Stress and memory in health and disease

*Impact on Alzheimer’s disease and memory mechanisms*

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Effects of corticosterone on mild auditory fear conditioning and extinction; role of sex and training paradigm

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Abstract

Multiple lines of evidence suggest that glucocorticoid hormones enhance memory consolidation of fearful events. However, most of these studies involve male individuals. Since anxiety, fear and fear-associated disorders present differently in male and female subjects we investigated in mice whether male and female mice perform differently in a mild, auditory fear conditioning task and tested the modulatory role of glucocorticoid hormones. Using an auditory fear conditioning paradigm with different foot shock intensities (0.1 mA, 0.2 mA and 0.4 mA) and frequencies (1x or 3x), we find that intraperitoneal injections with corticosterone (2 mg/kg) immediately after training, altered freezing behaviour when repeated foot shocks were applied, and that the direction of the effects were opposite in male and female mice. Effects were independent of foot shock intensity. In male mice, corticosterone consistently increased freezing behaviour in response to the tone, whereas in female mice, corticosterone reduced freezing behaviour twenty-four hours after training. These effects were not related to the phase of the oestrous cycle. In addition, corticosterone enhanced extinction learning for all tones, in both male and female mice. These results emphasise that glucocorticoid hormones influence memory consolidation and retrieval, and underscore sex-specific effects of glucocorticoid hormones in modulating conditioned fear responses.

Keywords: sex, stress, corticosterone, fear conditioning, consolidation, retrieval, extinction, estrous
1. Introduction

Memories for fearful events are generally retained well\(^1,2\). Extensive evidence from human and animal studies have associated stress hormones like glucocorticoids (corticosterone in rodents; cortisol in humans) with altered memory formation\(^3\). Glucocorticoid hormones are produced by the adrenal glands and their release is increased during and after exposure to stress or emotional experiences\(^4\). These hormones readily cross the blood-brain barrier and bind to mineralocorticoid (MR) and glucocorticoid (GR) receptors present in the brain. Via genomic and non-genomic effects, they can influence neural function and memory formation\(^5,6\). Many studies have reported that corticosterone, as well as synthetic GR agonists, can alter cognitive functions and e.g. enhance memory consolidation\(^1,2\). While these behavioural effects after short term exposure are usually adaptive in nature, prolonged exposure to elevated glucocorticoid hormones may be deleterious and has been associated with stress-associated disorders\(^7\).

Many studies on the effects of glucocorticoid hormones have predominantly used male individuals to investigate their effects\(^8\), while memory formation under the influence of stress, as well as many anxiety-, stress and psychiatric disorders are present with distinct sex differences in humans. For instance, the lifetime prevalence of post-traumatic stress disorder (PTSD) is two times higher in women than in men\(^9\). Animal studies have reported striking differences in stress-responsiveness between sexes. For example, female rodents have higher basal corticosterone levels than males\(^10-15\), and a higher binding capacity for hippocampal GRs\(^16\). In addition, female rodents express less MRs\(^17\) as well as a lower binding of corticosterone to these receptors\(^18,19\). Interestingly, chronic stress in male rats downregulates GR immunoreactivity in the dentate gyrus and CA1 region of the hippocampus, but increases GR binding in CA1 in females\(^20,21\). In these studies, MR binding was increased in the CA3 region of female, but not in male rats.

Together, these studies suggest sex differences in the action of glucocorticoids. Indeed, sex differences are also present in cognitive performance. Male rodents have been reported to perform better than females in spatial memory tasks like the Morris water maze (e.g.\(^22\)). Yet, after acute and chronic stress, performance of male rats in spatial memory tasks was impaired\(^23-25\), whereas female animals improved their spatial memory abilities following stress\(^26\). Sex-differences have also been reported in fear-related memory\(^27\). However, whether glucocorticoid effects on memory consolidation in auditory fear conditioning paradigm differs between male and female animals, and how different aspects of the training paradigm contribute to
these effects, remains largely elusive. We therefore systematically investigated in mice how these hormones regulate fear memory formation in the context of varying training intensities, and whether effects were different between male and female mice.

Figure 1. Training and testing paradigm cued fear conditioning. A. Mice are trained in a fear conditioning paradigm with one or three 30 second tones, coupled with a 2 second foot shock of varying intensity ("training"). Immediately following training, mice were injected i.p. with corticosterone (2 mg/kg) or saline. Twenty-four hours later, mice were introduced in a novel environment, and re-exposed to the same 30 second tone for 6 times ("retrieval"). B. The effects of sex, foot shock intensity and foot shock frequency on freezing behaviour after re-exposure to a single tone at retrieval. All mice received a saline injection following training. A main effect was observed for foot shock intensity (F(2,65)=40.89, p=0.001), foot shock frequency (F(1,65)=31.78, p=0.001), and sex (F(1,65)=14.01, p=0.001), and an interaction effect was found between sex x intensity (F(2,65)=3.33, p=0.05), and between sex x frequency x intensity (F(2,65)=4.18, p=0.02).
2. Results

2.1. Effects of shock intensity and frequency on freezing behaviour in male and female mice

Male and female mice were trained in an auditory fear conditioning paradigm with varying foot shock intensities (0.1 mA, 0.2 mA or 0.4 mA) and frequencies (1x or 3x), and freezing behaviour to the tone was measured twenty-four hours later in a novel context by exposing mice to six tones (Figure 1A). Both the foot shock intensity and the frequency of foot shocks at training together determined freezing behaviour at retrieval (Figure 1B). Twenty-four hours after training, the three foot-shock paradigm increased freezing levels relative to a single foot shock of the same intensity (frequency effect: $F(1,65)=31.78$, $p=0.001$). Increasing the foot shock intensity also increased the freezing levels in response to a tone twenty-four hours later, also in both sexes (intensity effect: $F(2,65)=40.89$, $p=0.001$). Female mice overall displayed more freezing behaviour than male mice during the retrieval phase (sex effect: $F(1,65)=14.01$, $p=0.001$; sex x intensity interaction effect: $F(2,65)=3.31$, $p=0.05$). Following a three foot-shock training paradigm with an intensity of 0.2 mA, female mice displayed increased freezing behaviour when compared to males (post hoc: $p<0.05$). However, following a three foot-shock training paradigm with an intensity of 0.4 mA, male and female mice again displayed comparable freezing levels (post hoc: $p>0.05$).

2.2. Effects of corticosterone treatment on freezing behaviour

To investigate the effects of glucocorticoids, corticosterone or control saline injections were given immediately following the training. Corticosterone significantly affected freezing behaviour to the first tone, but differently in male and female mice, and depending on the frequency of the foot shock (treatment x sex x frequency: $F(1,133)=10.46$, $p=0.002$) (Figure 2A). Post hoc testing only revealed an effect of treatment in male mice following three foot shocks ($p=0.049$).

Corticosterone also significantly affected freezing behaviour to the subsequent tones, as measured by the average freezing over the six tones (Figure 2B, Supplementary Figure + Table 1). Corticosterone induced an overall effect on freezing behaviour to the tones during retrieval, although differently in male and female mice (sex*treatment effect: $F(1,133)=17.21$, $p<0.001$) (Figure
These effects were irrespective of frequency or intensity of the training (treatment*frequency: F(1,133)=0.40, p=0.53; treatment*intensity: F(1,133)=0.80, p=0.45).

Figure 2. The effect of corticosterone on freezing behaviour. A. Freezing levels to the first tone. A significant interaction effect was observed between sex x frequency x treatment (F(11,133)=10.25, p=0.002), and corticosterone increased freezing only in male mice after a training paradigm with three foot shocks (p=0.049). B. Average freezing behaviour over the six tones. Corticosterone resulted in an overall effect on freezing behaviour to the tones during retrieval, although differently in male and female mice (sex*treatment effect: F(1,133)=17.21, p<0.001).
2.3. Effect of shock frequency (single vs. repeated foot shock) on corticosterone effects on freezing behaviour

After a single foot shock, the administration of corticosterone directly after training had no effect on the average freezing levels to the tones during the retrieval, regardless of the foot shock intensity or the sex of the animal, as indicated by comparable freezing levels to the tones (treatment effect: F(1,61)=0.28, p=0.60) (Figure 2B, Supplementary Figure + Table 1). When corticosterone was delivered after three foot shocks, it affected freezing at all shock intensities (treatment*frequency effect: F(5,72)=7.85, p<0.001) (Figure 2B), except for the 3 x 0.1 mA training paradigm, whereas no effect of corticosterone was observed in females (Figure 2A).

2.4. Role of sex in corticosterone-enhanced freezing behaviour

Interestingly, the effects of corticosterone on memory consolidation were sex-dependent. In male mice, corticosterone increased freezing to the tones at the 3 x 0.1 mA, 3 x 0.2 mA, and 3 x 0.4 mA training paradigms (Figure 2A). In female mice, corticosterone decreased freezing to the tones after 3 x 0.2 mA,
and 3 x 0.4 mA foot shocks, although no effect of corticosterone was observed at the 3 x 0.1 mA foot shock paradigm (Figure 2A).

2.5. Effect of oestrous cycle on corticosterone-induced freezing behaviour

No effect of oestrous cycle was observed on freezing behaviour, nor on the effects of corticosterone on freezing behaviour in female mice (main cycle effect: F(1,46)=1.13, p=0.29; treatment*cycle effect: F(1,46)=0.12, p=0.73) (data not shown).

2.6. Effect of corticosterone on extinction learning over the tones

The extinction of freezing responses after repeated tone-exposures, as measured by the difference in freezing level between tone 1 and tone 6, was different between the sexes. These effects also depended on training frequency and treatment (sex*frequency*treatment interaction effect: F(1,134)=4.8, p=0.03) (Figure 3). In male mice, corticosterone did not affect the extinction following a single foot shock. However, following three foot-shocks, corticosterone increased extinction over the tones in male mice, independent of foot shock intensity. In female mice, an effect of corticosterone treatment on extinction was observed following a single foot shock, independent of foot-shock intensity. Following three foot-shocks, corticosterone treatment no longer affected extinction levels.

3. Discussion

In this study we investigated whether male and female mice perform differently in a mild, auditory fear conditioning task with different foot shock intensities (0.1 mA, 0.2 mA, 0.4 mA) and frequencies (one or three times) and tested the modulatory role of glucocorticoids. We report that corticosterone treatment after training enhances freezing behaviour at retrieval in male mice, but reduces freezing in female mice. The effects of corticosterone treatment were only apparent after a three-times repeated foot shock paradigm, and not following a single foot shock, regardless of the foot shock intensity. Furthermore, corticosterone treatment increased extinction learning over the tones, in both male and female mice.
3.1. Sex differences

As expected, subjecting animals to higher foot shock intensities resulted in higher freezing levels. Likewise, exposure to three foot shocks at training also resulted in higher freezing levels at retrieval than training with a single foot shock of the same intensity. This illustrates that freezing behaviour at retrieval reflects the intensity of the learning experience at training, which could be an appropriate measure to assess the intensity of the memory, as suggested by previous studies.28

When comparable training parameters were applied, female mice always displayed higher freezing levels than male mice. Only after a three-times repeated foot shock of 0.4 mA did we not observe a difference between male and female mice. This may stem from a ceiling effect, as both sexes displayed relatively high freezing levels. For female mice, the freezing levels did not increase further between a three-times repeated foot shock of 0.2 mA and 0.4 mA, which may be attributed to a ceiling effect as well. For male mice, the increase in freezing between a three-times repeated foot shock of 0.2 mA and 0.4 mA was still substantial. These findings suggest that fear memory formation and consolidation might be different between the sexes. This could potentially be modulated by female sex hormone-dependent mechanisms (e.g. oestrogen and progesterone), that may influence plasticity-related associative fear memory. Indeed, a similar dimorphic pattern of corticosterone has been reported on a trace-conditioned eye blink response,29 and following a contextual training paradigm, females have also been reported to freeze more than males.30 However, in the current study, we did not observe any effect of oestrous cycle on freezing levels. Studies on sex differences following fear conditioning have been inconsistent, with studies reporting no effects of oestrous cycle on freezing behaviour,31 or decreased freezing levels in females.32–38 Although our study cannot explain the discrepancies between these studies, we speculate that they may arise from experimental variations within the fear-conditioning paradigm animal species or strain.

3.2. Corticosterone and freezing behaviour

Numerous studies have illustrated that glucocorticoids facilitate memory consolidation (e.g.2,3,39–42). Also in the current study, we find that corticosterone increases memory consolidation. The use of post-training administration of corticosterone, as opposed to corticosterone administration prior to training, suggests an effect on memory consolidation that is not confounded by possible effects on attentional, motivational or sensory-perceptual mechanisms, that
may have occurred when corticosterone treatment would have been given at the time of conditioning or testing. In both male and female mice, and regardless of foot shock intensity, corticosterone administration after training did not affect freezing levels at retrieval following a single foot shock. Yet, after three repeated foot shocks, corticosterone significantly increased freezing behaviour during retrieval. An exception was freezing behaviour in female mice after a three-times repeated foot shock of 0.1 mA. This effect can possibly be attributed to a floor effect, as freezing levels in control female mice were already very low. The difference between a single versus a repeated training paradigm is not merely due to the fact that a repeated foot shock induced overall higher freezing levels. For instance, freezing levels in females after a single 0.4 mA foot shock are higher than after 3 x 0.2 mA foot shock. Yet, corticosterone resulted in differences when the three-times repeated training paradigm is used. These results suggest that it is not the severity/adversity of the training paradigm per se that determines whether corticosterone alters freezing, but that it is the frequency with which the mouse is repeatedly exposed to the tone-foot shock that determines the effects of the hormone. Possibly, the learning component in a repeated foot shock paradigm is stronger than in a single foot shock paradigm, and such a paradigm may therefore be more susceptible to modulation by corticosterone. This notion is supported by the observation by Hui et al., showing that corticosterone did not enhance freezing behaviour following an unpaired presentation of the tone and foot shock, or the tone or the shock alone, indicating that a learning process is critical for corticosterone to have an effect.

### 3.3. Effect of sex on corticosterone-induced freezing behaviour

Interestingly, the effects of corticosterone on freezing behaviour were opposite in male and female mice. In agreement with previous literature, showing that corticosterone selectively enhances memory in male rats, we found that corticosterone enhanced freezing behaviour in male mice. These results are consistent with previous findings indicating that corticosterone, as well as drugs that selectively activate GRs, enhance memory consolidation for several types of training, including discrimination learning, inhibitory avoidance, contextual fear conditioning, water-maze spatial training, and appetitive conditioning. On the other hand, we found that post-training treatment with corticosterone reduced auditory freezing in female mice, providing evidence that also the glucocorticoid effects on memory in these paradigms are sex-dependent.

In agreement with these findings, previous studies have demonstrated
that in females, corticosterone also impairs memory formation in a contextual fear conditioning paradigm and in spatial memory tasks\textsuperscript{52,53}. These differences may arise from interactions between HPA axis signalling and female sex-hormone dependent pathways (e.g. oestrogen and/or progesterone signalling). Alternatively, the effects of corticosterone have been reported to follow a Yerkes-Dodson or inverted-U shape dose response relationship, in which optimal enhancing effects on memory are seen at midrange doses, whereas high doses are less effective or may even impair memory\textsuperscript{5}. As female mice have both higher basal corticosterone levels and a stronger corticosterone release upon a stressor\textsuperscript{54}, the currently used dose of corticosterone (2 mg/kg) may not have been effective in enhancing memory in females.

Interestingly, the use of oral contraceptives has been shown to affect HPA axis responsiveness during stress exposure in females, resulting in a blunted cortisol response and a lack of stress-induced effects on memory\textsuperscript{55,56}. Aside from sex-specific differences in stress sensitivity or responsivity\textsuperscript{57}, in the human population, the effects of stress-induced glucocorticoid release on memory may therefore differ between men and women in part because of the high use of oral contraceptives by females. In future studies, the use of oral contraceptives will be important to take into consideration for proper interpretation of the results.

\textbf{3.4. Corticosterone enhances extinction learning over the tones}

Previous research has shown that corticosterone facilitates the extinction process\textsuperscript{42,58,59}. Our present study shows that corticosterone treatment immediately after training enhanced the extinction of freezing over the tones 24 hours later, when differences in corticosterone levels have already ceased to exist between the groups. The training paradigm and sex also played a role in this extinction, as in male mice, only a three-times repeated foot shock paradigm resulted in enhanced extinction, whereas in females a single foot shock paradigm enhanced extinction, irrespective of foot shock intensity. The current study cannot clarify the nature of this interaction between sex and foot shock frequency. However, both in male and female mice, corticosterone treatment after training enhanced extinction learning, in a comparable way in both sexes. The stronger extinction following corticosterone treatment corresponds to other studies showing facilitated extinction of fear behaviour after corticosterone treatment in fear conditioning\textsuperscript{60-62} or appetitive operant conditioning tasks\textsuperscript{63}.

Interestingly, the corticosterone-induced effects on memory strength
(enhancing memory in males while impairing it in females) appear to differ from the effects on extinction, as in both male and female mice, corticosterone enhanced extinction learning. This may indicate the involvement of different brain areas in the effects of corticosterone. Numerous studies have shown that auditory fear conditioning is largely dependent on amygdala activation, whereas prefrontal cortex-amygdala circuits are essential for fear extinction learning\textsuperscript{64,65}. This suggests that corticosterone may have different effects in amygdala and / or prefrontal cortex in male and female mice, which could contribute to the divergent nature of the hormone effect on memory consolidation and extinction.

4. Conclusion

The results reported here add to existing evidence that corticosteroid hormones influence memory consolidation. These findings emphasise sex-specific effects of corticosterone in a mild auditory fear condition paradigm. Furthermore, corticosterone enhanced extinction of fearful memories to the same extent in male and female mice. Together, these data suggest that fear memories may be better retained in male animals when compared to female animals. They further emphasise the importance of studying both males and females in stress-related phenotypes and warrant more studies into the mechanisms that underlie sex differences.

5. Materials and Methods

5.1. Mice and breeding

All mice were kept under standard housing conditions (temperature 20-22 °C, 40-60% humidity) Standard chow and water were available \textit{ad libitum}, and mice were housed on a 12/12 h light/dark schedule (lights on at 8 a.m.) and a radio provided background noise\textsuperscript{66}. All experimental procedures were conducted under the national law and European Union directive 2010/63/EU on animal experiments, and were approved by the animal welfare committee of the University of Amsterdam. Male and female C57Bl/6 mice were bred in house. After weaning, mice were housed with 2-5 same sex littermates per cage until the start of experiments.
5.2. Fear conditioning

Three month (± 2 weeks) old male and female mice were tested in an auditory fear conditioning paradigm. Two weeks prior to fear conditioning, mice were housed individually. All experimental procedures occurred in the morning between 09.00 a.m. and 11.00 a.m. During testing, the mice were recorded by a camera connected to a computer with Ethovision software (version 6.1, Noldus, The Netherlands). Mice were placed in a square chamber with black walls (W x L x H: 30 x 24 x 26 cm) which had a stainless steel grid floor connected to a shock generator, and which had been cleaned with 1% acidic acid to create a recognisable odour trace. Mice were allowed to explore the context for three minutes, after which once or 3 times, a 30-second tone was applied (2.8 kHz, 76 dB), coupled to a 2-second foot shock (0.1, 0.2 or 0.4 mA) during the last 2 seconds of the tone, with an inter-tone-interval of 60 seconds (the “training” phase) (see Figure 1A). After the last tone-foot shock pairing, the mice remained in the chamber for 30 seconds. Twenty-four hours later, mice were introduced in a novel, circular box (diameter: 35 cm, transparent walls, sawdust floor) cleaned with 25% EtOH. After 3 minutes, a 30-second tone was applied for 6 times, with an inter-tone-interval of 60 seconds. Freezing behaviour during every trial was scored by an observer blind to the experimental condition, with “freezing” being defined as “no body movements except those related to breathing” (68). 4 to 15 mice (7 on average) were used (see Supplementary table 1 for the number of mice per group).

5.3. Corticosterone treatment

Corticosterone (Sigma; 16 mg/ml dissolved in 99.9% EtOH and diluted 40x in saline) or vehicle (2.5% EtOH in saline) were injected intraperitoneally, immediately after the training (final dose: 2 mg/kg, injection volume: 5 µl/g body weight).

5.4. Oestrous cycle determination

20 µl of 0.9% saline was used to elute cells from the female’s vagina, which were spread on a glass slide and analysed directly after sampling by means of a light microscope with a 10× total magnification. Cycle stage of every female was assessed and classified as “oestrous” or “non-oestrous”, as described previously (69). Detection of the oestrous phase was performed after fear conditioning. Seventeen mice were in oestrous, and 53 mice were in non-oestrous.
5.5. Statistical analyses

Data were analysed using SPSS 22.0 (IBM software). All data are expressed as mean ± standard error of the mean (S.E.M.). Data were considered statistically significant when p<0.05. Outliers were determined using a Grubb’s test. To determine the effects of treatment on freezing to the tones (Supplementary Figure 1, Supplementary Table 1), a two-way repeated measures ANOVA was performed using treatment (vehicle vs. CORT) as between-subject factors, and freezing behaviour to the different tones as the within-subject factor. A 2x3x2-way ANOVA was performed to assess the difference between groups accounting for sex, foot shock intensity and foot shock frequency. A 2x2x3x2-way ANOVA was performed to compare differences between groups accounting for treatment, sex, foot shock intensity and foot shock frequency.

6. Acknowledgements

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8. Supplementary data

Supplementary figure 1. Freezing behaviour in response to the six tones during retrieval following saline (black) or corticosterone (red) treatment. Corticosterone was given immediately following auditory fear conditioning, and 24 hours later freezing behaviour to six consecutive tones was measured. *: main treatment effect.

Figure continues on next page
Effects of sex and CORT on fear conditioning

Supplementary Figure 1. Freezing behaviour in response to the six tones during retrieval following saline (black) or corticosterone (red) treatment. Corticosterone was given immediately following auditory fear conditioning, and 24 hours later freezing behaviour to six consecutive tones was measured. *: main treatment effect.

Supplementary Figure 1 (continued).
**Supplementary table 1. Statistical tests corresponding to the graphs in Supplementary Figure 1.**

<table>
<thead>
<tr>
<th>Males</th>
<th>Females</th>
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| 1x0.1 mA | T: F (5, 55) = 3.30, p=0.01*  
Treatment: F (1, 11) = 1.323, p=0.27  
Saline group: N=6, corticosterone group: N=7 | T: F (5, 50) = 3.00, p=0.02*  
Treatment: F (1, 10) = 0.26, p=0.62  
Saline group: N=6, corticosterone group: N=6 |
| 3x0.1 mA | T: F (5, 60) = 13.66, p<0.001*  
Treatment: F (1, 12) = 7.68, p=0.02*  
Saline group: N=7, corticosterone group: N=7 | T: F (5, 40) = 19.57, p<0.001*  
Treatment: F (1, 8) = 1.09, p=0.33  
Saline group: N=5, corticosterone group: N=5 |
| 1x0.2 mA | T: F (5, 55) = 9.09, p<0.001*  
Treatment: F (1, 11) = 1.031, p=0.33  
Saline group: N=6, corticosterone group: N=7 | T: F (5, 40) = 15.91, p<0.001*  
Treatment: F (1, 8) = 0.09, p=0.78  
Saline group: N=5, corticosterone group: N=5 |
| 3x0.2 mA | T: F (5, 125) = 19.25, p<0.001*  
Treatment: F (1, 25) = 11.08, p<0.01*  
Saline group: N=12, corticosterone group: N=15 | T: F (5, 50) = 6.77, p<0.001*  
Treatment: F (1, 10) = 7.846, p=0.02*  
Saline group: N=6, corticosterone group: N=6 |
| 1x0.4 mA | T: F (5, 50) = 25.56, p<0.001*  
Treatment: F (1, 10) = 0.00, p=0.96  
Saline group: N=6, corticosterone group: N=6 | T: F (5, 50) = 3.38, p=0.01*  
Treatment: F (1, 10) = 1.54, p=0.24  
Saline group: N=6, corticosterone group: N=6 |
| 3x0.4 mA | T: F (5, 35) = 10.91, p<0.001*  
Treatment: F (1, 7) = 14.46, p=0.01*  
Saline group: N=4, corticosterone group: N=5 | T: F (5, 55) = 3.18, p=0.01*  
Treatment: F (1, 11) = 7.45, p=0.02*  
Saline group: N=7, corticosterone group: N=6 |

* indicates a significant main effect of either tone or treatment.