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mGlu5 inhibition in the basolateral amygdala prevents estrous cycle-dependent changes in cue-induced cocaine seeking

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Abstract

Drug associated cues are a common relapse trigger for individuals recovering from cocaine use disorder. Sex and ovarian hormones influence patterns of cocaine use and relapse vulnerability, with studies indicating that females show increased cue-induced craving and relapse vulnerability compared to males. In a rodent model of cocaine craving and relapse vulnerability, cue-induced cocaine seeking behavior following weeks of withdrawal from extended-access cocaine self-administration is higher in females in the estrus stage of the reproductive (estrous) cycle (Estrus Females) compared to both Males and females in all other stages (Non-Estrus Females). However, the neuronal substrates and cellular mechanisms underlying these sex differences is not fully understood. One region that contributes to both sex differences in behavioral responding and cue-induced cocaine seeking is the basolateral amygdala (BLA), while one receptor known to play a critical role in mediating cocaine seeking behavior is metabotropic glutamate receptor 5 (mGlu5). Here we assessed the effects of BLA mGlu5 inhibition following prolonged withdrawal from cocaine self-administration on observed estrous cycle-dependent changes in cue-induced cocaine seeking behavior. We found that BLA microinjections of the mGlu5 antagonist MTEP selectively reduced the enhanced cue-induced cocaine seeking normally observed in Estrus Females while having no effect on cocaine seeking in Males and Non-Estrus Females. These findings identify a unique interaction between cocaine-exposure, estrous cycle fluctuations and BLA mGlu5-dependent transmission on cue-induced cocaine seeking behavior.

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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.

CRediT authorship contribution statement

Claire M. Corbett: Data curation, Investigation, Writing – review & editing. **Emily N.D. Miller:** Investigation, Writing – review & editing. **Jessica A. Loweth:** Visualization, Formal analysis, Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

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Keywords

Sex differences; Estrous cycle; mGlu5 inhibition; Basolateral amygdala; Cue-induced cocaine seeking

1. Introduction

Cocaine use disorder is a chronic, relapsing disease that is prevalent in both males and females. However, sex differences in patterns of cocaine use and relapse vulnerability have been reported in both clinical and preclinical models [1–5]. In humans, female cocaine users report shorter periods of abstinence after initiating cocaine use [6,7], longer periods of cocaine use following relapse [8] and greater cocaine craving and depressive symptomatology [9] relative to men. In rodents, female rats acquire cocaine self-administration more readily than males when tested at low infusion doses [10,11] and are more motivated to obtain cocaine [12,13]. Female rats also show greater cocaine-primed reinstatement of previously extinguished cocaine seeking behavior compared to males [14] and develop addiction-like features earlier during withdrawal than males [15]. However, less is known regarding the cellular and molecular mechanisms driving these behavioral sex differences in patterns of cocaine use and relapse vulnerability. Such studies are necessary to identify therapeutic targets to effectively reduce craving and promote abstinence in recovering users of cocaine of both sexes.

Cues are one of the most common relapse triggers. In rodents, cue-induced cocaine seeking progressively increases during the first month of forced abstinence or withdrawal from extended-access cocaine self-administration, a phenomenon referred to as incubation of cue-induced cocaine craving [16–18]. Incubation of cue-induced cocaine craving has also been shown to occur in humans [19] and is thought to reflect increased relapse vulnerability. Our lab [20] and others [21,22] have identified sex differences in incubated cue-induced cocaine seeking in which withdrawal-dependent changes in cocaine seeking behavior are influenced by the rat reproductive (estrous) cycle. Following a few weeks of withdrawal from extended-access cocaine self-administration, female rats in the estrus stage of the cycle (Estrus Females) show enhanced cue-induced cocaine seeking compared to both Males and females in all other stages of the estrous cycle (Non-Estrus Females; [20,22]). In rats, estrus is the stage of the cycle when ovulation occurs and when estrogen and progesterone have just dropped from peak levels but the ratio of estradiol to progesterone remains elevated [23]. While no sex differences in cue-induced cocaine seeking are observed during early withdrawal [withdrawal day (WD) 1–2; [20,22], Estrus Females show enhanced cue-induced seeking behavior compared to both Males and Non-Estrus Females on WD15 that further increases following a more prolonged withdrawal period (WD48) [20]. These findings indicate that relapse vulnerability changes across the estrous cycle, an effect which intensifies over the first month and a half of withdrawal. However, the neuronal substrates and cellular mechanisms driving these observed estrous cycle-dependent changes in cue-induced seeking behavior remain unknown.

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One brain region known to play a critical role in cue-induced drug seeking behavior [24–27] and the expression of incubated cue-induced cocaine craving [28,29] is the basolateral amygdala (BLA). There are also known sex- and estrous cycle-dependent differences in excitatory synaptic transmission in the BLA [30] and BLA-dependent behaviors are influenced by fluctuations in ovarian hormones across the reproductive cycle in both humans and rats [31,32]. We have recently shown an increase in the spontaneous activity of BLA pyramidal neurons in anesthetized male rats following two to three weeks withdrawal from extended-access cocaine self-administration [33]. Further increasing BLA activity during early withdrawal by exposing animals to repeated restraint stress also enhances cue-induced cocaine seeking on WD15 compared to non-stressed controls [33]. Together, these findings indicate that the level of BLA neuronal activity may directly influence withdrawal-dependent changes in cue-induced cocaine seeking behavior, including observed estrous cycle-dependent changes in cue-induced cocaine seeking behavior following prolonged withdrawal [20,22].

One potential mechanism mediating changes in BLA activity and sex differences in cue-induced cocaine seeking behavior is signaling via metabotropic glutamate receptor 5 (mGlu5), a member of the group I metabotropic glutamate receptor (mGlu) family that couples to Gq/11 and is primarily expressed postsynaptically on BLA pyramidal neurons [34]. Previous studies have shown that mGlu5-dependent transmission regulates both BLA neuronal activity and sex differences in behavioral responding to cocaine. First, chronic stress exposure leads to an mGlu5-dependent increase in neuronal excitability of BLA pyramidal neurons in mice of unspecified sex [35]. Second, surface-bound estrogen receptors couple with mGlu5 in the nucleus accumbens (NAc) to initiate postsynaptic signaling cascades necessary to elicit estradiol-induced potentiation of both cocaine-induced locomotor activity and cocaine self-administration in ovariectomized female rats [36]. However, the role mGlu5-dependent transmission in the BLA plays in the expression of observed estrous cycle-dependent changes in cue-induced cocaine seeking behavior following prolonged withdrawal has not yet been investigated. Based on these previously published findings, we hypothesized that the enhanced seeking behavior observed in Estrus Females is mediated by mGlu5-dependent transmission in the BLA. To test this hypothesis, we assessed the effects of inhibiting mGlu5-dependent transmission in the BLA on cue-induced cocaine seeking following prolonged withdrawal from extended-access cocaine self-administration in both males and females and across the estrous cycle. These studies will advance our understanding of the mechanisms driving sex differences in cocaine craving and relapse vulnerability.

2. Materials and methods

2.1. Subjects and surgery

Adult male (250–275 g upon arrival) and female (225–250 g upon arrival) Sprague-Dawley rats were purchased from Envigo (Dublin, VA and Frederick, MD, respectively). Rats were housed on a reverse light dark cycle (lights off at 9:00AM, on at 9:00PM) with food and water freely available. Rats were group housed by sex during the initial acclimation period (5–7 days) and singly housed immediately following surgery and for the remainder of the

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experiment. Following the acclimation period, rats underwent dual surgery to implant both a catheter into the jugular vein and bilateral guide cannula as described previously [17,37, 38]. Briefly, rats were anesthetized with ketamine & xylazine (males: 80 mg/kg & 10 mg/kg, i.p.; females: 60 mg/kg & 7.5 mg/kg, i.p.) and a silastic catheter (Plastics One, Roanoke, VA) was first inserted into the right jugular vein and passed subcutaneously to the mid-scapular region. Rats were then placed in a stereotaxic instrument (Stoelting Co, Wood Dale, IL) with the incisor bar positioned at 0.0 mm. Rats were chronically implanted with bilateral guide cannula (22 gage, Plastics One) aimed at the BLA (AP, −2.7; ML, ±4.8 - ±5.0; DV, −7.7). Coordinates are in millimeters from bregma and skull. Guide cannulae were positioned 1 mm dorsal to the injection site and secured to the scalp with screws and dental cement. Following surgery, 22 gage obturators were placed into the guide cannulae flush to the guide tips. All procedures were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the USPHS Guide for Care and Use of Laboratory Animals.

2.2. Estrous cycle monitoring

Estrous cycle was determined in intact, freely cycling females throughout the course of the experiment as described previously [20]. Briefly, females were swabbed at the onset of the dark cycle (~9am-10am) and approximately 30 min before the start of any behavioral test to determine estrous cycle stage when each session began. Vaginal samples were collected by gently swabbing the vaginal canal using a saline-dipped cotton-tipped applicator and “smearing” samples on microscope slides [39]. Males were handled on an identical schedule. Slides were stained with toluidine blue and examined using light microscopy. Estrous cycle stage (metestrus, diestrus, proestrus, estrus) was determined as described in detail in Corbett et al., [20]. All female rats exhibited normal cycling (4 to 5 day cycles) throughout the study. Based on both our previous findings and others showing enhanced incubated cue-induced cocaine seeking in Estrus Females compared to both Non-Estrus Females and Males and no differences in cue-induced seeking behavior across females in metestrus, diestrus and proestrus [20,22], females were divided into Estrus and Non-Estrus (metestrus, diestrus, proestrus) groups on the seeking test day.

2.3. Cocaine self-administration

Self-administration began ~7 days following implantation of intravenous jugular catheters and indwelling guide cannulae aimed at the BLA. As described previously [18,20,33], animals underwent saline or cocaine self-administration for 6 h/day for 10 days under a fixed-ratio-1 (FR1) reinforcement schedule (0.5 mg/kg/infusion). Each session started after the onset of the dark cycle (~10:00AM) in operant chambers (MED Associates, St. Albans, VT) equipped with active and inactive nose-poke holes. Nose-pokes in the active hole responses turned on the infusion pump and led to the delivery of a 20 s light cue and a 20 s timeout period, while nose-pokes in the inactive hole was without consequence. For each rat/chamber, pump times ranged from approximately 2 to 3 s and were adjusted based on body weight to deliver 0.5 mg/kg/infusion in a 100 μl/kg volume to all rats.

2.4. Microinjections and cue-induced seeking tests

Following cocaine self-administration and prolonged withdrawal (>WD47), microinjections were performed 10 min prior to seeking tests. Microinjection needles (28 ga, Plastics One,

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Roanoke, VA) were connected via PE-20 tubing to Hamilton syringes (2.5 μl, Hamilton, Reno, NV). Blockers were removed and MTEP (3.0 μg/μl) or aCSF (Vehicle) was directly injected through the guide cannulae over a 1 min period followed by an additional 1 min to allow for diffusion. This dose was selected based on previous reports showing that a single BLA microinjection of MTEP at 3 μg/μl eliminates cue-induced reinstatement of ethanol reinstatement [40] and that microinjections of this dose into the NAc shell has no effect on locomotor activity [41]. Seeking tests were conducted between WD48 - WD50 to ensure enough animals were in Estrus and Non-Estrus at the time of the test. Males were tested on an identical schedule in order to have an even distribution across withdrawal days within all groups. Some animals received seeking tests again two weeks later (WD68–69) following a counter-balance design, as previously reported [17]. However, due to differences in cycle stage across the two testing periods, data was only included once from the first or second seeking test (rather than using a within-subjects design; see Data Analyses). During the cue-induced seeking test, nose-pokes in the active hole resulted in presentation of the light cue previously paired with cocaine, but no drug infusion. Responding in the inactive hole had no consequence and serves as a control for general activity level. The number of times an animal responds in the active hole in this drug-free state provides the operational measure of cue-induced cocaine craving [16,42].

2.5. Drugs

Cocaine HCl was generously provided by the National Institute on Drug Abuse Drug Supply Program. The selective mGlu5 negative allosteric modulator MTEP was purchased from Tocris and dissolved in sterile artificial cerebrospinal fluid (aCSF) at a concentration of 3 μg/μl.

2.6. Histology

Within a week of completing the cue-induced seeking test, rats were anesthetized with ketamine & xylazine (see Subjects & Surgery for dosing) and perfused transcardially with saline (0.9%) and 10% formalin. Brains were removed and stored in 10% formalin at room temperature for at least 24 to 48 h. Brains were then sectioned at a thickness of 40 μm on a cryostat, mounted on Superfrost Plus slides (VWR, Radnor, PA) and stained with cresyl violet. Brain sections were examined under a light microscope to determine placement of guide cannula and location of the tip of the microinjection needle using the rat brain atlas [43]. Any animals with placements outside of the BLA were excluded from the analyses.

2.7. Data analyses

Self-administration data were analyzed using one-way, two-way and between-within ANOVAs with sex/cycle (Males, Non-Estrus, Estrus) and future drug treatment (Vehicle, MTEP) as the between-subjects factors and self-administration day (1–10) as the within-subjects factor. Seeking test data (active and inactive hole responding) were analyzed using one-way ANOVAs to assess incubation of cocaine craving and between-within and two-way ANOVAs to assess effects of BLA MTEP microinjections on incubated craving. For these analyses, drug treatment (Vehicle, MTEP) and sex/cycle (Males, Non-Estrus, Estrus) were the between-subjects factors and time (10, 20, 30 min) was the within-subjects factor.

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Data analyses were performed using Statistica (Tibco) and Graphpad Prism. Statistical significance was set at $p < 0.05$. All data are expressed as the mean ± SEM.

3. Results

3.1. No sex differences in cocaine self-administration

Animals self-administered cocaine (0.5 mg/kg/infusion) under extended-access conditions (6 h/day for 10 days) known to produce incubation of cue-induced cocaine craving in both males and females [17,18,20,33]. Consistent with previously published findings from our lab [20] and others [22], no sex differences in cocaine intake or self-administration behavior (active and inactive hole responding) were observed across the 10 day self-administration period between males and females (Fig. 1). One-way ANOVAs conducted on average responding across the 10 day period revealed no significant effect of sex on active hole responding ($F_{1,34} = 0.08$, $p = 0.78$), number of infusions obtained ($F_{1,34} = 0.139$, $p = 0.71$), or inactive hole responding ($F_{1,34} = 2.49$, $p = 0.12$) (Fig. 1A). Similarly, the between-within ANOVA conducted on daily responding across the 10 day self-administration period with sex (males, females) as the between-subjects factor and self-administration day (1–10) as the within-subjects factor revealed no effect of sex or interaction between sex and self-administration day, respectively, on active hole responding ($F_{1,34} = 0.08$, $p = 0.77$; $F_{9,306} = 1.18$, $p = 0.30$), infusions obtained ($F_{1,34} = 0.138$, $p = 0.71$; $F_{9,306} = 0.82$, $p = 0.60$), and inactive hole responding ($F_{1,34} = 2.49$, $p = 0.12$; $F_{9,306} = 0.26$, $p = 0.98$ (Fig. 1B). Due to variation in responding across time, there was a significant effect of self-administration day on number of infusions obtained ($F_{9,306} = 2,73$, $p = 0.004$) and inactive hole responding ($F_{9,306} = 4.02$, $p < 0.001$) and a near-significant effect on active hole responding ($F_{9,306} = 1.834$, $p = 0.06$). Estrous cycle effects on cocaine self-administration were not assessed in this study as we have previously shown no effects of estrous cycle on cocaine self-administration across the entire 10 day period [20].

Based on our previous findings showing enhanced cue-induced cocaine seeking behavior in Estrus Females compared to both Non-Estrus Females and Males [20], all rats were assigned to one of six groups based on sex/cycle (Males, Non-Estrus Females, Estrus Females) and drug treatment (Vehicle, MTEP) on the day of the seeking test. To ensure that any observed group differences in seeking behavior cannot be attributed to group differences in self-administration behavior, we compared cocaine intake and behavioral responding during cocaine self-administration between these six groups (Fig. 2). Two-way ANOVAs conducted on average responding across the 10 days of self-administration with sex/cycle (Males, Non-Estrus, Estrus) and future drug treatment (Vehicle, MTEP) as the between-subjects factors revealed no significant main effect of sex/cycle, no main effect of drug treatment and no interaction between sex/cycle and drug treatment for the following measures, respectively: active-hole nose-poke responding ($F_{2,30} = 0.96$, $p = 0.39$; $F_{1,30} = 0.0056$, $p = 0.94$; $F_{2,30} = 1.33$, = 0.28), cocaine infusions obtained ($F_{2,30} = 0.11$, $p = 0.89$; $F_{1,30} = 0.56$, $p = 0.46$; $F_{2,30} = 1.58$, $p = 0.22$), and inactive-hole nose-poke responding ($F_{2,30} = 1.59$, $p = 0.22$; $F_{1,30} = 0.09$, $p = 0.77$; $F_{2,30} = 3.1$, $p = 0.06$) (Fig. 2A). Similarly, the between-within ANOVA conducted on daily responding across the 10 day self-administration period with sex/cycle (Males, Non-Estrus Females, Estrus Females) and treatment group (Vehicle, MTEP) as the

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between-subjects factor and self-administration day (1–10) as the within-subjects factor revealed no significant effects of sex/cycle or treatment group and no interaction between the two, respectively, on active hole responding ($F_{2,30} = 0.96$, $p = 0.39$; $F_{1,30} = 0.0056$, $p = 0.94$; $F_{2,30} = 1.33$, $p = 0.27$), infusions obtained ($F_{2,30} = 0.11$, $p = 0.898$; $F_{1,30} = 0.56$, $p = 0.46$; $F_{2,30} = 1.58$, $p = 0.22$), and inactive hole responding ($F_{2,30} = 1.58$, $p = 0.22$; $F_{1,30} = 0.092$, $p = 0.076$; $F_{2,30} = 3.07$, $p = 0.06$ (Fig. 2B). Due to variation in responding across time, there was a significant effect of self-administration day on all three measures (active hole: $F_{9,270} = 2.97$; $p = 0.002$; infusions: $F_{9,270} = 2.94$, $p = 0.002$; inactive hole: $F_{9,270} = 4.97$, $p < 0.001$) (Fig. 2B).

3.2. Effects of BLA mGlu5 inhibition on estrous cycle-dependent changes in cocaine seeking behavior

Following cocaine self-administration and prolonged withdrawal or forced abstinence (>WD47), the effects of BLA mGlu5 inhibition on estrous cycle-dependent changes in cue-induced cocaine seeking behavior were assessed. Animals were divided into groups based on sex/cycle stage on the day of the seeking test (Males, Non-Estrus Females, Estrus Females) and received microinjections of vehicle (aCSF) or MTEP (3 μg/μl) 10 min before the cue-induced seeking test. As a result, the following 6 groups were generated: Males/Vehicle, Males/MTEP, Non-Estrus/Vehicle, Non-Estrus/MTEP, Estrus/Vehicle, Estrus/MTEP. This experiment was designed to assess the effects of inhibiting mGlu5-dependent transmission in the BLA following prolonged withdrawal, when estrous cycle effects on cocaine seeking behavior have both emerged and are pronounced compared to earlier withdrawal time-periods (e.g., 2 weeks, the earliest time-point we have identified when these sex differences are present) [20,22]. As a result, these animals did not receive a seeking test or intra-BLA infusions on WD1, a time-point when there are no effects of sex or estrous cycle fluctuations on cue-induced cocaine-seeking behavior [20,22]. However, to confirm that all animals showed a time-dependent increase in or incubation of seeking behavior, we compared their seeking behavior to average seeking behavior on WD1 from a separate group of animals (collapsed across sex/cycle) that underwent an identical self-administration paradigm. As expected, all 6 groups showed a clear increase in or incubation of cue-induced seeking behavior following prolonged withdrawal compared to average seeking behavior on WD1 (Fig. 3A). The one-way ANOVA conducted on these data with treatment group as the between-group factor revealed a significant main effect ($F_{6,45} = 18.72$, $p < 0.0001$). Tukey post-hoc tests revealed a significant increase in cue-induced seeking behavior in the active hole following prolonged withdrawal (>WD47) in all 6 groups compared to active hole responses on WD1 (dotted line, Fig. 3A). However, as expected, no change in inactive hole responding was observed compared to WD1 levels ($F_{6,45} = 0.38$, $p = 0.89$; dotted line, Fig. 3B).

Following prolonged withdrawal in vehicle-treated animals, an increase in cue-induced seeking behavior was observed in vehicle-treated Estrus Females compared to vehicle-treated Males and Non-Estrus Females, replicating previous findings [20,22] (Fig. 3A). Interestingly, inhibition of mGlu5-dependent transmission in the BLA prevented the enhanced cue-induced seeking behavior normally observed in Estrus Females, while having no effect on seeking behavior in Males or Non-Estrus Females (Fig. 3A). The ANOVA

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conducted on active-hole responses with sex/cycle (Males, Non-Estrus, Estrus) and drug treatment (Vehicle, MTEP) as the between-subjected factors revealed a significant effect of sex/cycle ($F_{2,30} = 7.78$, $p = 0.002$), no effect of drug treatment ($F_{1,30} = 2.005$, $p = 0.17$), and a significant interaction between drug treatment and sex/cycle ($F_{2,30} = 6.31$, $p = 0.0052$). As expected, tukey post-hoc tests revealed a significant increase in cue-induced cocaine seeking in vehicle-treated Estrus Females compared to both vehicle-treated Males (*, $p = 0.0023$) and vehicle-treated Non-Estrus females (*, $p < 0.001$) (Fig. 3A) [20,22]. Compared to vehicle-treated controls, microinjections of MTEP had no effect on seeking behavior in Males ($p = 0.999$) and Non-Estrus Females ($p = 0.73$). However, inhibiting mGlu5 in the BLA during the estrus stage of the estrous cycle (Estrus Females) significantly decreased seeking behavior compared to vehicle-treated Estrus Females (#, $p = 0.014$) (Fig. 3A). Analyses of seeking behavior over the 30 min seeking test session with sex/cycle (Males, Non-Estrus, Estrus) and drug treatment (Vehicle, MTEP) as the between-subjects factors and time (10, 20, 30 min) as the within-subjects factor similarly revealed a significant effect of sex/cycle ($F_{2,30} = 7.78$, $p = 0.0019$), no significant effect of drug treatment ($F_{1,30} = 2.00$, $p = 0.17$), and a significant interaction between sex/cycle and drug treatment ($F_{2,30} = 6.31$, $p = 0.005$). Tukey post-hoc analyses revealed an overall increase in seeking behavior in Estrus Females that received vehicle compared to vehicle-treated groups (Males, $p = 0.002$; Non-Estrus Females, $p = 0.00097$) and MTEP-treated groups (Males, $p = 0.003$; Non-Estrus Females, $p = 0.014$; Estrus Females, $p = 0.014$) (Fig. 3A). While there was a significant effect of time due to changes in seeking behavior across the session ($F_{2,60} = 13.23$, $p < 0.001$), no significant interaction between sex/cycle, drug treatment and time was observed ($F_{4,60} = 1.75$, $p = 0.15$). In contrast, there were no effects of sex/estrous cycle or drug treatment on inactive hole responding (Fig. 3B). The two-way ANOVA conducted on average inactive hole responding with sex/cycle (Male, Non- Estrus, Estrus) and drug treatment (Vehicle, MTEP) as the between-subjects factors revealed no significant effect of sex/cycle ($F_{2,30} = 0.00053$, $p = 0.99$) or drug treatment ($F_{1,30} = 0.59$, $p = 0.45$) and no interaction between the two ($F_{2,30} = 0.45$; $p = 0.65$) (Fig. 3B). Following completion of the study, histology was conducted to confirm microinjection placements within the BLA. Only animals with bilateral microinjection placements within the BLA, as shown in Fig. 4, were included in the analyses described here. Together, these data indicate that inhibiting mGlu5-dependent transmission in the BLA selectively prevents the enhanced cue-induced cocaine seeking behavior normally observed in Estrus Females following prolonged withdrawal from extended-access cocaine self-administration. As such, these findings identify mGlu5-dependent signaling in the BLA as a critical mechanism underlying estrous cycle-dependent changes in cue-induced cocaine seeking behavior.

4. Discussion

This study investigated the effects of inhibiting mGlu5-dependent transmission in the BLA on estrous cycle-dependent changes in cue-induced cocaine seeking behavior. Interestingly, we found that inhibiting mGlu5-dependent transmission in the BLA selectively reduced cocaine seeking behavior in Estrus Females but had no effect on cocaine seeking in MTEP-treated Males or Non-Estrus Females. These findings indicate a unique interaction between estrous cycle stage, mGlu5-dependent signaling in the BLA, and cocaine seeking behavior.

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4.1. Sex differences in effects of BLA mGlu5 inhibition on cue-induced cocaine seeking behavior

Our results show clear sex differences in the effects of BLA mGlu5 inhibition on cue-induced cocaine seeking following prolonged withdrawal, with a reduction in seeking behavior observed only in both MTEP-treated Estrus Females and no reduction in MTEP-treated Males and MTEP-treated Non-Estrus Females. While this is the first study to assess the effects of inhibiting mGlu5-dependent transmission directly within the BLA on cocaine seeking behavior in either males or females, microinjections of an identical dose of MTEP (3 μg/μl) into the BLA prevents the reinstatement of previously extinguished cue-induced ethanol seeking in male rats [40]. These latter findings indicate that one injection at this dose can effectively block mGlu5 receptors and inhibit drug seeking behavior. Therefore, the lack of effect of MTEP microinjections in the BLA on cue-induced seeking behavior in Males and Non-Estrus Females is unlikely to be dose-specific. Instead, it is more likely that our findings reflect estrous cycle-dependent changes in mGlu5-dependent neurotransmission in this region. In addition, previous studies have shown that microinjections of this same dose of MTEP (3 μg/μl) into the NAc shell reduces ethanol seeking but has no effect on locomotor activity [41]. Similarly, we observed no effects of BLA MTEP microinjections on inactive hole responding across all three groups (Fig. 3), a control for general activity level. These findings argue against non-specific effects of BLA mGlu5 inhibition as the underlying cause for the observed reduction in cue-induced cocaine seeking behavior in Estrus Females.

It is well-established that in male rats, systemic or intra-accumbens mGlu5 inhibition via administration of negative allosteric modulators like MTEP reduces cocaine-primed reinstatement and cue-induced reinstatement of cocaine seeking behavior [44–46]. However, these studies were conducted using extinction-reinstatement models [47–50], which are known to involve different neural substrates than those in abstinence models like the incubation model [51–53]. Systemic administration of MTEP also reduces incubated context-induced cocaine seeking and inactive hole responding [54], but to our knowledge site-specific effects of mGlu5 inhibition on incubated cue-induced cocaine craving have not been assessed. Systemic MTEP administration also robustly reduces incubation of methamphetamine craving and significantly reduces inactive hole responding and locomotor activity, indicative of a reduction in general behavioral activity [55]. Interestingly, intra-NAc core injections of MTEP have no effect on incubation of cue-induced methamphetamine craving, indicating that systemic effects of MTEP on incubated craving may be driven in part by an overall reduction in activity or that MTEP may act within other brain regions to inhibit incubated cue-induced methamphetamine craving [55]. To our knowledge our findings here are the first to assess site-specific effects of mGlu5 inhibition on the expression of cue-induced cocaine seeking behavior in incubated rats following prolonged withdrawal from extended-access cocaine self-administration. While our findings do not address the effects of BLA MTEP on the development of sex differences in incubated cocaine seeking behavior (i.e., administering MTEP during both early and later withdrawal time-points), they identify clear sex differences in cellular mechanisms in the BLA contributing to cue-induced cocaine seeking at later withdrawal time-points.

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4.2. Potential mechanisms mediating effects of BLA mGlu5 inhibition on enhanced cue-induced cocaine seeking in estrus females

There are several potential mechanisms through which mGlu5-dependent transmission in the BLA could be contributing to the enhanced cue-induced cocaine seeking behavior observed following prolonged withdrawal in Estrus Females. It is well known that mGlu5 activation can indirectly enhance excitatory transmission of pyramidal neurons via endocannabinoid-dependent activation of presynaptic CB1 receptors on GABAergic interneurons and inhibition of GABA release onto pyramidal neurons [56]. This includes depolarization-induced suppression of inhibition (DSI), which has been demonstrated in a mechanically isolated BLA pyramidal neuron preparation [57], and heterosynaptic long-term depression (LTD). Heterosynaptic LTD has been demonstrated in brain regions such as the hippocampus and PFC [58,59], with recent evidence from electron microscopy studies for this form of LTD in the BLA [34]. One possibility is that mGlu5-dependent transmission in the BLA is enhanced in cocaine-exposed Estrus Females, leading to enhanced excitatory transmission via disinhibition of BLA pyramidal neurons. In turn, inhibiting mGlu5-dependent transmission in the BLA would prevent this disinhibition, which may contribute to the observed reduction in seeking behavior in MTEP-treated Estrus Females. Future studies should investigate alterations in BLA mGlu5-dependent synaptic transmission and synaptic plasticity across the estrous cycle in cocaine-exposed rats, including the role ovarian hormones play in modulating these measures.

Another potential mechanism that could be contributing to the observed sex differences in BLA mGlu5-dependent inhibition of cocaine seeking is changes in mGlu5 protein expression levels in the BLA. Previous studies have shown that chronic social isolation stress increases anxiety-like behavior, intrinsic excitability of BLA pyramidal neurons, and BLA mGlu5 protein expression levels in mice of unspecified sex [35]. Inhibiting mGlu5-dependent transmission prevents this stress-induced increase in both anxiety-like behavior and neuronal excitability of BLA pyramidal neurons [35]. Although effects of cocaine exposure on BLA mGlu5 protein expression levels have not been assessed in either males or females, previous studies have shown that cocaine can increase mGlu5 protein levels in other brain regions in male rats [53] and there are known baseline sex differences in mGlu5 protein expression levels in the hippocampus [60]. One possibility is that total and/or surface protein expression levels of mGlu5 are selectively increased in the BLA in cocaine-exposed Estrus Females, which may increase BLA activity and contribute to both the enhanced seeking behavior observed in Estrus Females and the selective reduction in seeking behavior in MTEP-treated Estrus Females.

In female rats, there is a unique connection between group I mGlus (mGlu1 & mGlu5) and estrogen receptors (ERs) that has been identified in brain regions such as the hippocampus and NAc. Specifically, estradiol has been shown to act on surface-bound ERs that are coupled to group 1 mGlus [61]. Coupling of ERs/group 1 mGlus is regulated by the structural membrane protein caveolin and by palmitoylation, as estrogen receptors must be palmitoylated to signal on the cell surface [36,62]. ER-dependent activation of group I mGlus then results in the activation of Gq-mediated signaling cascades and alterations in synaptic transmission via endocannabinoid-dependent inhibition of presynaptic GABA

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release onto pyramidal neurons [36]. Importantly, estradiol-dependent potentiation of behavioral responding to cocaine in ovariectomized female rats is mediated by estrogen receptor-coupled mGlu5 receptors in NAc medium spiny neurons [63,64], effects which are endocannabinoid-dependent [65]. While this mechanism has not been identified or investigated in the BLA, ERα and ERβ are both expressed in this region and manipulations like chronic stress exposure can alter both mRNA and protein expression levels of ERα and ERβ as well as anxiety-and depression-like behavior [66]. These findings indicate that alterations in ER levels in the BLA can influence behavioral responding. Future studies should investigate the effects of cocaine exposure and estrous cycle fluctuations on mRNA and protein expression levels of ERα and ERβ as well as physical interactions and signaling between ERs and Group I mGlus. Based on previous reports in other brain regions [36], changes in ER and mGlu5 coupling in BLA pyramidal neurons across the estrous cycle could potentially influence mGlu5-dependent transmission and contribute to the selective reduction in cue-induce cocaine seeking in MTEP-treated Estrus Females observed here.

5. Conclusions

This study is the first to assess the effects of BLA mGlu5 inhibition on cue-induced cocaine seeking following prolonged withdrawal in both males and females and across the estrous cycle. Our findings indicate that mGlu5-dependent transmission in the BLA mediates the enhanced seeking behavior observed in Estrus Females following prolonged withdrawal from extended-access cocaine self-administration. These studies are the first to identify cellular and synaptic mechanisms underlying sex differences in cocaine seeking behavior and indicate that sex and estrous cycle must be taken into account when developing effective treatment strategies to promote abstinence in individuals recovering from cocaine use disorder.

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Data availability

Data will be made available on request.

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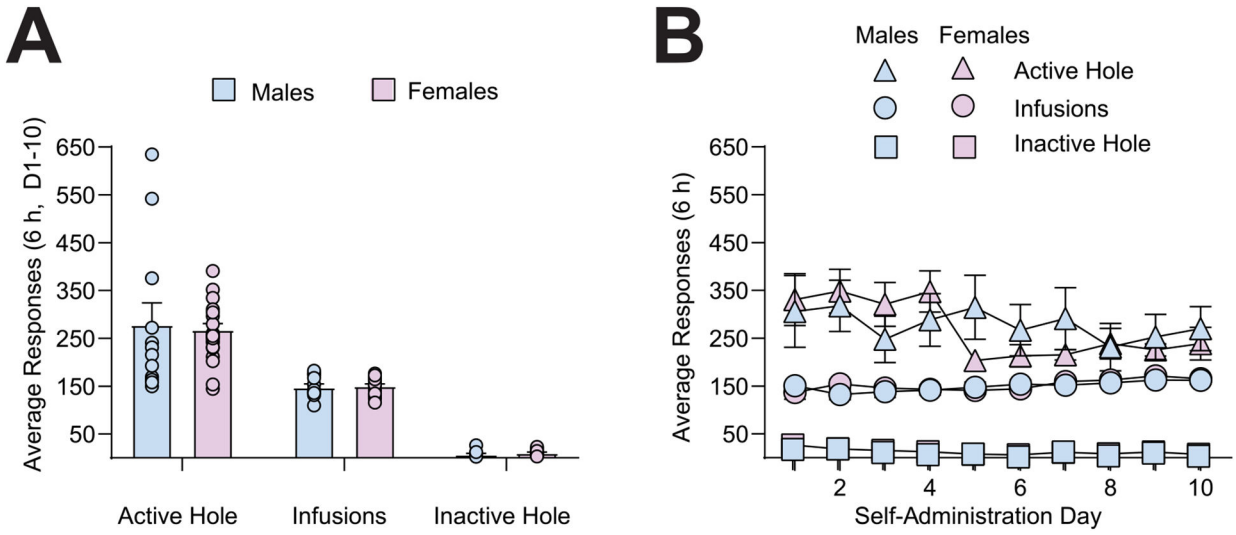

Fig. 1.

Males and females showed similar levels of cocaine intake and responding during cocaine self-administration. Consistent with our previous findings [20], no group differences between males and females were observed for average (**A**) or daily (**B**) active hole responding, cocaine infusions obtained and inactive hole responding across the ten days of cocaine self-administration. Data are shown as mean ± SEM. $n = 12$ males and $n = 24$ females.

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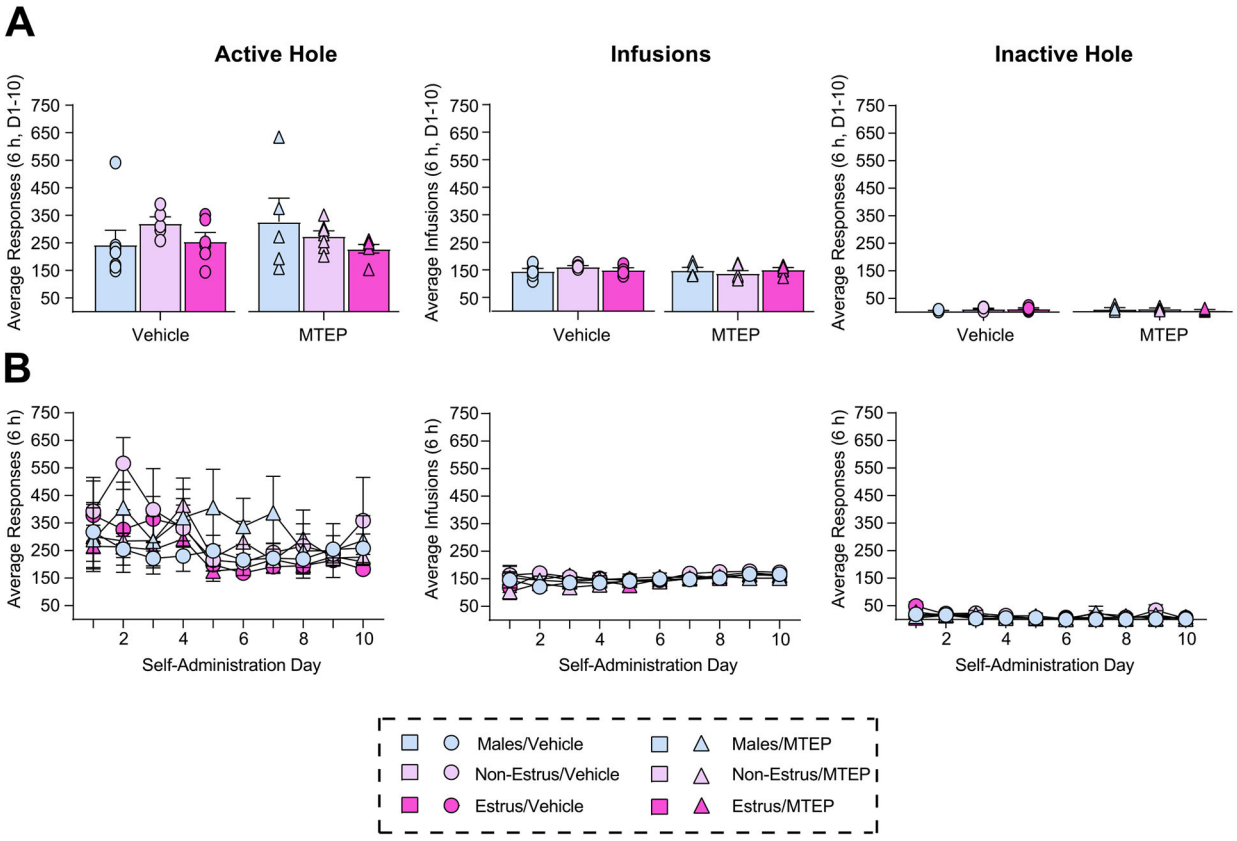

Fig. 2.

No group differences in cocaine self-administration between animals tested across different cycle stages and with different drug treatments. Animals were divided into 6 groups based on sex/cycle stage (Males, Non-Estrus Females, Estrus Females) and treatment (Vehicle or MTEP) on the day of the future seeking test. No group differences in average (**A**) or daily (**B**) active hole responding, cocaine infusions obtained or inactive hole responding were observed during self-administration, indicating that any observed group differences in incubated cocaine seeking behavior cannot be attributed to differences in cocaine intake or self-administration behavior. Data are shown as mean ± SEM. n = 5–7 rats per group.

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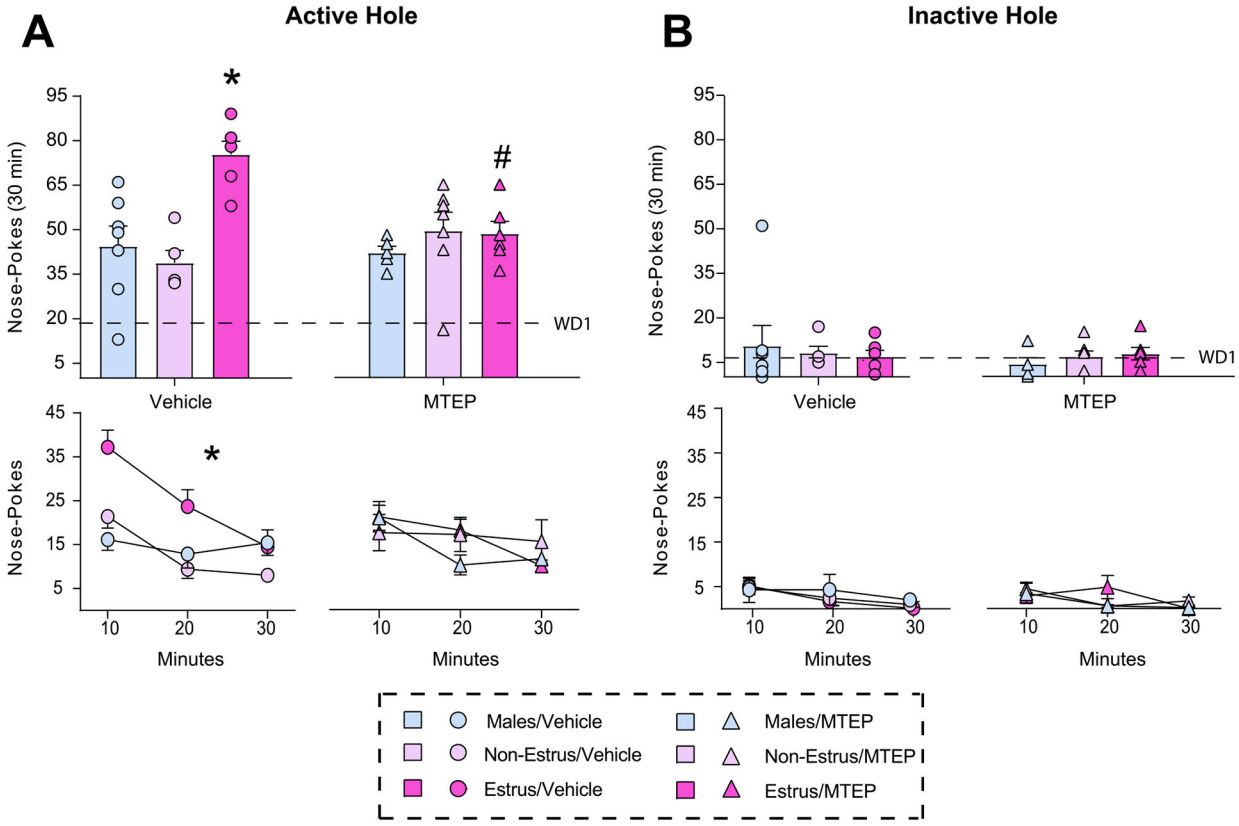

Fig. 3.

Inhibition of mGlu5-dependent transmission in the BLA selectively prevents the enhanced cue-induced cocaine seeking behavior normally observed in Estrus Females following prolonged withdrawal while having no effect on seeking behavior in both Males and Non-Estrus Females at this time-point. Top: Average active (**A**) and inactive (**B**) hole responding across the 30 min seeking test (mean ± SEM). All groups showed a time-dependent increase in or incubation of cue-induced cocaine seeking compared to average active hole responding on WD1, with no changes in inactive hole responding over time (dashed line). As expected, vehicle-treated Estrus Females showed enhanced cue-induced seeking behavior compared to vehicle-treated Non-Estrus Females and Males (*$p < 0.05$). Microinjections of MTEP into the BLA prevented the enhanced seeking behavior normally observed in Estrus Females (#, $p < 0.05$, vs. Estrus Vehicle) while having no effect on cocaine seeking in MTEP-treated Males and Non-Estrus Females. **Bottom:** Time-course of active (**A**) and inactive (**B**) hole responding across the 30 min seeking test. *$p < 0.05$, vs. vehicle-treated Non-Estrus Females & Males averaged across all time-bins. $n = 5–7$ rats per group.

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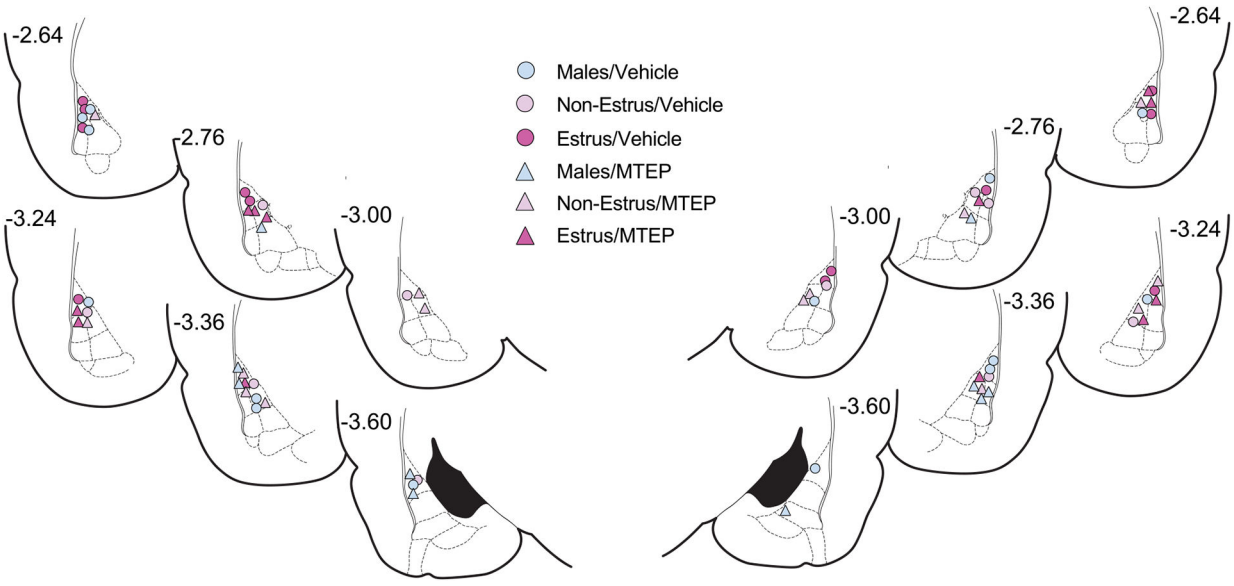

Fig. 4.

BLA Histology. Coronal sections adapted from Paxinos & Watson, 2007 [43] showing microinjection placements for vehicle- and MTEP-treated animals. Numbers above each section indicate distance (in mm) from bregma.