Estrous cycle stage gates the effect of stress on reward learning

Morgan P. Johnston[1], Brandon I. Garcia-Castañeda[1], Leonor G. Cedillo[1], Sachi K. Patel[1], Victoria S. Vargas[1], Matthew J. Wanat[1]

Author Affiliations:

[1]Department of Neuroscience and Developmental Biology, University of Texas at San Antonio, San Antonio, TX, USA.

Corresponding Author:

Matthew J. Wanat
Department of Neuroscience, Developmental and Regenerative Biology
University of Texas at San Antonio
Matthew.wanat@utsa.edu

Author Contributions:

MPJ and MJW designed the experiments. MPJ, BIG, LGC, SKP, and VSV collected the data. MJP analyzed the data. MJP and MJW wrote the manuscript. We would like to thank Claire Stelly for providing training with the behavioral task.

Funding and Disclosure:

This work was supported by National Institutes of Health grants MH127466 (MJW) and MH133062 (MJW). The work was additionally supported by the National Science Foundation Graduate Research Fellowship Program (MPJ and BIG). The authors declare no conflict of interest.

Abstract:

Stress produces transient physiological responses that lead to long-lasting changes in cue-driven behavior. In particular, a single exposure to stress facilitates reward learning in male rats. Since stress can produce distinct behavioral phenotypes between males and females, it is critical to additionally determine how stress impacts reward learning in females. To address this, female rats were exposed to restraint stress immediately prior to training on an appetitive Pavlovian conditioning task with food rewards. Females were categorized based on their estrous cycle stage on the first day of Pavlovian conditioning. A single exposure to stress enhanced conditioned responding in non-estrus females but suppressed conditioned responding in estrus females. Therefore, a single stress experience produced opposing effects on cue-driven behavior depending upon the estrous cycle stage. In contrast, both estrus and non-estrus rats exposed to repeated prior stress exhibited an increase in conditioned responding relative to animals that underwent a single stress exposure. We further examined if the distal stress experience subsequently impacted extinction and the ability to learn a new cue-reward association. Prior stress did not affect extinction, though estrus and non-estrus rats exposed to repeated prior stress exhibited higher levels of conditioned responding to the novel cue-reward pairing. Taken together, our data demonstrate that the influence of stress on reward learning is impacted acutely by the estrous cycle as well as by one's prior history with stress.

Introduction:

Stress produces transient physiological responses which can lead to long-lasting behavioral changes. These persistent behavioral alterations can arise from stress impacting learning processes. Across species, stress elicits greater activation of the hypothalamic-pituitary-adrenal axis in females relative to males [1,2]. This increased stress responsivity is thought to contribute to the higher incidences of stress-related disorders in women compared to men [1-3]. While prior studies have characterized the effect of stress on learning cue-outcome associations in males, it is also critical to determine how stress affects learning in females [4-13]. The impact of stress on learning has traditionally been studied in the context of stress-enhanced fear/threat learning [4-10,14-25]. In female rodents, a single stress enhances Pavlovian conditioned responding to cues associated with a foot shock [20,22-24]. Multiple exposures to stress can further enhance fear/threat learning [26]. Robust behavioral responding to aversive cues may initially appear beneficial, as it helps avoid threats in one's environment. However, this stress-enhanced responding is accompanied by negative consequences. For example, stressed females also exhibit deficits during extinction [8,15,17,19-21,27].

Considerably less is known about the impacts of stress on reward learning. In human studies that included women but did not separate data by gender, stress prior to conditioning

results in enhanced reward learning compared to controls [28-32]. A study that examined women specifically found that individuals who exhibited the greatest stress response also displayed the highest degree of conditioned responding to a reward [32]. Furthermore, stress experience produces deficits in extinction to a reward-paired cue in humans [33,34]. Interestingly, others have found that stress has no impact on reward learning in human women or female rats [35-39]. These conflicting findings may be due to an interaction of cycling hormones and stress.

Hormonal cycles, such as the menstrual cycle in humans and the estrous cycle in rodents, can modulate reward learning [40-47]. Women in the late luteal phase of the menstrual cycle during training on a probabilistic learning task display greater reward conditioning relative to those in the late follicular phase [40]. In rats, females trained during estrus (the rodent analog of the late luteal phase) display greater cue-reward associations [41,42,48]. This enhanced association is evident a month after the initial conditioning session, demonstrating that estrous stage during the formation of cue-reward associations can have a long-lasting impact on behavior [42]. While the estrous cycle and stress separately influence reward learning, it is unclear how the two may interact to affect cue-reward associations.

To address this, we exposed naturally cycling female rats to either a single or repeated stress (or control procedures) prior to training on an appetitive Pavlovian conditioning task. During Pavlovian training sessions, rats learned to associate an audio cue with the delivery of a food reward. We subsequently altered the training parameters to assess how the distal stress experience affected the extinction of the previously established cue-reward relationship as well as the capacity to learn a novel cue-reward association. By monitoring the estrous cycle throughout training we determined how the stress interacted with the cycle stage to influence reward learning.

Methods

I. Subjects

The University of Texas at San Antonio Institutional Animal Care and Use Committee approved all procedures. Adult (60-70 days old) female Sprague Dawley rats (Charles Rivers and Inotiv) were pair-housed upon arrival, allowed *ad libitum* access to water and chow, and maintained on a 12 hr light/dark cycle. Prior to behavioral procedures, rats were either single- or pair-housed.

II. Behavioral procedures

Rats were placed on mild dietary restriction to 90% of their free feeding weight, allowing for a weekly increase of 1.5%. Rats were regularly handled and estrous smears were obtained

for one week prior to onset of behavioral experiments and after each Pavlovian conditioning session. All behavioral sessions occurred during the rats' light cycle. Pavlovian conditioning occurred in operant boxes (MedAssociates) with a grid floor, a house light, a recessed food tray equipped with an infrared beam-break detector, and a white noise and tone generator. Rats underwent one habituation session in which they were placed in an operant box and received 20 un-signaled food pellets (45 mg, BioServ) delivered at a 90 s variable interval.

IIa. Restraint stress and control procedures

Rats were randomly divided into four treatment groups: single stress, single control, repeated stress, and repeated control. Prior to the first Pavlovian conditioning session, rats in the single stress/control groups underwent one restraint stress or control procedure (Fig. 1A). The stress procedure involved confining the rats to a clear acrylic tail vein restrainer (Braintree Scientific) for 20 min [13,49]. For the control procedure, rats were placed in a clean, empty cage for 20 min [13]. Both control and stress procedures took place in an isolated room, distinct from where Pavlovian conditioning sessions would subsequently occur. To avoid potential confounds due to social transmission of stress, cagemates underwent the same stress/control procedures[50-54]. Additionally, stress and control treatments were administered on separate days. Single stress/control rats began the first Pavlovian conditioning session 20 min after the conclusion of stress or control procedure. Repeated control/stress treatment animals underwent stress or control procedure once a day for five consecutive days (**Fig. 2A** and **Fig. 3A**). A subset of repeated control rats underwent a handle only control procedure. These rats were removed from their cage, weighed, and placed back in their home cage once a day for five days. However, we found no significant difference between our standard control procedure and the handle only control procedure (main effect $F_{(1,25)} = 1.6$, $p = 0.2$), so those rats are combined as the repeated control groups. The first Pavlovian conditioning session began 20 min after the conclusion of the last stress or control procedure.

IIb. Pavlovian conditioning and contingency change

After their final stress or control treatment, rats were transferred to a novel, clean recovery cage for 5 min. They were then placed in the operant chamber for 15 min prior to beginning Pavlovian conditioning, for a total of 20 min between stress/control procedure and onset of Pavlovian conditioning [13]. Prior research in male rats demonstrated that stress-enhanced conditioned responding was evident 20 min after stress, but absent 2 hrs after stress [13]. Rats underwent an additional 9 Pavlovian training sessions, for a total of 10 Pavlovian conditioning sessions. Importantly, stress or control procedures were only administered prior to the first session. Each Pavlovian training session consisted of 50 trials. In each trial, rats were

presented with a 5 s white noise conditioned stimulus (CS+) which co-terminated with the delivery of a single food pellet unconditioned stimulus (US) and a 4.5 s illumination of the tray light. Trials were separated by a 55 ± 15 s intertrial interval (ITI) (**Fig. 1B**).

A subset of rats underwent further conditioning sessions where the cue-reward contingencies were altered. In these training sessions, the white noise cue that had previously indicated a reward was no longer rewarded (CS+ → CS-; CS- trial), while a novel audio cue (tone, 5 s) now predicted a reward delivery (new CS+; CS+ trial). Trials were separated by a 55 ± 15 s ITI. Training sessions following the cue-reward contingency change consisted of 25 CS- trials and 25 CS+ trials presented in a pseudo-random order. Rats underwent five of these additional training sessions. Following the completion of Pavlovian training, rats were assessed for anxiety-like behavior and general locomotor activity. Rats were placed in an elevated zero maze apparatus for 5 min. Total distance travelled and percent time spent in open and closed arms were measured.

IIc. Behavioral analysis

We recorded head entries into the food tray across training sessions. Conditioned responding was quantified as the change in rate of head entries during the 5 s CS relative to the 5 ss preceding the CS [13,55-58]. Response latency was calculated as the time difference between CS onset and the first head entry during the CS. The rate of head entries when the cue was not playing (non-CS head entry rate) served as a measure of general motor activity. Conditioned responding to the CS- during the cue-reward contingency change sessions was normalized to the level of responding during the first contingency change session (conditioning session 11) to account for the differences in conditioned responding between treatment groups.

III. Vaginal cytology and Estrous Cycle Tracking

Vaginal cells were obtained via lavage, which entails flushing saline into the vaginal canal using a pipette. The saline with the vaginal sample was then placed on a slide, allowed to air dry, and then stained with crystal violet [59]. The resulting estrous smears were then examined via light microscopy. The person evaluating the samples was blind to the stress status of the rat. Samples with >80% nucleated epithelial cells were considered proestrus, >80% cornified epithelial cells were estrus, >80% leukocytes were diestrus, and an approximately equal mixture of all described cells were metestrus [48,59].

Estrous smears were obtained daily for one week prior to behavioral experiments and after each Pavlovian conditioning session. On days in which rats underwent Pavlovian conditioning, estrous smears were obtained 30-60 min after the completion of the training session to minimize any potential impact of the estrous smear collection on conditioned

responding. Females were categorized based on the estrous stage on the first day of Pavlovian conditioning. Rats were categorized as estrus or non-estrus, consistent with others (**Supplemental Fig**. **1**)[60-63].

IV. Statistical analysis

Statistical analyses were performed using GraphPad Prism. A mixed-effects model fit was used to analyze effects on behavioral measures. Significance was set to α=0.05 for all tests. All data are graphed as mean ± SEM.

Results

Single stress exposure regulates conditioned responding in an estrous cycle-dependent manner.

Stress and the estrous cycle independently affect cue-outcome associations, but how the two may interact to regulate reward learning remains unknown [8,15,20-22,41-44,47]. To address this, naturally cycling female rats received either a single stress exposure (20 min restraint) or control procedure (20 min in an empty cage) 20 min prior to their first Pavlovian conditioning session (**Fig**. **1A)**. During the Pavlovian training sessions, rats were presented with a 5 s audio cue which co-terminated with the delivery of a food pellet (**Fig**. **1B**). Prior research in male rats utilizing the same stress and conditioning paradigms found that a single stress enhances conditioned responding [13]. However, we found no effect of stress on conditioned responding, latency, or non-CS head entry rate when all females were analyzed together (Stress: n = 34; Control: n = 38) (**Fig**. **1C,F,I**). Given that the estrous cycle can influence cue-reward associations, we next separated the data based on the estrous stage each rat was in during their first Pavlovian conditioning session (Stress Non-estrus: n = 20; Stress Estrus: n=10; Control Non-estrus: n = 21; Control Estrus: n = 16) [41-43]. We found that a single stress enhanced conditioned responding in non-estrus females (**Fig**. **1D**). There was also an interaction effect of stress and session on the latency to enter the food port and non-CS head entry rate in non-estrus rats (**Fig**. **1G,J**). In contrast, we observed the opposite effect when stress was administered to females in estrus, as this led to a persistent decrease in conditioned responding (**Fig**. **1E**). These findings demonstrate that a single exposure to stress impacts cue-driven behavior in an estrous-dependent manner.

Repeated stress further enhances conditioned responding across the estrous cycle.

Repeated stress can further potentiate stress-enhanced fear learning, compared to a single stress [26]. Therefore, we next examined the effect of repeated restraint stress on reward learning (Non-estrus: n = 15; Estrus: n = 12). In the repeated stress group, restraint stress was administered daily for five consecutive days and Pavlovian conditioning began 20 min after the

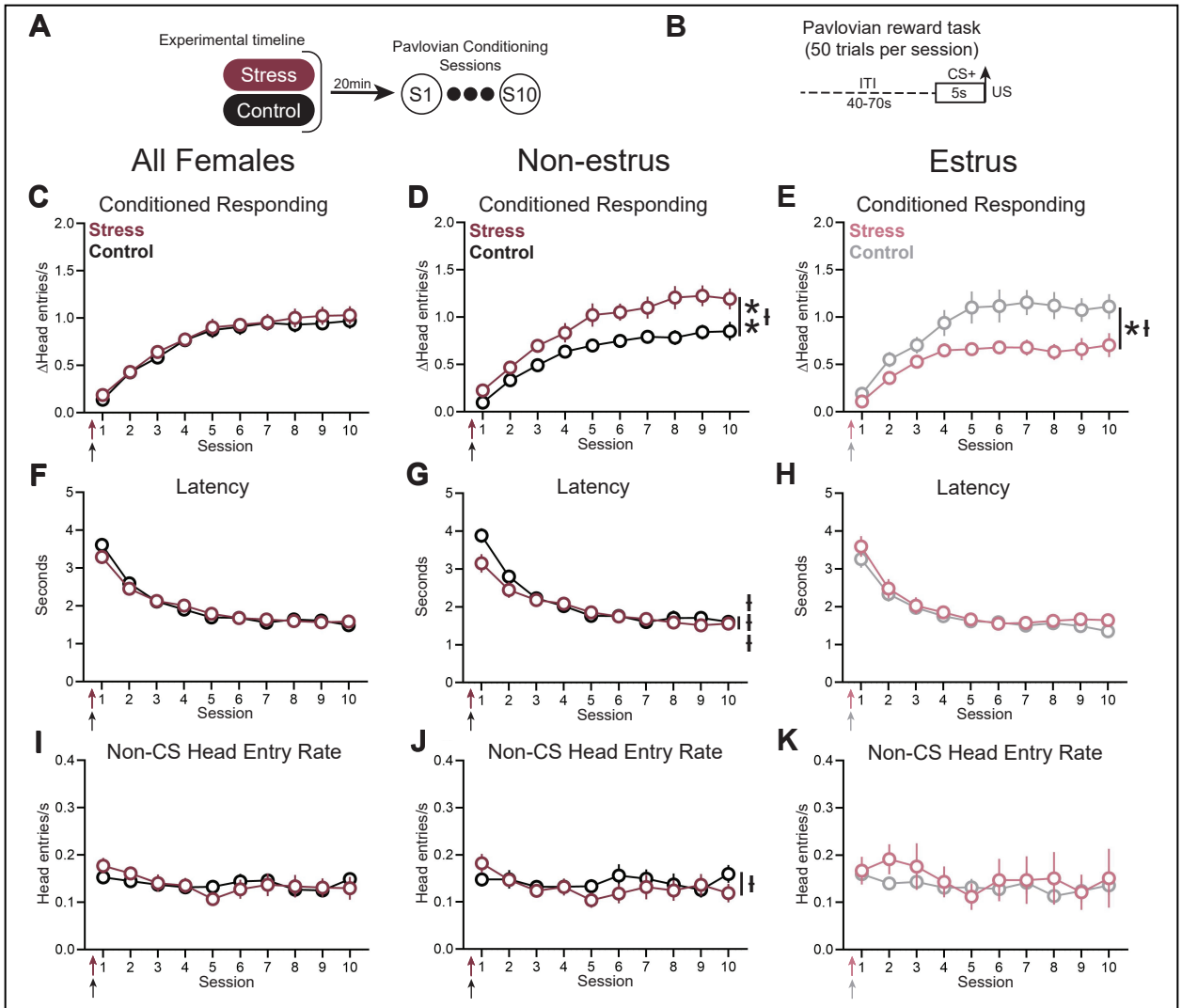

Figure 1: Estrous-dependent effect of a single stress exposure on conditioned responding. (A) Experimental timeline. Rats undergo a single restraint stress or control procedure prior to their first Pavlovian conditioning session. (B) Trial structure. (C) No effect of stress on conditioned responding when examining female rats across cycle stages (stress: n = 34; control: n = 38). (D) Stress enhanced conditioned responding in rats that underwent stress and the first Pavlovian conditioning session during a non-estrus phase of the estrous cycle (main effect of stress: $F_{(1,42)} = 10.69$, ** p = 0.002,; interaction of session x stress: $F_{(9,351)} = 2.0$, ǂ p = 0.04; non-estrus stress: n = 20; non-estrus control: n = 21). (E) Stress suppressed conditioned responding in rats that underwent their first Pavlovian conditioning session in estrus (main effect of stress: $F_{(1,25)} = 5.9$, * p = 0.02; interaction of session x stress: $F_{(9,218)} = 2.021$; ǂ p = 0.04; estrus stress: n = 10; estrus control: n = 16). (F) No effect of stress on response latency across all female rats. (G) Effect of stress on response latency in non-estrus rats (interaction of session x stress: $F_{(9,351)} = 3.5$, ǂǂǂ p = 0.0004). (H) No effect of stress on response latency in estrus rats. (I) Stress did not affect the non-CS head entry rate across all female rats. (J) Effect of stress on the non-CS head entry rate in non-estrus rats (interaction of session x stress: $F_{(9,351)} = 2.1$, ǂ p = 0.03). (K) No effect of stress on non-CS head entry rate in estrus rats.

conclusion of the final stress exposure (**Fig. 2A**). When compared to a single stress exposure, repeated stress resulted in a further enhancement of conditioned responding in both non-estrus and estrus rats (**Fig. 2B-C**). Repeated stress also decreased the latency to enter the food port

i n both non-

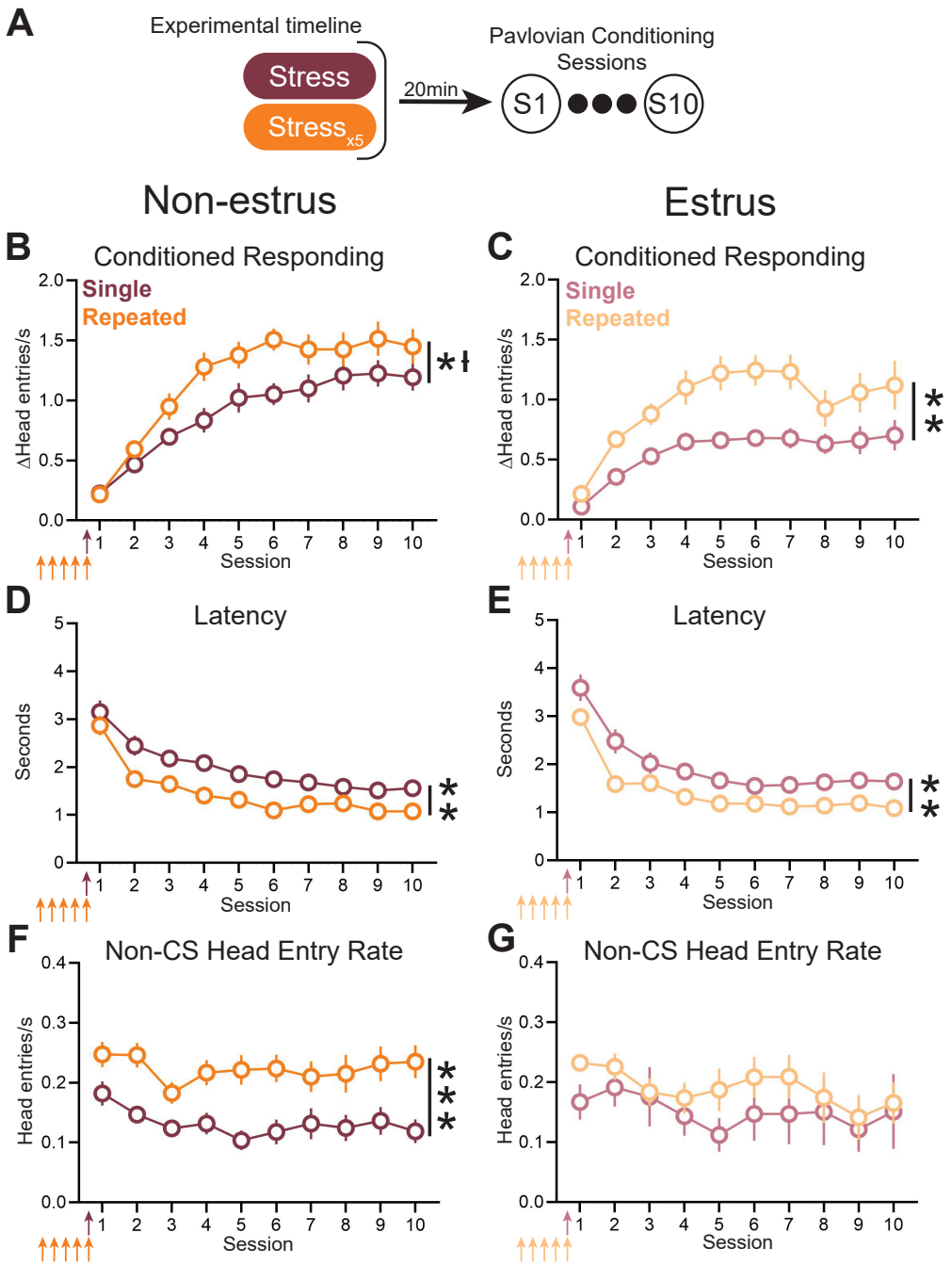

Figure 2: Repeated prior stress enhanced conditioned responding in estrus and non-estrus rats. (A) Experimental timeline. (B,C) Repeated stress enhanced conditioned responding relative to a single stress in non-estrus rats (main effect of repeated stress: $F_{(1,38)} = 5.3$, * $p = 0.027$, interaction of session and repeated stress: $F_{(9,308)} = 2.20$, ł $p = 0.02$; non-estrus single stress: n = 20; non-estrus repeated stress: n = 15) and estrus rats (main effect of repeated stress: $F_{(1,21)} = 12.4$, ** $p = 0.002$, Estrus single stress: n = 10; Estrus repeated stress: n = 12). (D,E) Repeated stress decreased the responses latency in non-estrus rats (main effect of repeated stress: $F_{(1,38)} = 8.4$, ** $p = 0.006$) and estrus rats (main effect of repeated stress: $F_{(1,21)} = 14.6$, ** $p = 0.001$). (F,G) Repeated stress increased the non-CS head entry rate compared to single stress treatment only in non-estrus rats (main effect of repeated treatment: $F_{(1,38)} = 16.5$, *** $p = 0.0002$).

estrus and estrus rats (**Fig**. **2D-E**). Additionally, repeated stress increased the non-CS head entry rate non-estrus rats only (**Fig**. **2F**). Interestingly, non-estrus rats exposed to repeated control treatments exhibited enhanced conditioned responding and reduced latency relative to non-estrus rats exposed to a single control treatment (**Supplemental Fig**. **2**). The repeated control treatment did not alter conditioned responding in estrus rats (**Supplemental Fig. 2**). Therefore, repeated administration of control procedures alone can produce similar behavioral effects to stress in non-estrus rats. Taken together, our data suggest that repeated stress administration enhances reward associations, compared to a single stress, but also increases general activity in non-estrus rats.

We next directly compared the behavior of rats that received repeated stress relative to those that received repeated control procedures. When we did not separate our data according to the estrous cycle, we found no main effect of repeated stress (n = 29) compared to repeated control (n = 27)(**Fig**. **3B,E,H**). However, there was an interaction effect of stress and session on conditioned responding (**Fig. 3B**). This result was driven by the non-estrus females, which also exhibited an interaction effect of stress and session on conditioned responding (**Fig. 3C**). Repeated stress additionally decreased the latency in estrus rats and increased non-CS head entry rate in non-estrus rats (**Fig**. **3G,I**).

The state of the animal when it is initially acquiring cue-outcome associations influences the resulting cue-driven behavior [13,43,64]. For example, when male rats are stressed 20 min prior to Pavlovian conditioning, they display long-lasting enhanced conditioned responding[13]. However, if stress is administered 2 hrs prior to conditioning, this enhancement is lost[13]. Accordingly, our initial analysis of rats undergoing repeated stress/control treatments focused on the estrous cycle stage on the last treatment day and first day of Pavlovian conditioning. However, we additionally examined whether the estrous cycle stage on the first day of the stress/control treatment influenced conditioned responding. As our repeated stress and control procedures lasted five days and the estrous cycle is a 4-5 day cycle, one might expect that many rats were in the same stage during their first and last day of stress or control procedure [48,59]. Indeed, 70% of repeated control rats and 66% of repeated stress rats were in the same estrus or non-estrus category on their first and last day of control/stress treatment. Previous research illustrates that stress can dysregulate the estrous cycle [65]. When analyzing the vaginal cytology of repeated control/stress rats, we observed a subset of rats would become “stuck” in a particular stage, with 48% stuck in estrus and 52% in non-estrus (**Supplemental Fig. 3B**). Interestingly, 62.1% of repeated stress rats became stuck while only 11.1% of repeated control rats became stuck (**Supplemental Fig. 3C**). Further, once a rat became stuck in a particular stage, it had an 80% chance of remaining in that stage until the stress/control treatment ended (data not shown). As noted above, greater than two thirds of rats were in the

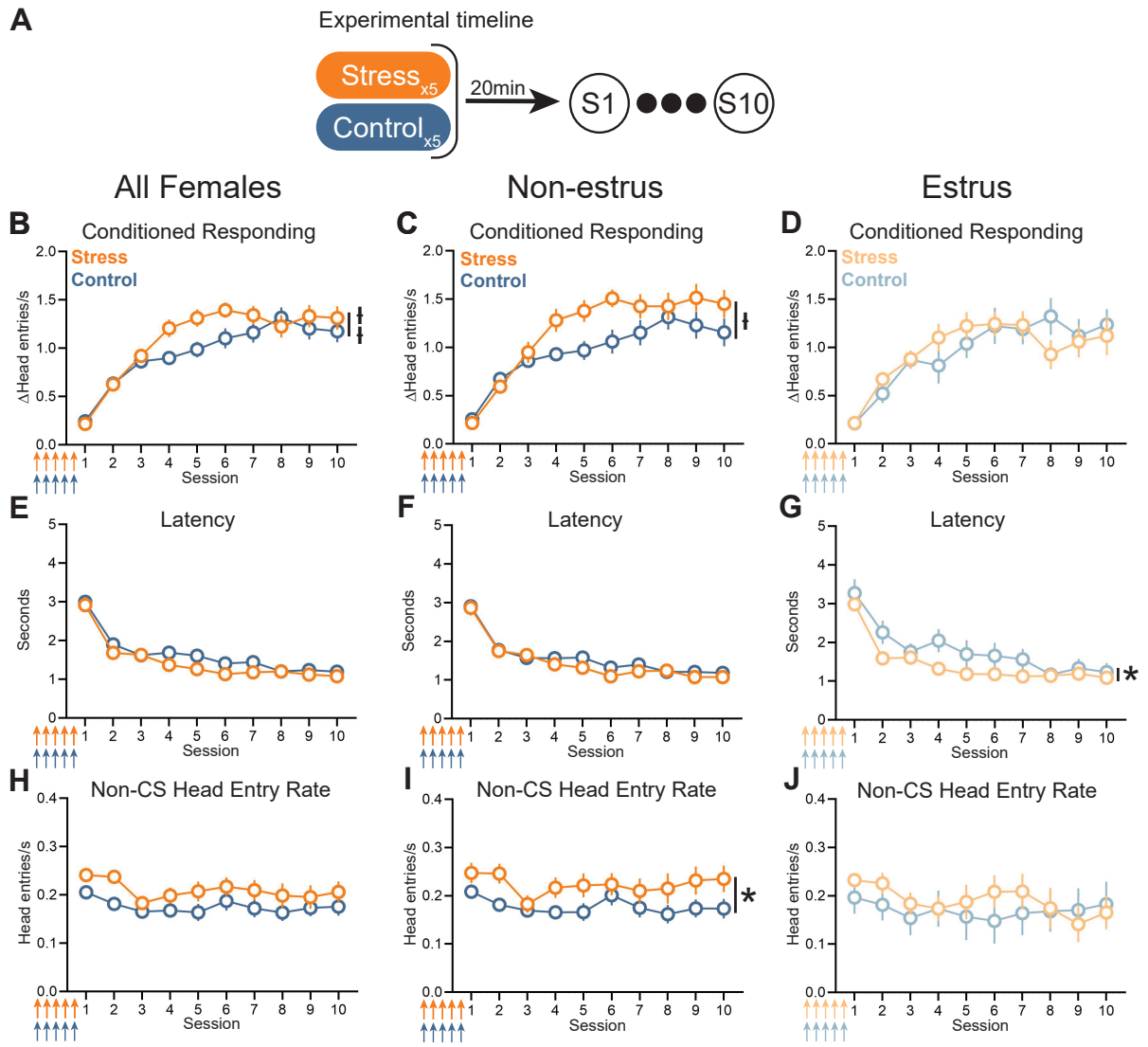

Figure 3: Repeated stress relative to repeated control treatment enhanced conditioned responding only in non-estrus rats. (A) Experimental timeline. (B) Effect of repeated stress on conditioned responding when examining female rats across cycle stages (interaction of session x stress: $F_{(9,464)} = 2.8$, ǂ $p = 0.005$; repeated stress: n = 29; repeated control: n = 27). (C) Repeated stress enhanced conditioned responding compared to repeated control treatment in non-estrus rats (interaction of session x stress: $F_{(9,302)} = 2.5$, ǂ $p = 0.01$; non-estrus repeated stress: n = 15; non-estrus repeated control: n = 20). (D) No effect of repeated stress relative to repeated control treatment on conditioned responding in estrus rats (estrus repeated stress: n = 12; estrus repeated control: n = 7). (E-G) Effect of repeated stress relative to repeated control treatment on response latency across all females (E), rats in non-estrus (F), and rats in estrus (G, main effect of stress: $F_{(1,17)} = 4.7$, * $p = 0.04$). (H-J) Effect of repeated stress relative to repeated control treatment on the non-CS head entry rate across all females (H), rats in non-estrus (I, main effect of stress: $F_{(1,35)} = 4.3$, * $p = 0.04$), and rats in estrus (J).

same estrous category on the first and last day of stress/control administration. As such, the behavioral findings are similar whether data is analyzed by estrous stage during the first or fifth day of stress/control treatment (**Supplemental Fig. 4**).

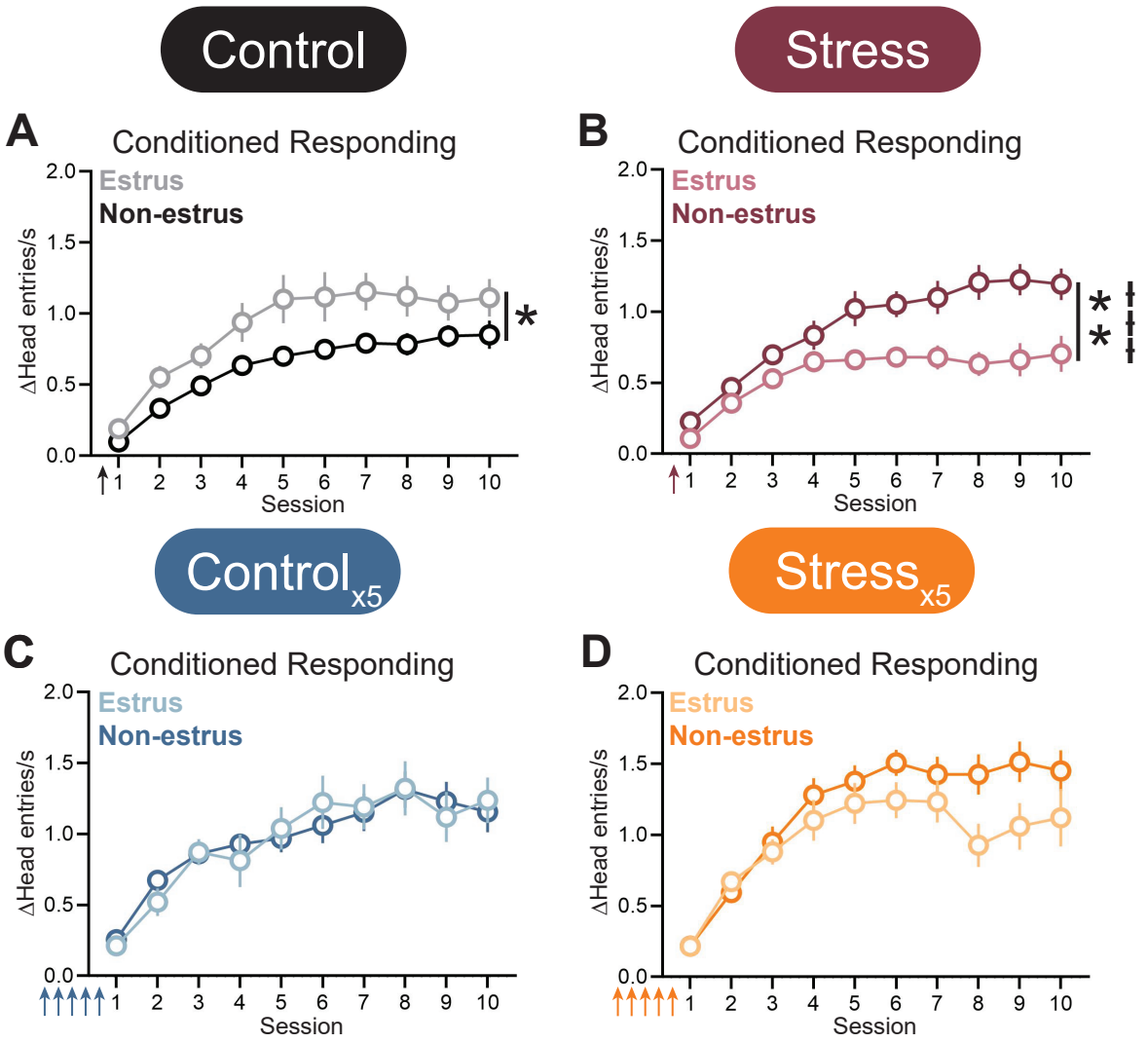

Figure 4: Effect of estrous stage on conditioned responding within manipulation groups. (A) In animals undergoing a single control treatment, estrus rats exhibited a higher level of conditioned responding relative to non-estrus rats (main effect of stage: $F_{(1,35)} = 6.9$, * $p = 0.01$). (B) In animals undergoing a single stress treatment, non-estrus rats exhibited a higher level of conditioned responding relative to estrus rats (main effect of stage: $F_{(1,32)} = 9.2$, ** $p = 0.005$; interaction of session x stage: $F_{(9,256)} = 4.0$, ǂǂ $p < 0.0001$). (C-D) No effect of estrous stage on conditioned responding in repeated control (C) or repeated stress rats (D).

Repeated control/stress treatment abolishes estrous-dependent effects of stress

To further investigate the effect of the estrous cycle on cue-reward associations, we re-examined our data by comparing rats in estrus and non-estrus stages within each manipulation - single control, single stress, repeated control, and repeated stress (**Fig. 4**; **Supplemental Fig. 5**). In animals undergoing a single control treatment, estrus rats exhibited a higher level of conditioned responding relative to non-estrus rats, consistent with others (**Fig. 4A**) [41,42]. However, in animals underling a single stress treatment, non-estrus rats display greater

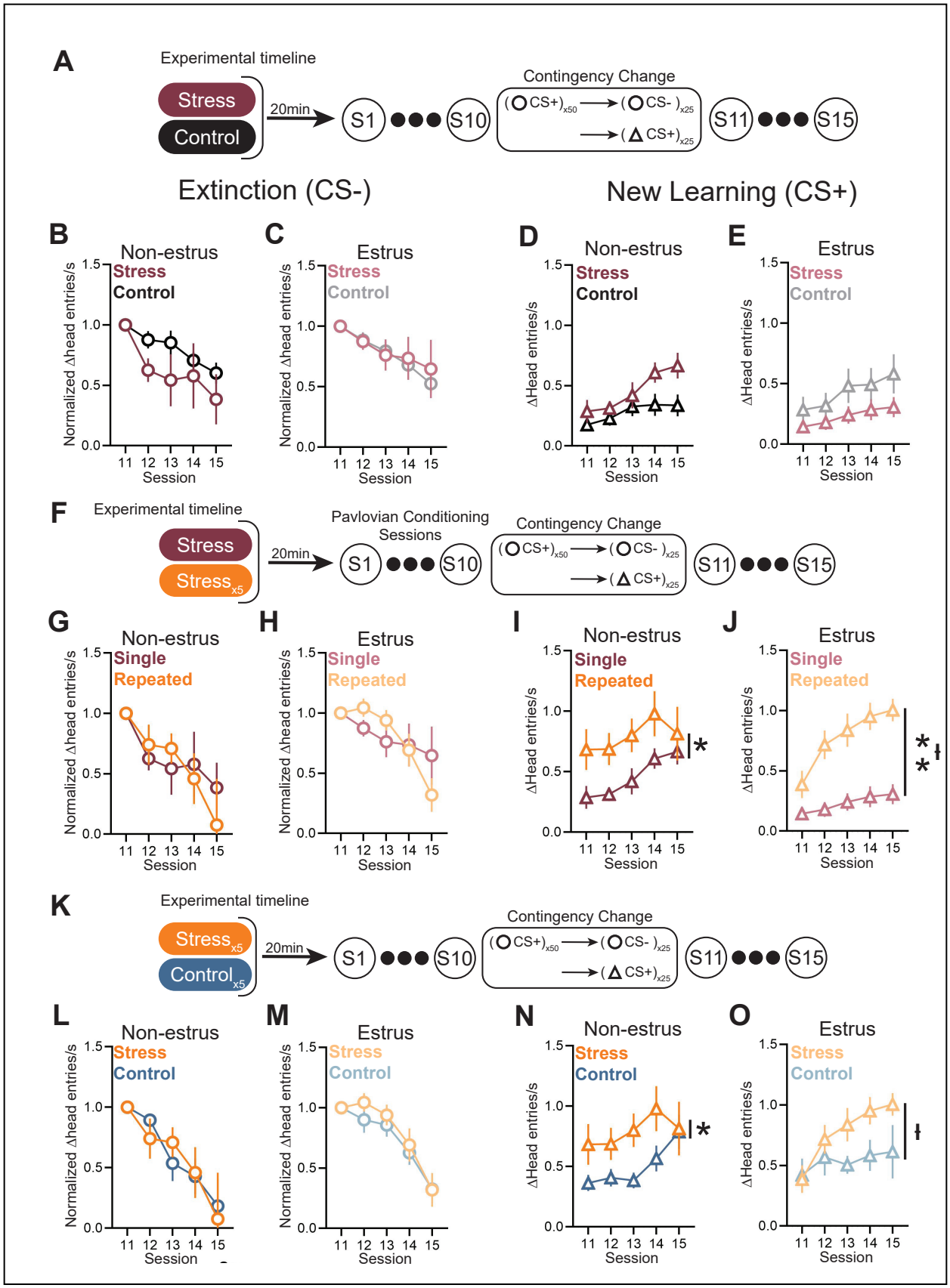
A
Experimental timeline
Stress
Control
20min
S1
S10
Contingency Change
(CS+)x50
(CS-)x25
(CS+)x25
S11
S15
Extinction (CS-)
New Learning (CS+)
B
Non-estrus
Stress
Control
Normalized Δhead entries/s
Session
C
Estrus
Stress
Control
D
Non-estrus
Stress
Control
ΔHead entries/s
E
Estrus
Stress
Control
F
Experimental timeline
Stress
Stressx5
Pavlovian Conditioning Sessions
G
Non-estrus
Single
Repeated
H
Estrus
Single
Repeated
I
Non-estrus
Single
Repeated
J
Estrus
Single
Repeated
K
Experimental timeline
Stressx5
Controlx5
L
Non-estrus
Stress
Control
M
Estrus
Stress
Control
N
Non-estrus
Stress
Control
O
Estrus
Stress
Control

Figure 5: Temporally distal repeated stress facilitates new reward learning without affecting extinction. (A) Experimental timeline. (B-C) A single stress treatment (non-estrus: n = 6; estrus: n = 5) did not affect extinction relative to control treated rats (non-estrus: n = 8; estrus: n = 6). (D-E) A single stress treatment did not affect conditioned responding to a novel CS+ relative to control treated rats. (F) Experimental timeline. (G-H) Repeated stress treatment (non-estrus: n = 6; estrus: n = 10) did not affect extinction relative to rats receiving a single stress treatment . (I-J) Repeated stress enhanced conditioned responding to a new CS+ relative to single stress in both non-estrus rats (I, main effect of repeated stress: $F_{(1,10)} = 5.6$, $p = 0.04$) and estrus rats (J, main effect of repeated stress: $F_{(1,13)} = 11.9$, ** $p = 0.004$; interaction of session x repeated stress: $F_{(4,50)} = 3.1$, ł $p = 0.02$). (K) Experimental timeline. (L-M) Repeated stress treatment did not affect extinction relative to the repeated control treatment. (N-O) Repeated stress enhanced conditioned responding to a new CS+ relative to the repeated control treatment in both non-estrus rats (L, main effect of repeated stress: $F_{(1,14)} = 5.7$, * $p = 0.03$) and estrus rats (interaction of session x repeated stress: $F_{(4,58)} = 2.7$, ł $p = 0.04$).

conditioned responding relative to estrus rats (**Fig**. **4B**). There is no effect of estrous stage on latency and non-CS head entry rate in single stress/control treatment rats, demonstrating that the estrous cycle selectively impacts cue-driven behavior (**Supplemental Fig**. **5**). In contrast to the rats that underwent a single stress/control treatment, those that received repeated stress/ control exhibited no difference in conditioned responding between estrus and non-estrus groups (**Fig**. **4 C-D; Supplemental Fig. 5**). Our findings demonstrate that the estrous cycle gates the effect of a single stress experience on conditioned responding, but this estrous-dependent effect is absent in rats that had received multiple prior exposures to stressful stimuli.

Temporally distal repeated stress facilitates new reward learning without affecting extinction.

Stress impairs extinction in fear conditioning tasks [8,11,17,19-21,27]. This suggests that prior stress exposure can have long-lasting impacts on flexible behavior temporally distant from when the stress was experienced. Here, we examined if stress experience prior to the first Pavlovian conditioning session would impact extinction as well as the ability to learn a new cue-reward association. After the initial 10 Pavlovian conditioning sessions in which the rats were trained to associate a white noise with a food reward, the rats underwent a cue-reward contingency change. In the next five sessions, the previously rewarded white noise had no consequence (CS-) and a novel tone resulted in food delivery (CS+) (**Fig**. **5A,F,K**). We found no significant effect of stress on extinction (**Fig. 5; Supplemental Figs. 6-7**). However, in rats that were in a non-estrus stage on the first day of Pavlovian conditioning, repeated stress enhanced conditioned responding to a new CS+ relative to both a single stress (**Fig**. **5I**) and repeated control treatments (**Fig. 5N**). Similarly, repeated stress rats in estrus exhibited enhanced conditioned responding to a new CS+ relative to single stress estrus rats (**Fig**. **5J)**. Even though the rats exhibited a persistent effect of manipulation on new learning, this did not translate to alterations in anxiety-like behavior or general activity (**Supplemental Fig. 8**). Collectively, our results illustrate that repeated stress enhances conditioned responding (**Fig. 2**) and subsequently facilitates the ability to acquire a new cue-reward association (**Fig. 5**).

Discussion

Prior research demonstrates that a single stress exposure enhances conditioned responding to a reward-paired cue in male rats [13]. Here, we found that a single stress experience did not affect conditioned responding when examining female rats across all estrous cycle stages. However, this initial analysis obscured the opposing behavioral effect of stress on estrus and non-estrus rats. Specifically, female rats that began Pavlovian conditioning during non-estrus stages exhibited a stress-mediated *enhancement* in conditioned responding. In contrast, females rats that began Pavlovian conditioning during estrus exhibited a stress-mediated *suppression* in conditioned responding. Prior research performed in stress-naïve animals found higher levels of conditioned responding in estrus rats [42], which mirrors our findings from rats that underwent a single control treatment. Therefore, the effect of a single stress experience functionally inverts how the estrous cycle stage impacts cue-driven behavior in reward-based tasks.

Repeated prior stress facilitates learning in fear/threat conditioning tasks [26]. Consistent with these findings, we found that repeated prior stress enhanced conditioned responding toward reward-predictive cues in both estrus and non-estrus rats when compared to rats receiving a single stress exposure. Furthermore, there was no difference in cue-driven behavior when comparing estrus and non-estrus rats that underwent the repeated stress treatment. These findings indicate that repeated stress experience blunts the influence of the estrous cycle on the behavioral effects of stress. We argue these results could explain why some human studies have not reported that cycle stage interacts with stress to modulate cue-based actions. A challenge with human research relative to preclinical studies is that it can be difficult to control for prior stress experience. Additionally, women and girls, compared to men and boys, consistently report higher daily stress levels, are more likely to experience stress, and are more likely to perceive an event as stressful [66-76]. Thus, human studies examining those with a menstrual cycle will likely include subjects with prior stress experience. Indeed, human studies that monitor the menstrual cycle often do not report an effect of cycle stage on stress-induced changes in cued associations [30,32,40,77-80]. Taken together, these findings highlight the importance of understanding when behavior is, and is not, gated by the estrous and menstrual cycles.

Based on the findings from stress-enhanced fear/threat learning models, one might expect that prior stress will alter behavioral responding during extinction [11,12,20]. In support, administering a cold pressor test prior to training on a reward-based task produced a transient deficit in performance during early extinction in humans [34]. However, neither a single nor repeated stress (compared to relevant control procedures) impacted extinction to the previously

rewarded cue. Therefore, the impact of prior stress on extinction may depend on task-specific parameters such as the stressor used and whether the cue predicted an aversive or rewarding outcome. While stress did not affect extinction in our task, we instead found that prior repeated stress treatment enhanced conditioned responding (1) to the initial cue-reward pairing and (2) to a novel cue-reward pairing that was temporally distal to the stress exposure. This suggests that repeated prior stress experience coupled with reward learning primes rats to rapidly acquire a new cue-outcome association.

While the data presented here provides valuable insight into the effects of stress and the estrous cycle on cue-reward associations, future studies are needed to identify the neural systems responsible for these behavioral outcomes. The mesolimbic dopamine system is well-positioned to mediate the interaction between stress, learning, and the estrous cycle. Dopamine release in the ventral striatum is necessary for Pavlovian conditioning to occur and dopamine is released in the ventral striatum as a result of restraint stress [81-84]. Furthermore, dopamine neuron activity fluctuates throughout the estrous cycle [41,61,85-88]. Additionally, we previously found that dopamine signaling in the ventral lateral striatum is necessary for stress-enhanced conditioned responding to a reward-paired cue in male rats [13]. Thus, dopamine signaling in the ventral striatum is well positioned to be the mechanism by which a single stress enacts estrous-dependent changes in reward conditioning in female rats as well.

In conclusion, we found that a single stress elicited divergent effects on reward learning in an estrous-dependent manner. However, repeated stress enhanced conditioned responding, compared to a single stress, in an estrous-independent manner. Alterations in reward processing is a common symptom in many stress-related disorders, which disproportionately affect women [72,89-95]. As such, increasing research is needed to examine the impact of stress on cue-outcome associations in female subjects. The data presented here highlights the need for continued inclusion of female subjects and increased attention to the role of cycling hormones in pre-clinical studies investigating the effect of stress on behavioral outcomes.

References:

1 Oyola MG, Handa RJ. Hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes: sex differences in regulation of stress responsivity. Stress. 2017;20(5):476-94.

2 Sze Y, Brunton PJ. Sex, stress and steroids. Eur J Neurosci. 2020;52(1):2487-515.

3 Leistner C, Menke A. Hypothalamic-pituitary-adrenal axis and stress. Handb Clin Neurol. 2020;175:55-64.

4 Shors TJ. Acute stress rapidly and persistently enhances memory formation in the male rat. Neurobiol Learn Mem. 2001;75(1):10-29.

5 Shors TJ, Weiss C, Thompson RF. Stress-induced facilitation of classical conditioning. Science. 1992;257(5069):537-9.

6 Wilson LM, Wilson JR, Dicara LV. Facilitation of Pavlovian conditioned cardiodecelerations following preshock in immobilized rats. Physiol Behav. 1975;15(6):653-8.

7 Rau V, Fanselow MS. Exposure to a stressor produces a long lasting enhancement of fear learning in rats. Stress. 2009;12(2):125-33.

8 Binette AN, Liu J, Bayer H, Crayton KL, Melissari L, Sweck SO, et al. Parvalbumin-Positive Interneurons in the Medial Prefrontal Cortex Regulate Stress-Induced Fear Extinction Impairments in Male and Female Rats. J Neurosci. 2023;43(22):4162-73.

9 Chakraborty P, Chattarji S. Timing is everything: differential effects of chronic stress on fear extinction. Psychopharmacology (Berl). 2019;236(1):73-86.

10 Zhang W, Rosenkranz JA. Repeated restraint stress enhances cue-elicited conditioned freezing and impairs acquisition of extinction in an age-dependent manner. Behav Brain Res. 2013;248:12-24.

11 Hoffman AN, Lorson NG, Sanabria F, Foster Olive M, Conrad CD. Chronic stress disrupts fear extinction and enhances amygdala and hippocampal Fos expression in an animal model of post-traumatic stress disorder. Neurobiol Learn Mem. 2014;112:139-47.

12 Long VA, Fanselow MS. Stress-enhanced fear learning in rats is resistant to the effects of immediate massed extinction. Stress. 2012;15(6):627-36.

13 Stelly CE, Tritley SC, Rafati Y, Wanat MJ. Acute Stress Enhances Associative Learning via Dopamine Signaling in the Ventral Lateral Striatum. J Neurosci. 2020;40(22):4391-400.

14 Peyrot C, Provencher J, Duplessis Marcotte F, Cernik R, Marin MF. Using unconditioned responses to predict fear acquisition, fear extinction learning, and extinction retention patterns: Sex hormone status matters. Behav Brain Res. 2024;459:114802.

15 Hoffman AN, Armstrong CE, Hanna JJ, Conrad CD. Chronic stress, cyclic 17beta-estradiol, and daily handling influences on fear conditioning in the female rat. Neurobiol Learn Mem. 2010;94(3):422-33.

16 Mirshekar M, Abrari K, Goudarzi I, Rashidy-Pour A. Systemic administrations of beta-estradiol alleviate both conditioned and sensitized fear responses in an ovariectomized rat model of post-traumatic stress disorder. Neurobiol Learn Mem. 2013;102:12-9.

17 Shafia S, Nikkhah F, Akhoundzadeh K. Effect of combination fluoxetine and exercise on prefrontal BDNF, anxiety-like behavior and fear extinction in a female rat model of post-traumatic stress disorder (PTSD): a comparison with male animals. Behav Brain Funct. 2023;19(1):1.

18 Shanazz K, Dixon-Melvin R, Nalloor R, Thumar R, Vazdarjanova AI. Sex Differences In Avoidance Extinction After Contextual Fear Conditioning: Anxioescapic Behavior In Female Rats. Neuroscience. 2022;497:146-56.

19 Torres-Rodriguez O, Ortiz-Nazario E, Rivera-Escobales Y, Velazquez B, Colon M, Porter JT. Sex-dependent effects of microglial reduction on impaired fear extinction induced by single prolonged stress. Front Behav Neurosci. 2022;16:1014767.

20 Hassien AM, Shue F, Bernier BE, Drew MR. A mouse model of stress-enhanced fear learning demonstrates extinction-sensitive and extinction-resistant effects of footshock stress. Behav Brain Res. 2020;379:112391.

21 Sanders MJ, Stevens S, Boeh H. Stress enhancement of fear learning in mice is dependent upon stressor type: Effects of sex and ovarian hormones. Neurobiol Learn Mem. 2010;94(2):254-62.

22 Daws SE, Joseph NF, Jamieson S, King ML, Chevere-Torres I, Fuentes I, et al. Susceptibility and Resilience to Posttraumatic Stress Disorder-like Behaviors in Inbred Mice. Biol Psychiatry. 2017;82(12):924-33.

23 Przybyl KJ, Jenz ST, Lim PH, Ji MT, Wert SL, Luo W, et al. Genetic stress-reactivity, sex, and conditioning intensity affect stress-enhanced fear learning. Neurobiol Learn Mem. 2021;185:107523.

24 Brooks JB, Robinson PK, Warner S, Halder P, Trask S. Stress-enhanced fear learning can be reduced with unconditional stimulus deflation with constraints. Behav Brain Res. 2025;481:115438.

25 Adkins JM, Halcomb CJ, Rogers D, Jasnow AM. Stress and sex-dependent effects on conditioned inhibition of fear. Learn Mem. 2022;29(9):246-55.

26 Conoscenti MA, Weatherill DB, Huang Y, Tordjman R, Fanselow MS. Isolation of the differential effects of chronic and acute stress in a manner that is not confounded by stress severity. Neurobiol Stress. 2024;30:100616.

27 Baran SE, Armstrong CE, Niren DC, Hanna JJ, Conrad CD. Chronic stress and sex differences on the recall of fear conditioning and extinction. Neurobiol Learn Mem. 2009;91(3):323-32.

28 Cavanagh JF, Frank MJ, Allen JJ. Social stress reactivity alters reward and punishment learning. Soc Cogn Affect Neurosci. 2011;6(3):311-20.

29 Yang X, Nackley B, Friedman BH. Comparison between the Effects of Acute Physical and Psychosocial Stress on Feedback-Based Learning. Brain Sci. 2023;13(8).

30 Lighthall NR, Gorlick MA, Schoeke A, Frank MJ, Mather M. Stress modulates reinforcement learning in younger and older adults. Psychol Aging. 2013;28(1):35-46.

31 Petzold A, Plessow F, Goschke T, Kirschbaum C. Stress reduces use of negative feedback in a feedback-based learning task. Behav Neurosci. 2010;124(2):248-55.

32 Boyle CC, Stanton AL, Eisenberger NI, Seeman TE, Bower JE. Effects of stress-induced inflammation on reward processing in healthy young women. Brain Behav Immun. 2020;83:126-34.

33 Schwabe L, Wolf OT. Socially evaluated cold pressor stress after instrumental learning favors habits over goal-directed action. Psychoneuroendocrinology. 2010;35(7):977-86.

34 Schwabe L, Wolf OT. Stress prompts habit behavior in humans. J Neurosci. 2009;29(22):7191-8.

35 Ossewaarde L, Qin S, Van Marle HJ, van Wingen GA, Fernandez G, Hermans EJ. Stress-induced reduction in reward-related prefrontal cortex function. Neuroimage. 2011;55(1):345-52.

36 Cunningham S, Mazurka R, Wynne-Edwards KE, Milev RV, Pizzagalli DA, Kennedy S, et al. Cortisol reactivity to stress predicts behavioral responsivity to reward moderation by sex, depression, and anhedonia. J Affect Disord. 2021;293:1-8.

37 Zhang Y, Chen F, Yu M, Li Y, Chen S, Choudhary MI, et al. Sex differences in reward-based operant conditioning performance and neurotransmitter changes following chronic sleep restriction stress in rats. Behav Brain Funct. 2025;21(1):3.

38 Dougherty R, Thrailkill EA, Mohammed Z, VonDoepp S, Hilton-Vanosdall E, Charette S, et al. Acute stress facilitates habitual behavior in female rats. Physiol Behav. 2024;275:114456.

39 Derman RC, Lattal KM. Persistent effects of acute trauma on Pavlovian-to-instrumental transfer. Front Behav Neurosci. 2022;16:1028262.

40 Banis S, Lorist MM. The combined effects of menstrual cycle phase and acute stress on reward-related processing. Biol Psychol. 2017;125:130-45.

41 Calipari ES, Juarez B, Morel C, Walker DM, Cahill ME, Ribeiro E, et al. Dopaminergic dynamics underlying sex-specific cocaine reward. Nature communications. 2017;8:13877.

42 Johnson AR, Thibeault KC, Lopez AJ, Peck EG, Sands LP, Sanders CM, et al. Cues play a critical role in estrous cycle-dependent enhancement of cocaine reinforcement. Neuropsychopharmacology. 2019;44(7):1189-97.

43 Hilz EN, Agee LA, Jun D, Monfils MH, Lee HJ. Estrous cycle state-dependent renewal of appetitive behavior recruits unique patterns of Arc mRNA in female rats. Front Behav Neurosci. 2023;17:1210631.

44 Hilz EN, Lee HJ. Estradiol and progesterone in female reward-learning, addiction, and therapeutic interventions. Front Neuroendocrinol. 2023;68:101043.

45 Yoest KE, Cummings JA, Becker JB. Estradiol, dopamine and motivation. Cent Nerv Syst Agents Med Chem. 2014;14(2):83-9.
46 Corbett CM, Bozarth SL, West EA. Effects of sex and estrous cycle on action-outcome contingencies. Behav Brain Res. 2025;477:115317.
47 Hilz EN, Smith RW, Hong YJ, Monfils MH, Lee HJ. Mapping the estrous cycle to context-specific extinction memory. Behav Neurosci. 2019;133(6):614-23.
48 Ajayi AF, Akhigbe RE. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. Fertil Res Pract. 2020;6:5.
49 Wanat MJ, Bonci A, Phillips PE. CRF acts in the midbrain to attenuate accumbens dopamine release to rewards but not their predictors. Nat Neurosci. 2013;16(4):383-5.
50 Bruchey AK, Jones CE, Monfils MH. Fear conditioning by-proxy: social transmission of fear during memory retrieval. Behav Brain Res. 2010;214(1):80-4.
51 Kim EJ, Kim ES, Covey E, Kim JJ. Social transmission of fear in rats: the role of 22-kHz ultrasonic distress vocalization. PLoS One. 2010;5(12):e15077.
52 Carnevali L, Montano N, Tobaldini E, Thayer JF, Sgoifo A. The contagion of social defeat stress: Insights from rodent studies. Neurosci Biobehav Rev. 2020;111:12-18.
53 Toyoshima M, Okuda E, Hasegawa N, Kaseda K, Yamada K. Socially Transferred Stress Experience Modulates Social Affective Behaviors in Rats. Neuroscience. 2022;502:68-76.
54 Gomez-Sotres P, Skupio U, Dalla Tor T, Julio-Kalajzic F, Cannich A, Gisquet D, et al. Olfactory bulb astrocytes link social transmission of stress to cognitive adaptation in male mice. Nat Commun. 2024;15(1):7103.
55 Fonzi KM, Lefner MJ, Phillips PEM, Wanat MJ. Dopamine Encodes Retrospective Temporal Information in a Context-Independent Manner. Cell reports. 2017;20(8):1765-74.
56 Lefner MJ, Dejeux MI, Wanat MJ. Sex Differences in Behavioral Responding and Dopamine Release during Pavlovian Learning. eNeuro. 2022;9(2).
57 Lefner MJ, Stelly CE, Fonzi KM, Zurita H, Wanat MJ. Critical periods when dopamine controls behavioral responding during Pavlovian learning. Psychopharmacology (Berl). 2022;239(9):2985-96.
58 Stelly CE, Girven KS, Lefner MJ, Fonzi KM, Wanat MJ. Dopamine release and its control over early Pavlovian learning differs between the NAc core and medial NAc shell. Neuropsychopharmacology. 2021;46(10):1780-87.
59 Cora MC, Kooistra L, Travlos G. Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. Toxicol Pathol. 2015;43(6):776-93.

60 Becker JB, Ramirez VD. Sex differences in the amphetamine stimulated release of catecholamines from rat striatal tissue in vitro. Brain Res. 1981;204(2):361-72.

61 Dazzi L, Seu E, Cherchi G, Barbieri PP, Matzeu A, Biggio G. Estrous cycle-dependent changes in basal and ethanol-induced activity of cortical dopaminergic neurons in the rat. Neuropsychopharmacology. 2007;32(4):892-901.

62 Roberts DC, Bennett SA, Vickers GJ. The estrous cycle affects cocaine self-administration on a progressive ratio schedule in rats. Psychopharmacology (Berl). 1989;98(3):408-11.

63 Morissette M, Di Paolo T. Sex and estrous cycle variations of rat striatal dopamine uptake sites. Neuroendocrinology. 1993;58(1):16-22.

64 Blair RS, Acca GM, Tsao B, Stevens N, Maren S, Nagaya N. Estrous cycle contributes to state-dependent contextual fear in female rats. Psychoneuroendocrinology. 2022;141:105776.

65 Poitras M, Lebeau M, Plamondon H. The cycle of stress: A systematic review of the impact of chronic psychological stress models on the rodent estrous cycle. Neurosci Biobehav Rev. 2024;162:105730.

66 Association AP. in *Stress in America* (APA.org, 2023).

67 Medaris A. Women say they're stressed, misunderstood, and alone. 11/1/2023. https://www.apa.org/topics/stress/women-stress.

68 Matud MP. Gender differences in stress and coping styles. Personality and Individual Differences. 2004;37(7):1401-15.

69 Feingold A. Gender differences in personality: a meta-analysis. Psychol Bull. 1994;116(3):429-56.

70 Fischer AH, Rodriguez Mosquera PM, van Vianen AE, Manstead AS. Gender and culture differences in emotion. Emotion. 2004;4(1):87-94.

71 Ollendick TH, Yang B, Dong Q, Xia Y, Lin L. Perceptions of fear in other children and adolescents: the role of gender and friendship status. J Abnorm Child Psychol. 1995;23(4):439-52.

72 McLean CP, Asnaani A, Litz BT, Hofmann SG. Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. J Psychiatr Res. 2011;45(8):1027-35.

73 Kelly MM, Tyrka AR, Anderson GM, Price LH, Carpenter LL. Sex differences in emotional and physiological responses to the Trier Social Stress Test. J Behav Ther Exp Psychiatry. 2008;39(1):87-98.

74 Sandanger I, Nygard JF, Sorensen T, Moum T. Is women's mental health more susceptible than men's to the influence of surrounding stress? Soc Psychiatry Psychiatr Epidemiol. 2004;39(3):177-84.

75 Altemus M, Sarvaiya N, Neill Epperson C. Sex differences in anxiety and depression clinical perspectives. Front Neuroendocrinol. 2014;35(3):320-30.

76 Smith KB, Murack M, Ismail N. The sex-dependent and enduring impact of pubertal stress on health and disease. Brain Res Bull. 2023;200:110701.

77 Treadway MT, Admon R, Arulpragasam AR, Mehta M, Douglas S, Vitaliano G, et al. Association Between Interleukin-6 and Striatal Prediction-Error Signals Following Acute Stress in Healthy Female Participants. Biol Psychiatry. 2017;82(8):570-77.

78 Seo D, Jia Z, Lacadie CM, Tsou KA, Bergquist K, Sinha R. Sex differences in neural responses to stress and alcohol context cues. Hum Brain Mapp. 2011;32(11):1998-2013.

79 Nillni YI, Rasmusson AM, Paul EL, Pineles SL. The Impact of the Menstrual Cycle and Underlying Hormones in Anxiety and PTSD: What Do We Know and Where Do We Go From Here? Curr Psychiatry Rep. 2021;23(2):8.

80 Potenza MN, Hong KI, Lacadie CM, Fulbright RK, Tuit KL, Sinha R. Neural correlates of stress-induced and cue-induced drug craving: influences of sex and cocaine dependence. Am J Psychiatry. 2012;169(4):406-14.

81 Tsai HC, Zhang F, Adamantidis A, Stuber GD, Bonci A, de Lecea L, et al. Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning. Science. 2009;324(5930):1080-4.

82 Darvas M, Wunsch AM, Gibbs JT, Palmiter RD. Dopamine dependency for acquisition and performance of Pavlovian conditioned response. Proc Natl Acad Sci U S A. 2014;111(7):2764-9.

83 Saunders BT, Richard JM, Margolis EB, Janak PH. Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. Nat Neurosci. 2018;21(8):1072-83.

84 Anstrom KK, Miczek KA, Budygin EA. Increased phasic dopamine signaling in the mesolimbic pathway during social defeat in rats. Neuroscience. 2009;161(1):3-12.

85 Perez SM, Chen L, Lodge DJ. Alterations in dopamine system function across the estrous cycle of the MAM rodent model of schizophrenia. Psychoneuroendocrinology. 2014;47:88-97.

86 Becker JB. Gender differences in dopaminergic function in striatum and nucleus accumbens. Pharmacol Biochem Behav. 1999;64(4):803-12.

87 Shanley MR, Miura Y, Guevara CA, Onoichenco A, Kore R, Ustundag E, et al. Estrous Cycle Mediates Midbrain Neuron Excitability Altering Social Behavior upon Stress. J Neurosci. 2023;43(5):736-48.

88 Zhang D, Yang S, Yang C, Jin G, Zhen X. Estrogen regulates responses of dopamine neurons in the ventral tegmental area to cocaine. Psychopharmacology (Berl). 2008;199(4):625-35.

89 Whitton AE, Treadway MT, Pizzagalli DA. Reward processing dysfunction in major depression, bipolar disorder and schizophrenia. Current opinion in psychiatry. 2015;28(1):7-12.

90 Rupprechter S, Stankevicius A, Huys QJM, Steele JD, Series P. Major Depression Impairs the Use of Reward Values for Decision-Making. Sci Rep. 2018;8(1):13798.

91 Pizzagalli DA, Iosifescu D, Hallett LA, Ratner KG, Fava M. Reduced hedonic capacity in major depressive disorder: evidence from a probabilistic reward task. J Psychiatr Res. 2008;43(1):76-87.

92 Mukherjee D, Lee S, Kazinka R, T DS, Kable JW. Multiple Facets of Value-Based Decision Making in Major Depressive Disorder. Sci Rep. 2020;10(1):3415.

93 Halahakoon DC, Kieslich K, O'Driscoll C, Nair A, Lewis G, Roiser JP. Reward-Processing Behavior in Depressed Participants Relative to Healthy Volunteers: A Systematic Review and Meta-analysis. JAMA Psychiatry. 2020;77(12):1286-95.

94 Goschke T. Dysfunctions of decision-making and cognitive control as transdiagnostic mechanisms of mental disorders: advances, gaps, and needs in current research. Int J Methods Psychiatr Res. 2014;23 Suppl 1(Suppl 1):41-57.

95 Caceda R, Nemeroff CB, Harvey PD. Toward an understanding of decision making in severe mental illness. J Neuropsychiatry Clin Neurosci. 2014;26(3):196-213.