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# The estrous cycle has no effect on incubation of methamphetamine craving and associated Fos expression in dorsomedial striatum and anterior intralaminar nucleus of thalamus

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## Abstract

Relapse is a major challenge in treating drug addiction, and drug seeking progressively increases after abstinence, a phenomenon termed “incubation of drug craving”. Previous studies demonstrated both sex differences and an effect of estrous cycle in female rats in incubation of cocaine craving. In contrast, while incubation of methamphetamine craving is similar across sexes, whether estrous cycle plays a role in this incubation has yet to be fully addressed. Moreover, whether neural mechanisms underlying incubation of methamphetamine craving differ across estrous cycles is largely unknown. To address these gaps, we first compared methamphetamine self-administration, and methamphetamine seeking on both abstinence days 1 and 28 between male rats and female rats across the estrous cycle. Next, we examined neuronal activation associated with incubated methamphetamine seeking in dorsomedial striatum (DMS) and lateral portion of the anterior intralaminar nucleus of thalamus (AIT-L), two brain areas previously implicated in incubation of methamphetamine craving. We found no effect of sex or estrous cycle on methamphetamine self-administration and methamphetamine seeking on abstinence days 1 and 28. We also found no effect of sex or estrous cycle on the number of Fos-expressing cells in DMS or AIT-L following methamphetamine seeking test. Taken together, our results showed that methamphetamine self-administration and incubation of methamphetamine craving was not dependent on sex or estrous cycles under our experimental condition, and the role of DMS and



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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

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AIT-L in incubation of methamphetamine craving may be similar across sexes and across estrous cycles in female rats.

**Keywords**

Methamphetamine; Craving; Estrous cycle; Sex; Striatum; Thalamus

## Introduction

Relapse during abstinence, often triggered by drug-associated cues, is a major challenge in treating drug addiction, including methamphetamine [1–3]. In rats, cue-induced methamphetamine seeking progressively increases after abstinence [4,5]. This incubation of methamphetamine craving has also been demonstrated in methamphetamine-dependent individuals [6]. Preclinical work from us and others has previously identified circuit, synaptic, and molecular mechanisms underlying this incubation of methamphetamine craving primarily in male rats [7,8]. For example, we demonstrated a critical role of dorsal striatum in this incubation in male rats [9]. A growing number of studies also began to examine neuroadaptations associated with incubation of methamphetamine craving in female rats [10–17], but only one published study has taken estrous cycle into consideration [10].

In humans, sex differences in methamphetamine craving and relapse are unclear based on the mixed findings across different studies [18,19], and the effect of the estrous cycle in women on methamphetamine relapse has not been directly investigated. In rats, there are also no clear conclusions regarding sex differences in methamphetamine seeking across different animal models of relapse. For example, some studies reported higher methamphetamine seeking in female than male rats after methamphetamine-primed [20–24], cue- [25], stress-induced [25], and yohimbine-induced reinstatement [26], while other studies found either no sex difference [26–28] or higher methamphetamine seeking in male than female rats after context-induced reinstatement [29]. In addition, in studies that considered the effect of estrous cycle, they found that estrous cycle has no effect on methamphetamine-primed [22,25,30] or context-induced reinstatement of methamphetamine seeking [29].

Regarding incubation of methamphetamine craving, previous studies reported no sex differences in incubation of methamphetamine craving after either forced abstinence [10,12,14,26] or voluntary abstinence [31,32], similar to incubation studies using heroin [31] and oxycodone [33]. These findings are in contrast with previous preclinical studies demonstrating higher cue-induced cocaine seeking in female rats than male rats after forced abstinence [34–36]. Sex differences in incubation of cocaine craving are indeed attributed to the effect of estrous cycle with estrus females exhibiting higher cue-induced cocaine seeking after prolonged abstinence than non-estrus females [34–36]. Further, recent studies demonstrated sex- and estrous cycle-dependent neural mechanisms underlying incubation of cocaine craving [37–39]. For example, inhibition of metabotropic glutamate receptor 5 (mGlu5) in basolateral amygdala (BLA) prevents incubated cocaine seeking in

estrus-female, but not in non-estrus female or male rats [37]. However, whether estrous cycle impacts incubation of methamphetamine craving has yet to be fully addressed [10]. Moreover, previous studies demonstrated sex differences in transcription regulations [12,14–16] and synaptic plasticity [10,17] across different brain regions after prolonged abstinence from methamphetamine self-administration in rats, but it is unknown whether there are estrous cycle-dependent neuronal mechanisms associated with incubation of methamphetamine craving.

In this study, we aimed to extend previous methamphetamine incubation studies comparing sexes [10,12,14,15,17,31,32] and assess whether estrous cycles in female rats impact incubation of methamphetamine craving at the behavioral level. We also began to explore whether neural mechanisms underlying this incubation are estrous cycle-dependent by focusing on neuronal activation, assessed by Fos expression, in dorsomedial striatum (DMS) and lateral anterior intralaminar nucleus of thalamus (AIT-L) associated with incubated methamphetamine seeking. We chose these two brain regions based on our previous study demonstrating a critical role of projections from AIT-L to DMS in incubation of methamphetamine craving in male rats [9,40].

## Materials and methods

### Subjects

We used Sprague-Dawley rats (Charles River). Male rats weighed 275–300 g prior to surgery and female rats weighed 175–200 g prior to surgery. Rats were maintained under a reverse 12:12-h light/dark cycle with food and water freely available. We kept the rats 4 per cage prior to surgery and then housed them individually after surgery. We performed the experiments under the protocols approved by the University of Maryland College Park Animal Care and Use Committee and in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health).

### Intravenous surgery

We anesthetized the rats with isoflurane (5 % induction, 2–3 % maintenance) and inserted silastic catheters into the rats' jugular veins as previously described [40,41]. We injected the rats with ketoprofen (2.5 mg/kg, s.c.) after surgery to relieve pain and inflammation; we allowed them to recover 5–7 days before methamphetamine self-administration training. During the recovery and training phases, we flushed the catheters every 24–48 h with gentamicin (Hospira; 4.8 mg/mL) dissolved in sterile saline.

### Apparatus

We trained the rats in self-administration chambers located inside sound-attenuating cabinets and controlled by a Med Associates (Georgia, VT) system. Each chamber has two levers located 8–9 cm above the floor. During self-administration training, presses on the retractable (active) lever activated the infusion pump (which delivered a methamphetamine infusion); presses on the stationary (inactive) lever were not reinforced with the drug. For methamphetamine intravenous infusions, we connected each rat's catheter to a liquid swivel (Instech) via polyethylene-50 tubing, protected by a metal spring. We then attached the

liquid swivel to a 20-ml syringe via polyethylene-50 tubing and to a 22-gauge modified needle (Plastics One, VA).

### Methamphetamine self-administration training

We used a training procedure similar to those in previous studies [11, 40]. We trained the rats to self-administer methamphetamine for 6 h per day (six 1-h sessions with 10 min off in between each session), under a fixed-ratio-1 (FR1) with a 20-s timeout reinforcement schedule. Methamphetamine was obtained from the National Institute on Drug Abuse Drug Supply Program, dissolved in sterile saline (10 mg/ml), and delivered at a dose of 0.1 mg/kg/infusion over 3.5 s (0.10 ml/infusion). We trained the rats for 10 sessions over an 11-day period (off day between 5th and 6th day). We used Brevital (3 to 4 mg/kg) to check catheter patency for low responders during the training.

The daily training sessions started at the onset of the dark cycle and began with the extension of the active lever and the illumination of the red house light. The house light remained on for the duration of each 1-h session for a total of six 1-h sessions. During training, active lever presses led to the delivery of a methamphetamine infusion and a compound 5-s tone-light cue (the tone and light modules were located above the active lever). During the 20-s timeout, we recorded the non-reinforced lever presses. We set 15 infusions as the maximum for each 1-h session to prevent overdose. The red house light was turned off, and the active lever retracted after the rats received the maximum number of infusions or at the end of the 6-h session.

### Abstinence phase

During the abstinence phase, we housed the rats individually in the animal facility and handled them 2–3 times per week.

### Methamphetamine seeking test (Seeking test)

We conducted all seeking tests (2 h) immediately after the onset of the dark cycle. The sessions began with the extension of the active lever and the illumination of the red house light, which remained on for the duration of the session. Active lever presses during testing [the operational measure of drug seeking in incubation of craving studies [42–44] resulted in contingent presentations of the tone-light cue, previously paired with methamphetamine infusions, but did not result in drug infusions. Inactive lever presses were used as a measure of non-specific activity and/or response generalization [42,43].

### Estrous cycle monitoring

To habituate female rats to vaginal swabbing, we started collecting vaginal swabs three days before the start of self-administration. We then monitored the estrous cycle during self-administration training (10 days), between abstinence days 24–29, and before seeking tests on abstinence days 27, 28, or 29. We tested both male and female rats across abstinence days 27–29 to balance the number of female rats within each estrous cycle stage. Before self-administration training and during the abstinence phase, we took vaginal swabs at the onset of the dark cycle. During self-administration training, we swabbed the rats daily 5 min before the start of the training session. On the testing day, we swabbed the rats 30

min before the start of the seeking test. Vaginal swabs were collected with saline-dipped cotton applicators as described previously [34,36,45,46]. Briefly, we gently inserted the cotton tip (< 1 cm) into the vaginal canal and performed one circular motion. We then collected the vaginal sample by rolling the cotton tip onto a glass microscope slide. After staining the slides with toluidine blue, we analyzed vaginal cytology using the Nikon DS-Fi3 camera attached to an inverted Nikon Eclipse Ti2 Series microscope. We classified each vaginal sample into four phases. Proestrus was classified based on the presence of ≥70 % of nucleated epithelial cells. Estrus was classified based on the presence of ≥70 % of non-nucleated cornified cells. Metestrus was classified based on the presence of an equal distribution of non-nucleated cornified cells and leukocytes, with or without the presence of nucleated epithelial cells. Diestrus was classified based on the presence of ≥70 % of leukocytes [45,47,48]. Representative toluidine blue staining of vaginal swabs in female rats was presented in our recent publication [46]. Note that 1 out of 170 vaginal samples (during self-administration) were classified as unidentified phase due to the low number of cells, and we excluded this sample from subsequent statistical analysis. Male rats were handled on an identical schedule.

## Fos immunohistochemistry

We perfused rats immediately after seeking tests on abstinence days 27–29. We anesthetized the rats with isoflurane and perfused them transcardially with ~100 ml of 0.1 M sodium phosphate (PBS) followed by 400 ml of 4 % paraformaldehyde (PFA) in PBS. We extracted the brains and postfixed them in 4 % PFA for 2 h, then transferred them to 30 % sucrose in PBS for 48 h at 4 °C. We froze the brains on dry ice and kept them at −80 °C until sectioning. We cut serial coronal sections (40 μm) using a Leica Microsystems cryostat and preserved the sections in cryoprotectant (20 % glycerol and 2 % DMSO in PBS, pH 7.4).

For Fos immunohistochemistry, we processed 1-in-5 series of sections from each rat for immunochemical detection of Fos. Free-floating sections were repeatedly rinsed in PBS (3 × 10 min washes) and incubated with 3 % Normal Goat Serum (NGS) in PBS with 0.25 % Triton X-100 for 1 h at room temperature. Next, we incubated the sections with anti-c-Fos primary antibody (1:5000, Cat #5348, Cell Signaling, RRID: AB_10557109) diluted in 3 % NGS in PBS with 0.25 % Triton X-100 overnight at 4 °C. The sections were then washed with PBS (3 × 10 min washes) and incubated with biotinylated goat anti-rabbit secondary antibody (1:600, Vector Laboratories, Cat# BA-1000, RRID: AB_2313606) diluted in 1 % NGS in PBS with Triton X-100 for 2 h at room temperature. Next, we washed the sections with PBS (3 × 10 min washes) and incubated the sections with avidin-biotin-peroxidase complex (ABC, ABC Elite Kit, #PK6100, Vector Laboratories) for 1 h at room temperature. We then washed the sections with PBS (3 × 10 min washes) and developed the sections in 3,3′-Diaminobenzidine (DAB) for 100 s. We washed the section in PBS (4 × 5 min) and mounted the section on glass slides (Fisherbrand™ Superfrost™ Plus Microscope Slides, Cat #12-550-15). Once dried, the slides were dehydrated in a series of ethanol (30 %, 60 %, 90 %, 95 %, 100 %, 100 %) and cleaned with Citrisolv (Fisher Scientific). We then cover-slipped slides with Permount (Fisher Scientific)

### Image acquisition and analysis

We digitally captured bright-field images of Fos immunoreactive (IR) cells in DMS and AIT-L using a Nikon DS-Fi3 camera attached to an inverted Nikon Eclipse Ti2 Series microscope. We captured and analyzed the images using NIS-Elements (Nikon, 5.20.00) at 10X magnification in a blind manner. We used an automatic counting method to quantify Fos-IR cells (inter-rater reliability between H.L and X.L $r = 0.97$, $p < 0.001$). For each rat, we analyzed two brain sections (4 hemispheres) in brain regions listed above in the following bregma levels: DMS, +1.6 to +1.2 mm; AIT-L, −2.4 to −2.7 mm.

### Experimental procedure

We performed intravenous surgery on 7 male rats and 17 female rats and trained them to self-administer methamphetamine for 10 days as described above. To minimize the carryover effect of extinction learning, we tested rats for methamphetamine seeking in a 30-min session on abstinence day 1. On abstinence days 27, 28, or 29, we tested rats for methamphetamine seeking in a 2-h session. We monitored their estrous cycles by vaginal swabs 3 days before the start of self-administration training, during the self-administration training, between abstinence days 24–28, and 30 min before seeking tests. Male rats were handled on an identical schedule. For the analysis of methamphetamine seeking data, we combined proestrus, diestrus, and metestrus together as the non-estrus stage, to be consistent with previous literature on cocaine and heroin seeking [34–37,49–51]. To ensure that there were enough estrus and non-estrus females, we tested rats for methamphetamine seeking on abstinence days 27, 28, or 29 (referred to as abstinence day 28 below). We anesthetized the experimental rats, perfused them, and extracted their brains immediately after seeking tests on abstinence day 28. We then processed brains for Fos immunohistochemistry and quantified the number of Fos-expressing cells in DMS and AIT-L.

### Statistical analysis

We analyzed the data with SPSS (version 27). We first tested the data for sphericity and homogeneity of variance (Levene's test). If the data did not violate sphericity or homogeneity of variance, we used parametric tests to analyze the data, including one-way ANOVA, repeated two-way ANOVA, repeated three-way ANOVA, or *t*-test as appropriate. If the sphericity assumption was not met, we used Greenhouse-Geisser correction to adjust the degrees of freedom. We followed up on significant interactions with the Tukey HSD post hoc test, or *t*-test, as appropriate. If homogeneity of variance was violated, we used the Kruskal-Wallis H test or the *t*-test with unequal variance. We followed up on the significant effects with pairwise comparison in the SPSS output sheets or *t*-tests with unequal variance. For the repeated measures analyses of the training data, we replaced 14 outlier values of inactive lever presses with the group mean for a given training day. We also removed outlier values from data presentation. We defined outliers as 3 median absolute deviations (MADs) above the group median [52], and we replaced 1 outlier (the highest value above the threshold) for each training day. In addition, we used the data from the day 1 seeking test and the first 30 min of the day 28 seeking test to calculate the incubation slope (Fig. 4). The equation is "Incubation slope = (active lever presses on abstinence day 28 – active lever presses on abstinence day 1)/(28 – 1)". Finally, we indicate the between and within-subject

factors of the different analyses in the Results section. All statistical comparisons are listed in Tables S1 and S2.

## Results

### Methamphetamine self-administration (Figs. 1–3)

Male and female rats escalated methamphetamine intakes across training days and preferred active lever associated with methamphetamine infusions over the non-reinforced inactive lever (Fig. 1). We analyzed the infusions using Training Day as a within-subject factor and Sex (Male, Female) as a between-subject factor (Fig. 1A). We found a significant main effect of Training Day ($F_{3.6,79.0} = 31.495$, $p < 0.001$) and a significant interaction between these two factors ($F_{3.6,79.0} = 3.58$, $p = 0.012$), but no significant main effect of Sex ($p > 0.05$). The significant interaction was due to some of the male rats having difficulty learning the operant task during the first few training days, and post hoc analyses showed no significant sex differences in infusions across training days. We also analyzed the average daily infusion using Sex as a between-subject factor and found no effect of Sex (Fig. 1B, $p > 0.05$). Taken together, these data showed that there were no sex differences during methamphetamine self-administration, which was further supported by analysis of lever presses described below.

We analyzed the lever presses using Training Day and Lever as within-subject factors and Sex as a between-subject factor (Fig. 1A). We found a significant main effect of Training day ($F_{2.1,46.0} = 4.467$, $p = 0.016$) and Lever ($F_{1,22} = 46.347$, $p < 0.001$), but no significant main effect of Sex ($p > 0.05$). We also found a significant interaction between Training day and Lever ($F_{2.1,45.7} = 6.528$, $p = 0.003$), but no significant interaction between Training day and Sex, Lever and Sex, or interaction among these three factors ($p > 0.05$). Further, we analyzed the average daily lever presses using Lever as a within-subject factor and Sex as a between-subject factor (Fig. 1B). We found a significant main effect of Lever ($F_{1,22} = 45.326$, $p < 0.001$), but no main effect of Sex or an interaction between these two factors ($p > 0.05$).

Next, we analyzed the infusions and level presses within female rats across different estrous cycle stages during methamphetamine self-administration (Fig. 2). To examine whether the estrous cycle could differentially impact the acquisition and maintenance of methamphetamine self-administration, we analyzed the data from the first five (Fig. 2A) and the last five (Fig. 2B) training days separately. To account for individual variability, we also analyzed the data normalized to the average infusion (or active/inactive lever presses) across 5 training days within each rat. Our analysis included the Cycle stage as the between-subject factor (proestrus, estrus, metestrus, diestrus).

During the first five days, our analysis of both raw data and normalized data showed no significant effect of Cycle stage on infusions or active lever presses ($p > 0.05$). However, we found a significant effect of Cycle stage on inactive lever presses (raw data: $F_{3,81} = 5.522$, $p = 0.002$; normalized data: $F_{3,81} = 2.919$, $p = 0.039$). For the raw inactive lever presses, the post hoc analysis revealed that inactive lever presses in the proestrus stage were significantly higher than those in estrus ($p = 0.047$) and diestrus stage ($p < 0.001$). For normalized

inactive lever presses, the post hoc analysis revealed that normalized inactive lever presses in the proestrus stage were significantly higher than those in the diestrus stage ($p = 0.023$). The higher inactive lever presses during the proestrus stage were possibly due to more rats being in the proestrus stage during their first (9 out of 17 rats) and second self-administration training day (8 out of 17 rats). During the last five training days, our analysis of both raw data and normalized data showed no significant effect of Cycle stage for infusion or inactive lever presses ($p > 0.05$). For active lever presses, we found a significant effect of Cycle stage on the raw active lever presses ($H_3$=8.052 $p = 0.045$), but not on normalized active lever presses ($p > 0.05$), indicating that individual variabilities contributed to the statistical significance observed in the analysis of raw active lever presses. Taken together, our data demonstrated no effect of the estrous cycle on methamphetamine intake throughout self-administration in female rats.

Finally, we analyzed the percentage of time in each cycle stage during the first and last five days of methamphetamine self-administration using Cycle stage as a within-subject factor (Fig. 3). During the first five days, we found a significant effect of Cycle stage ($F_{1.9,31.1}$ = 6.189, $p = 0.006$). Post-hoc analyses showed that the percentage of time in the proestrus, estrus, and diestrus stage was significantly higher than in the metestrus stage. During the last five days, we found no significant effect of Cycle stage ($p > 0.05$). These data suggest that methamphetamine self-administration altered the estrous cycling patterns in female rats.

### Methamphetamine seeking test (Fig. 4)

We found no difference in methamphetamine seeking between male and female rats, or between non-estrus and estrus female rats on abstinence day 1 or day 28. We analyzed the lever presses with the between-subject factor of Sex (Male, Female) or Cycle stage (Non-estrus, Estrus) and the within-subject factor of Lever. Comparing sexes (Fig. 4Aa and 4Ab), we found significant main effects of Lever (abstinence day 1: $F_{1,22} = 16.495$, $p < 0.001$; abstinence day 28: $F_{1,22} = 47.837$, $p < 0.001$), but no significant effects of Sex or interaction between Lever and Sex ($p > 0.05$). Comparing cycle stages (Fig. 4Ba and 4Bb), we found significant main effects of Lever (abstinence day 1: $F_{1,15} = 17.139$, $p < 0.001$; abstinence day 28: $F_{1,14} = 21.807$, $p < 0.001$), but no significant effects of Cycle stage or interaction between Lever and Cycle stage ($p > 0.05$).

Finally, to examine the effect of Sex or Cycle stage on incubation of methamphetamine craving, we analyzed the active lever presses and the incubation slopes calculated from the seeking test on abstinence day 1, and the first 30 min of the seeking test on abstinence day 28 (Fig. 4Ac and Fig. 4Bc). For active lever presses, we used the between-subject factor of Sex or Cycle Stage and the within-subject factor of Abstinence day (1, 28). We observed a significant main effect of Abstinence day (Left panel of Fig. 4Ac: $F_{1,22} = 65.674$, $p < 0.001$; Left panel of Fig. 4Bc: $F_{1,14} = 12.17$, $p = 0.004$), but no significant main effect of Sex (or Cycle stage) or significant interaction between Abstinence day and Sex (or Cycle stage) ($p > 0.05$). For incubation slopes, we used Sex or Cycle stage as a between-subject factor and found no effect of Sex (Right panel of Fig. 4Ac) or Cycle stage (Right panel of Fig. 4Bc) on the incubation slopes ($p > 0.05$).

### Fos expression in DMS and AIT-l after 2-h seeking test on abstinence day 28 (Fig. 5)

Finally, we found no difference in the number of Fos-expressing cells in either DMS or AIT-L between males and females, or between non-estrous and estrous females. We analyzed the number of Fos-expressing cells with the between-subject factor of Sex (Male, Female) or Cycle stage (Non-estrus, Estrus), and observed no significant effect of either factor ($p > 0.05$).

## Discussion

In this study, we compared incubation of methamphetamine craving and associated Fos expression in DMS and AIT-L between male and female rats, and across estrous cycle stages within female rats. We found no sex differences in methamphetamine self-administration, incubation of methamphetamine craving, or the number of Fos-expressing cells in DMS or AIT-L associated with methamphetamine seeking on abstinence day 28. Within female rats, we observed no difference in methamphetamine self-administration across estrous cycle stages, and both incubation of methamphetamine craving and associated Fos expression in DMS and AIT-L on abstinence day 28 were similar between non-estrus and estrus female rats. Together, our findings demonstrated that neither sex nor estrous cycle impacted incubation of methamphetamine craving and associated Fos expression in DMS and AIT-L under our experimental conditions.

### Effect of sex and estrous cycle on incubation of methamphetamine craving

Our finding about the lack of sex differences in methamphetamine self-administration is consistent with some studies [17,20,26,28,31,32, 53–58], but other studies found either a higher methamphetamine intake in male than female rats [10,12,14,59] or the opposite [22,29, 60]. These inconsistent findings can be due to several factors in the experimental designs, such as methamphetamine dose, duration of self-administration training, reinforcement schedules, and a combination with other behavioral procedures. In contrast, our finding about the lack of sex differences in incubation of methamphetamine craving is consistent with all previous methamphetamine incubation studies using either forced abstinence [10,12,14,26] or voluntary abstinence [31,32].

Regarding the estrous cycle, we found methamphetamine intake during methamphetamine self-administration was similar across different estrous cycle stages, which is in line with a previous study using a short-access methamphetamine self-administration procedure at a lower dose (0.05 mg/kg) [61]. Further analysis of vaginal cytology collected during methamphetamine self-administration revealed that the percentage of time in the metestrus stage was the shortest on average during the first five training days, which fits the pattern of natural cycling in female rats [62,63]. However, there were considerable variations among individual rats regarding the percentage of time in proestrus, estrus, and diestrus stages. During the last five training days, we found no effect of Cycle stage on the percentage of time. Across all training days, some rats spent more than 50 % of the time in proestrus and estrus stage, indicating that these rats may not be cycling anymore. These data together suggest that methamphetamine self-administration altered the cycling patterns in female rats, which is consistent with earlier studies showing that the non-contingent

administration of methamphetamine disrupts the estrous cycle in female rats [64] and long-term methamphetamine use in humans disrupts the menstrual cycles [65]. Therefore, we cannot rule out the possibility that disrupted or halted cycling masks the effect of estrous cycle during methamphetamine self-administration.

Consistent with previous work demonstrating no effect of estrous cycle on methamphetamine-primed reinstatement of methamphetamine seeking [22,25], we found that methamphetamine seeking was similar between non-estrus and estrus females on both abstinence day 1 and 28, and the incubation slope was also similar between non-estrus and estrus females. Together, these data indicated that estrous cycle had no effect on incubation of methamphetamine craving under our experimental condition. This finding is in contrast with previous cocaine studies that consistently demonstrated potentiated cocaine seeking in estrus compared with non-estrus females [34–36], providing additional evidence that cocaine and methamphetamine, despite both being psychostimulants, rely on different neural mechanisms to elicit drug relapse [7,66].

One limitation of our study is that we used a within-subject design to assess incubation of methamphetamine craving. Although we limited the test duration to 30 min on abstinence day 1 to minimize the carryover effect of extinction learning, we cannot rule out the possibility that testing rats during a specific estrous cycle stage on abstinence day 1 can impact the subsequent seeking test on abstinence day 28. For example, the high estrogen/progesterone levels during the proestrus stage have been shown to influence extinction learning during both fear [67,68] and appetitive conditioning [69,70]. Another limitation is that due to the inherent challenge to sample across four estrous cycle stages over a short period (e.g., between abstinence days 27 to 29), we had no metestrus rats and only 2 proestrus rats in the non-estrus group for the later seeking test. Therefore, it is possible that there are differences in methamphetamine seeking (or Fos expression) among proestrus, metestrus, and diestrus females after prolonged abstinence. However, we expect no cycle stage-dependent difference between metestrus and diestrus females because of the similar hormone levels between these two stages in rodents [71,72]. Previous studies also showed no differences across proestrus, metestrus, and diestrus in cue-induced cocaine seeking after abstinence [34,35,49,50,73]. Nevertheless, our data cannot fully rule out the potential difference between proestrus and other cycle stages.

### Effect of sex and estrous cycle on Fos expression associated with incubated methamphetamine seeking

We extended our previous work focusing on the role of AIT-L to DMS in incubation of methamphetamine craving in male rats [40] and demonstrated that neither DMS nor AIT-L exhibited sex-dependent or estrous cycle-dependent differences in Fos expression associated with methamphetamine seeking on abstinence day 28. This finding suggests that the role of DMS and AIT-L in incubation of methamphetamine craving, in terms of circuit activity, may generalize across sexes and estrous cycle stages. It is important to note that previous studies have demonstrated sex differences in dorsal striatum at both the biochemical and molecular levels associated with the dopaminergic system after prolonged abstinence from methamphetamine self-administration [14, 16]. Although the impact of estrous cycles on

these neuroadaptations after methamphetamine self-administration has not been investigated, previous work has shown that electrophysiological properties in dorsal striatum vary throughout the estrous cycle in naïve rodents [74–76]. Similarly, amphetamine-induced dopamine release in rat striatum also differs across the estrous cycle [77]. Therefore, it is possible that distinct molecular mechanisms in dorsal striatum underlie a similar level of Fos expression associated with incubated methamphetamine seeking across sexes and estrous cycle.

In addition, one study recently found increased Fos immunoactivity in dorsal striatum associated with methamphetamine-primed reinstatement of methamphetamine seeking in female rats, compared with male rats [23]. However, the use of different animal relapse models (extinction-based vs. abstinence-based) [78] precludes the validity of direct comparisons between these studies. For example, pharmacological inactivation of prelimbic cortex decreases both cue-induced and methamphetamine-primed reinstatement of methamphetamine seeking [79,80], but pharmacological inactivation of dorsomedial prefrontal cortex has no effect on incubated methamphetamine seeking [81].

Finally, a main limitation of our Fos study is that we did not include a No-test control group to measure the baseline Fos expression without the methamphetamine seeking test. However, our previous work using the same behavioral procedure has demonstrated an increased number of Fos-expressing cells in rats tested incubated methamphetamine seeking compared to rats from the No-test group in AIT-L of both male [40] and female rats [11], and DMS of male rats [9]. Regarding Fos expression in DMS of female rats, we performed Fos immunohistochemistry on brain sections collected from a previous experiment [11]. We observed a significantly higher number of Fos-expressing cells in DMS of females after the incubated methamphetamine seeking test (209.8 ± 19.8, $n = 5$), compared with females in the No-test group (2.2 ± 1.5, $n = 5$, $p < 0.001$, unpublished data). In addition, the number of Fos-expressing cells in No-test females was low in both AIT-L (2.7 ± 0.5) and DMS (2.2 ± 1.5) with little variability, suggesting there is no effect of estrous cycle on the baseline Fos expression. Taken together, these data support the conclusion that there is neuronal activation, assessed by Fos expression, in both DMS and AIT-L associated with incubated methamphetamine seeking in male and female rats.

## Conclusions

Consistent with previous studies [10,12,14,26,31,32], we showed no sex difference in incubation of methamphetamine craving at the behavioral level. Extending these studies, we demonstrated the time-dependent increase of methamphetamine seeking after abstinence was independent of the estrous cycles, and Fos expression in DMS and AIT-L was independent of either sex or estrous cycles. Overall, our data suggest that in contrast to cocaine [34–39], the estrous cycle is not a critical factor that influences incubation of methamphetamine craving and the role of AIT-L and DMS in methamphetamine relapse, at least in terms of circuit activity, might generalize across sexes and estrous cycles in female rats.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Data availability

Data will be made available on request.

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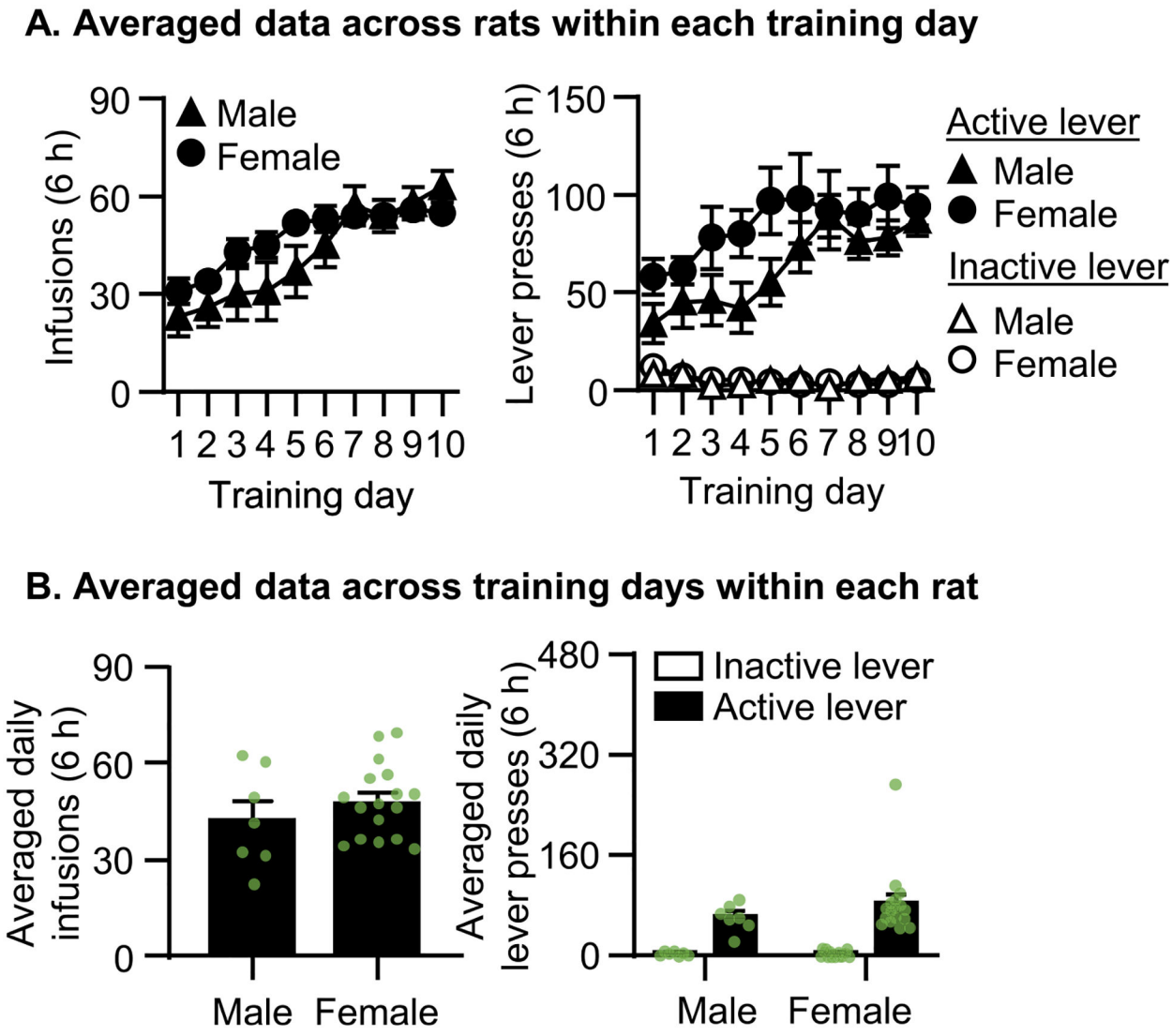


**Fig. 1. Sex had no effect on methamphetamine self-administration.**

**(A)** Methamphetamine self-administration in male and female rats. Data are mean ± SEM number of methamphetamine (0.1 mg/kg/infusion) infusions, and active and inactive lever presses across rats within each 6-h daily self-administration session. **(B)** Data are mean ± SEM number of methamphetamine infusions, and active and inactive lever presses across all ten 6-h daily self-administration sessions within each rat. Green circles represent data for the individual rat. n (male) = 7 and n (female) = 17.

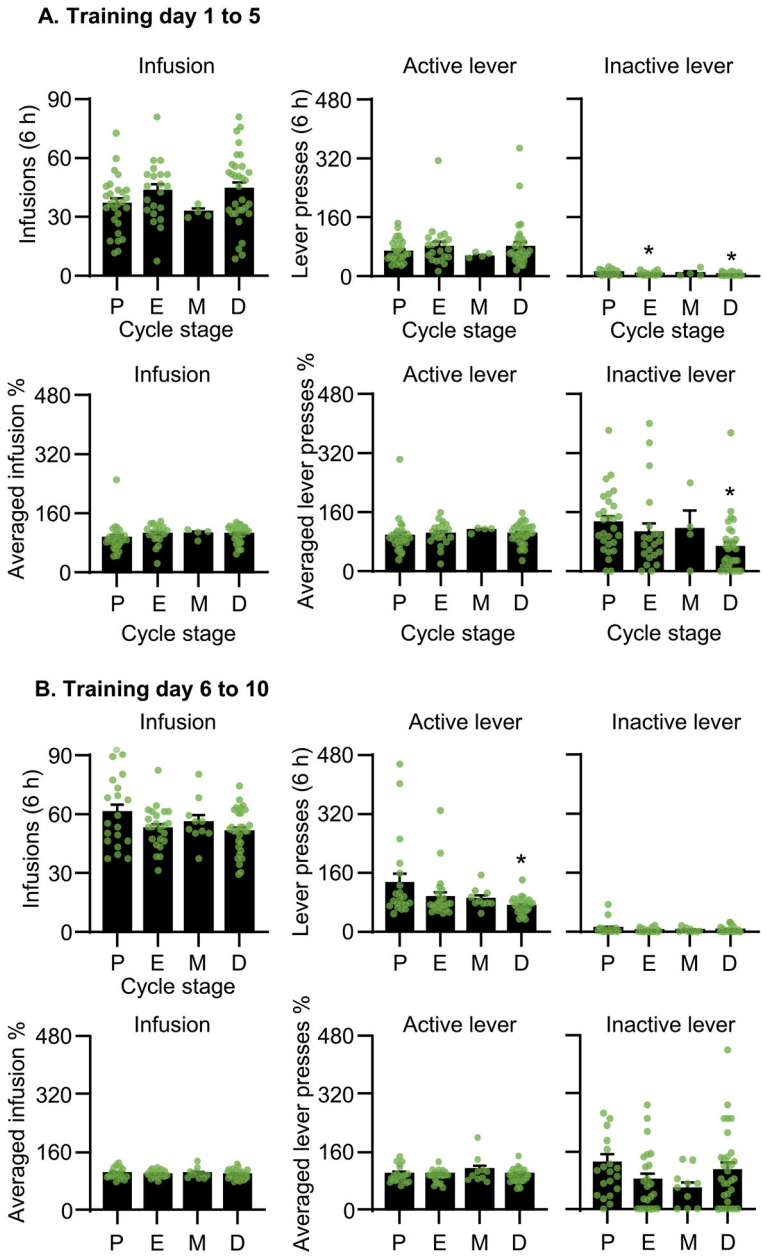


**Fig. 2. Estrous cycle had no effect on methamphetamine self-administration.**

**(A)** Data are mean ± SEM number of methamphetamine infusions, active and inactive lever presses (or normalized to the 5-day average within each rat) across proestrus, estrus, metestrus, and diestrus stages over the first five days of self-administration sessions. Green circles represent individual data for each cycle stage. *Different from proestrus, $p < 0.05$. n (proestrus) = 28, n (estrus) = 21, n (metestrus) = 4, n (diestrus) = 32 from all 17 female rats. (B) Data are mean ± SEM number of methamphetamine infusions, active and inactive lever presses (or normalized to the 5-day average within each rat) across proestrus, estrus, metestrus, and diestrus stages over the last five days of self-administration sessions. Green circles represent individual data for each cycle stage. *Different from proestrus, $p < 0.05$. n

(proestrus) = 20, n (estrus) = 23, n (metestrus) = 10, n (diestrus) = 31 from all 17 female rats.

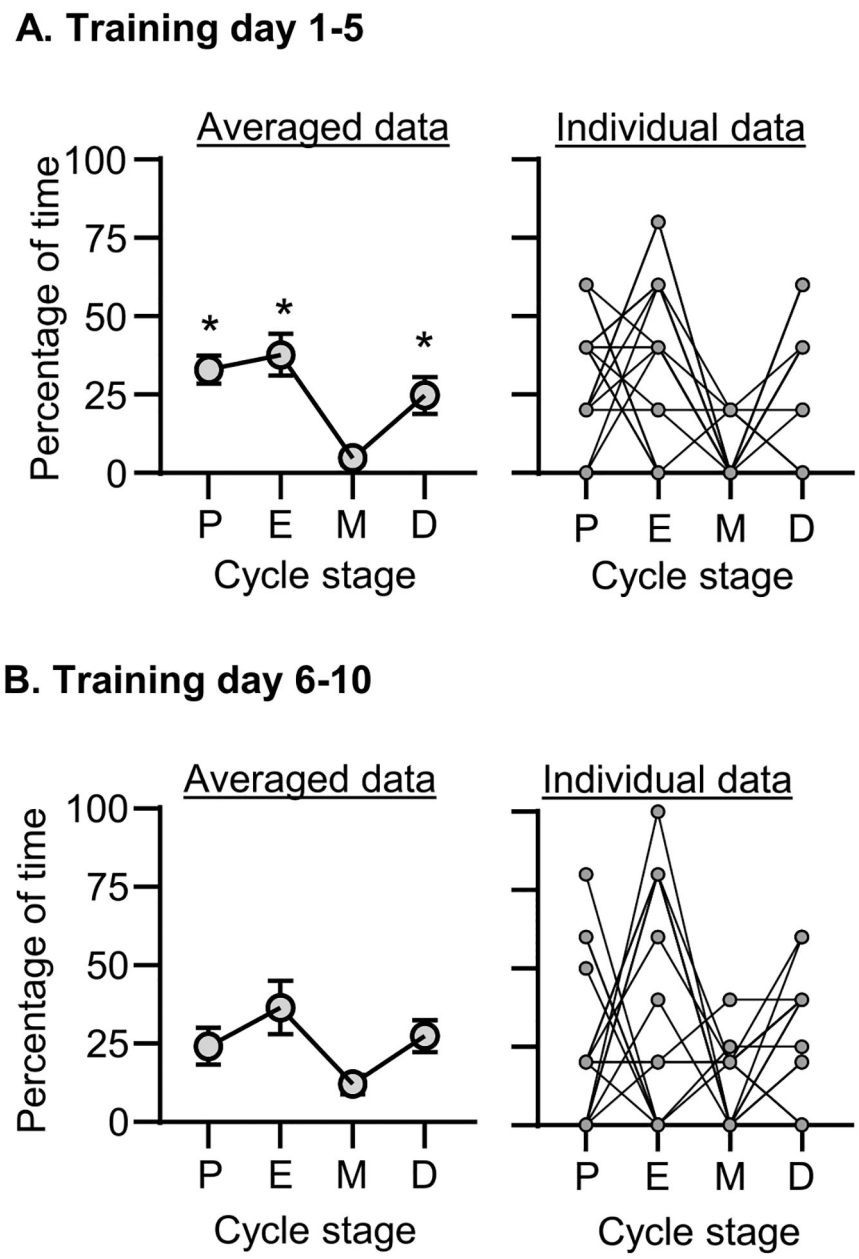


**Fig. 3. Percentage of time in each estrous cycle stage during methamphetamine self-administration.**

**(A)** Training day 1–5. The percentage of time in each estrous cycle was presented as mean ± SEM across rats (left panel) and data for individual rats (right panel). *Different from metestrus, $p < 0.05$, $n = 17$. **(B)** Training day 6–10. The percentage of time in each estrous cycle was presented as mean ± SEM across rats (left panel) and data for individual rats (right panel). $n = 17$.

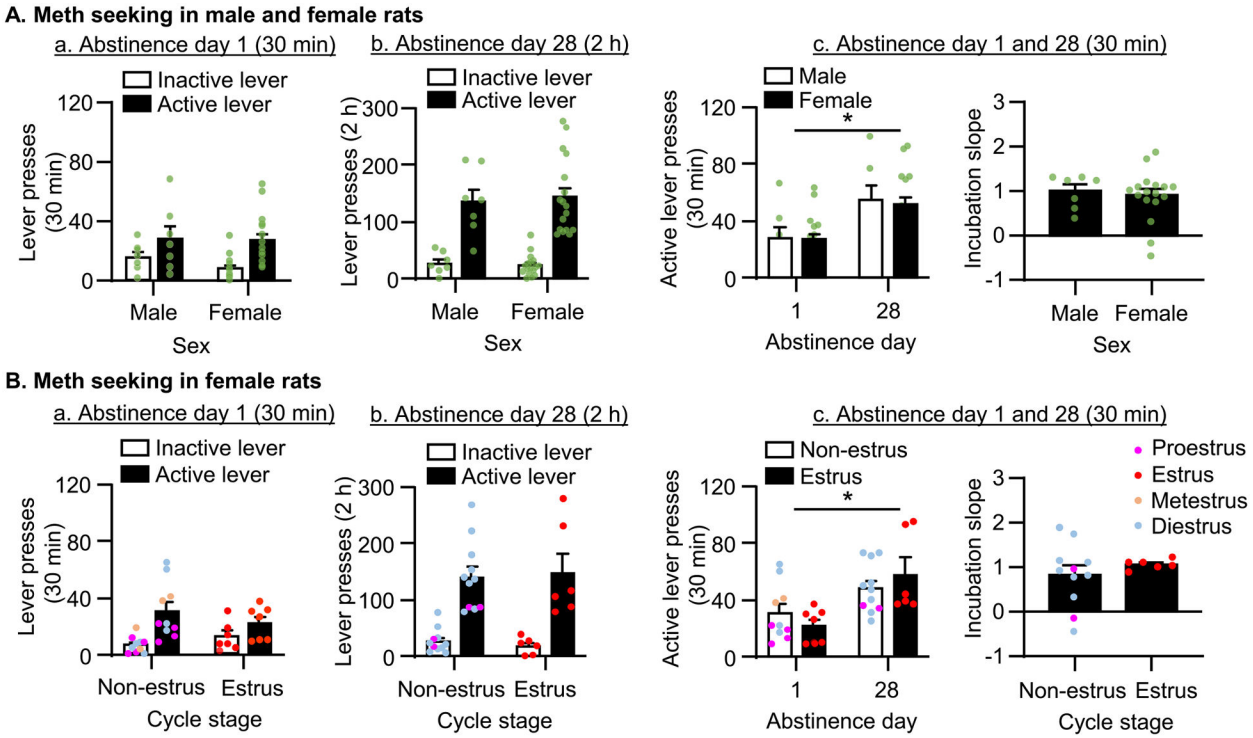


**Fig. 4. Sex or estrous cycle had no effect on incubation of methamphetamine craving.**

**(A-B)** Seeking test on abstinence days 1 and 28 in male and female rats. a) Data are mean ± SEM of lever presses on the previously active and inactive lever during the 30-min seeking test on abstinence day 1. b) Data are mean ± SEM of lever presses on the previously active and inactive lever during the 2-h seeking test on abstinence day 28. c) Left panel: Data are mean ± SEM of lever presses on the previously active and inactive lever during the 30-min seeking test on abstinence day 1 and the first 30 min of the 2-h seeking test on abstinence day 28. Right panel: Data are mean ± SEM of calculated incubation slopes using data from the 30-min seeking test on abstinence day 1 and the first 30 min of the seeking test on abstinence day 28. Incubation slope = active lever presses on abstinence day 28 – active lever presses on abstinence day 1/(28–1). Green circles represent data for the individual rat. Magenta circles represent individual data from female rats in the proestrus stage; red circles represent individual data from female rats in the estrus stage; orange circles represent individual data from female rats in the metestrus stage; blue circles represent individual data from female rats in the diestrus stage. *Main effect of Abstinence Day, $p < 0.05$. n (male) = 7 and n (female) = 17. Abstinence day 1: n (non-estrus) = 10 and n (estrus) = 7. Abstinence day 28: n (non-estrus) = 11 and n (estrus) = 6.

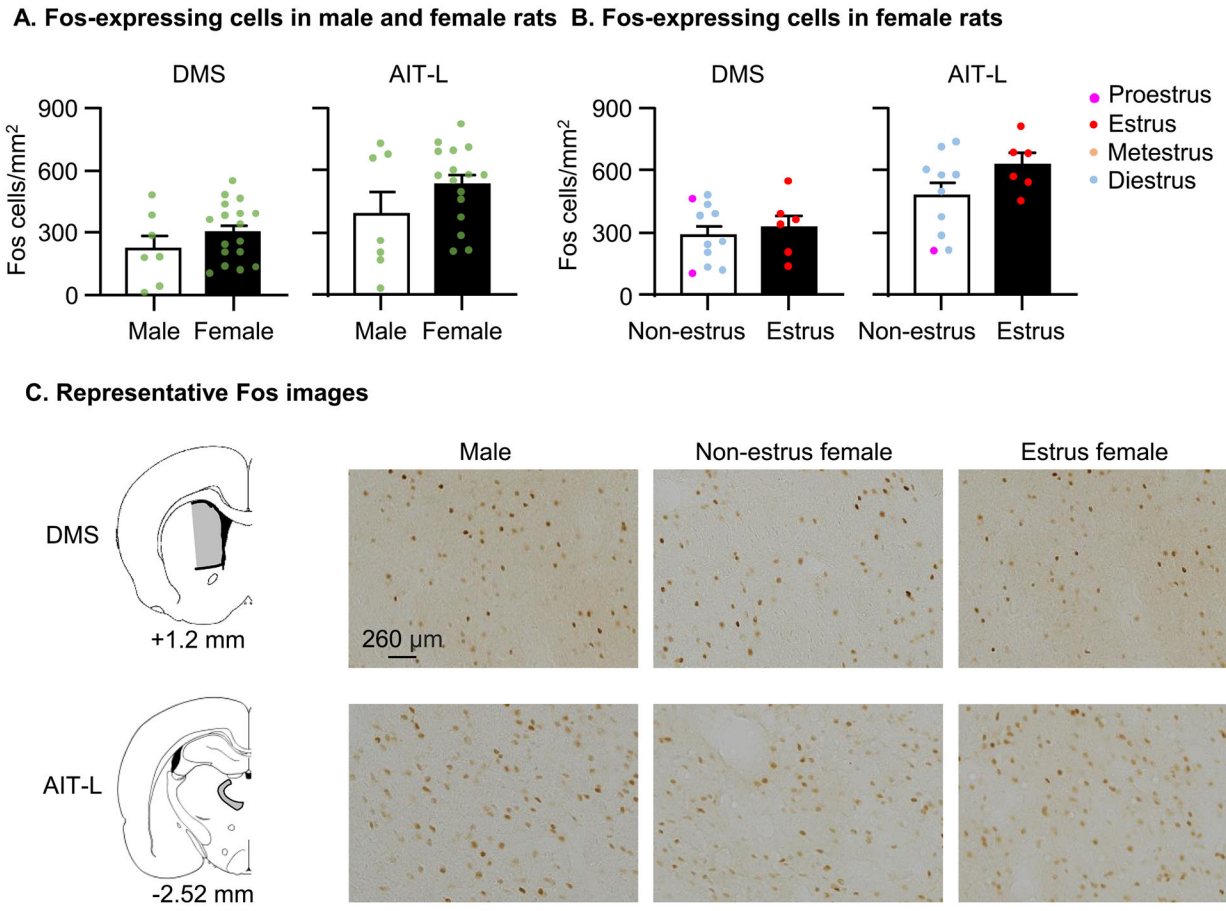


**Fig. 5. Sex or estrous cycle had no effect on Fos-expression associated with incubated methamphetamine seeking in DMS or AIT-L.**

Data are mean ± SEM of the number of Fos-expressing cells/mm$^2$ for each brain region in male and female rats **(A)** or in female rats across the estrous cycle **(B)**. Green circles represent data for the in-dividual male or female rat. Magenta circles represent individual data from female rats in the proestrus stage; red circles represent individual data from female rats in the estrus stage; orange circles represent individual data from female rats in the metestrus stage; blue circles represent individual data from female rats in the diestrus stage. n (male) = 7, n (females) = 17, n (non-estrus) = 11, and n (estrus) = 6. **(C)** Representative Fos image for each brain region.