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Archival Report

Incubation of Cocaine Craving After Intermittent-Access Self-administration: Sex Differences and Estrous Cycle

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ABSTRACT

BACKGROUND: Studies using continuous-access drug self-administration showed that cocaine seeking increases during abstinence (incubation of cocaine craving). Recently, studies using intermittent-access self-administration showed increased motivation to self-administer and seek cocaine. We examined whether intermittent cocaine self-administration would potentiate incubation of craving in male and female rats and examined the estrous cycle's role in this incubation.

METHODS: In experiment 1, male and female rats self-administered cocaine either continuously (8 hours/day) or intermittently (5 minutes ON, 25 minutes OFF × 16) for 12 days, followed by relapse tests after 2 or 29 days. In experiments 2 and 3, female rats self-administered cocaine intermittently for six, 12, or 18 sessions. In experiment 4, female rats self-administered cocaine continuously followed by relapse tests after 2 or 29 days. In experiments 3 and 4, the estrous cycle was measured using a vaginal smear test.

RESULTS: Incubation of cocaine craving was observed in both sexes after either intermittent or continuous drug self-administration. Independent of access condition and abstinence day, cocaine seeking was higher in female rats than in male rats. In both sexes, cocaine seeking on both abstinence days was higher after intermittent drug access than after continuous drug access. In female rats, incubation of craving after either intermittent or continuous drug access was significantly higher during estrus than during non-estrus; for intermittent drug access, this effect was independent of the training duration.

CONCLUSIONS: In both sexes, intermittent cocaine access caused time-independent increases in drug seeking during abstinence. In female rats, the time-dependent increase in drug seeking (incubation) is critically dependent on the estrous cycle phase.

Keywords: Cocaine self-administration, Estrous cycle, Incubation of craving, Intermittent access, Relapse, Sex differences

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Drug addiction is characterized by high relapse rates during abstinence (1,2). In humans, relapse can be precipitated by exposure to cues associated with drug use (3,4). Gawin and Kleber (5) proposed that cue-induced cocaine craving progressively increases during abstinence. In rats, a similar phenomenon called "incubation of craving" was observed after forced abstinence from cocaine self-administration (6,7) and other addictive drugs (8–10). Incubation of drug craving during the 6 months of abstinence was recently demonstrated in human cocaine users (11).

In previous incubation studies, cocaine was continuously available for the duration of the daily sessions (9,12,13). However, human cocaine users self-administer cocaine intermittently, with large drug doses separated by long intervals between intoxicating events (14), a drug-taking practice that induces spikes of cocaine brain concentrations. Based on these clinical observations, Zimmer *et al.* (15) developed an intermittent-access cocaine self-administration procedure in rats that causes binge-like self-administration behavior and spiking brain cocaine levels (15). In this procedure, rats have 5 minutes of cocaine access followed by 25 minutes of timeout during daily sessions (typically 6 hours/day) (15–22). Several studies have shown that rats trained under the intermittent-access procedure showed strong motivation to take and seek cocaine, as assessed in progressive-ratio, resistance-to-punishment, economic-demand, and extinction-reinstatement procedures (15,18–20,22–26). Most recently, James *et al.* (27) showed that in a direct comparison between intermittent and continuous cocaine access, the motivation to take and seek cocaine in these procedures is higher in the intermittent-access condition. These authors also showed that cue-induced reinstatement after short or prolonged home-cage

SEE COMMENTARY ON PAGE e53

forced abstinence was higher in the intermittent-access condition; however, the relevance of these data to incubation of cocaine craving is unknown because there was no evidence for time-dependent increases in cue-induced reinstatement after extinction in either access condition. Thus, it is unknown whether intermittent-access cocaine self-administration would potentiate incubation of cocaine craving.

Another factor that can affect incubation of cocaine craving, and more generally relapse to cocaine seeking, is sex (28,29). Human correlational studies suggest that periods of cocaine abstinence are shorter in women (30–33) and that women report stronger craving to cues associated with cocaine than men (33–37). There is also evidence for sex differences in cocaine self-administration and relapse/reinstatement in preclinical studies. Female rats acquire cocaine self-administration at a faster rate than male rats (38,39), exhibit a higher cocaine-primed reinstatement response than male rats (40,41), and show stronger incubation of cocaine craving (29).

Sex differences in cocaine relapse may be influenced by the estrous cycle and ovarian hormones. In human studies, women have a decrease in desire (craving) to smoke cocaine during the luteal phase (42,43). The luteal phase is characterized by a high level of progesterone, which decreases cocaine's subjective effects (42,44), suggesting a protective effect of progesterone. Preclinical studies in rats report similar effects. Female rats in estrus show higher cocaine seeking after periods of abstinence (29,45), extinction (40), and cocaine-induced reinstatement (46,47) than female rats in non-estrus or male rats. Suppression of endogenous hormones (estrogen and progesterone) by ovariectomy decreases cocaine-induced reinstatement compared with sham rats, while chronic estradiol treatment in ovariectomized rats restores cocaine reinstatement to levels similar to those of sham rats (48,49). Moreover, the role of sex and ovarian hormones in cocaine's behavioral effects have been demonstrated using conditioned place preference (CPP) and locomotor sensitization procedures. Female rats show higher locomotor activity for acute and repeated cocaine administration than male rats (50–53) and exhibit a CPP at a low cocaine dose that does not cause CPP in male rats (54). In ovariectomized female rats, estradiol treatment increases the magnitude of cocaine CPP and locomotor sensitization (54–57). Finally, in choice self-administration procedures (cocaine vs. food), female rats are more likely to choose cocaine than male rats (6,58,59), and estradiol treatment increases cocaine choice in both gonadectomized male rats and female rats (60,61). Together, the data suggest that in both human and rodent models, the estrous cycle contributes to cocaine taking and seeking.

In experiment 1, we found that intermittent cocaine access caused stronger incubation of craving than continuous cocaine access, an effect that was more pronounced in female rats. However, in experiment 2, we did not observe the incubation effect in female rats trained under the intermittent-access condition, suggesting that we overlooked key factors regulating incubated cocaine craving in female rats. Therefore, in experiment 3, we investigated two possible factors: estrous cycle phase and training duration. We found that after intermittent-access cocaine self-administration, incubation of cocaine craving is critically dependent on the estrous cycle's phase but not on training duration. Therefore, in experiment 4, we determined the generality of this effect to female rats trained under the continuous cocaine access condition.

METHODS AND MATERIALS

For information on subjects, see the Supplement.

Experiment 1: Effect of Intermittent-Access Cocaine Self-administration on Incubation of Craving in Male and Female Rats

The goal of experiment 1 was to determine whether intermittent cocaine access would increase incubation of cocaine craving and whether there are sex differences in this effect.

Cocaine Self-administration. Rats were trained to self-administer cocaine in operant conditioning chambers equipped with two levers, two cue lights above the levers, a tone, and a house light. Each session started by inserting the two levers and illuminating the house light. Pressing on the active lever delivered a cocaine infusion (0.1 mL/3.5 seconds; 0.75 mg/kg body weight/infusion) and a compound tone/light cue for 3.5 seconds, followed by a 3.5-second timeout during which lever pressing was not reinforced. Pressing on the inactive lever had no programmed consequence. The rats were first trained to self-administer cocaine on a fixed ratio 1 schedule over 4 days for 2 hours/day (maximum infusions = 20). Next, the rats self-administered cocaine either continuously or intermittently for 8 hours/day for 12 days. In the continuous-access condition, the rats had free access to the drug during the daily sessions. In the intermittent-access condition, the rats had access to cocaine during 16 five-minute ON periods that were separated by 25-minute OFF periods (15), corresponding to 80 minutes of cocaine access during the 8-hour daily session. At the onset of each 5-minute ON period, the lever extended and the house light was turned on; at the end of the 5-minute access period the levers retracted and the house light was turned off.

Abstinence Phase. During this phase, the rats were housed in the animal facility and were handled two times per week.

Relapse Test. The rats tested on abstinence day 2 or 29 were matched for their cocaine intake during training (intermittent access: n = 25 males/n = 24 females; continuous access: n = 27 males/n = 23 females). On test day, the rats were placed in the same chambers where they previously self-administered cocaine. The relapse test was conducted under extinction conditions in the presence of the drug-associated cues during a single 3-hour session. The session began with the illumination of the house light and the extension of the active and inactive levers. Active lever presses resulted in contingent presentations of the tone/light cue for 3.5 seconds, but no cocaine, while inactive lever presses had no programmed consequence. The number of active lever presses is the operational measure of drug seeking in incubation studies (8,10,62).

Experiment 2: Independent Replication

The original goal of experiment 2 was to replicate the data in experiment 1 and characterize Fos expression in different brain areas during relapse tests performed 2 or 29 days after intermittent-access cocaine self-administration in female rats. The procedures for cocaine self-administration, abstinence, and relapse tests were as in experiment 1, except that the relapse tests lasted 90 minutes, an optimal time for detecting Fos protein expression in the brain.

Experiment 3: Effect of Duration of Self-administration Training and Estrous Cycle on Incubation of Craving in Female Rats After Intermittent-Access Cocaine Self-administration

Experiment 2 did not replicate the strong incubation effect in female rats after intermittent cocaine access observed in experiment 1. Therefore, in experiment 3, we investigated two possible factors: estrous cycle phase and training duration. We manipulated the amount of cocaine intake by training the female rats for six, 12, or 18 sessions.

Cocaine Self-administration and Abstinence Phase. Female rats went through the same procedures of self-administration, abstinence, and relapse tests (3 hours) as described in experiment 1, except that the number of intermittent self-administration sessions varied and the estrous cycle was monitored after the relapse tests by vaginal swab. The female rats self-administered cocaine for either six (n = 16), 12 (n = 14), or 18 (n = 16) sessions and tested for relapse on either abstinence day 2 or abstinence day 29. These rats received vaginal cytological tests (see Supplement).

Experiment 4: Effect of the Estrous Cycle on Incubation of Craving in Female Rats After Continuous-Access Cocaine Self-administration

The goal of experiment 4 was to examine whether the estrous cycle contributes to incubation of craving after continuous-access cocaine self-administration.

Cocaine Self-administration and Abstinence Phase. Female rats (n = 46) went through the same procedures as described in experiment 1 for continuous-access cocaine self-administration, abstinence, and relapse tests (3 hours). Additionally, these rats received vaginal cytological tests (see Supplement).

For information on statistical analyses, see the Supplement.

RESULTS

Experiment 1: Effect of Intermittent Cocaine Self-administration on Incubation of Craving in Male and Female Rats

Cocaine Self-administration. During the acquisition phase in sessions 1 to 4 (2 hours/day continuous access) there were no sex differences in the number of infusions, the frequency of infusions (infusions/min), and active and inactive lever presses (Figure 1B–E). Results showed that escalation of cocaine intake of days was observed under both access conditions, that total daily drug intake and the number of active lever presses were higher in the continuous-access condition, that infusion rate per minute and the number of inactive lever presses were higher in the intermittent-access condition, and that there were no sex differences in drug self-administration under either access condition. The mixed analysis of variance for total drug intake that included the between-subjects factors of access condition (intermittent, continuous) and sex (male, female) and the within-subjects factor of session showed significant effects of access condition ($F_{1,95}$ = 153.8, $p < .001$) and session ($F_{11,1045}$ = 80.7, $p < .001$) but no effect of sex ($p > .1$). There was also a significant access condition-by-session interaction ($F_{11,1045}$ = 5.9, $p < .001$) due to stronger escalation in the continuous-access condition. The analysis of infusion rate showed significant effects of access condition ($F_{1,95}$ = 434.4, $p < .001$) and session ($F_{11,1045}$ = 48.2, $p < .001$) but no effects of sex ($p > .1$). The statistical results of active and inactive lever presses are described in Supplemental Table S1.

Relapse Tests. Independent of the abstinence day and sex, cocaine seeking was higher after intermittent drug access than after continuous drug access (Figure 1F). Additionally, independent of the access condition and abstinence day, cocaine seeking was higher in female rats than in male rats. Finally, independent of the access condition and sex, cocaine seeking was higher on abstinence day 29 than on abstinence day 2 (incubation of craving). The mixed analysis of variance of lever presses, which included the between-subjects factors of access condition, sex, and abstinence day (2,29) and the within-subjects factor of lever (active, inactive), showed significant effects of access condition ($F_{1,91}$ = 33.5, $p < .001$), sex ($F_{1,91}$ = 16.4, $p < .001$), abstinence day ($F_{1,91}$ = 56.4, $p < .001$), and lever ($F_{1,91}$ = 304.3, $p < .001$) but no significant interactions among access condition, sex, and abstinence day.

Experiment 2: Independent Replication

We attempted to replicate the finding of strong incubation of cocaine craving in female rats after intermittent-access cocaine self-administration. As in experiment 1, the female rats escalated their daily cocaine intake and active lever presses under the intermittent-access training condition (Figure 2; see Supplemental Table S1 for statistical results). Unexpectedly, we observed no evidence for incubation of cocaine craving with a similar number of active lever presses during the different test days (Figure 2E). The statistical analysis that included the between-subjects factor of abstinence day and the within-subjects factor of lever showed a significant effect of lever ($F_{1,16}$ = 60.8, $p < .001$) but no significant effect of abstinence day or an interaction between the two factors (p values $>.05$).

Experiment 3: Effect of Duration of Cocaine Self-administration Training and the Estrous Cycle on Incubation of Craving in Female Rats

To understand the lack of incubation effect in experiment 2, we examined two experimental factors. First, we investigated possible role of the duration of intermittent-access cocaine

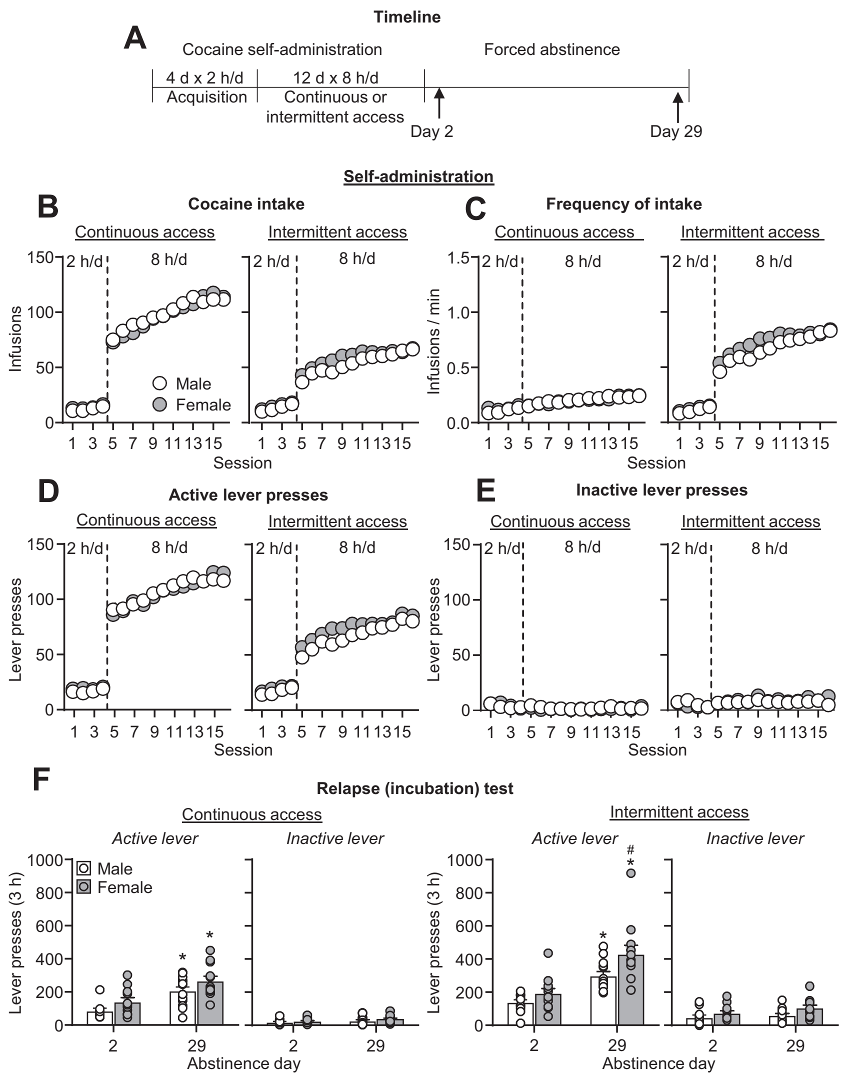

Figure 1. Continuous and intermittent cocaine self-administration and cocaine seeking for male and female rats. **(A)** Timeline of the experiment. **(B)** Total cocaine intake. Mean ± SEM number of infusions per session (continuous access: n = 27 males, n = 23 females; intermittent access: n = 25 males, n = 24 females). **(C)** Frequency of intake. Mean ± SEM number of infusions per minute of cocaine access per session. **(D)** Active lever presses. Mean ± SEM number of active lever presses per session of self-administration. **(E)** Inactive lever presses. Mean ± SEM number of inactive lever presses per session of self-administration. Left in all graphs (sessions 1–4): 2-hour continuous-access self-administration acquisition sessions; right in all graphs (sessions 6–14): 8-hour self-administration sessions. **(F)** Relapse (incubation) test. Mean ± SEM number of active and inactive lever presses per session (continuous access: males, n = 13 for day 2 and n = 14 for day 29; females, n = 11 for day 2 and n = 12 for day 29; intermittent access: males, n = 12 for day 2 and n = 13 for day 29; females, n = 12 for day 2 and day 29). *Different from day 2 within each sex, $p < .05$. #Different from male rats on day 29 of abstinence, $p = .05$.

self-administration training. The 12 sessions of intermittent access may have been a threshold number of cocaine exposure, resulting in lack of incubation in experiment 1. Second, we tested whether the estrous cycle regulates cocaine seeking during the relapse tests, because previous studies found significant effects of estrous cycle phases on cocaine-seeking behavior (29,40,45). Accordingly, by chance, during the relapse tests many rats in experiment 1 may have been in the critical estrous cycle phase, most likely estrus, while those in experiment 2 may have been in non-estrus.

Cocaine Self-administration. As in experiments 1 and 2, the female rats escalated their daily cocaine intake under the intermittent-access training condition. This escalation had occurred independent of the number of training sessions, and no significant effect of the number of training sessions was observed on the active and inactive levers (Figure 3B–D; see Supplemental Table S1 for statistical results).

Relapse Tests: Training Duration and Estrous Cycle. The training duration had no effect on the emergence of incubation of cocaine craving (Figure 3E). The statistical analysis that included the between-subjects factor of training duration (six, 12, 18 sessions) and abstinence day and the within-subjects factor of lever showed significant effects of abstinence day ($F_{1,40}$ = 18.2, $p < .001$) and lever ($F_{1,40}$ = 157.8, $p < .001$), and an interaction between these two factors ($F_{1,40}$ = 18.2, $p < .001$), but no effect on training duration or interactions between this factor and the other factors (p values $>.05$).

We found no differences between diestrus or proestrus (data not shown), confirming results from previous studies (29,47). Thus, we combined the diestrus and proestrus data (non-estrus) for data analysis. Across the three groups assigned to the different number of training sessions, the vaginal cytological analysis identified 13 rats in non-estrus and 10 rats in estrus on abstinence day 2, and 16 and 7 rats,

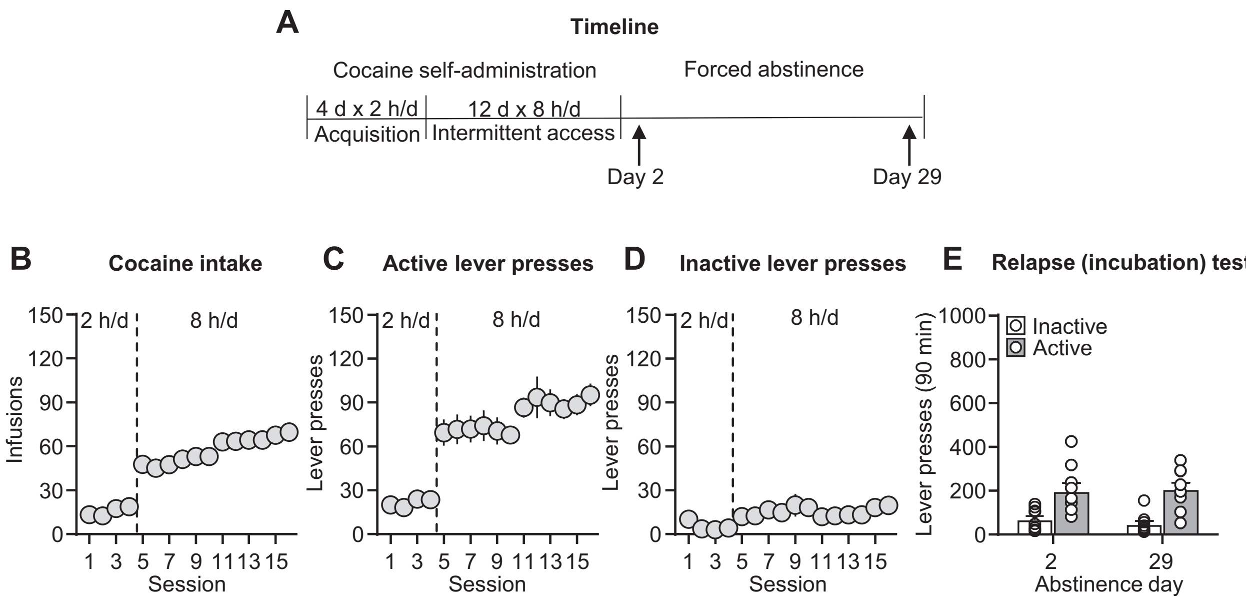

Figure 2. Lack of effect of incubation of cocaine craving in female rats after intermittent-access cocaine self-administration. **(A)** Timeline of the experiment. **(B)** Total cocaine intake. Mean ± SEM number of infusions per session. **(C)** Active lever presses. Mean ± SEM number of active lever presses per session of self-administration. **(D)** Inactive lever presses. Mean ± SEM number of inactive lever presses per session of self-administration. Left in the graph (sessions 1–4): 2-hour continuous-access self-administration acquisition sessions; right in the graph (sessions 6–14): 8-hour self-administration sessions (n = 18). **(E)** Relapse (incubation) test. Mean ± SEM number of active and inactive lever presses per session (n = 9 day 2 and 29).

respectively, on abstinence day 29. Because there were no differences in lever presses during the relapse tests among the three training-duration groups, we combined them for the statistical analysis of the effect of estrous cycle on incubation of cocaine craving. The estrous cycle phase had no effect on nonincubated cocaine seeking on abstinence day 2 but had a strong effect on the higher or incubated cocaine seeking on abstinence day 29 (Figure 3F). The statistical analysis that included the between-subjects factors of abstinence day and estrus phase (estrus, non-estrus) and the within-subjects factor of lever showed significant effects of abstinence day ($F_{1,42}$ = 48.9, $p < .001$), estrus phase ($F_{1,42}$ = 21.4, $p < .001$), and lever ($F_{1,42}$ = 194.9, $p < .001$), and significant interactions between estrus phase and abstinence day ($F_{1,42}$ = 12.5, p = .001) and among estrus phase, abstinence day, and lever ($F_{1,42}$ = 7.6, p = .009). Last, mean daily cocaine intake in the estrus and non-estrus groups was not associated with lever presses during the relapse tests (data not shown).

Experiment 4: Effect of the Estrous Cycle on Incubation of Craving in Female Rats After Continuous Cocaine Self-administration

Cocaine Self-administration. The female rats escalated their daily cocaine intake under the continuous-access training condition (Figure 4B; see Supplemental Table S1 for statistical results).

Relapse Tests. As in experiment 3, the estrous cycle phase had no effect on nonincubated cocaine seeking on abstinence day 2 but had a significant effect on incubated cocaine seeking on day 29 (Figure 4E). The mixed analysis of variance, which included the between-subjects factors of abstinence day and estrus phase (estrus, non-estrus) and the within-subjects factor of lever, showed significant effects of abstinence day ($F_{1,37}$ = 28.9, $p < .001$), estrus phase ($F_{1,37}$ = 7.9, p = .008), and lever ($F_{1,37}$ = 171.8, $p < .001$), and a significant interaction between estrus phase and abstinence day ($F_{1,37}$ = 7.2, p = .011) and among estrus phase, abstinence day, and lever ($F_{1,37}$ = 5.3, p = .027).

DISCUSSION

We examined the effect of intermittent cocaine self-administration on incubation of cocaine craving in male and female rats. There are four main findings in our study. First, incubation of cocaine craving during abstinence was observed after intermittent-access drug self-administration, extending previous reports using the classical continuous-access training procedure (9,13,63). Second, in both male and female rats and independent of the abstinence day, intermittent-access cocaine self-administration increased relapse to cocaine seeking. Intermittent cocaine access also increased inactive lever presses during testing; the reasons for this effect are unknown. Third, independent of the abstinence day and training conditions, lever responding in the relapse tests was higher in female rats than in male rats, extending previous results on increased relapse vulnerability in female rats in rat models (29,31,40). Most important, independent of the cocaine access condition, incubation of cocaine craving was more pronounced during estrus than non-estrus. This finding suggests a critical role of ovarian hormones in incubation of craving.

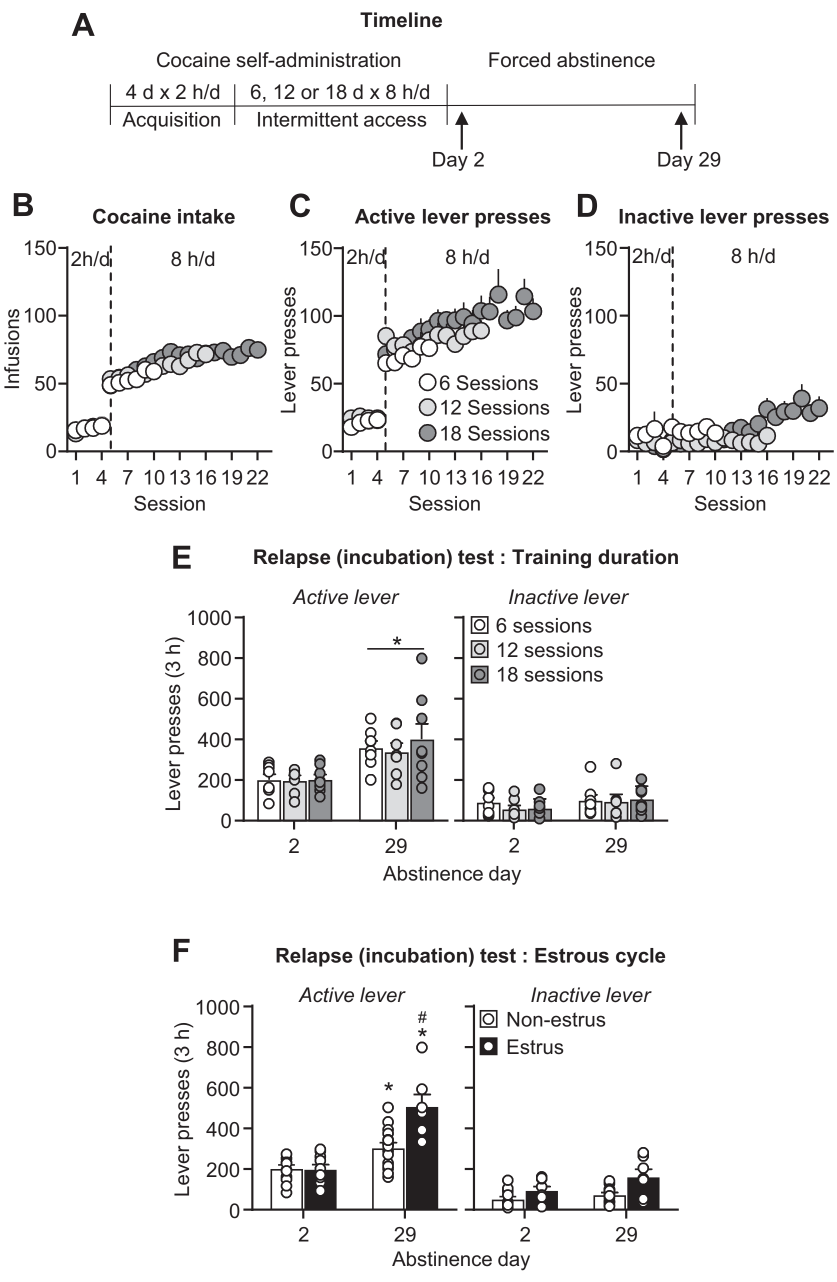

Figure 3. Effect of duration of intermittent-access cocaine self-administration training and estrous cycle on incubation of craving in female rats. **(A)** Timeline of the experiment. **(B)** Total cocaine intake. Mean ± SEM number of infusions per session. **(C)** Active lever presses. Mean ± SEM number of active lever presses per session of self-administration. **(D)** Inactive lever presses. Mean ± SEM number of inactive lever presses per session of self-administration. **(E)** Relapse (incubation) test: training duration. Mean ± SEM number of active and inactive lever presses per session (six sessions: $n = 8$, 12 sessions: $n = 7$, 16 sessions: $n = 8$ for day 2 and day 29). **(F)** Relapse (incubation) test: estrous cycle. Mean ± SEM number of active and inactive lever presses per session (non-estrus: $n = 13/16$ [diestrus: $n = 6/10$, proestrus: $n = 7/6$] and estrus $n = 10/7$ for days 2/29, respectively). *Different from day 2 within each training condition or estrous phase, $p < .05$. #Different from non-estrus at day 29 of abstinence, $p < .05$.

Sex Differences in Cocaine Self-administration and Seeking

In experiment 1, we found that both male and female rats increased cocaine intake over the sessions under either continuous- or intermittent-access training conditions. These results extend previous findings on escalation of cocaine intake in male and female rats under continuous-access conditions (49,64) and male rats under intermittent-access conditions (24–26). Under both access conditions, we found no sex differences in cocaine self-administration. These results are not consistent with previous reports on higher cocaine intake in female rats than in male rats trained under continuous-access conditions (39,65,66). The reasons for these different results are unknown and may be due to differences in the parameters of cocaine self-administration training in our studies versus these previous studies (e.g., unit dose, duration of daily session, number of training days). However, our negative results are consistent with recent studies showing lack of sex differences in extended access methamphetamine self-administration (67,68).

Under both access conditions, lever responding in the relapse tests was higher in female rats than in male rats. These results agree with those from previous studies on increased

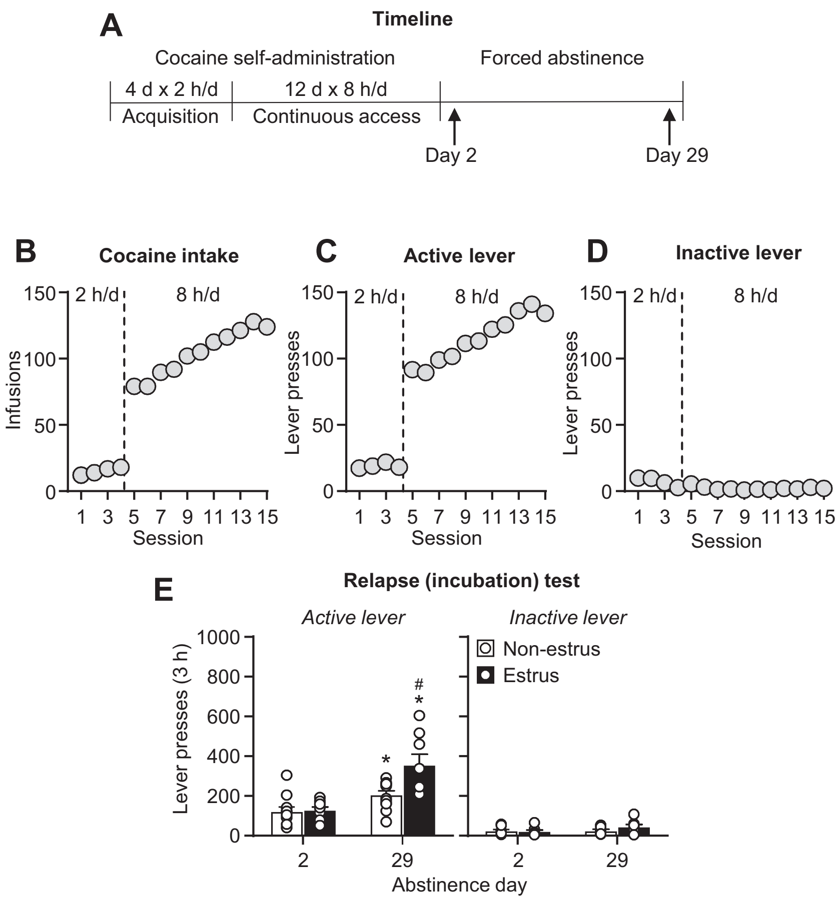

Figure 4. Effect of estrous cycle on incubation of craving after continuous-access cocaine self-administration in female rats. **(A)** Timeline of the experiment. **(B)** Total cocaine intake. Mean ± SEM number of infusions per session. **(C)** Active lever presses. Mean ± SEM number of active lever presses per session of self-administration. **(D)** Inactive lever presses. Mean ± SEM number of inactive lever presses per session of self-administration. **(E)** Relapse (incubation) test. Mean ± SEM number of active and inactive lever presses per session. Non-estrus: n = 12 (diestrus: n = 6, proestrus: n = 6) for day 2 and n = 12 (diestrus: n = 8, proestrus: n = 4) for day 29; estrus: n = 9 for day 2 and n = 8 for day 29. *Different from day 2 within estrus phase, $p < .05$. #Different from non-estrus at day 29 of abstinence, $p < .05$.

resistance to extinction and cue- and cocaine priming–induced reinstatement in female rats (40,41,46,47,69,70). Similar results have been extended to other drugs, showing increased cue- and drug priming–induced reinstatement of methamphetamine and nicotine seeking in female rats (71–73); however, see Venniro *et al.* (68) for lack of sex differences in incubation of methamphetamine craving. Together, our results extend previous results on sex differences in relapse to cocaine seeking (29,31,41) and highlight the importance of sex as a biological variable in cocaine relapse studies.

Role of the Estrous Cycle in the Incubation of Cocaine Craving

In experiment 2, we failed to replicate experiment 1 finding on incubation of craving in female rats after intermittent-access self-administration training. To address this unexpected finding, we investigated the role of two factors: total drug exposure and estrous cycle phase. As for the first factor, 12 daily sessions of 8-hour intermittent-access cocaine self-administration corresponds to 80 min/day of cocaine access and may have been a threshold level of cocaine exposure to induce incubation of cocaine craving. In this regard, parametric studies in male rats have shown that incubation of cocaine craving, as assessed in extinction and cue-induced reinstatement tests, is less robust after 10 days of 2-hour continuous-access daily sessions than after 10 days of 6-hour daily sessions (8). We found that six, 12, or 18 sessions of intermittent-access cocaine self-administration produced similar levels of incubation, indicating that the discrepant results between experiments 1 and 2 are not due to the duration of the training phase or amount of drug intake during this phase. Regarding the second factor, as discussed in the introduction, several studies have shown a role of ovarian hormones and estrous cycle in cocaine self-administration (38,39,49,52,65,66,74,75) and reinstatement of cocaine seeking after continuous-access drug self-administration (40,46–48,76,77). We found potentiation of incubation of cocaine craving during estrus, an effect that was observed after either continuous- or intermittent-access cocaine self-administration. Thus, the lack of incubation in experiment 2 may be due to a high proportion of rats being tested during non-estrus on day 29. However, it cannot be ruled out that other unknown experimental factors contributed to the negative results in experiment 2, because in experiments 3 and 4 we observed modest but statistically significant incubation in non-estrus rats. These results suggest that incubation of cocaine craving occurs during both estrus and non-estrus, but this incubation is weaker and less robust during non-estrus. Another reason for the replication failure in experiment 2 could be the use of different testing durations (90 minutes vs. 180 minutes in experiment 1). However, this possibility can be ruled out, because as shown in Supplemental Figure S1, reliable incubation was observed in experiment 1 (and experiments 3 and 4) when we analyzed the first 90-minute data in these experiments.

Our data for the relapse tests on abstinence day 29 are consistent with previous findings showing that female rats in estrus show higher cocaine-induced reinstatement and are more resistant to extinction than female rats in non-estrus (40,46,47). In contrast, we did not observe any differences in cocaine seeking on abstinence day 2 between the rats in estrus and non-estrus. Previous studies found that the estrus phase is associated with increased cocaine seeking during early abstinence extinction sessions (29,47). The reasons for these different results are unknown, and it cannot be ruled out that the lack of effect of estrus phase on day 2 is due to a floor effect or to differences in the training procedures used: extended 8-hour access in our study versus 2-hour limited access in the previous studies (see above). Additionally, cocaine exposure can disrupt the reproductive cycle in rodents and humans (43,78), and in rats, the dysregulation of the estrous cycle lasts for up to 18 days after withdrawal from cocaine (79). Therefore, the negative results regarding the estrous cycle's role in cocaine seeking during early abstinence should be interpreted with caution.

A methodological issue to consider in experiment 3 is that we determined the estrus phase based on a single-day cytology analysis that is less reliable than multiple-day analysis. However, it is unlikely that this methodological issue confounds data interpretation because we replicated and extended experiment 3 results in two experiments in which we determined the estrus phase based on multiple-day analysis. In experiment 4, we showed a critical role of estrus phase in incubation after continuous-access cocaine self-administration. Additionally, in an experiment in which we only determined cocaine seeking 29 days after intermittent-access cocaine self-administration, we found higher lever presses during estrus versus non-estrus (Supplemental Figure S2).

Finally, a future research question regards the mechanisms of the estrus phase's role in incubation of craving. In this regard, a potential mechanism is potentiation of activity of dopaminergic projections from the ventral tegmental area to the nucleus accumbens during estrus. This projection plays a critical role in relapse/reinstatement of cocaine seeking (80,81). An early rat study showed potentiation of amphetamine-induced striatal dopamine release and stereotypy during estrus (82). More recently, a mouse study showed that dopamine neuron activity in the ventral tegmental area-to-nucleus accumbens projection and cocaine's rewarding effects in the CPP procedure are potentiated during estrus (83).

Conclusions

Our results demonstrate that the phenomenon of incubation of cocaine craving generalizes to female and male rats trained under the intermittent-access drug self-administration procedure (15). Additionally, in both male and female rats, intermittent-access cocaine self-administration potentiates cocaine seeking during both early and late abstinence. Finally, we demonstrated a critical relationship between the phase of the estrous cycle and the magnitude of incubation of craving in female rats after both continuous and intermittent self-administration, a finding that may mediate sex differences in cocaine seeking. Thus, to the degree that results from animal models generalize to humans (84–87), our findings implicate the phase of the menstrual cycle as a risk factor for relapse in women and, therefore, should be taken into consideration in the development of relapse prevention treatments. A translational question for future research is whether incubation of cocaine craving, recently demonstrated in humans using an electroencephalography-based measure (11), is modulated by the menstrual cycle.

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CN, TIR, AP, SM, AH, and Z-BY carried out the experiments; CN and TIR performed data analysis. CN, MMC, AH, YS, and SI designed the study. CN, YS, and SI wrote the manuscript. All authors critically reviewed the content and approved the final version before submission.

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ARTICLE INFORMATION

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REFERENCES

1. Hunt WA, Barnett LW, Branch LG (1971): Relapse rates in addiction programs. J Clin Psychol 27:455–456.
2. Sinha R (2011): New findings on biological factors predicting addiction relapse vulnerability. Curr Psychiatry Rep 13:398–405.
3. O'Brien CP, Childress AR, McLellan AT, Ehrman R (1992): Classical conditioning in drug-dependent humans. Ann N Y Acad Sci 654:400–415.
4. Wikler A (1973): Dynamics of drug dependence. Implications of a conditioning theory for research and treatment. Arch Gen Psychiatry 28:611–616.
5. Gawin FH, Kleber HD (1986): Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. Arch Gen Psychiatry 43:107–113.
6. Grimm JW, Hope BT, Wise RA, Shaham Y (2001): Neuroadaptation. Incubation of cocaine craving after withdrawal. Nature 412:141–142.
7. Tran-Nguyen LT, Fuchs RA, Coffey GP, Baker DA, O'Dell LE, Neisewander JL (1998): Time-dependent changes in cocaine-seeking behavior and extracellular dopamine levels in the amygdala during cocaine withdrawal. Neuropsychopharmacology 19:48–59.
8. Lu L, Grimm JW, Hope BT, Shaham Y (2004): Incubation of cocaine craving after withdrawal: A review of preclinical data. Neuropharmacology 47(suppl 1):214–226.

9. Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y (2011): Neurobiology of the incubation of drug craving. Trends Neurosci 34:411–420.
10. Venniro M, Caprioli D, Shaham Y (2016): Animal models of drug relapse and craving: From drug priming-induced reinstatement to incubation of craving after voluntary abstinence. Prog Brain Res 224:25–52.
11. Parvaz MA, Moeller SJ, Goldstein RZ (2016): Incubation of cue-induced craving in adults addicted to cocaine measured by electroencephalography. JAMA Psychiatry 73:1127–1134.
12. Szumlinski KK, Shin CB (2018): Kinase interest you in treating incubated cocaine-craving? A hypothetical model for treatment intervention during protracted withdrawal from cocaine. Genes Brain Behav 17:e12440.
13. Wolf E, Kuhn M, Normann C, Mainberger F, Maier JG, Maywald S, *et al.* (2016): Synaptic plasticity model of therapeutic sleep deprivation in major depression. Sleep Med Rev 30:53–62.
14. Allain F, Minogianis EA, Roberts DC, Samaha AN (2015): How fast and how often: The pharmacokinetics of drug use are decisive in addiction. Neurosci Biobehav Rev 56:166–179.
15. Zimmer BA, Dobrin CV, Roberts DC (2011): Brain-cocaine concentrations determine the dose self-administered by rats on a novel behaviorally dependent dosing schedule. Neuropsychopharmacology 36:2741–2749.
16. Allain F, Roberts DCS, Levesque D, Samaha AN (2017): Intermittent intake of rapid cocaine injections promotes robust psychomotor sensitization, increased incentive motivation for the drug and mGlu2/3 receptor dysregulation. Neuropharmacology 117:227–237.
17. Calipari ES, Ferris MJ, Siciliano CA, Zimmer BA, Jones SR (2014): Intermittent cocaine self-administration produces sensitization of stimulant effects at the dopamine transporter. J Pharmacol Exp Ther 349:192–198.
18. Calipari ES, Ferris MJ, Zimmer BA, Roberts DC, Jones SR (2013): Temporal pattern of cocaine intake determines tolerance vs sensitization of cocaine effects at the dopamine transporter. Neuropsychopharmacology 38:2385–2392.
19. Calipari ES, Jones SR (2014): Sensitized nucleus accumbens dopamine terminal responses to methylphenidate and dopamine transporter releasers after intermittent-access self-administration. Neuropharmacology 82:1–10.
20. Calipari ES, Siciliano CA, Zimmer BA, Jones SR (2015): Brief intermittent cocaine self-administration and abstinence sensitizes cocaine effects on the dopamine transporter and increases drug seeking. Neuropsychopharmacology 40:728–735.
21. Roberts DC, Gabriele A, Zimmer BA (2013): Conflation of cocaine seeking and cocaine taking responses in IV self-administration experiments in rats: Methodological and interpretational considerations. Neurosci Biobehav Rev 37:2026–2036.
22. Zimmer BA, Oleson EB, Roberts DC (2012): The motivation to self-administer is increased after a history of spiking brain levels of cocaine. Neuropsychopharmacology 37:1901–1910.
23. Allain F, Bouayad-Gervais K, Samaha AN (2018): High and escalating levels of cocaine intake are dissociable from subsequent incentive motivation for the drug in rats. Psychopharmacology (Berl) 235:317–328.
24. Allain F, Samaha AN (2018): Revisiting long-access versus short-access cocaine self-administration in rats: Intermittent intake promotes addiction symptoms independent of session length [published online ahead of print Jun 19]. Addict Biol.
25. Kawa AB, Bentzley BS, Robinson TE (2016): Less is more: Prolonged intermittent access cocaine self-administration produces incentive-sensitization and addiction-like behavior. Psychopharmacology (Berl) 233:3587–3602.
26. Singer BF, Fadanelli M, Kawa AB, Robinson TE (2018): Are cocaine-seeking "habits" necessary for the development of addiction-like behavior in rats? J Neurosci 38:60–73.
27. James MH, Stopper CM, Zimmer BA, Koll NE, Bowrey HE, Aston-Jones G (2018): Increased number and activity of a lateral subpopulation of hypothalamic orexin/hypocretin neurons underlies the expression of an addicted state in rats [published online ahead of print Aug 7]. Biol Psychiatry.
28. Carroll ME, Lynch WJ (2016): How to study sex differences in addiction using animal models. Addict Biol 21:1007–1029.
29. Kerstetter KA, Aguilar VR, Parrish AB, Kippin TE (2008): Protracted time-dependent increases in cocaine-seeking behavior during cocaine withdrawal in female relative to male rats. Psychopharmacology (Berl) 198:63–75.
30. Becker JB, Perry AN, Westenbroek C (2012): Sex differences in the neural mechanisms mediating addiction: A new synthesis and hypothesis. Biol Sex Differ 3:14.
31. Lynch WJ, Roth ME, Carroll ME (2002): Biological basis of sex differences in drug abuse: Preclinical and clinical studies. Psychopharmacology (Berl) 164:121–137.
32. McKay JR, Rutherford MJ, Cacciola JS, Kabasakalian-McKay R, Alterman AI (1996): Gender differences in the relapse experiences of cocaine patients. J Nerv Ment Dis 184:616–622.
33. Robbins SJ, Ehrman RN, Childress AR, O'Brien CP (1999): Comparing levels of cocaine cue reactivity in male and female outpatients. Drug Alcohol Depend 53:223–230.
34. Fox H, Sinha R (2014): The role of guanfacine as a therapeutic agent to address stress-related pathophysiology in cocaine-dependent individuals. Adv Pharmacol 69:217–265.
35. Fox HC, Sofuoglu M, Morgan PT, Tuit KL, Sinha R (2013): The effects of exogenous progesterone on drug craving and stress arousal in cocaine dependence: Impact of gender and cue type. Psychoneuroendocrinology 38:1532–1544.
36. Li CS, Kemp K, Milivojevic V, Sinha R (2005): Neuroimaging study of sex differences in the neuropathology of cocaine abuse. Gend Med 2:174–182.
37. Potenza MN, Hong KI, Lacadie CM, Fulbright RK, Tuit KL, Sinha R (2012): Neural correlates of stress-induced and cue-induced drug craving: Influences of sex and cocaine dependence. Am J Psychiatry 169:406–414.
38. Hu M, Crombag HS, Robinson TE, Becker JB (2004): Biological basis of sex differences in the propensity to self-administer cocaine. Neuropsychopharmacology 29:81–85.
39. Lynch WJ, Carroll ME (1999): Sex differences in the acquisition of intravenously self-administered cocaine and heroin in rats. Psychopharmacology (Berl) 144:77–82.
40. Kippin TE, Fuchs RA, Mehta RH, Case JM, Parker MP, Bimonte-Nelson HA, *et al.* (2005): Potentiation of cocaine-primed reinstatement of drug seeking in female rats during estrus. Psychopharmacology (Berl) 182:245–252.
41. Lynch WJ, Carroll ME (2000): Reinstatement of cocaine self-administration in rats: Sex differences. Psychopharmacology (Berl) 148:196–200.
42. Evans SM, Haney M, Foltin RW (2002): The effects of smoked cocaine during the follicular and luteal phases of the menstrual cycle in women. Psychopharmacology (Berl) 159:397–406.
43. Sofuoglu M, Dudish-Poulsen S, Nelson D, Pentel PR, Hatsukami DK (1999): Sex and menstrual cycle differences in the subjective effects from smoked cocaine in humans. Exp Clin Psychopharmacol 7:274–283.
44. Sofuoglu M, Mitchell E, Kosten TR (2004): Effects of progesterone treatment on cocaine responses in male and female cocaine users. Pharmacol Biochem Behav 78:699–705.
45. Carroll ME, Anker JJ (2010): Sex differences and ovarian hormones in animal models of drug dependence. Horm Behav 58:44–56.
46. Feltenstein MW, Byrd EA, Henderson AR, See RE (2009): Attenuation of cocaine-seeking by progesterone treatment in female rats. Psychoneuroendocrinology 34:343–352.
47. Feltenstein MW, See RE (2007): Plasma progesterone levels and cocaine-seeking in freely cycling female rats across the estrous cycle. Drug Alcohol Depend 89:183–189.
48. Anker JJ, Larson EB, Gliddon LA, Carroll ME (2007): Effects of progesterone on the reinstatement of cocaine-seeking behavior in female rats. Exp Clin Psychopharmacol 15:472–480.

49. Larson EB, Anker JJ, Gliddon LA, Fons KS, Carroll ME (2007): Effects of estrogen and progesterone on the escalation of cocaine self-administration in female rats during extended access. Exp Clin Psychopharmacol 15:461–471.
50. Craft RM, Stratmann JA (1996): Discriminative stimulus effects of cocaine in female versus male rats. Drug Alcohol Depend 42:27–37.
51. Glick SD, Hinds PA (1984): Sex differences in sensitization to cocaine-induced rotation. Eur J Pharmacol 99:119–121.
52. Hu M, Becker JB (2008): Acquisition of cocaine self-administration in ovariectomized female rats: Effect of estradiol dose or chronic estradiol administration. Drug Alcohol Depend 94:56–62.
53. van Haaren F, Meyer ME (1991): Sex differences in locomotor activity after acute and chronic cocaine administration. Pharmacol Biochem Behav 39:923–927.
54. Russo SJ, Festa ED, Fabian SJ, Gazi FM, Kraish M, Jenab S, *et al.* (2003): Gonadal hormones differentially modulate cocaine-induced conditioned place preference in male and female rats. Neuroscience 120:523–533.
55. Cummings JA, Jagannathan L, Jackson LR, Becker JB (2014): Sex differences in the effects of estradiol in the nucleus accumbens and striatum on the response to cocaine: Neurochemistry and behavior. Drug Alcohol Depend 135:22–28.
56. Perrotti LI, Russo SJ, Fletcher H, Chin J, Webb T, Jenab S, *et al.* (2001): Ovarian hormones modulate cocaine-induced locomotor and stereotypic activity. Ann N Y Acad Sci 937:202–216.
57. Russo SJ, Jenab S, Fabian SJ, Festa ED, Kemen LM, Quinones-Jenab V (2003): Sex differences in the conditioned rewarding effects of cocaine. Brain Res 970:214–220.
58. Perry AN, Westenbroek C, Becker JB (2013): The development of a preference for cocaine over food identifies individual rats with addiction-like behaviors. PLoS One 8:e79465.
59. Perry AN, Westenbroek C, Jagannathan L, Becker JB (2015): The roles of dopamine and alpha1-adrenergic receptors in cocaine preferences in female and male rats. Neuropsychopharmacology 40:2696–2704.
60. Bagley JR, Adams J, Bozadjian RV, Bubalo L, Ploense KL, Kippin TE (2019): Estradiol increases choice of cocaine over food in male rats. Physiol Behav 203:18–24.
61. Kerstetter KA, Ballis MA, Duffin-Lutgen S, Carr AE, Behrens AM, Kippin TE (2012): Sex differences in selecting between food and cocaine reinforcement are mediated by estrogen. Neuropsychopharmacology 37:2605–2614.
62. Li X, Caprioli D, Marchant NJ (2015): Recent updates on incubation of drug craving: A mini-review. Addict Biol 20:872–876.
63. Dong Y, Taylor JR, Wolf ME, Shaham Y (2017): Circuit and synaptic plasticity mechanisms of drug relapse. J Neurosci 37:10867–10876.
64. Ahmed SH, Koob GF (1998): Transition from moderate to excessive drug intake: Change in hedonic set point. Science 282:298–300.
65. Carroll ME, Morgan AD, Lynch WJ, Campbell UC, Dess NK (2002): Intravenous cocaine and heroin self-administration in rats selectively bred for differential saccharin intake: Phenotype and sex differences. Psychopharmacology (Berl) 161:304–313.
66. Roth ME, Carroll ME (2004): Sex differences in the escalation of intravenous cocaine intake following long- or short-access to cocaine self-administration. Pharmacol Biochem Behav 78:199–207.
67. Venniro M, Zhang M, Caprioli D, Hoots JK, Golden SA, Heins C, *et al.* (2018): Volitional social interaction prevents drug addiction in rat models. Nat Neurosci 21:1520–1529.
68. Venniro M, Zhang M, Shaham Y, Caprioli D (2017): Incubation of methamphetamine but not heroin craving after voluntary abstinence in male and female rats. Neuropsychopharmacology 42:1126–1135.
69. Becker JB (2016): Sex differences in addiction. Dialogues Clin Neurosci 18:395–402.
70. Feltenstein MW, Henderson AR, See RE (2011): Enhancement of cue-induced reinstatement of cocaine-seeking in rats by yohimbine: Sex differences and the role of the estrous cycle. Psychopharmacology (Berl) 216:53–62.
71. Cox BM, Young AB, See RE, Reichel CM (2013): Sex differences in methamphetamine seeking in rats: Impact of oxytocin. Psychoneuroendocrinology 38:2343–2353.
72. Feltenstein MW, Ghee SM, See RE (2012): Nicotine self-administration and reinstatement of nicotine-seeking in male and female rats. Drug Alcohol Depend 121:240–246.
73. Holtz NA, Lozama A, Prisinzano TE, Carroll ME (2012): Reinstatement of methamphetamine seeking in male and female rats treated with modafinil and allopregnanolone. Drug Alcohol Depend 120:233–237.
74. Grimm JW, See RE (1997): Cocaine self-administration in ovariectomized rats is predicted by response to novelty, attenuated by 17-beta estradiol, and associated with abnormal vaginal cytology. Physiol Behav 61:755–761.
75. Jackson LR, Robinson TE, Becker JB (2006): Sex differences and hormonal influences on acquisition of cocaine self-administration in rats. Neuropsychopharmacology 31:129–138.
76. Doncheck EM, Urbanik LA, DeBaker MC, Barron LM, Liddiard GT, Tuscher JJ, *et al.* (2018): 17β-Estradiol potentiates the reinstatement of cocaine seeking in female rats: role of the prelimbic prefrontal cortex and cannabinoid type-1 receptors. Neuropsychopharmacology 43:781–790.
77. Larson EB, Roth ME, Anker JJ, Carroll ME (2005): Effect of short- vs. long-term estrogen on reinstatement of cocaine-seeking behavior in female rats. Pharmacol Biochem Behav 82:98–108.
78. Chen CJ, Vandenbergh JG (1994): Effect of chronic cocaine on reproduction in female house mice. Pharmacol Biochem Behav 48:909–913.
79. King TS, Canez MS, Gaskill S, Javors MA, Schenken RS (1993): Chronic cocaine disruption of estrous cyclicity in the rat: Dose-dependent effects. J Pharmacol Exp Ther 264:29–34.
80. Schmidt HD, Anderson SM, Famous KR, Kumaresan V, Pierce RC (2005): Anatomy and pharmacology of cocaine priming-induced reinstatement of drug seeking. Eur J Pharmacol 526:65–76.
81. Shalev U, Grimm JW, Shaham Y (2002): Neurobiology of relapse to heroin and cocaine seeking: A review. Pharmacol Rev 54:1–42.
82. Becker JB, Cha JH (1989): Estrous cycle-dependent variation in amphetamine-induced behaviors and striatal dopamine release assessed with microdialysis. Behav Brain Res 35:117–125.
83. Calipari ES, Juarez B, Morel C, Walker DM, Cahill ME, Ribeiro E, *et al.* (2017): Dopaminergic dynamics underlying sex-specific cocaine reward. Nat Commun 8:13877.
84. Epstein DH, Preston KL, Stewart J, Shaham Y (2006): Toward a model of drug relapse: An assessment of the validity of the reinstatement procedure. Psychopharmacology (Berl) 189:1–16.
85. Heilig M, Epstein DH, Nader MA, Shaham Y (2016): Time to connect: Bringing social context into addiction neuroscience. Nat Rev Neurosci 17:592–599.
86. Reiner DJ, Fredriksson I, Lofaro OM, Bossert JM, Shaham Y (2018): Relapse to opioid seeking in rat models: Behavior, pharmacology and circuits. Neuropsychopharmacology 44:465–477.
87. Sinha R, Shaham Y, Heilig M (2011): Translational and reverse translational research on the role of stress in drug craving and relapse. Psychopharmacology (Berl) 218:69–82.