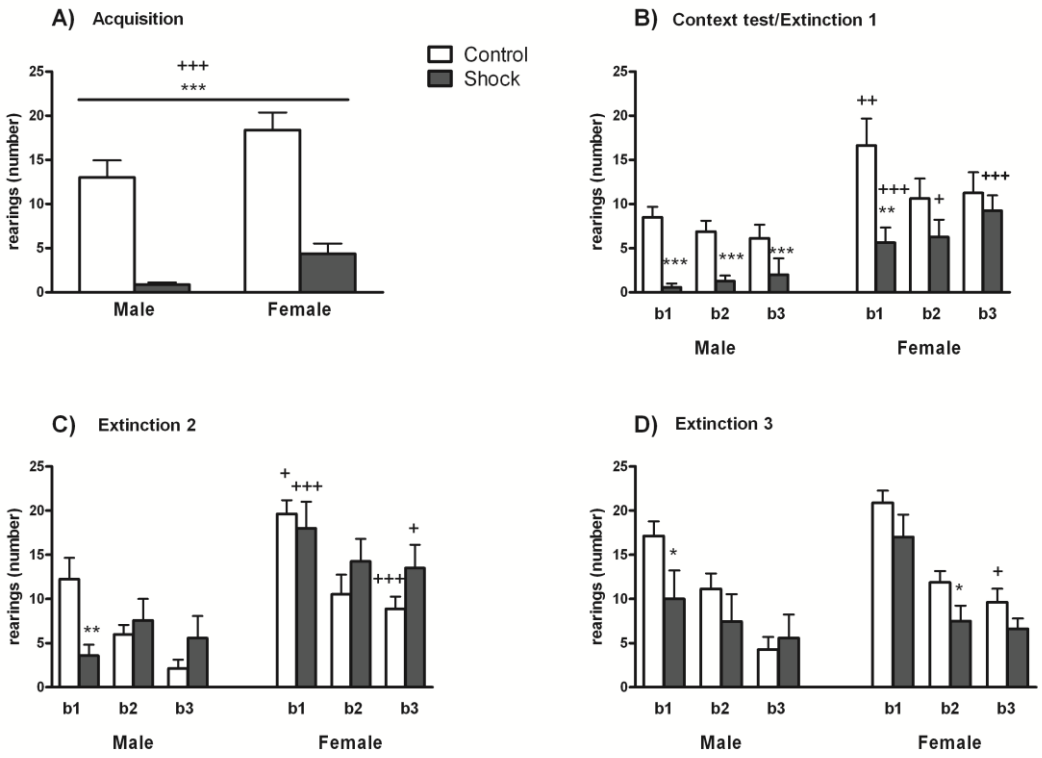

Figure 2

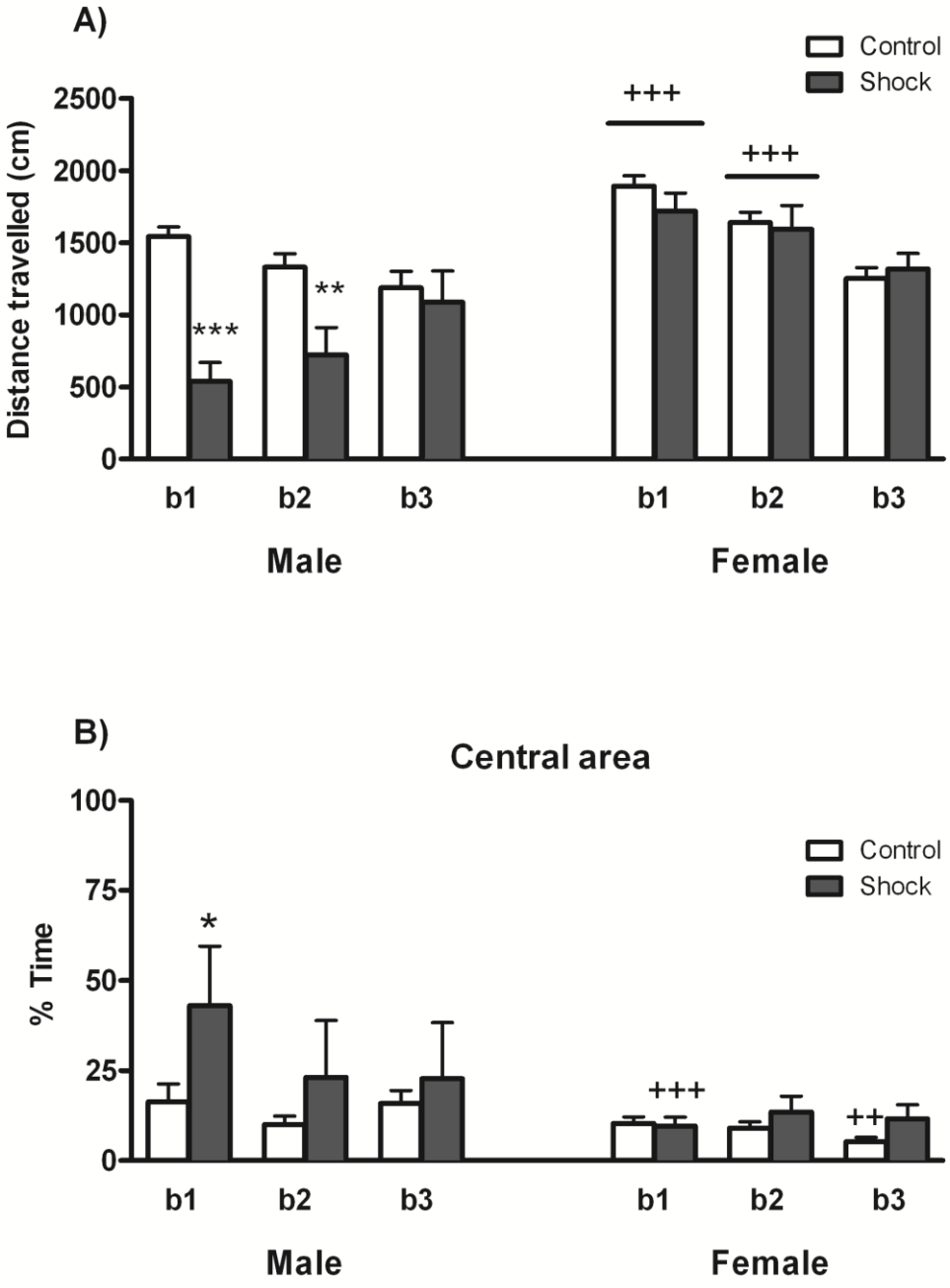

Figure 3

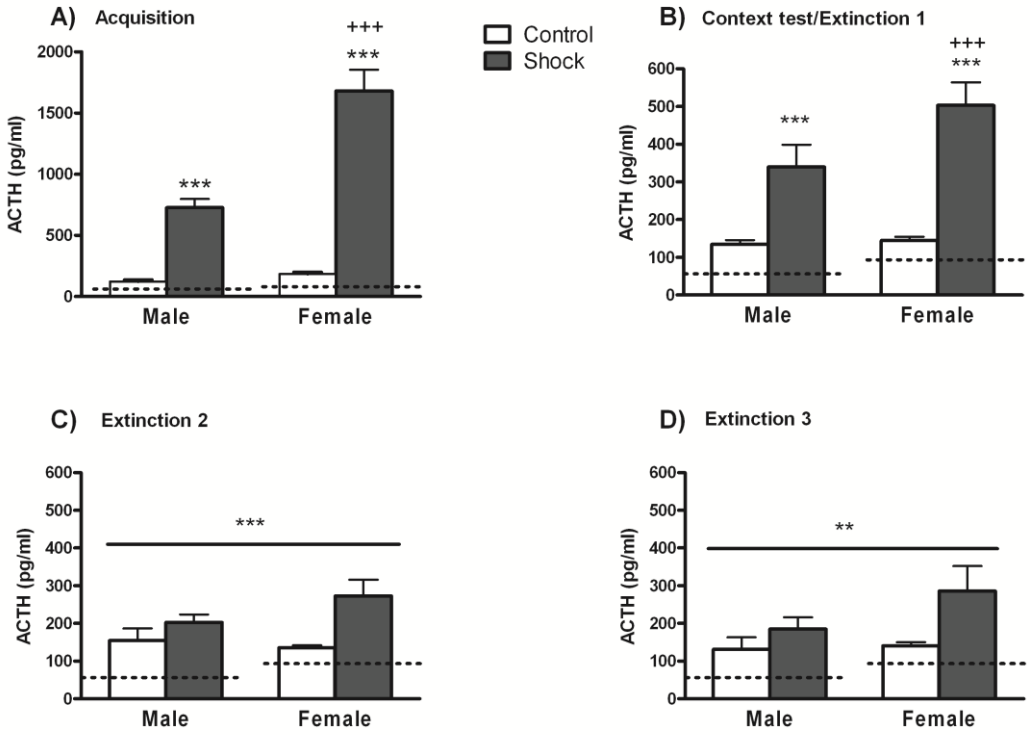

Figure 4

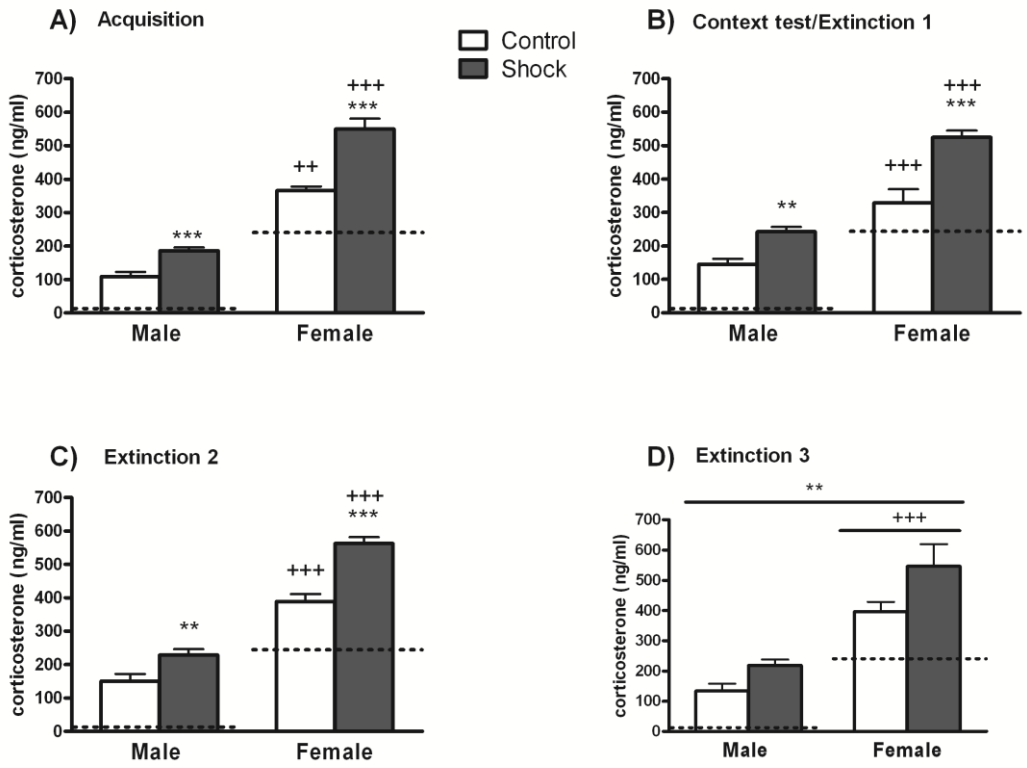

Figure 5

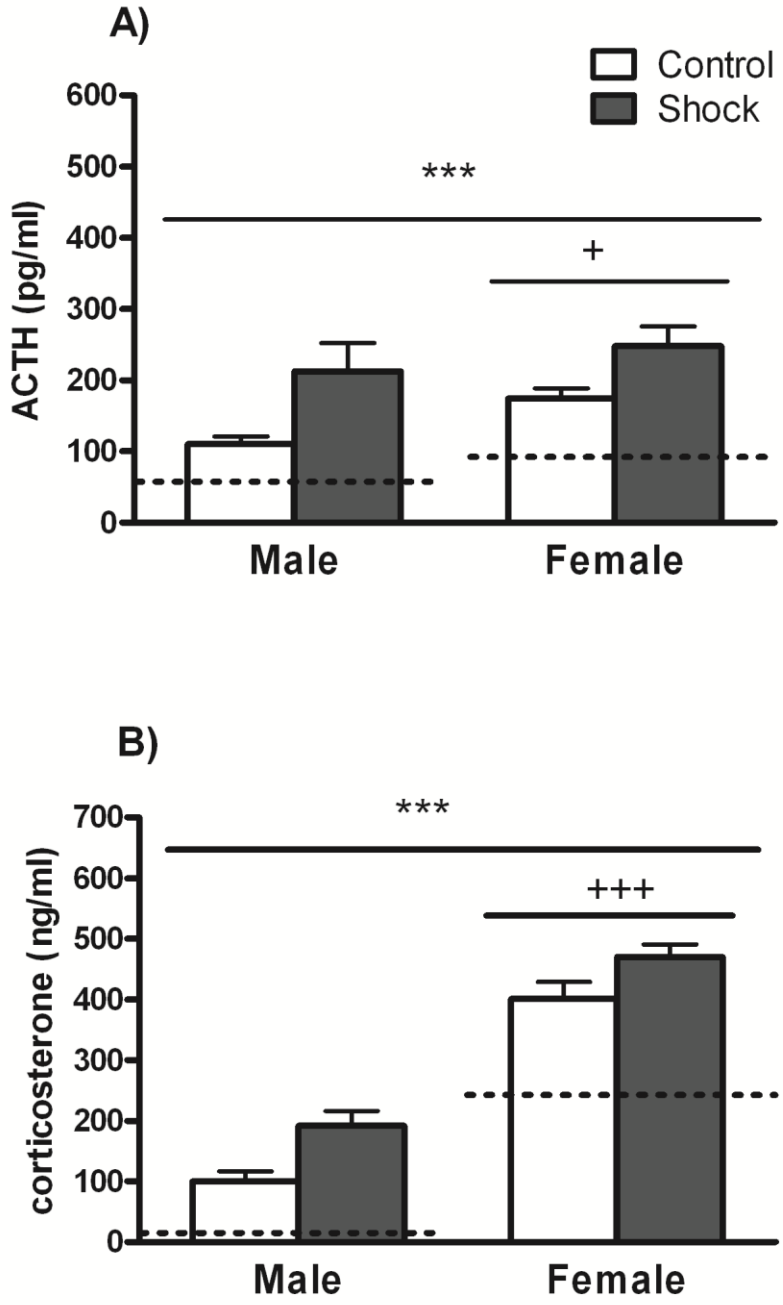

Figure 6

Research highlights:

- Shock-induced contextual fear conditioning measured by freezing was greater in males than in females.
- HPA response to contextual fear conditioning (acquisition, test and extinction) was stronger in females than in males.
- Shock-induced generalization of fear to an open-field was only observed in males.