Neuroscience

Sex Differences in Discrimination Behavior and Orbitofrontal Engagement During Context-Gated Reward Prediction

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Abstract

Animals, including humans, rely on contextual information to interpret ambiguous stimuli. Impaired context processing is a hallmark of several neuropsychiatric disorders, including schizophrenia, autism, PTSD, and addiction. While sex differences in the prevalence and manifestations of these disorders are well established, potential sex differences in context processing remain uncertain. Here we examined sex differences in the contextual control over cue-evoked reward seeking and its neural correlates, in rats. Male and female rats were trained in a bidirectional occasion-setting preparation in which the validity of two reward-predictive cues was informed by the presence, or absence, of a background contextual feature (A:X+ / X-/ A:Y-/ Y+). Females were significantly slower to acquire contextual control over cue-evoked reward seeking. However, once established, the contextual control over behavior was more robust in female rats; it showed less within-session variability (less influence of prior reward) and greater resistance to acute stress. This superior contextual control achieved by females was accompanied by an increased activation of the orbitofrontal cortex compared to males. Critically, these behavioral and neural sex differences were specific to the contextual modulation process and not observed in simple, context-independent, reward prediction tasks. These results indicate a sex-biased trade-off between the speed of acquisition and the robustness of performance in the contextual modulation of cued reward seeking. The different distribution of sexes along the Fast learning ↔ Steady performance continuum might reflect different levels of engagement of the orbitofrontal cortex, and might have implications for our understanding of sex differences in psychiatric disorders.

eLife assessment

This **valuable** manuscript reveals sex differences in bi-conditioning Pavlovian learning and conditional behavior. Males learn hierarchical context-cue-outcome associations more quickly, but females show more stable and robust task performance. These sex differences are related to cellular activation in the orbitofrontal cortex. Although the evidence for the claims is **solid**, the claim of sex differences in context-dependent discrimination behaviour is not fully supported by the data. Nevertheless, the results will be of interest to many behavioural neuroscientists, particularly those who investigate sex-specific behaviours.

Introduction

Pavlovian learning is often conceived as the formation of a binary association between two events, where event #1 (the stimulus) evokes the anticipation of event #2 (the outcome). Under this strict definition, the background circumstances (the context) in which those events occur play little role. However, this simple form of learning is often augmented by higher level cognitive systems that allow animals (including humans) to learn and retrieve context-specific associations (e.g., the word “shot” might evoke different representations in a bar vs. a vaccine clinic) [1–3].

Deficits in contextual modulation are observed in several neuropsychiatric disorders including schizophrenia, autism, post-traumatic stress disorders (PTSD), and substance use disorders (SUD) [4–11], and may contribute to the intrusive thoughts and maladaptive (situation-inappropriate) behaviors that characterize these disorders. Importantly, the prevalence and symptomatology of these disorders vary between sexes. For example, the incidence of schizophrenia and autism is higher in men [12] while women are more at risk of developing SUD or PTSD following initial drug use or trauma [13, 14]. This disparity might be due, in part, to subtle sex differences in executive strategies [15–17]. Stress, a contributing factor in all aforementioned disorders, can further exacerbate these sex differences, resulting in sex-specific cognitive vulnerabilities [17–19]. Sex differences in executive functions and sensitivity to stress have been reproduced in animal models using behavioral assays of attention, impulsivity, and working memory [16, 20, 21]. Whether these sex differences extend to the contextual modulation of associative predictions remains unknown.

In animal models, the contextual modulation of associative predictions can be studied in occasion-setting preparations. In positive occasion-setting, animals learn that a target cue (X) results in a reward outcome (+) only when that cue is accompanied by a contextual feature (A); the same cue presented in absence of this contextual feature remains without consequence (A:X+ / X-). Conversely, in negative occasion-setting, a target cue results in reward, except when accompanied by a specific contextual feature (A:X-/ X+). In both preparations, the contextual feature is thought to hierarchically modulate (positively or negatively) the association between the target cue and the outcome [3, 22]. Strong evidence for this hierarchical modulation is the fact that a contextual feature can simultaneously function as a positive modulator for one target cue and as a negative modulator for another target cue (A:X+ / X-/ A:Y-/ Y+). In such bidirectional occasion-setting procedures all cues and contexts have equal probabilities of reward, therefore, accurate reward predictions cannot be achieved via simple associative summation but instead require hierarchical contextual modulation.

Compared to simple forms of associative learning, the neural bases of context-gated associative predictions (occasion-setting) remain severely understudied. Although recent studies have implicated the orbitofrontal cortex (OFC) in occasion-setting [23–25], most of the behavioral and neurobiological occasion-setting studies have exclusively used male subjects and/or did not explicitly address sex as a biological variable.

Therefore, the purpose of this study was two-fold: 1) investigate sex differences in the (bidirectional) contextual gating of reward prediction and its sensitivity to stress, and 2) investigate sex differences in OFC engagement during the contextual gating of reward predictions. We found that female rats were slower than males to acquire discriminated (context-gated) reward-seeking. However, once established, context-gated performance was more stable in females; it showed less variability within a session and was more resistant to acute stress. Moreover, we showed that this superior contextual control achieved by females was accompanied by an increased activation of the orbitofrontal cortex.

Results

Male and female rats were randomly assigned to one of four Pavlovian discrimination tasks outlined in **Figure 1A**. In these tasks [adapted from 26], all rats were exposed to two auditory cues (X and Y, 10s) presented within two distinct, alternating, visual contexts (A and noA, 2min). All rats received similar amount of reward, and produced a similar behavioral response (magazine approach). However, the different reward contingencies featured in these tasks presumably promoted the emergence of different associative architectures for reward prediction (**Fig. 1B**).

In the **Ctx-dep. O1** task, cue X was rewarded only in context A and cue Y was rewarded only in context noA; all rewarded trials resulted in the same outcome (O1). Accurate reward prediction in this task critically relies on the context-informed hierarchical modulation associative predictions (i.e occasion-setting). The **Ctx-dep. O1/O2** task featured similar contingencies with the exception that the two rewarded trials (A:X and Y) resulted in two different outcomes (O1 and O2, respectively). This allowed for distinct context-outcome associations (Ctx.A→O1 and Ctx.noA→O2), as well as distinct cue-outcome associations (X→O1 and Y→O2). As a result, context-sensitive performance could potentially be achieved via simple (nonhierarchical) summation process. In the **Simple discrimination** task, cue X was always rewarded and cue Y was never rewarded, regardless of context. Finally, in the **No discrimination** task, both cues X and Y were probabilistically rewarded in both contexts. Importantly, the only task that unambiguously engages context-gated reward predictions (or contextual occasion setting) is the first task: context-dependent discrimination with a single outcome (ctx-dep. O1).

Male rats acquire context-regulation over reward-seeking faster than females

Fig.1 C-D displays the course of acquisition of discriminated Pavlovian approach in all four tasks. In the context-dependent discrimination with a single outcome (Ctx dep. O1), Linear Mixed Models (LMM) analysis revealed a significant Trial x Session interaction, ($F(17, 181.488) = 73.343$, $P < 0.001$), as rats gradually learned to respond more during rewarded than non-rewarded trials. Critically, the analysis also revealed a significant Sex x Trial x Session interaction ($F(17, 181.488) = 4.683$, $P < 0.001$). Post-hoc comparisons revealed that males and females displayed similar levels of responding to rewarded trials (Ps > 0.260), but females maintained higher responding during non-rewarded trials, causing both sexes to differ in this measure from session 40 onwards (Ps < 0.047).

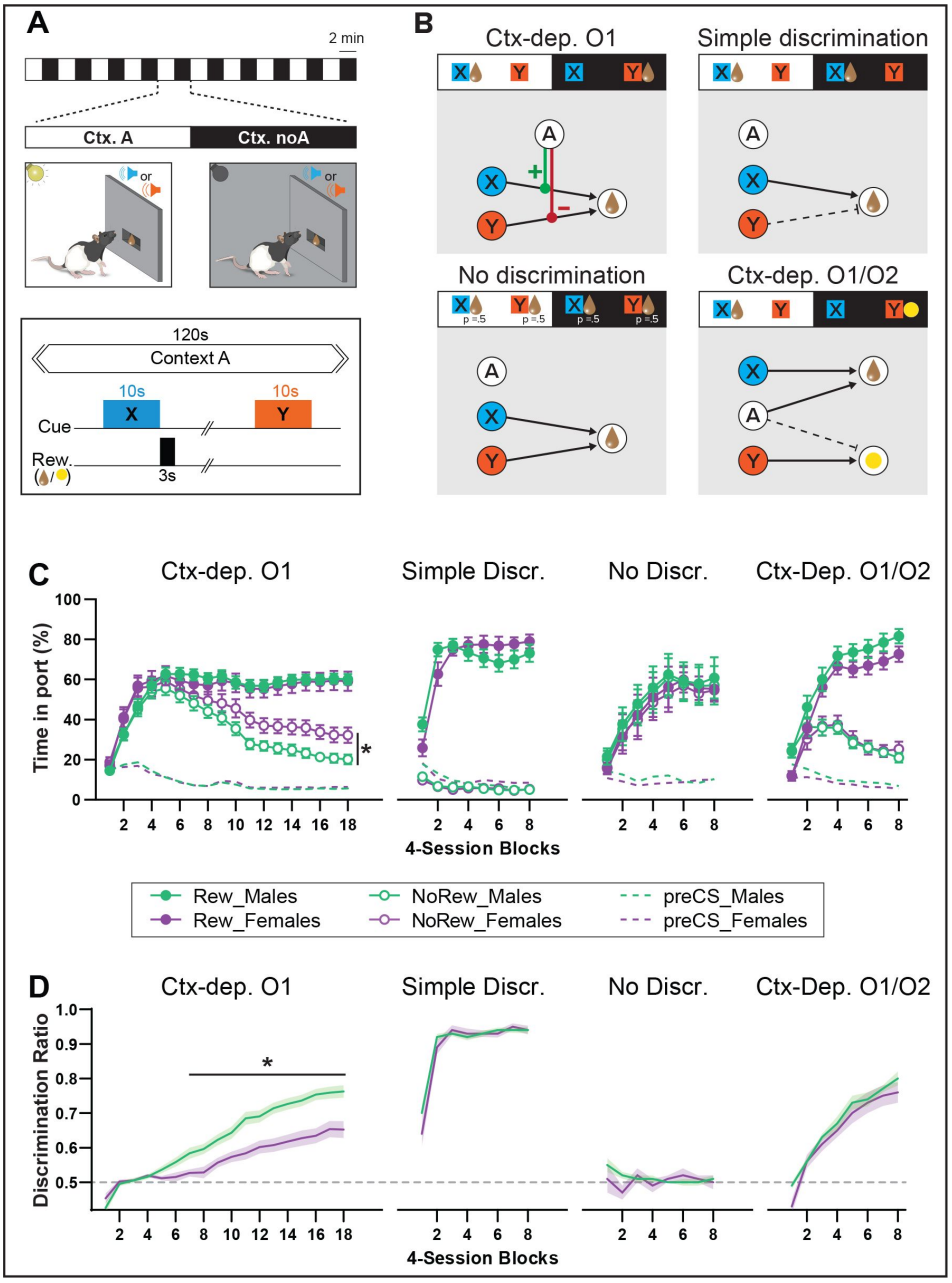

Fig. 1.

Sex differences in the acquisition of context-gated but not context-independent reward predictions.

A Apparatus and general task design. During each session, the local context alternates every 2min between a Ctx.A (houselight on) and Ctx.noA (houselight off). Embedded in these context cycles, brief auditory stimuli (X or Y; 10s) are presented and potentially followed by reward (chocolate milk or banana pellets). **B** Reward contingencies and corresponding associative structures for each training condition. Ctx-dep. O1: the local context informs the predictive status of the ambiguous reward cues X and Y. Simple discrimination: reward cues are not ambiguous (only X is rewarded) and the local context is irrelevant. No Discrimination: reward cues X and Y are potentially rewarded and the local context is irrelevant. Ctx-dep. O1/O2: Context-dependent discrimination with differential outcomes; in this case, the local context is relevant but may contribute to context-sensitive performance via simple (non-hierarchical) associations (summation of cue- and context-evoked predictions). **C** Time in port (% cue time) during rewarded and non-rewarded trials across acquisition for male and female rats trained in each task. **D** Discrimination ratio across acquisition for male and female rats trained in each task. Error bars and error bands indicate ± s.e.m. *: P < 0.05, t-tests

To facilitate comparisons between groups, we calculated for each animal a discrimination ratio, defined as the time in port during rewarded trials divided by the total time in port during all trials (**Fig 1D**). With the exception of animals trained in the No Discrimination task (for which discrimination between rewarded and non-rewarded trials was impossible by design), all training groups demonstrated a gradual increase in discrimination ratio (main Session effect: No Discr. $P = 0.477$; all other groups $Ps < 0.001$). Importantly, only in Ctx-dep.O1 did we detect a main effect of Sex ($F(1, 69.876) = 15.185$, $P < 0.001$) and a Sex x Session interaction ($F(17, 365.569) = 3.054$, $P < 0.001$). Indeed, acquisition of discriminated performance was faster in males, causing significant sex differences from session 28 onwards ($Ps < 0.014$). No main effect or interaction with Sex was detected for any other training group ($Ps > 0.305$).

We next calculated the proportion of male and female rats that successfully acquired discriminated responding during the training period (**Fig. 2**). The criterion for successful discrimination was defined as a time in port during rewarded trials that exceeded the time in port during non-rewarded trials by at least 50% (Time rew ≥ 1.5* Time nonrew, which corresponds to a discrimination ratio ≥ 0.6) for a minimum of 4 out of 5 consecutive sessions. Rats in the Ctx-dep. discrimination groups had to reach this criterion for both cues to be considered "discriminators" (A:X ≥ 1.5* X and Y ≥ 1.5 A:Y). A significant sex difference in the proportion of discriminators was observed in the Ctx-dep. O1 group ($\chi 2 = 5.35$; $P = 0.021$), as a greater proportion of males reached criterion (**Fig. 2 B-C**). No sex differences were observed in the proportion of discriminators in any other tasks.

We then analyzed the number of sessions required for rats to reach the discrimination criterion (**Fig. 2D**). Rats that failed to reach the discrimination criterion were excluded from this analysis. A Kruskal-Wallis one-way ANOVA revealed a significant effect of the behavioral task ($H (2) = 87.361$, $P < 0.001$) with different sessions-to-criterion for all the discrimination tasks (Ctx-dep. O1 > Ctx-dep. O1/O2 > Simple; $Ps < 0.001$, Dunn's tests). Sex differences in the speed of acquisition (i.e. sessions-to-criterion) were analyzed separately within each task. In Ctx-dep. O1, males were significantly faster to reach the discrimination criterion ($T(38) = 2.502$, $P = 0.017$). No sex differences in the speed of acquisition were observed in the other tasks ($Ps \geq 0.175$).

These results indicate that female rats were generally slower to acquire contextual control over cue-evoked reward-seeking. This sex difference in the development of discriminated performance was observed only in Ctx-dep. O1, the only task that unambiguously engages contextual modulation of reward predictions. Note that estrous cycle did not affect performance in female discriminators (**Fig. S1**).

Trial history influences context-gated reward-seeking in male but not female rats

The delivery of reward, while critical for learning, can also interfere with the expression of context-dependent discrimination [27]. Therefore, we investigated to which extent within-session reward history (i.e. prior rewarded or non-rewarded trials) influenced performance in the Ctx-dep. O1 task, in male and female discriminators (**Fig. 3**). Performance was analyzed over 10 consecutive sessions (sessions 71-80; 800 trials total). The first trial of a session was excluded as it was not preceded by any trial. For the remaining 790 trials (79 trials x 10 sessions), we determined if the trial was preceded by a rewarded or non-rewarded trial. Responding during non-rewarded and rewarded trials was analyzed separately.

Fig. 3A shows the time in port during non-rewarded trials, as a function of the outcome of the previous trial (prior reward, or non-reward). A RM-ANOVA revealed a main effect of Prior Rewards ($F(1, 38) = 27.144$; $P < 0.001$) as prior rewards increased responding during subsequent non-rewarded trials. The analysis showed no main effect of Sex ($F(1, 38) = 1.783$; $P = 0.190$), but a marginal Sex x Prior Reward interaction ($F(1, 38) = 4.015$; $P = 0.052$), as males were slightly more

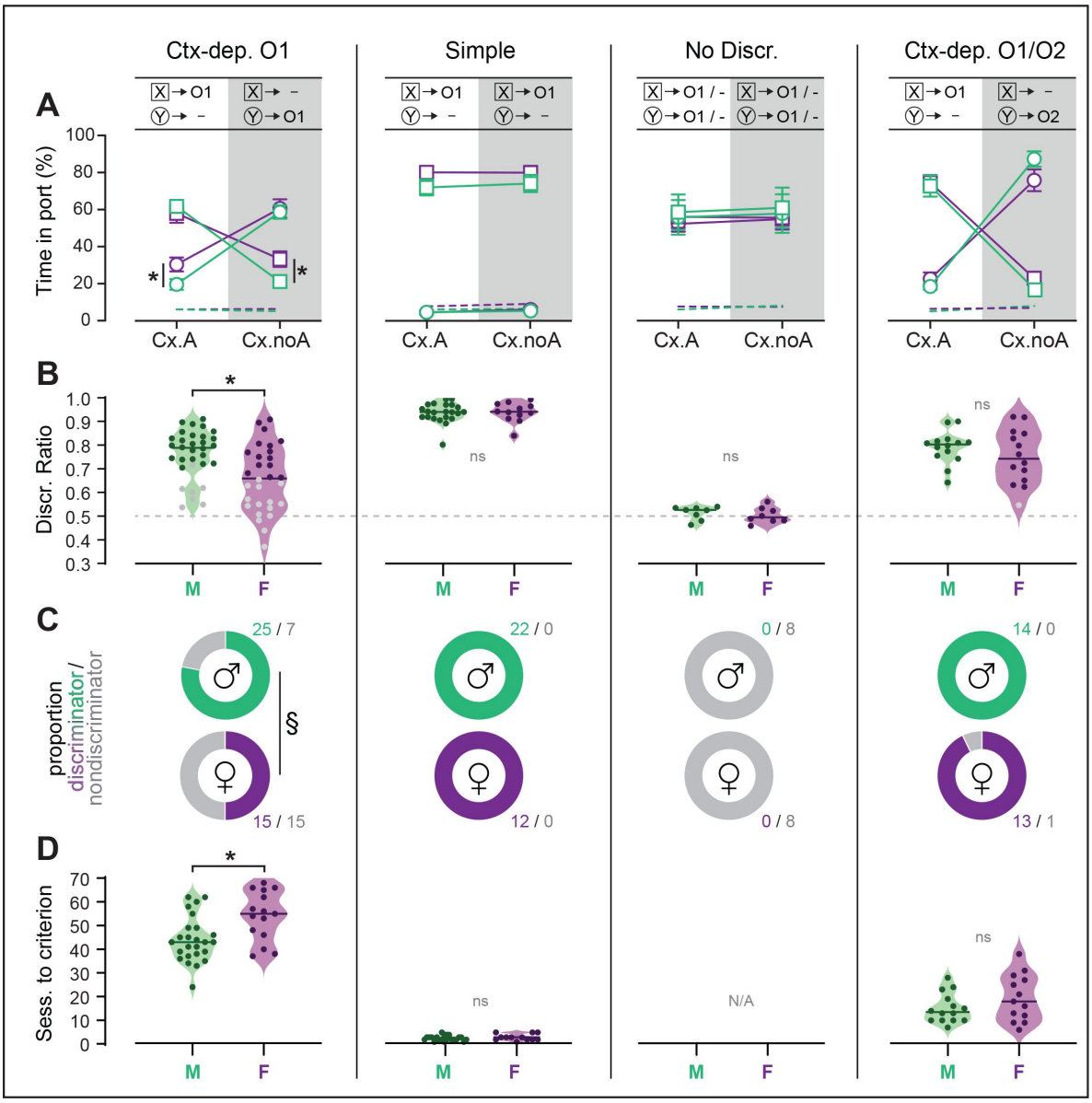

Figure 2.

Individual differences in the acquisition of discriminated Pavlovian approach.

A Time in port (% cue time) during the four different trial types (A:X / X / A:Y / Y) at the end of the training period (last 3 days average) for male and female rats trained in each discrimination task. **B** Discrimination ratio at the end of the training period (last 3 days average) for male and female rats trained in each discrimination task. Dark symbol: discriminator; Grey symbol: non-discriminator. Horizontal bar = group median; Dashed horizontal line represents the indifference level (no discrimination). **C** Proportion of male and female rats classified as discriminator or non-discriminator in each task. **D** Speed of acquisition (sessions to criterion) for discriminator rats, in each task. Horizontal bar = group median *: P < 0.05 Males vs. Females, t-tests; §: P < 0.05 chi-square test.

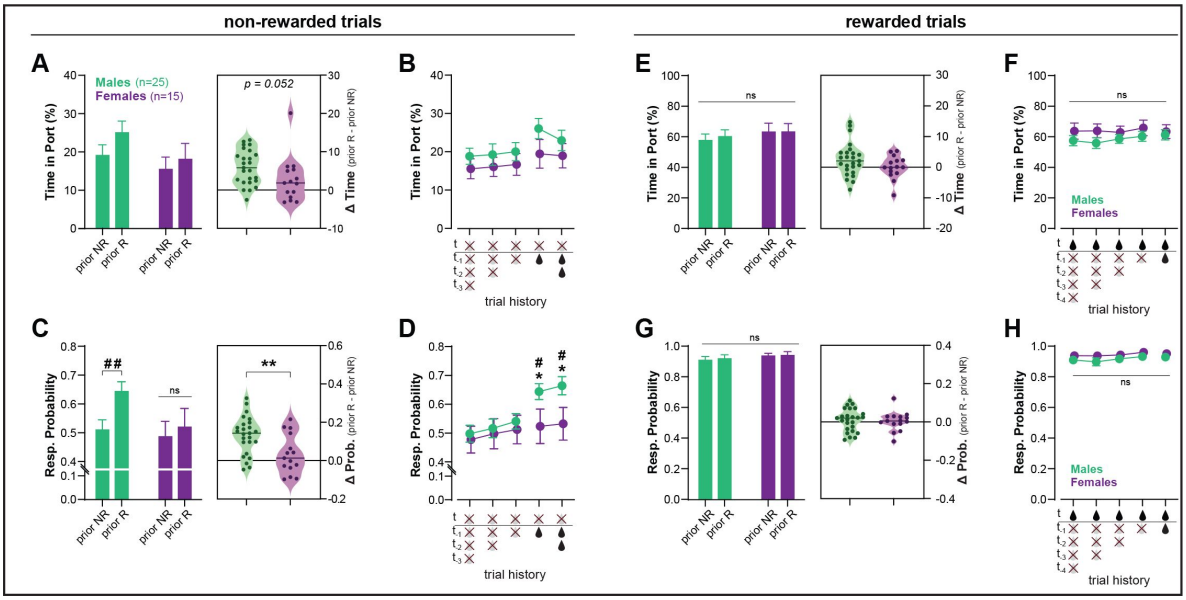

Fig. 3.

Recent rewards disrupt contextual control of reward seeking in male, but not female, rats.

A Time in port (% cue time) during non-rewarded trials as a function of prior trial (rewarded: prior R, or non-rewarded: prior NR) for male and female rats trained in Ctx-dep. O1 task. Inset: increase in responding caused by prior reward for male and female rats (horizontal bar = median difference score). **B** Time in port during non-rewarded trials as a function of prior consecutive non-rewarded or rewarded trials. **C** Response probability on non-rewarded trials as a function of prior trial (rewarded: prior R, or non-rewarded: prior NR). Inset: increase in the probability of responding caused by prior reward for male and female rats (horizontal bar = median difference score). **D** Response probability on non-rewarded trials as a function of prior consecutive non-rewarded or rewarded trials. **E** Time in port (% cue time) during rewarded trials as a function of prior trial (rewarded: prior R, or non-rewarded: prior NR). Inset: difference in responding caused by prior reward for male and female rats. **F** Time in port during rewarded trials as a function of prior consecutive non-rewarded or rewarded trials. **G** Response probability on rewarded trials as a function of prior trial (rewarded: prior R, or non-rewarded: prior NR). Inset: difference in the probability of responding caused by prior reward for male and female rats. **H** Response probability on rewarded trials as a function of prior consecutive non-rewarded or rewarded trials. Error bars indicate ± s.e.m. * P < 0.05 Males vs. Females; # P < 0.05; ## < 0.01 Prior R vs. prior NR; t-tests

influenced by prior rewards. The effect of trial history appears to be carried exclusively by the outcome of the previous trial (t-1) (no effect of consecutive prior rewards or non-rewards; Ps ≥ 0.147, Bonferroni t-tests; **Fig. 3B**).

We then analyzed another metric of conditioned responding: the response probability (i.e. probability subject enters the reward port during cue presentation). We reasoned that this metric might be more prone to detect brief lapses in discrimination performance (false alarms). **Fig. 3C** shows the probability of responding to a non-rewarded cue as a function of the outcome of the prior trial (prior reward or non-reward). A RM-ANOVA revealed a main effect of Prior Reward ($F(1, 38) = 25.607$; $P < 0.001$), and a significant Sex x Prior Reward interaction ($F(1, 38) = 9.247$; $P = 0.004$). In males, prior reward increased the probability of responding on subsequent non-rewarded trials ($T(38) = 43.752$; $P < 0.001$) while in females, trial history had no effect ($T(38) = 1.631$; $P = 0.209$). As a result, males and females differed in their probability of responding to non-rewarded trials when those trials were preceded by reward ($T(38) = 4.508$; $P = 0.040$). Here again we found no influence of the number of consecutive past reward or non-rewards (Ps ≥ 0.710, **Fig. 3D**).

Responding to rewarded cues remained high throughout the sessions and was not influenced by trial history (Ps ≥ 0.113); moreover no main effect or interaction with Sex was observed on these rewarded trials (Ps ≥ 0.127) (**Fig. 3E-H**). These results indicate that male rats have difficulty inhibiting responding to non-rewarded (contextually irrelevant) cues following a recent reward. In contrast, female rats maintained high discrimination performance throughout the session and recent rewards had little to no effect on their discrimination accuracy.

Acute stress disrupts context-gated reward-seeking in male but not female rats

We then tested the effect of an acute stress (90-min restrain) on discrimination performance (expressed as discrimination ratio), for all training groups (**Fig. 4**). With the exception of the No Discrimination group, acute stress reduced discrimination accuracy in all tasks (main Stress effect: Ps ≤ 0.004). However, only in the Ctx-dep. O1 task did we observe a significant Stress x Sex interaction $F(1, 24) = 5.665$; $P = 0.026$) as only males showed reduced discrimination performance following acute stress (Stress vs. Baseline; Males: $T(14) = 5.165$; $P < 0.001$; Females: $T(10) = 1.290$; $P = 0.209$). No main effect or interaction with Sex was observed in any other task (Ps ≥ 0.178). Therefore, although acute stress disrupted discrimination performance in all tasks, females trained in Ctx-dep. O1 appear to be protected from this disrupting effect of stress.

Female rats show higher orbitofrontal engagement during context-gated reward predictions

For a subset of animals, we quantified c-Fos-immunoreactive (cfos+) cells in the OFC (medial, ventral and lateral subregions) following regular behavior testing (no-stress condition). We also quantified cfos+ in two control regions, M1J and S1J, where we did not expect task-specific activation. Compared to baseline (homecage controls), behavioral testing increased cfos+ expression in all regions of interest, regardless of task (**Fig. 5**; homecage c-Fos+ counts are shown but not included in the following statistical analyses). A RM-ANOVA revealed a main effect of Region $F(4, 192) = 85.037$; $P ≤ 0.001$) and a significant Task x Sex x Region interaction ($F(12, 192) = 1.9$; $P = 0.036$). Follow-up planned contrast analysis compared males and females within each task and brain region. In the Ctx-dep. O1 task, females showed increased c-fos+ expression compared to males, in all OFC subregions ($T(14) ≥ 2.387$; $P ≤ 0.032$). No sex differences were found in any other task (Ps ≥ 0.255). Moreover, no sex differences were found in the control regions M1J and S1J, regardless of task (Ps ≥ 0.139).

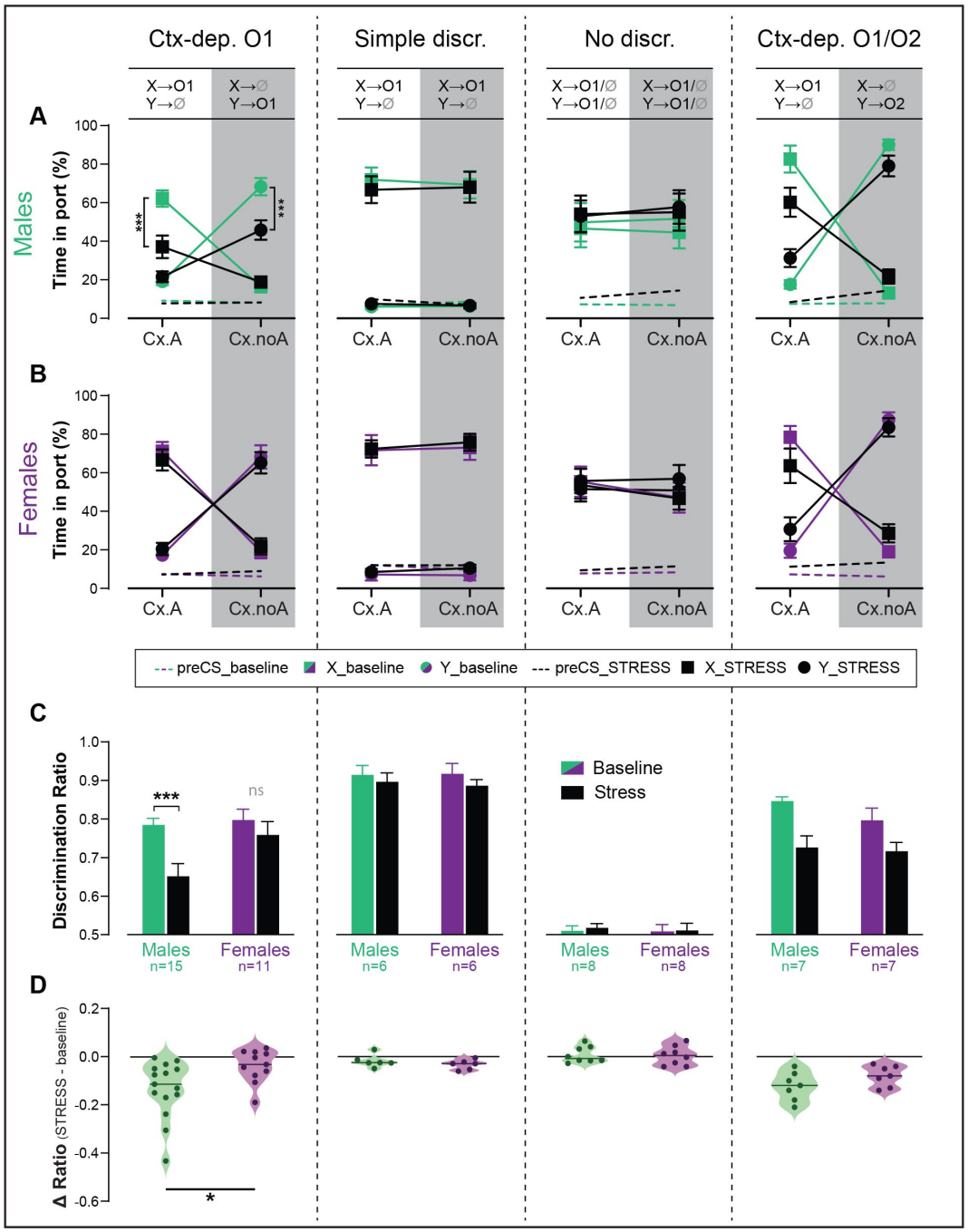

Fig. 4.

Acute stress disrupts context-gated reward seeking in male but not female rats

A-B Time in port (% cue time) during the four different trial types (A:X / X / A:Y / Y) at baseline and after acute stress for male (**A**) and female (**B**) rats trained in each discrimination task. **C** Discrimination ratio at baseline and after acute stress, for male and female rats trained in each discrimination task. **D** Stress-induced disruption in discrimination performance (stress – baseline) for male and female rats trained in each discrimination task (horizontal bar = median difference score). Error bars indicate ± s.e.m; * P < 0.05; *** P < 0.001, t-tests

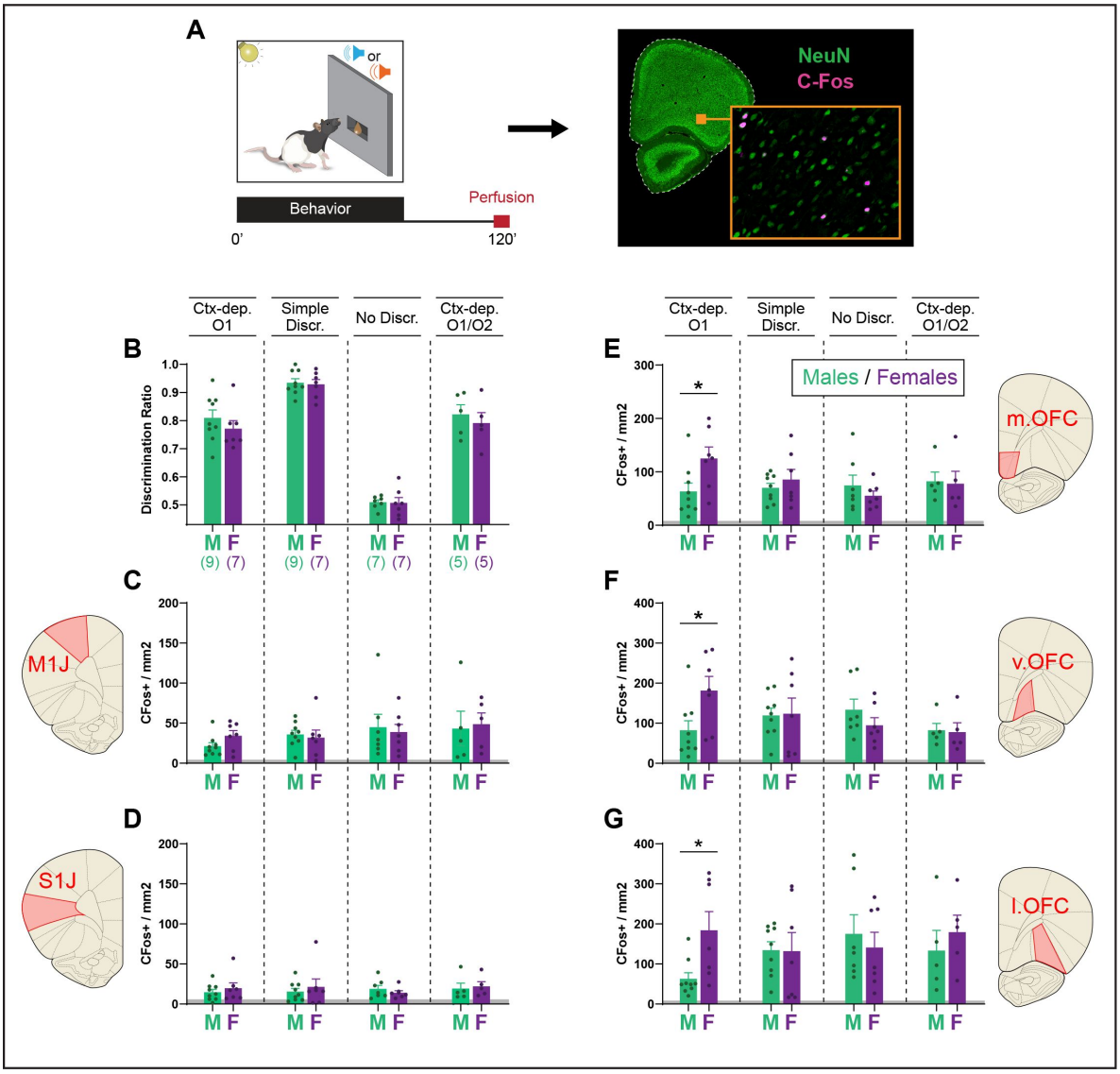

Fig. 5.

Female rats show higher orbitofrontal engagement during context-gated reward seeking.

A Experimental design. Brains were collected 2h after the start of the behavioral session. Brain sections were stained for c-Fos (activity marker) and NeuN (general neuronal marker). **B** Behavioral performance (discrimination ratio) ratio during the final session, before tissue collection for male and female rats trained in each task. **C-G** c-Fos+ density in the primary jaw motor cortex (**C**), primary jaw somatosensory cortex (**D**), medial orbitofrontal cortex (**E**), ventral orbitofrontal cortex (**F**), and lateral orbitofrontal cortex (**G**) for males and females trained in each discrimination task. Error bars indicate ± s.e.m. Gray bands indicate average c-fos+ density for animals who remained in their home cage prior to tissue collection (homecage controls; n=5). *: P < 0.05 Males vs. Females, t-tests.

Discussion

We investigated sex differences in contextual modulation of cue-evoked reward predictions and its neural correlates. Male and female rats were trained on a context-dependent discrimination task (Ctx-dep. O1) in which a contextual feature informs the validity of two reward cues (i.e. bidirectional occasion-setting). Overall, males acquired discriminated, context-gated behavior faster than females. However, once established, contextual control over behavior was more robust in females; it was more stable within a session (less influence of prior reward) and more resistant to acute stress. This superior contextual control achieved by females was accompanied by elevated c-Fos expression in all OFC subregions (medial, ventral and lateral). Critically, no sex differences (behavioral or neurobiological) were observed among animals trained on simpler discrimination tasks that do not engage contextual gating (Simple Discrimination, No discrimination, Ctx-dep. O1/O2).

For Ctx-dep. O1, context is an occasion-setter, i.e. a stimulus that hierarchically modulates the associative strength between a target cue and its outcome. However, our task differs from classic occasion-setting preparations in several ways. Historically, Pavlovian modulation has been studied in preparations in which the modulating stimuli (the occasion-setter) exerts unidirectional modulation, either strengthening (A:X+ / X-) or weakening (A:X-/ X+) a cue-outcome association. In such preparations, the occasion-setter might also establish a direct association (excitatory or inhibitory) with the outcome [1, 28, 29], thereby entangling the contributions of hierarchical modulation and direct stimuli-evoked predictions. To circumvent this issue, occasion-setting preparations commonly introduce a temporal gap (5-30s) between the termination of a discrete occasion-setting stimulus and its target cue. This approach has generally been successful in limiting direct associations between the occasion-setter and the outcome, instead promoting hierarchical modulation processes [30, 31, but see 32]. This temporal gap however introduces a working-memory requirement (animals must remember if the target cue was preceded by the occasion-setter) which complicates results interpretations. Here, we used a bidirectional occasion-setting preparation with a single reward outcome (Ctx-dep. O1) as an alternative approach to investigate contextual modulation [26]. Here, contextual feature (A) positively modulates one target association (A:X+ / X-) and negatively modulates another association (A:Y-/ Y+). Critically, there are no direct informative associations between contextual feature A and the single reward outcome (rewards are equally frequent in the presence or absence of A), so accurate discrimination necessitates hierarchical modulation [22, 33]. Moreover, the contextual feature precedes and overlaps with the target cue, thereby eliminating working-memory requirements. Thus, sex differences observed in Ctx-dep. O1, strongly indicate sex differences in hierarchical (context-informed) associative modulations. Alternatively, males and females might differ in terms of attentional resources. Indeed, unlike Simple Discrimination or No discrimination tasks, Ctx-dep. O1 requires high multimodal attention (animals must simultaneously attend to the visual context and auditory cues). However, no sex differences were observed in context-dependent discrimination with two outcomes (Ctx-dep. O1/O2), a task that shares similar attentional requirements with Ctx-dep. O1, but does not necessarily engage hierarchical contextual modulation (context potentially informing predictions via direct context-outcome associations). Together, these results indicate that the sex differences observed here are not attributable to simple associative, motivational, working-memory, or attentional processes, but are specific to the neurocomputational operations required for the hierarchical, contextual control of behavior.

Sex differences in context-dependent behaviors have previously been reported in fear conditioning preparations where females often display generalized fear responses that evade precise contextual control [34–37]. Elevated baseline anxiety and fear reactivity in females might contribute to this relative failure to regulate fear responses [38, 39, but see 40]. However, we show here that reduced contextual control in females (at least in the early stages of acquisition) is not limited to aversive behaviors but extends to appetitive, reward-seeking

behaviors. This suggests a broader, domain-general, delay in the engagement of contextual, top-down regulation processes in female rodents. This is consistent with higher sign-tracking tendencies in female rats [41–45] —another form of appetitive behavior that reflects reduced top-down cognitive control (opposed to goal-tracking behavior) [46–48]. Note that the reduced top-down contextual control displayed by females in our study was limited to delayed acquisition. Provided sufficient training, females actually established more robust contextual control over cue-evoked reward seeking than males.

The biological basis for the delayed acquisition of contextual control in females is unknown. Although estrous cycle had no effect on the expression of context-dependent behavior in female discriminators, fluctuating sex hormones (and resulting neurobiological changes) could have contributed to the slower acquisition of contextual control in females. Indeed, learning the hierarchical contextual rules for reward prediction is a complex and lengthy process that probably requires distributed plasticity across several brain regions including the striatum, amygdala, hippocampus and neocortex [3, 49]. Estrous cycle affects synaptic physiology and plasticity at all these sites via changes in dopamine reuptake [50, 51], neuronal excitability [52–56], spine density [57, 58], gene expression [59, 60], or neurogenesis [61]. This dynamic neurobiological environment might constitute a challenge for females attempting to solve the Ctx-dep. O1 task, and might contribute to their delayed acquisition of contextual control. Interestingly, this dynamic neurobiological environment might also contribute to the more stable performance ultimately achieved by females. Indeed, in deep learning models, the injection of random "noise" in (artificial) neural networks during training is commonly used to build redundancy and increase network performance and stability [62, 63]. This *'robustness through perturbations'* approach often comes at the expense of learning speed, similar to what we observed in females. This hypothesis of a role of cyclical sex hormones in the trade-off between learning speed and performance stability remains to be confirmed experimentally.

Regardless of the precise mechanism, our results indicate that, compared to male rats, females ultimately achieved more stable contextual control over cued reward-seeking; their behavior remained context-regulated under stress or after recent rewards. This behavioral sex difference was accompanied by neural sex differences. Compared to males, females displayed increased OFC activation during Ctx-dep. O1. Importantly, no behavioral or neural sex differences were observed for any other task. Together, these results strongly implicate the OFC in the sex-biased expression of contextual modulation of reward-seeking. This is consistent with recent studies demonstrating a critical role of the OFC in hierarchical control over cue-evoked behaviors [23–25]. More generally, the OFC is proposed to encode task structure including context-dependent contingencies [64– 66]. Accordingly, the OFC appears critical for decision-making when optimal choice requires knowledge of the broader task structure (i.e. when stimuli cannot be taken at face value) [67, 68]. Although causality remains to be established, our data suggest that the increased orbitofrontal engagement displayed by females might contribute to their resilient contextual regulation of behavior.

The contextual regulation of behavior is at the heart of cognitive control [69]. Deficits in this process are integral to the pathophysiology of numerous neuropsychiatric disorders [4]. Although extrapolation of animal data to humans requires caution, this study might offer insight into the biological sex differences observed in certain disorders. For instance, the initial delay in establishing contextual control observed here in females might contribute to the rapid progression of SUD or PTSD observed in women following initial drug use or trauma [13, 14, 70, 71]. Conversely, the more stable contextual control ultimately achieved by females after extended training observed here might contribute to the better prognosis for women following behavioral and cognitive treatments for these disorders [71–73], and might be due, in part, to increased OFC engagement [74].

Methods

Subjects

Subjects were male and female Long Evans rats (Charles Rivers), 8-10 weeks at arrival. Rats were housed in same-sex pairs in a temperature-controlled vivarium (22 °C) with a 12-hr light/dark cycle; behavioral experiments were conducted during the light cycle. Rats were given free access to water in their home cage for the duration of the experiments. After a week of acclimation to the colony room, rats were mildly food-restricted to maintain ~95% of age-matched free-feeding weights. All experimental procedures were conducted in accordance with UCSB Institutional Animal Care and Use Committees and the US National Institute of Health guidelines.

Apparatus

Behavioral training was conducted in 12 identical conditioning chambers enclosed in individual sound- and light-attenuating cubicles (Med Associates, St. Albans, VT). Six chambers were dedicated to male subjects and six chambers were dedicated to female subjects; animals of both sexes were trained simultaneously. A recessed reward delivery port was located at the center of the front panel and was connected to a pellet dispenser and a syringe pump for liquid reward delivery (syringe pump located outside of the sound-attenuating cubicle). Subjects' presence in the reward port was detected by the interruption of an infrared beam. Two ceiling-facing chamber lights located on the front and back internal walls of the cubicle provided diffuse chamber illumination and were used to manipulate the background visual context. Auditory stimuli were delivered via a white noise speaker and a clicker (76dB each), located in the back and front panel, respectively. A fan mounted on the cubicle provided ventilation and low background noise. A computer equipped with Med-PC software (Med Associates) controlled all experimental programs and data collection.

Rewards

Food rewards consisted of a 45mg banana-flavored food pellet (BioServe Dustless Precision Pellets, F0059 formula) or a 0.17mL bolus of sweetened chocolate milk (2 parts chocolate Nesquik® + 1 part water + 5% sucrose w/v) delivered over 3s. These rewards are highly discriminable but equicaloric and were equally preferred by rats in preliminary studies. The identity of the rewarding outcomes was counterbalanced within behavioral tasks and within sexes.

Discrimination Training

Rats were randomly assigned to one of four Pavlovian discrimination conditions: Context dependent discrimination with single outcome (Ctx-dep. O1, n = 30F + 32M), Context dependent discrimination with dual outcome (Ctx-dep. O1/O2, n = 14F + 14M), Simple discrimination (Simple, n = 12F + 22M), or No Discrimination (NoDiscr., n = 8F + 8M). Animals were individually placed in the conditioning chambers for 80-min training sessions 5-6 days/week. During each session, the visual background –the local context– alternated every two minutes between flashing houselights (0.25s on, 0.25s off; Context A) or darkness (Context noA). Within each context phase, two 10-s auditory cues, X or Y (white noise or clicker, counterbalanced), were presented individually, in two separate trials, and were potentially followed by reward. The presentation of each cue within a context phase was selected semi-randomly, so that the identity of the first cue had no incidence on the identity of the second cue (i.e. the same cue could repeat twice within a context phase, so the outcome of the first trial did not inform the outcome of the second trial). Each session consisted of 40 context cycles and 80 cue presentations (i.e. 80 trials) with an average intertrial of 50s ± 20s (rectangular distribution). The reward contingencies in each task are outlined in **Figure 1**.

Context dependent discrimination with single outcome (Ctx-dep. O1)

In this task, the reward-predictive validity of each cue was informed by the context, so that cue X was rewarded only in Ctx.A and cue Y was rewarded only in Ctx.noA. All rewarded trials resulted in the same reward outcome (O1). Considered individually, both cues and both contexts have equal probabilities of resulting in the same outcome, therefore accurate performance in this task cannot be understood in terms of summed associative strength between cues and contexts. Instead, accurate performance in this task critically relies on contextual modulation (i.e. occasion-setting). During the first 35 training sessions, rats experienced an equal number of rewarded and unrewarded trials (20 trials for each context/cue combination per session). On the 36th session and for the remainder of the experiment, the relative proportion of rewarded trials was reduced (5 A:X+; 15 X-; 15 A:Y-; 5 Y+) in an effort to promote discrimination.

Context dependent discrimination with dual outcome (Ctx-dep. O1/O2)

This task followed similar contingencies as previously described, with the exception that the two rewarded trial types (A:X and noA:Y) resulted in two different food outcomes (O1 and O2, respectively). This has profound implications for the learning processes engaged in the task. The delivery of different outcomes in different contexts allows for distinct context-outcome associations (Ctx.A → O1 and Ctx.noA → O2), as well as distinct cue-outcome associations (X → O1 and Y → O2). These different associative structures could potentially facilitate discrimination performance via simple summation process.

Simple Discrimination

Cue X was always rewarded and cue Y was never rewarded, regardless of the context. Presumably, this training protocol promotes accurate reward prediction via simple associative learning (context-independent predictions).

No Discrimination

Both cues X and Y were probabilistically rewarded in both context (the background context provides no disambiguating information). Presumably, this training protocol promotes reward prediction via simple associative learning (context-independent predictions) although the reward cues retain some ambiguity.

Assessment of Estrous Cycle

Estrous cycle phases were monitored via vaginal cytology for a subset of females (n = 10) over 15 consecutive days (sessions 66-80). Immediately after the behavioral session, the tip of a saline-moistened cotton swab was inserted into the vagina and rotated to dislodge cells from the vaginal wall. Swabs were immediately rolled onto a glass slide, and the samples were preserved with a spray fixative (M-FixTM, MilliporeSigma) without allowing the cells to dry. Slides were then stained with a modified Papanicolau staining procedure as follows: 50% ethyl alcohol, 3 min; tap water, 10 dips (×2); Gill's hematoxylin 1, 6 min; tap water, 10 dips (×2); Scott's water, 4 min; tap water, 10 dips (×2); 95% ethyl alcohol, 10 dips (×2); modified orange-greenish 6 (OG-6), 1 min; 95% ethyl alcohol, 10 dips; 95% ethyl alcohol 8 dips; 95% ethyl alcohol 6 dips; modified eosin azure 36 (EA-36), 20 min; 95% ethyl alcohol, 40 dips; 95% ethyl alcohol, 30 dips; 95% ethyl alcohol, 20 dips; 100% ethyl alcohol, 10 dips (×2); xylene, 10 dips (×2); coverslip immediately. Samples were observed with light microscopy (4x). Four criteria were used to determine the specific estrus cycle: 1. cell density, 2. percentage of nucleated epithelial cells, 3. cornified epithelial cells, and 4. Percentage of leukocytes [75–77]. Each sample was independently rated by two trained observers (S.P. and A.M.), blind to the behavioral data. Inter-rater agreement was high (88.2%). Only behavioral sessions for which both raters agreed on the estrous phase were selected for analysis. As a result, 2-4 behavioral sessions were considered for each subject at each phase of the

estrous cycle. Behavioral data on these sessions were averaged to express a single data point for each rat at each stage of the estrous cycle. Females that did not show a regular cycle were excluded from the analysis (n = 2).

Acute Restraint Stress

Before testing the effect of acute stress on discrimination performance, the relative proportion of rewarded trials was adjusted in all groups to match the proportion of rewarded trials in the context-dependent discrimination with a single outcome (Ctx-dep. O1). As a result, rewarded cues became less frequent and represented only 1/4 of the trials. For all groups, these rewarded trials were equally distributed across both contexts. After 5-6 sessions of adjustment to these new conditions, we tested the effect of an acute stress on discrimination performance in all training groups. Acute stress was induced by restraining rats in standard Plexiglas rat restraint tubes for 90 min, in a novel brightly lit room. To account for the size differences between male and females, two different sized restraint tubes were used. After placing the rats in the tube, the length of the restrainer was adjusted to immobilize the rat without causing pain or interfering with animals' breathing. After 90 min of restraint, the rats were returned to their homecage and left undisturbed for 5 min before being placed in the conditioning chambers for a normal behavioral session.

cFos Immunofluorescence

Following stress test, animal were retrained for 5-6 sessions, under standard conditions (no stress; equal number of rewards for all training groups). After their last behavioral session, animals remained in the conditioning chambers for 40 min (120 min total); they were then deeply anesthetized with a lethal dose of Euthasol and perfused transcardially with ice-cold 1x PBS followed by ice-cold 4% PFA in PB. Brains were extracted, post-fixed (4% PFA overnight) and cryoprotected (30% sucrose + 0.1% sodium azide, 1x PBS) until processed for immunostaining. Brains were frozen and sectioned into 40µm with a sliding cryostat (Leica CM1860). Free floating coronal sections were washed for 10 min in PBS, followed by a 2-h incubation in a blocking solution (5% NGS, 0.2% Triton X-100 in 1x PBS) at room temperature. Sections were then incubated with rabbit anti-cFos (1:1000, Cell Signaling, CAT#: 2250) and mouse anti-NeuN (neuron-specific protein; 1:2000 or 1:3000, Novus, CAT#: NBP1-92693) primary antibodies, in the blocking solution, for 20 h a 4°C. Following primary antibody incubation, sections were washed in PBS 3 times for 10 min and then incubated with anti-rabbit Alexa Fluor® 647-conjugated secondary antibody (far-red) (1:250, Jackson ImmunoResearch, CAT#: 111-605-144) and anti-mouse Alexa Fluor® 488-conjugated secondary antibody (green) (1:500, Jackson ImmunoResearch, CAT#: 115-545-003) for 90 min at room temperature. Sections were then washed in PBS three times for 10 min, mounted on slides and immediately coverslipped with a hardset mounting medium (Vector laboratories, CAT#: H-1700). Images were acquired at a 10x magnification, using a Keyence BZ-X fluorescence microscope.

Brain regions of interest for c-Fos positive neurons quantification included the medial, ventral, and lateral orbitofrontal cortex (mOFC, vOFC, and lOFC). Additionally, two control regions were included, for which we did not expect task-specific activation: the jaw primary motor (M1J) and somatosensory cortex (S1J). These regions of interests were manually isolated from the original images, using only the green / NeuN channel (the far-red / c-fos channel was temporarily disabled for blinding purposes). A custom ImageJ script (National Institute of Health) was then used to count c-Fos positive neurons in the selected regions and normalize this count to the surface area of the selected region (c-Fos positive neurons / mm2). For each brain region, c-Fos positive neurons were counted bilaterally from 2-4 sections per animal and a mean count was computed for each animal.

Statistical analysis

Data were collected over three replication cohorts, each composed of approximately equal number of male and female rats. Cue-evoked reward seeking was quantified primarily as the percentage of time a rat spent in the food cup during the last 5 seconds of cue presentation. Discrimination accuracy was expressed as a discrimination ratio, defined as the time in port during rewarded trials divided by the total time in port during all trials. A ratio of 0.5 indicates equal responding during rewarded and non-rewarded trials (i.e. no discrimination) whereas a ratio of 1 indicates responding exclusively during rewarded trials (i.e. perfect discrimination). Time in port and discrimination ratio during acquisition were aggregated in 4-session bins. These longitudinal repeated measures were analyzed with Linear Mixed-Models (LMM) with a Restricted Maximum Likelihood (REML) estimation method. All covariance structures were explored and the best fitting model was determined by the lower Akaike information criterion score [78, 79]. The selection of a covariance structure influenced the degrees of freedom of the model, possibly resulting in non-integer values. The remaining of the analyses consisted generally of mixed-models repeated measures (RM) ANOVAs with Sex and/or Task as a between-subject factor, and Trial history, Stress, or Brain region as within-subject factors. The Geisser-Greenhouse correction was applied when data lacked sphericity, possibly resulting in non-integer degrees of freedom. Post-hoc or planned contrast pairwise comparisons were carried with Bonferroni-corrected t-tests. When appropriate, nonparametric tests were conducted and consisted of the Kruskal-Wallis H test followed by post-hoc Bonferroni-corrected Dunn's pairwise comparisons. All tests were two-tailed, significance was assessed against a type I error rate of 0.05. Statistical analyses were conducted with SPSS Statistics package (version 28.0.0.0; IBM SPSS). Graphs were generated using GraphPad Prism 9.

Authors contributions

Experimental design: RK, SP, AM; Behavior testing and data collection: AM, SP, BW, JC; Estrous cycle monitoring (vaginal cytology): AM, SP; Immunostaining: SP, AM, BW, JC; Cell counting: AM, RK; Data analysis: RK, AM, SP; Manuscript preparation: RK, AM, SP

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Competing Interests

The authors have nothing to disclose.

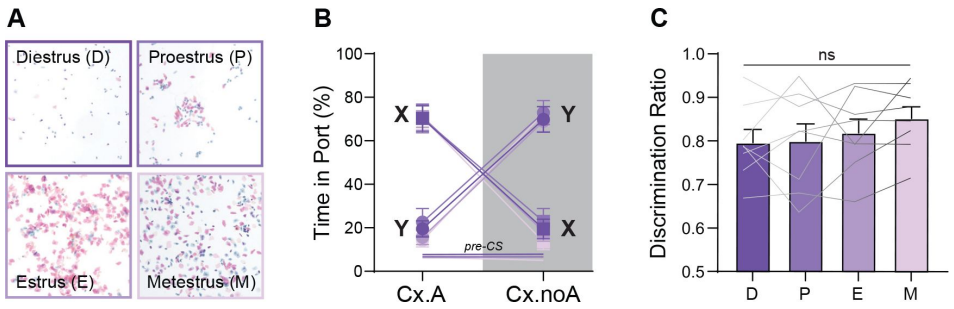

Fig. S1

Estrus cycle does not influence the expression of context-gated reward seeking

A. Vaginal cytology was used to track the stage of estrous cycle in a subset of female discriminators trained in the Ctx-dep. O1 task (n=8). **B.** Time in port during the four trial types (A:X / X / A:Y / Y) was not influenced by the estrous cycle (Estrous Stage $F(3, 21) = 0.450$; $P = 0.720$; Estrous Stage x Trial: $F(2.258, 15.808) = 1.293$; $P = 0.305$). **C.** Discrimination ratio was not influenced by the estrous cycle ($F(3, 21) = 1.601$; $P = 0.219$). Error bars indicate ± s.e.m.

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Reviewer #1 (Public Review):

Summary:
Peterson et al., present a series of experiments in which the Pavlovian performance (i.e. time spent at a food cup/port) of male and female rats is assessed in various tasks in which context/cue/outcome relationships are altered. The authors find no sex differences in context-irrelevant tasks and no such differences in tasks in which the context signals that different cues will earn different outcomes. They do find sex differences, however, when a single outcome is given and context cues must be used to ascertain which cue will be rewarded with that outcome (Ctx-dep O1 task). Specifically, they found that males acquired the task faster,

but that once acquired, the performance of the task was more resilient in female rats against exposure to a stressor. Finally, they show that these sex differences are reflected in differential rates of c-fos expression in all three subregions of rat OFC, medial, lateral, and ventral, in the sense that it is higher in females than males, and only in the animals subject to the Ctx-dep O1 task in which sex differences were observed.

Strengths:
• Well-written.
• Experiments elegantly designed.
• Robust statistics.
• Behaviour is the main feature of this manuscript, rather than any flashy techniques or fashionable lab methodologies, and luckily the behaviour is done really well.
• For the most part I think the conclusions were well supported, although I do have some slightly different interpretations to the authors in places.

Weaknesses:
1. With regards to the claim (page 4 of pdf), I think I can see what the authors are getting at when they claim "Only Ctx-dep.01 engages context-gated reward predictions", because the same reward is available in each context, and the animal must use contextual information to determine which cue will be rewarded. In other words, it has a discriminative purpose. In Ctx-dep.O1/O2, however, although the context doesn't serve a discriminative purpose in the sense that one cue will always earn a unique outcome, regardless of context, the fact that these cues are differentially rewarded in the different context means that animals may well form context-gated cue-outcome associations (e.g. CtxA-(CS1-O1), CtxnoA-(CS2-O2)). Moreover, the context is informative in this group in telling the animal which cue will be rewarded, even prior to outcome delivery, such that I don't think contextual information will fade to the background of the association and attention be lost to it in the way, say Mackintosh (1975) might predict. Therefore, I don't think this statement is correct.

2. I think the results shown in Figure 1 are very interesting, and well supported by the statistics. It's so nice to see a significant interaction, as so many papers try to report these types of effects without it. However, I do wonder how specific the results are to contextual modulation. That is, should a discriminative discrete cue be used instead of each context (e.g. CS1 indicates CS2 earns O1, CS3 indicates CS4 earns O1), would female rats still be as slow to learn the discrimination?

3. Pages 8-9 of pdf, where the biological basis or the delayed acquisition of contextual control in females is considered, I find this to be written from a place of assuming that what is observed in the males is the default behaviour. That is, although the estrous cycle and its effects on synaptic plasticity/physiology may well account for the results, is there not a similar argument to be made for androgens in males? Perhaps the androgens also somehow alter synaptic plasticity/physiology, leading to their faster speed, reduced performance stability, and increased susceptibility to stress.

4. In addition, the OFC - which is the brain region found to have differential expression of c-fos in males and females in Figure 5 - is not explicitly discussed with regard to the biological mechanisms of differences, which seems odd.

Reviewer #2 (Public Review):

Summary:
A bidirectional occasion-setting design is used to examine sex differences in the contextual modulation of reward-related behaviour. It is shown that females are slower to acquire contextual control over cue-evoked reward seeking. However, once established, the contextual control over behaviour was more robust in female rats (i.e., less within-session

variability and greater resistance to stress) and this was also associated with increased OFC activation.

Strengths:
The authors use sophisticated behavioural paradigms to study the hierarchical contextual modulation of behaviour. The behavioural controls are particularly impressive and do, to some extent, support the specificity of the conclusions. The analyses of the behavioural data are also elegant, thoughtful, and rigorous.

Weaknesses:
My primary concern is that the authors' claim of sex differences in context-dependent discrimination behaviour is not fully supported by their data.

First, the basic behavioural effect does not seem to replicate across experiments. The authors first show sex differences in the % time in food port and the discrimination ratio (Figures 1 and 2) such that males show better context-dependent discrimination than females (group ctx-dep O1). However, this difference is not observed in the baseline condition group in the next experiment, which investigates the effect of acute stress on context-gated reward seeking: "In Figure 4, we observe no difference between males versus females in group "ctx-dep O1".

Second, I am not fully convinced by the authors' assertion that the results are specific to the contextual modulation process. The authors' main conclusions are derived from comparing a group trained with the differential outcome procedure (group cxt-dep O1/O2) and a group with the non-differential outcome procedure (group cxt-dep O1). However, importantly, a different number of training sessions was used for ctx-dep O1/O2 and ctx-dep O1. Is it not possible that sex differences could have emerged with additional training in the cxt-dep O1/O2 group? Moreover, the authors also seem to assume that rats are not using a contextual strategy in the context-dep O1/O2 condition (i.e., rats use instead distinct context-outcome associations) but what is the evidence for this? Also, the authors argue that the impact of stress is specific to the hierarchical contextual modulation of behaviour however inspection of Figure 4A suggests that there may also be an effect of stress on the context-dependent O1/O2 group.

I also had some minor issues with how the authors interpreted some of the findings. First, it is shown that recent rewards disrupt contextual control of reward seeking in male, but not female, rats. That is, in males, prior reward increased the probability of responding on subsequent non-rewarded trials but trial history had no effect in females. How do the authors reconcile this finding with the quicker acquisition and better discrimination that is observed in males? It is not evident to me how males can have difficulty inhibiting responding to non-rewarded cues following recent reward yet still show better discrimination throughout training.

Finally, the authors argue that the contextual control over behaviour was more robust in female rats as females show less within-session variability and greater resistance to stress. What evidence is there that the restraint stress procedure causes a similar stress response in both sexes?

Reviewer #3 (Public Review):

Summary:
This manuscript reports an experiment that compared groups of rats acquisition and performance of a Pavlovian bi-conditional discrimination, in which the presence of one cue, A, signals that the presentation of one CS, X, will be followed by a reinforcer and a second CS, Y, will be nonreinforced. Periods of cue A alternated with periods of cue B, which signaled the opposite relationship, cue X is nonreinforced, and cue Y is reinforced. This is a conditional discrimination problem in which the rats learned to approach the food cup in the presence of

each CS conditional on the presence of the third background cue. The comparison groups consisted of the same conditional discrimination with the exception that each CS was paired with a different reinforcer. This makes the problem easier to solve as the background is now priming a differential outcome. A third group received simple discrimination training of X reinforced and Y nonreinforced in cues A and B, and the final group was trained with X and Y reinforced on half the trials (no discrimination). The results were clear that the latter two discrimination learning procedures resulted in rapid learning in comparison to the first. Rats required about 3 times as many 4-session blocks to acquire the bi-conditional discrimination than the other two discrimination groups. Within the biconditional discrimination group, female and male rats spent the same amount of time in the food cup during the rewarded CS, but females spent more time in the food cup during CS- than males. The authors interpret this as a deficit in discrimination performance in females on this task and use a measure that exaggerates the difference in CS+ and CS_ responding (a discrimination ratio) to support their point. When tested after acute restraint stress, the male rats spent less time in the food cup during the reinforced CS in comparison to the female rats, but did not lose discrimination performance entirely. The was also some evidence of more fos-positive cells in the orbitofrontal cortex in females, but this difference was of degree.

Overall, I think the authors were successful in documenting performance on the biconditional discrimination task. Showing that it is more difficult to perform than other discriminations is valuable and consistent with the proposal that accurate performance requires encoding of conditional information (which the authors refer to as "context"). There is evidence that female rats spend more time in the food cup during CS-, but I hesitate to agree that this is an important sex difference. There is no cost to spending more time in the food cup during CS- and they spend much less time there than during CS+. Males and females also did not differ in their CS+ responses, suggesting similar levels of learning. A number of factors could contribute to more food cup time in CS-, such as smaller body size and more locomotor activity. The number of food cup entries during CS+ and CS- was not reported here. Nevertheless, I think the manuscript will make a useful contribution to the field and hopefully lead readers to follow up on these types of tasks.

One area for development would be to test the associative properties of the cues controlling the conditional discrimination, can they be shown to have the properties of Pavlovian occasion-setting stimuli? Such work would strengthen the justification/rationale for using the terms "context" and "occasion setter" to refer to these stimuli in this task in the way the authors do in this paper.

Strengths:
- Nicely designed and conducted experiment.
- Documents performance difference by sex.

Weaknesses:
- Overstatement of sex differences.
- Inconsistent, confusing, and possibly misleading use of terms to describe/imply the underlying processes contributing to performance.