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No effect of sex and estrous cycle on the fear potentiated startle response in rats

Yulong Zhao, Elisabeth Y. Bijlsma*, Monika P. Verdouw, Lucianne Groenink

Division of Pharmacology, UIPS (Utrecht Institute of Pharmaceutical Sciences), Universiteitsweg 99, 3584 CG Utrecht, Netherlands

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ABSTRACT

The prevalence of anxiety disorders is higher in women than in men. Yet preclinical studies on anxiety are mostly performed in male subjects. This may have limited our understanding of mechanisms contributing to anxiety disorders. Since fear conditioning is considered an important factor in the etiology of anxiety disorders, the present study aimed to investigate the effect of sex and estrous cycle on conditioned fear and the anxiolytic effect of benzodiazepines in rats.

We measured the fear-potentiated startle response in male and female rats during different estrous cycle stages and performed a replication study in a separate cohort. In addition, we assessed the response to diazepam (0–3.0 mg/kg IP) and chlordiazepoxide (0–10 mg/kg IP) in male and female rats in proestrous/estrous and diestrous stage.

Our results showed that there were no sex differences in the expression of fear-potentiated startle. The estrous cycle also did not affect the fear-potentiated startle response. In addition, male and female rats did not differ in their fear-potentiated startle response following treatment with either diazepam or chlordiazepoxide.

In conclusion, the current study shows that male and female rats do not differ in their conditioned fear response and the responsiveness to benzodiazepines. The results further indicate that conditioned fear-related processes are not affected by gonadal hormone fluctuations in this paradigm. These findings may suggest that the higher prevalence of anxiety disorders in women more likely results from differences in responding to previous experiences or differences in other predisposing factors, rather than differences in conditioned fear per se.

1. Introduction

Fear conditioning is considered an important factor in the etiology of anxiety disorders and posttraumatic stress disorders [1]. In several case-control studies, individuals with panic disorder [2,3] or posttraumatic stress disorder [4,5] showed enhanced conditioned fear relative to healthy controls. The prevalence of anxiety disorders and posttraumatic stress disorder is up to twice as high in women compared to men [6–8]. Furthermore, sex differences have also been reported in drug responsiveness during the treatment of anxiety disorders [9–11]. Nevertheless, currently, the clinical treatment is similar between women and men [12]. Several preclinical studies have reported sex differences in animal anxiety-related behavioral models as well (reviewed in [13]). Yet, most preclinical studies on anxiolytic drug action have been performed in male animals only. This approach may have hampered a better understanding of mechanisms contributing to the actions of anxiolytics.

The fluctuation of gonadal hormones during the estrous cycle of female rats, especially estrogen and progesterone, may contribute to the sex differences in anxiety-like behavior. Female rats normally have an estrous cycle of approximately four to five days, which comprises four stages: an estrous, metestrous, diestrous, and proestrous stage. During the proestrous stage and the first half of estrous stage levels of progesterone and estrogen are increased to their peak, relative to the other stages [14]. In several behavioral tests, including the elevated plus maze [15–18], open field [19], and black and white box [20], female rats in proestrus or estrus showed reduced levels of anxiety relative to rats in metestrus and diestrus as well as male rats. Also, treatment with estrogen or progesterone had anxiolytic-like effects in a variety of tests, such as the elevated plus maze [21] and conditioned fear extinction [22].

The fear-potentiated startle test is a tool to measure the expression of conditioned fear. In this test, the startle response is enhanced when the startle-eliciting noise is presented in the presence of a previously conditioned aversive stimulus (typically a shock-paired cue light) [23]. The test is considered a reliable tool for detecting anxiolytic properties

* Corresponding author.
E-mail address: e.y.bijlsma@uu.nl (E.Y. Bijlsma).

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of agents [24,25] and has high translational value for studying fear and anxiety related processes (reviewed in [26]). Only a few studies have reported on sex differences in the rat fear-potentiated startle test and findings are conflicting. It has been reported a higher fear-potentiated startle response in female rats [27,28], whereas the absence of sex differences has also been found [29,30]. In addition, the effect of estrous cycle stage and gonadal hormone levels on fear-potentiated have hardly been studied. In female rats, both estrogen and progesterone peak during proestrus and drop quickly during the estrus [14]. One study compared female rats in different estrous cycle stages and did not find any differences [29]. With regard to the direct effects of gonadal hormone administration, studies indicate that estrogen enhances fear-potentiated startle in female rats [29] whereas progesterone administration does not affect fear-potentiated startle in female rats [31]. However, the addition of progesterone to estrogen treatment has been reported to counteract the anxiogenic effect of estrogen treatment [29]. Given the limited and conflicting results, we performed an exploratory study to examine potential sex differences in the fear-potentiated startle test and included estrous cycle as an experimental factor in the study design to objectively assess the effect of estrous cycle on sex differences in fear responding.

The benzodiazepines diazepam and chlordiazepoxide are frequently used as a short-term treatment anxiolytic in clinical practice [32]. They enhance the effect of the neurotransmitter gamma-aminobutyric acid (GABA) at the GABAA receptor through binding to the benzodiazepine recognition site on the GABAA receptor, resulting in anxiolytic effects [33]. Till now, only limited number of studies have investigated sex differences in the anxiolytic effects of benzodiazepines in humans and these studies consistently report that no sex differences were detectable [34–37]. However, sex differences in response to benzodiazepines have been reported regarding electroencephalogram, psychomotor impairments, and cognitive impairments, with drug effects being stronger in women than men [9,38,39]. Also, benzodiazepine dependency seems to occur more often in women than men [9]. In rodents, several studies studied sex differences in the anxiolytic effects of benzodiazepines but showed inconsistent results. Female rats appeared less sensitive to the anxiolytic effects than male rats in defensive burying test [40,41], but no sex differences [42,43], or even higher sensitivity of female rats [44], have been found in other tests. In male rats, diazepam has an anxiolytic effect in the fear-potentiated startle test [45–47]. Studies with male rats also show a clear anxiolytic effect of chlordiazepoxide on fear-potentiated startle response [48]. However, the effects of both diazepam and chlordiazepoxide on fear-potentiated startle have not been tested in female rats. Interestingly, allopregnanolone, a metabolite synthesized from progesterone, acts as a potent positive allosteric modulator of the GABAA receptors [49], enhancing their function (reviewed in [50]). These studies indicate that female rats may react differently to benzodiazepines than male rats depending on their estrous cycle stage.

In the present study, we investigated the effect of sex and estrous cycle on the fear potentiated startle response in rats. We also determined potential sex differences in the anxiolytic effect of diazepam and chlordiazepoxide and studied the effect of the estrous cycle thereon. We performed an exploratory study in which we measured the effect of all four estrous stages on fear-potentiated startle and compared the responses to those of male rats. For the replication study and pharmacological studies, we selected the two estrous stages that were most likely to affect fear-potentiated startle and drug effects.

2. Materials and methods

2.1. Animals and housing

A total of 48 female rats and 44 male rats (Wistar, Charles River Laboratories, Leiden, Netherlands) were tested in the present study. For the exploratory experiment (1 A) and the diazepam experiment (1B), the sample size was calculated on the basis of a previous study in our lab [27]. Since that study showed that the variation in fear-potentiated startle responses was larger in female than male rats, a bigger sample size was used for the female rat group than for the male rat group (16 females versus 12 males). For the replication experiment (2 A) and the chlordiazepoxide experiment (2B), the sample size was calculated on the basis of the data obtained during experiment 1. Since variation in female rats was not different from male rats, the same sample size was used for female and male rats in these experiments (32 females and 32 males). The replication study and chlordiazepoxide experiments were performed in two cohorts (16 females and 16 males in each cohort). Rats were six weeks old on arrival. They were housed on a reversed day-night cycle (lights on from 6P.M. till 6A.M.) in groups of four (groups of three for male rats in the exploratory experiment) in a temperature (21 °C ± 2) and humidity (55% ± 5) controlled room. Rats were randomly allocated to the cages upon arrival. Female and male rats were housed in the same room, in separate cages. To reduce the influence of external factors, each female cage was coupled to a male cage which was placed next to each other. Experimental procedures of these coupled cages were synchronized. In addition, within these cages, each female rat corresponded to a specific male rat in the coupled cage (during the exploratory experiment one of the two middle-numbered females in each cage was not coupled to a male rat). Food and water were available *ad libitum* in the home cages. Rats were allowed to acclimate to the environment for one week before the start of the experiments. The experiments were carried out during the dark phase of the day-night cycle between 8:30 A.M. and 12:30 P.M. The study was approved by the ethical committee of animal experiments in Utrecht (DEC, Utrecht University, The Netherlands).

2.2. Estrous cycle

The stage of the estrous cycle in female rats was determined by vaginal cytology. Using a plastic loop (inoculation loops 1 µl, VWR, VWR International B.V., United States), a vaginal smear was obtained. The loop was dipped in phosphate buffered saline (BioWhittaker, Lonza B.V., Belgium) and then inserted into the vagina and gently rubbed against the vaginal wall. Cells were smeared on a glass slide in a drop of saline. After air drying, the cells were stained with crystal violet (0.1%, Sigma) for 1 min. Stained cells were analyzed for estrous cycle stages via light microscopy by determining the predominant types of cells. Distinctions were as follows: proestrus: many nucleated epithelial cells and some epithelial cells; estrus: many epithelial cells and some nucleated epithelial cells; metestrus: some epithelial cells and some leukocyte cells; diestrus: many leukocyte cells and several nucleated epithelial and epithelial cells [51]. After the acclimation week, vaginal smears were taken daily from all female rats around 11:00 A.M. to determine their estrous cycle stages and to confirm the stability of their cycle during the experiment. Of note, on the day of fear-potentiated startle training or test, vaginal smears from trained or tested female rats were taken after the session to avoid interference with the fear-potentiated startle procedure. The results of smears in the exploratory experiment were verified by a second observer to confirm a correct detection of estrous cycle stages. Female rats in the proestrous and estrous stages were placed in one group since the higher hormone levels in these two stages are most likely to influence fear- potentiated startle and drug effect. The replication study and pharmacological studies compared females in the proestrous/estrous stage with females in the diestrous stage.

2.3. Startle apparatus

Eight startle devices were used simultaneously (SR-lab, San Diego Instruments, San Diego CA, USA). The startle devices consisted of a Plexiglas cylinder (9 cm diameter and 20 cm length) placed on a Plexiglas base. Each startle device was placed in a ventilated sound

attenuated cubicle. Cage movements were measured with a piezoelectric film attached to the Plexiglas base of the startle device. A calibration system (San Diego Instruments) was used to ensure comparable startle magnitudes across the eight devices throughout the experiment. Startle stimuli (95 dB, 100 dB, and 110 dB), consisting of 50 ms white-noise bursts were presented through a piezoelectric tweeter situated 15.2 cm from the top of the cylinder. Startle amplitudes were sampled each millisecond during a period of 65 ms beginning at the onset of the startle stimulus. During all training and test sessions, a background noise of 70 dB was presented to drown out noises originating from outside the individual cubicles. The light stimulus was delivered by light in the ceiling situated 15.2 cm from the top of the cylinder. There was no background illumination in any of the experiments.

2.4. Fear-potentiated startle training and test

Four days before the first fear-potentiated startle training, animals were habituated to the startle set-up during a habituation session (30 startle stimuli, 10 × 95, 100, and 110 dB, ISI 30 s). During this session, no grid floors were used.

During the fear-potentiated startle training, rats were placed in the startle chambers, and after an acclimation period of 5 min, ten light-shock pairings were presented with an average interval of 4 min (range, 3–5 min). A 0.6 mA shock was presented during the last 500 ms of the 3700 ms light period.

During the fear-potentiated startle test, rats were placed in the startle chambers, and after an acclimation period of 5 min, ten startle stimuli of 110 dB were presented for habituation (ISI 30 s), followed by 60 startle stimuli at an ISI of 30 s, 20 each at 95, 100 and 110 dB. Half of the 60 startle stimuli were presented during the last 50 ms of a 3250 ms light cue (cued trials), the other half were delivered in darkness (non-cued trials). These six different trial types were presented in a balanced, irregular order throughout the test. Expression of fear- potentiated startle response was analyzed on absolute startle values, and percent fear potentiation [(cued-non-cued)/non-cued*100%] when needed. The minimum interval between two fear-potentiated startle tests was four days since each female rat and the corresponding male rat were tested once during a 4-days cycle.

2.5. Drugs

Chlordiazepoxide HCl (2.5, 5, and 10 mg/kg) was purchased from Pharbita B.V. (Zaandam, The Netherlands), dissolved in 0.9% sodium chloride and administered i.p. in a volume of 2 ml/kg 20 min before testing. Diazepam (0.3, 1, 3 mg/kg) was purchased from BUFA B.V. (Uitgeest, The Netherlands), suspended in Gelatine Mannitol (0.5%/5%) and administered i.p. in a volume of 2 ml/kg 30 min before testing.

2.6. Experimental procedures

2.6.1. Experiment 1A – exploratory experiment

The exploratory experiment was performed to explore the sex differences in fear-potentiated startle and the influence of estrous cycle thereon. All female rats (and the corresponding male rats) were tested four times, once in each stage of the estrous cycle. Each test was preceded by a training session, 24 h earlier.

2.6.2. Experiment 1B – sex differences in the response to diazepam

This experiment was performed to study the anxiolytic effect of diazepam on the expression of fear-potentiated startle in both female and male rats and the effect of estrous cycle stage thereon. The experiment was set up as a cross-over design with two arms: 1. Dose-response study in proestrous/estrous stage and 2. Dose-response study in diestrous stage, both with four different doses of diazepam (including vehicle) according to a Latin square design. Rats in each cage were allocated to one of the two arms in such a way that all rats could be tested simultaneously, but during their assigned estrous cycle stage. However, 50% of the female rats became acyclic after the first half of the experiment. Thus, rats were only tested in one arm of the initially planned cross-over experiment. Each test was preceded by a training session 24 h earlier.

2.6.3. Experiment 2A – replication experiment

This experiment was performed to test the hypotheses of sex differences and the influence of estrous cycle following from the results of the exploratory experiment. All female rats and the corresponding male rats were tested twice during the experiment, once in proestrous/estrous stage and once in diestrous stage. Each test was preceded by a training session 24 h earlier.

2.6.4. Experiment 2B – sex differences in the response to chlordiazepoxide

In this experiment, the effect of chlordiazepoxide on the expression of fear-potentiated startle was studied in both female and male rats. The experiment was set up as a cross-over design with two arms: 1. Dose-response study in proestrous/estrous stage and 2. Dose-response study in diestrous stage, both with four different doses of chlordiazepoxide (including vehicle) according to a Latin square design. Rats in each cage were allocated to one of the two arms in such a way that all rats could be tested simultaneously, but during their assigned estrous cycle stage. Each test was preceded by a training session 24 h earlier.

Experiments 1 and 2 were not blinded to experimenters.

2.7. Statistical analyses

We used SPSS (IBM SPSS Statistics version 24, Inc., Chicago, IL) for statistical analyses. Female rats, which were acyclic or not in the expected cycle stages when tested, were excluded from statistical analyses. Accordingly, data of two female rats were excluded from the analysis of the replication experiment. Data of three female rats were excluded from the analysis of the diazepam experiment. Data of five female rats were excluded from the analysis of the chlordiazepoxide experiment. Their corresponding male rats were also excluded. All data were analyzed using repeated measures ANOVAs with trial type (cued versus non-cued) and intensity (three levels: 95 dB, 100 dB, and 110 dB) as within-subjects factors, and sex (female versus male) as a between-subjects factor. Data of female rats was analyzed separately to determine if the estrous cycle influenced the sex differences in fear-potentiated startle response and the drug responsiveness. Specific additional factors per experiment: Experiment 1 A: cycle (three levels: proestrous/estrous stage, metestrous stage, and diestrous stage) as a within- subjects factor; Experiment 2 A: cycle (proestrous/estrous stage versus diestrous stage) as a within- subjects factor; Experiment 1B and Experiment 2B: cycle (proestrous/estrous stage versus diestrous stage) as a between-subjects factor and drug (four levels: vehicle and three doses) as a within-subjects factor. Simple contrasts were performed to compare drug treatments with the corresponding vehicle group. A Greenhouse-Geisser correction was applied if sphericity was violated.

3. Results

3.1. Vaginal smears

Female rats showed a 4-days estrous cycle, in which we detected three stages that could reliably be identified: proestrous stage-transition stage-estrous stage on the first day of their cycle, metestrous stage on the second day of their cycle, diestrous stage during the last two days of their cycle. A detailed overview of consecutive estrous cycles for each female rat can be found in Supplement I.

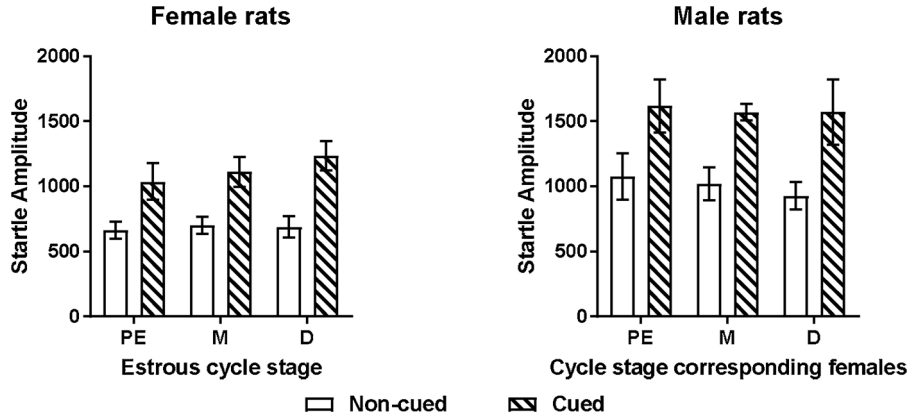

Fig. 1. Responses to non-cued and cued trials of female rats (N = 16) and the corresponding male rats (N = 12) in proestrous/estrous stage (PE), metestrous stage (M), and diestrous stage (D), with estrous cycle as a within-subjects factor. Data are shown as mean startle amplitudes (± SEM).

3.2. Experiment 1A – exploratory experiment on sex differences in fear-potentiated startle response

As shown in Fig. 1, fear potentiation was successfully induced (cue, $F1,26 = 64.1$, $p < 0.001$). Overall analyses showed that the differential response to non-cued and cued trials was not

affected by sex (cue x sex, $F1,26 = 1.14$, $p = 0.3$). The mean startle response, however, was lower in female rats than in male rats (sex, $F1,26 = 6.8$, $p = 0.015$). Separate analyses of the data obtained from female rats showed that the estrous cycle did not differently affect the response to non- cued and cued trials (cue x cycle, $F2,30 = 1.9$, $p = 0.2$). The different stages of the estrous cycle also did not affect the mean startle response (cycle, $F2,30 < 1$).

3.3. Experiment 2A – replication experiment on sex differences in fear-potentiated startle response

As shown in Fig. 2, the results of the replication study are comparable to those of experiment 1 A. Fear potentiation was successfully induced (cue, $F1,56 = 81.1$, $p < 0.001$) and the differential response to non-cued and cued trials was not affected by sex (cue x sex, $F1,56 = 1.3$, $p = 0.3$). In this experiment, the magnitude of the mean startle response did not differ between female and male rats (sex, $F1,56 < 1$). Separate analyses of data obtained from female rats showed that the differential response to non-cued and cued trials was not affected by estrous cycle (cue x cycle, $F1,28 < 1$). Stages of the estrous cycle also did not affect the mean startle response (cycle, $F1,28 < 1$)

3.4. Experiment 1B – sex differences in the response to diazepam

As shown in Fig. 3B, the effect of diazepam on non-cued and cued trials was not affected by sex (dose x cue x sex $F3,60 = 1.2$, $p = 0.3$). Diazepam did not selectively alter the response to cued trials (dose x cue $F3, 60 = 2.5$, $p = 0.092$, $\varepsilon = 0.75$), but reduced the mean startle response (dose, $F3,60 = 10.0$, $p < 0.001$, $\varepsilon = 0.77$). This reduction in mean startle response was similar for males and females (dose x sex $F3,60 = 1.1$, $p = 0.4$; Fig. 3B) and was significant at the 1.0 mg/kg and 3.0 mg/kg doses relative to vehicle treatment (simple contrasts: 1.0 mg/kg, $F1,20 = 26.6$, $p < 0.001$; 3.0 mg/kg, $F1,20 = 24.0$, $p < 0.001$; Fig. 3A).

Although there was no significant dose * sex interaction, further analyses were done on the effect of diazepam in both sexes separately to reassure that diazepam had an effect in both males and females. These separate analyses confirmed that diazepam decreased the mean startle response in both males and females, an effect that was significant at the 1.0 mg/kg and 3.0 mg/kg doses (effect dose: males $F_{3,18} = 5.2$, $p = 0.006$, simple contrasts: 1 mg/kg vs vehicle $p = 0.006$, 3 mg/kg vs vehicle $p = 0.004$; Females: effect dose $F_{3,22} = 4.7$, $p = 0.008$, simple contrasts: 1 mg/kg vs vehicle $p = 0.002$, 3 mg/kg vs vehicle $p = 0.015$).

An additional analysis was performed to determine whether the estrous cycle affected the effects of diazepam in the fear-potentiated startle test in female rats. This analysis showed that the effect of diazepam on the response to non-cued and cued trials was dependent on the estrous cycle (dose x cue x cycle $F3,33 = 6.9$, $p = 0.001$). Further analyses showed that only the effect of diazepam on the response to cued trials was differently affected by the estrous cycle stages (dose x

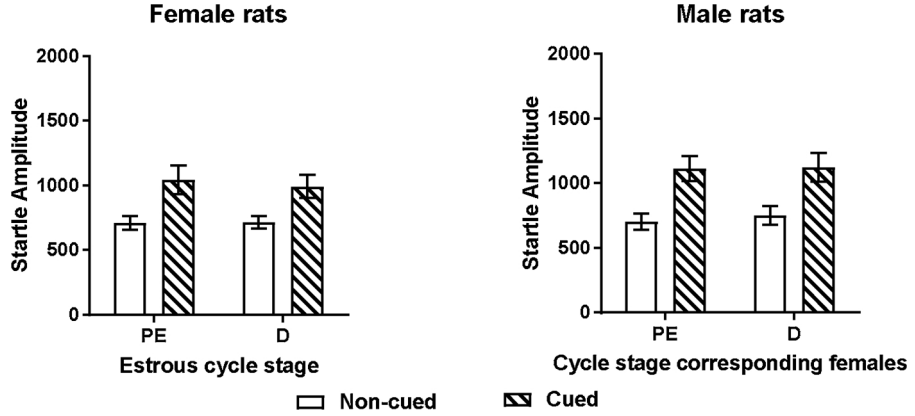

Fig. 2. Responses to non-cued and cued startle trials of female rats (N = 30) and their corresponding male rats (N = 30) in proestrous/estrous stage (PE) and diestrous stage (D), with estrous cycle as a within-subjects factor. Data are shown as mean startle amplitudes (± SEM).

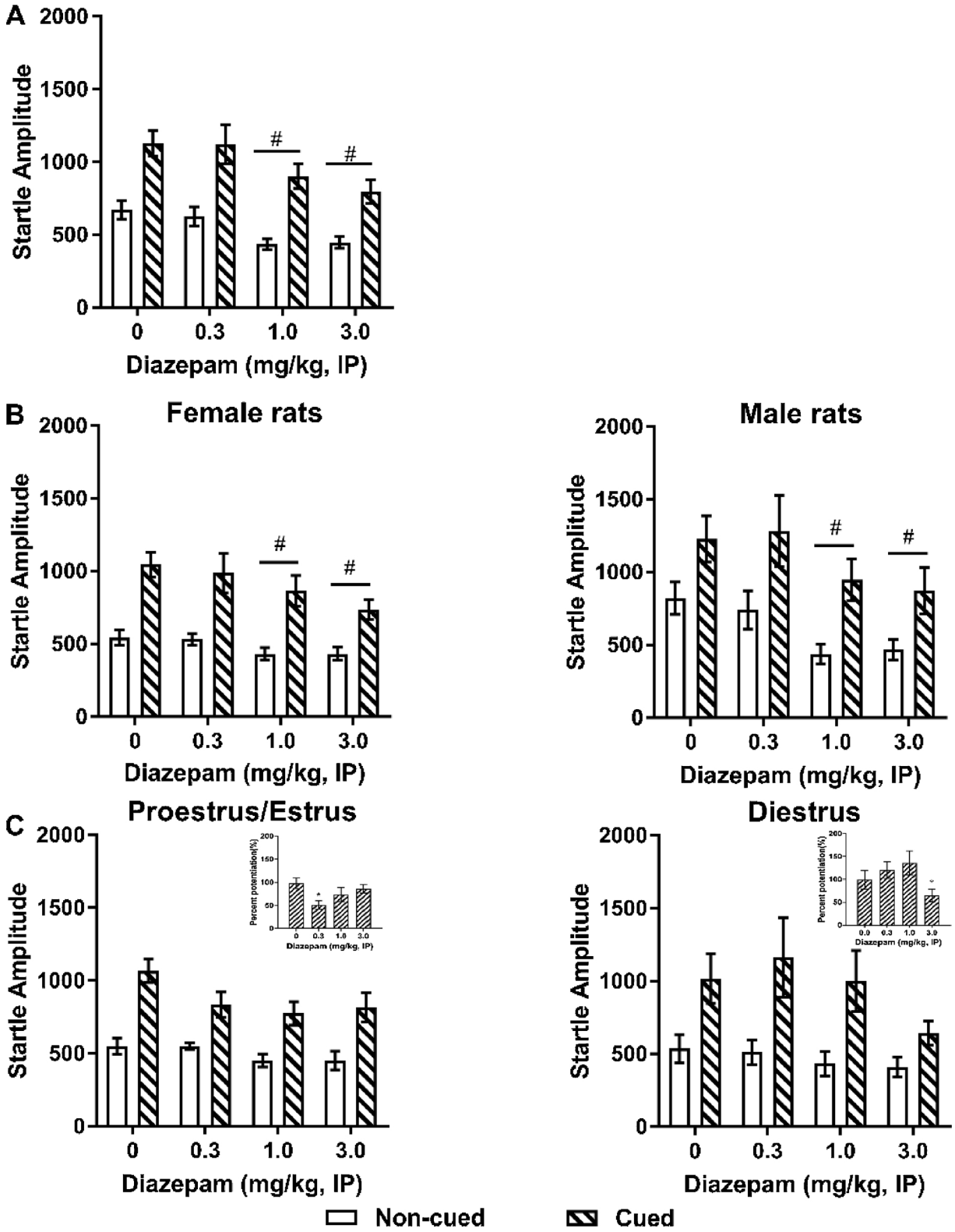

Fig. 3. The effect of diazepam on the response to non-cued and cued trials (A, N = 24). The effect of diazepam on the response to non-cued and cued trials split by sex (B, female rats, left panel, N = 13; the corresponding male rats, right panel, N = 11). The effect of diazepam on the response to non-cued and cued trials in female rats split by estrous cycle stage (C, proestrous/estrous stage [left panel; n = 7]; diestrous stage [right panel; n = 6]). Inset in C, the effect of diazepam on percent fear potentiation in proestrous/estrous stage (n = 7) and diestrous stage (n = 6). Data are presented as mean startle amplitudes (± SEM) and mean percent fear potentiation (± SEM, inset in C). * $p < 0.05$ compared to the vehicle condition. # represents significantly reduced mean startle response ($p < 0.05$) compared to the vehicle condition.

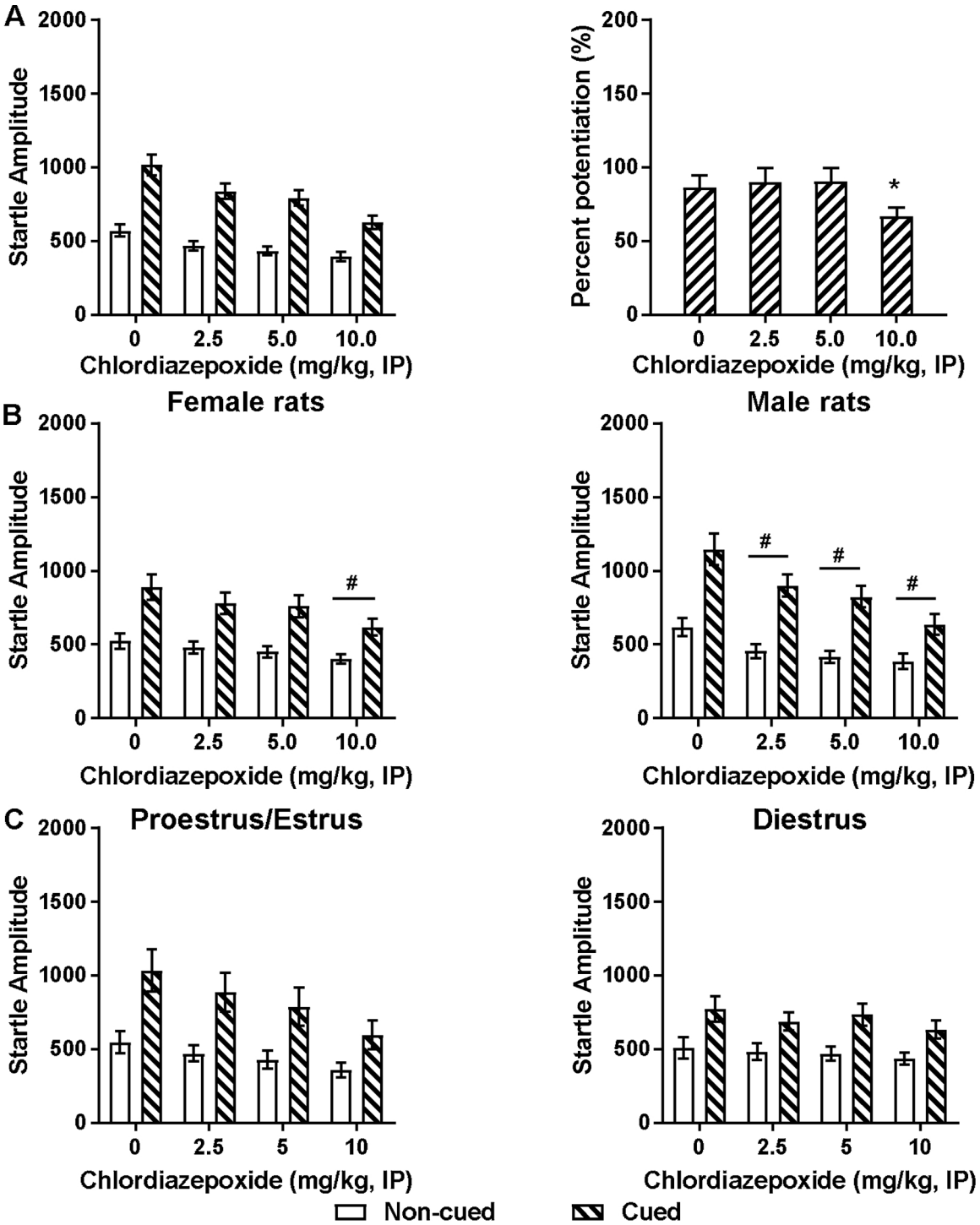

Fig. 4. The effect of chlordiazepoxide (CDP) on the response to non-cued and cued trials (A, left panel, N = 54) and on the percent fear potentiation (A, right panel, N = 54). The effect of CDP on the response to non-cued and cued trials split by sex (B, female rats, left panel, N = 27; the corresponding male rats, right panel, N = 27). The effect of CDP on the response to non-cued and cued trials of female rats split by estrous cycle stage (C, proestrous/estrous stage [left panel, n = 12]; diestrous stage [right panel, n = 15]). Data are presented as mean startle amplitudes (± SEM) and mean percent fear potentiation (± SEM, right panel in A). * $p < 0.05$ compared to the vehicle condition. # represents significantly reduced mean startle response compared to the vehicle condition.

cycle for cued trials, $F3,33 = 3.4$, $p = 0.029$; dose x cycle for non-cued trials, $F3,33 < 1$). However, this could not be ascribed to a particular cycle stage (dose effect, proestrus/estrus $F3,18 = 3.0$, $p = 0.060$; diestrus $F3,15 = 4.5$, $p = 0.071$, $\varepsilon = 0.42$), making it difficult to further interpret the cycle-dependent effect of diazepam. Analysis of the percent fear potentiation, however, did provide further clarification. The effect of diazepam on percent fear potentiation was dependent on estrous cycle (dose x cycle, $F3,33 = 7.7$, $p < 0.001$; inset Fig. 3C). More specifically, diazepam reduced the percent fear-potentiation of females in proestrus/estrus following the 0.3 mg/kg dose (dose, $F3,18 = 4.3$, $p = 0.019$, simple contrasts: 0.3 mg/kg, $F1,6 = 16.4$, $p = 0.007$), while treatment with 1.0 mg/kg diazepam increased the percent fear-potentiation female rats in diestrus (dose, $F3,15 = 5.6$, $p = 0.009$, simple contrasts: 1.0 mg/kg, $F1,5 = 15.2$, $P = 0.011$).

3.5. Experiment 2B – sex differences in the response to chlordiazepoxide

As shown in Fig. 4A, the effect of chlordiazepoxide in the fear-potentiated startle test was dependent on trial type (dose x cue F3, 147 = 4.9, p = 0.006, ε = 0.80). This differential effect of chlordiazepoxide on the response to non-cued and cued trials was not affected by sex (dose x cue x sex F3,147 < 1). Chlordiazepoxide significantly reduced the response to both cued (F3, 147 = 10.8, p < 0.001, ε = 0.67) and non-cued trials (F3, 147 = 7.0, p < 0.001, ε = 0.84) at all doses tested (simple contrasts: cued trials, 2.5 mg/kg F1,49 = 4.4, p = 0.042; 5.0 mg/kg F1,49 = 4.3, p = 0.050; 10 mg/kg F1,49 = 16.6, p < 0.001; non-cued trials, 2.5 mg/kg F1,49 = 5.6, p = 0.022; 5.0 mg/kg F1,49 = 12.0, p = 0.001; 10 mg/kg F1,49 = 11.8, p = 0.001; Fig. 4A). Analysis of the effect of chlordiazepoxide on percent potentiation showed that chlordiazepoxide significantly reduced percent fear potentiation at 10 mg/kg (dose F3,147 = 3.1, p = 0.037, ε = 0.81; simple contrasts: 10 mg/kg, F1,49 = 7.0, p = 0.011; insert Fig. 4A).

Although there was no significant dose * cue * sex interaction, further analyses were done on the effect of diazepam on the fear-potentiated startle response in both sexes separately to reassure that diazepam had an effect in both males and females (dose * cue interaction: males $F_{3,69}$ = 6.6, p = 0.001; females $F_{3,69}$ = 2.8, p = 0.049).

The effect of chlordiazepoxide on the mean startle response was affected by sex (dose x sex F3, 147 = 2.959, p = 0.034; Fig. 4B). In male rats, chlordiazepoxide significantly reduced the mean startle response at all doses tested (dose F3,72 = 9.3, p = 0.001, ε = 0.63; simple contrasts: 2.5 mg/kg, F1,24 = 5.5, p = 0.028; 5.0 mg/kg, F1,24 = 13.0, p = 0.001; 10 mg/kg, F1,24 = 12.8, p = 0.002),whereas in female rats only the highest dose of chlordiazepoxide significantly reduced the mean startle response (dose F3,72 = 3.7, p = 0.031, ε = 0.69; simple contrasts: 10 mg/kg, F1,24 = 5.0, p = 0.034).

A separate analysis of the data obtained from female rats showed that the estrous cycle did not affect the actions of chlordiazepoxide in the fear-potentiated startle test, neither those on different trial types nor on mean startle response (dose x cue x cycle F3,72 = 1.4, p = 0.2; dose x cycle F3,72 = 2.5, p = 0.063; Fig. 4C).

4. Discussion

In the present study, we found that the fear-potentiated startle response is not affected by sex or estrous cycle. Following treatment with either diazepam or chlordiazepoxide also no sex differences were observed in the expression of fear-potentiated startle.

So far, only a few studies have explored potential sex differences in fear-potentiated startle and results have been conflicting. Some studies have reported a lack of sex differences, both in rats [29,30] and mice [52]. Others have reported increased fear potentiated startle in female rats compared to males [27,28]. In humans fear-potentiated startle studies are also conflicting; some studies showed increased fear-potentiated startle in women and girls [53–55] and others showed no sex differences [56–58]. In the current study, our findings were consistent across multiple experiments, in which animals were tested repeatedly. Throughout these experiments the fear-potentiated startle responses of male and female rats were consistent and similar. Hence, our findings further strengthen the hypothesis that there are no sex differences in the fear-potentiated startle response in rats.

One factor that may have contributed to the conflicting results reported on sex differences in fear-potentiated startle is the estrous cycle. In the present study, we did not find any differences in fear-potentiated startle responding between the different estrous cycle stages in any of the experiments. These findings are consistent with an earlier study that showed no differences in fear-potentiated startle between proestrous and estrous stage [29]. Ferreira et al. [59] recently reported a similar lack of estrous cycle effects in female rats selected for low anxiety. Interestingly, female rats selected for high anxiety, showed a higher fear-potentiated startle response during estrus [59]. Studies on the fear-potentiated startle response following systemic administration of gonadal hormones in ovariectomized rats do suggest a hormone-dependent modulation of the fear- potentiated startle response. estradiol benzoate increased fear-potentiated startle, an effect that could be normalized by progesterone [29]. Progesterone treatment however, does not seem to have an effect on its own [60]. However, since the physiological state of ovariectomized rats is different from that in naturally cycling rats and the levels of hormones after systemic administration are probably far higher than that seen in naturally cycling rats, it is hard to compare these different approaches. Findings from the present and previous studies in naturally cycling rats suggest that under physiological conditions, gonadal hormone fluctuations across the estrus cycle do not affect fear-potentiated startle in females. Our findings are consistent with studies in humans, that show no differences in acquisition and expression of conditioned fear between estrous cycle stages [61–64].

Although several studies have shown sex differences in brain structures relevant for fear- potentiated startle, including for example the expression of GABAA receptors in the amygdala [65–67] and low estrogen during early follicular phase has been associated with greater activation of neural fear networks in response to aversive stimuli in human subjects [62], apparently these observed neurophysiological sex differences and estrous cycle dependent effects do not result in net differences in the acquisition and expression of fear-potentiated startle response between sexes. The consistency between rodent and human data with respect to the effects of gonadal hormones on conditioned fear-potentiated startle strengthens the translational value of the fear-potentiated startle paradigm. Moreover, this consistency suggests that for this cross- species measurement of fear, estrous cycle stage does not have to be taken into account, at least when looking at drug-free baseline fear responding. It must be mentioned, however, that sex differences have been reported in unconditioned affective startle paradigms measuring anxiety-related processes in both rodents [27,68] and humans [69,70]. Although the reported findings

in humans are inconsistent (see e.g., [35,37,71,72]).

Apart from the estrous cycle, methodological factors may also affect the level of conditioned fear expression and reveal sex differences. First, the behavioral procedure used to measure conditioned fear responses may be a very relevant factor for the detection of sex differences. Next to the fear-potentiated startle response, conditioned fear responses are regularly studied by the quantification of freezing behavior [73,74], tachycardia responses [75], and ultrasonic vocalization [76,77]. Most research on sex differences has been done on freezing behavior and these studies show mixed results with regard to sex differences (reviewed in [78]). A recent study suggests that the freezing response does not capture conditioned fear adequately in females as many of them engage in conditioned darting, which interferes with the freezing response [79]. In contrast to freezing behavior, the fear-potentiated startle response is not affected by this typical female behavior and, therefore, could yield more reliable information about potential sex differences in fear processing.

Secondly, in rodents, females outperform males in non- aversive conditioning [80–82]. However, when an aversive component is introduced, it has been suggested that males learn the association faster (reviewed in [83]). In both situations, however, males and females reach the same level of fear expression after sufficient training. In addition, both in humans and rodents, female subjects seem better in discriminating between safe and potential threatening stimuli, especially when levels of estrogen are high [29,61,62,64,68,62]. So, depending on the averseness of the unconditioned stimulus, the number of pairings between the conditioned and unconditioned stimulus (CS-US pairings), the level of extinction during fear-potentiated startle testing, the eventual level of fear expression may differ between males and females, and subsequently the manifestation of sex differences may differ.

Although the present study indicates that there are no sex differences in conditioned fear expression, it would be important to include fear extinction in future fear-potentiated startle research. Literature indicates that extinction recall is significantly decreased in women with lower level of gonadal hormones, especially estrogen, compared to men and women with higher levels of gonadal hormones [62–64]. These findings suggest that lower gonadal hormone level may impair fear extinction in women, which seems to be a vulnerability factor for the development of posttraumatic stress disorder [84].

We also determined potential sex differences in the anxiolytic effect of diazepam and chlordiazepoxide and studied the effect of the estrous cycle thereon. The current study did not show any sex differences in the effects of diazepam and chlordiazepoxide on the fear- potentiated startle response. To the best of our knowledge, sex differences in the response to benzodiazepines have not been investigated in fear conditioning paradigms in rats. In humans, some fear-potentiated startle studies did include both men and women in pharmacological studies with benzodiazepines. Of note, not all these studies involved classical fear conditioning. In line with the current findings, those studies consistently showed that the effects of benzodiazepines are not sex dependent: Baas et al. [34] reported a lack of sex differences in the effect of alprazolam on cued conditioned fear in a classical fear-potentiated startle paradigm; Patrick et al., [36] showed no sex differences in the effects of diazepam in an unconditioned fear-potentiated startle paradigm that used pictures with a negative valence as aversive stimulus; and both Riba et al. [37] and Grillon et al. [35] reported a lack of sex differences in the effect of alprazolam in an unconditioned context-potentiated ('threat of fear') startle paradigm. On the other hand, Hellewell et al. [85] did find that diazepam was able to decrease the skin conductance response in women, but not men, throughout a fear conditioning and extinction paradigm.

In contrast to the cued fear response, the mean startle response was more strongly affected by chlordiazepoxide in males compared to females; that is, in males mean startle response was already significantly decreased at a lower dose. Although this finding may seem a-specific at first sight, it may be very relevant for pathological anxiety, as over-generalization of conditioned fear has been proposed as an important hallmark of pathological anxiety [86]. In the current study, male rats showed a stronger increase in mean startle response following fear-potentiated startle training than females, whereas they did not differ in shock reactivity.

de Jongh et al. [27] reported a similar finding with male rats showing higher contextual fear after fear conditioning in the fear-potentiated startle test than females. Accordingly, several studies reported an increased acquisition of contextual conditioned fear in male compared to female rats, as measured with conditioned freezing [87,88]. Thus, several lines of evidence suggest that this increased mean startle response in males may reflect increased contextual conditioned fear in males, compared to females [89]. This may explain why chlordiazepoxide selectively decreased mean startle response in male but not in female rats.

A more specific look at the effect of estrous cycle on fear-potentiated startle in this study showed that the effect of diazepam on fear potentiation appeared to be dependent on estrous cycle stage. This suggests that fluctuations in gonadal hormones may play a role in the sensitivity towards diazepam. This idea is supported by several studies showing that administration of estrogen or progesterone can modulate the level of GABAA receptor binding by benzodiazepines in fear-related brain regions, although the direction of effect varies under influence of estrogen and/or progesterone [90–93]. Second, a study in human subjects showed that a lower level of allopregnanolone, an active metabolite of progesterone, was associated with anxiogenic symptoms [94]. These findings suggest that the anxiolytic effect of diazepam could be facilitated during proestrous/estrous stage. However, it is currently unclear why above-mentioned sex-dependent effects would differ between diazepam and chlordiazepoxide. Both chlordiazepoxide and diazepam act as a positive allosteric modulator of the GABAA receptor and we are not aware of any differences in pharmacodynamics, like receptor binding or intracellular pathway activation that could explain these differences. Also, the pharmacokinetic profiles of these drugs do not provide a satisfactory explanation [95–98]. However, since almost half of the female rats in the diazepam study became a-cyclic, the study with diazepam may have been underpowered, which may have contributed to the observed differential effects of diazepam and chlordiazepoxide.

Limitations

Some methodological issues may hamper the interpretation of the results. First, we performed our study on reversed day-night cycle to best mimic the human situation and vaginal smears were taken during the dark phase of the dark-night cycle. In contrast to previous studies in which vaginal cytology was determined in the light phase, we were unable to histologically differentiate between proestrous and estrous stage and to clearly detect four different estrous stages (See Supplement 1 for overview of determined estrous cycle stages in individual females). Since transition from proestrous to estrous takes place during the dark phase, we may have regularly detected this transition phase instead of clearly differentiated proestrous and estrous stages [51]. Therefore, it cannot be fully excluded that we missed the differential responding between the high estrogen condition during proestrus and low estrogen conditions in the other stages of the estrous cycle. It would be interesting to confirm our findings in a set up where discrimination between those estrous cycle stages is possible.

Another difficulty in our study is the inconsistency in overall startle responding between exploratory experiment and replication experiment. As discussed earlier, the sex differences in overall startle responding could well reflect increased contextual conditioned fear in males.

However, sex differences in startle amplitude may also be due to body weight differences [99,100]. It cannot fully be excluded that the sex difference in overall startle response was related to differences in body weight between males and females (+100 g in the exploratory experiment vs 70 g in the replication experiment), but the fact that during habituation sessions no sex differences were observed argues against this.

A third possible limitation of our study is the limited effect of both diazepam and chlordiazepoxide on the fear-potentiated startle response compared to previous reports (e.g. [45–48]). We cannot fully exclude that, due to these limited effects, we missed out on sex differences in the effect of diazepam and chlordiazepoxide on the fear-potentiated startle response. Importantly, from a translational point of view, our findings may not be that surprising. It has been reported repeatedly that benzodiazepines consistently decrease contextual conditioned fear, rather than cue conditioned fear, in human fear-potentiated startle paradigms, which is consistent with our data [34,35].

In conclusion, we showed that males and females do not differ in their conditioned fear response as measured with fear-potentiated startle, or its sensitivity to benzodiazepines. Future studies have to clarify whether this is also true for – putative – anxiolytics targeting different receptors and/or neurotransmitter systems. In addition, the observed sex differences in contextual anxiety would be an interesting subject of further study. The current findings suggest that it is not necessary to control for estrous cycle stage when studying fear expression in this paradigm. Based on these findings it may be suggested that the higher prevalence of anxiety disorders in women more likely results from differences in responding to previous (stressful) experiences or differences in fear extinction, rather than differences in fear conditioning per se.

Conflict of interest

The authors declare no conflict of interest in this research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbr.2018.05.022.

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