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**Opioid and sucrose craving are accompanied by unique behavioral and affective profiles
after extended abstinence in male and female rats**

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49 **Abstract**

50 Incubation of craving refers to the intensification of drug seeking behavior in response to
 51 reward-paired cues over the course of abstinence. In rodents, craving and drug-seeking
 52 behaviors have been measured by an increase in lever pressing in the absence of reinforcer
 53 availability in response to cue presentations. However, craving in rodents is difficult to define
 54 and little is known about the behavioral signatures that accompany increased drug-seeking
 55 behavior measured by lever pressing. The affective components of relapse are also important,
 56 but understudied in rodents. Hormonal fluctuations influence craving for psychostimulants, but
 57 little is known about the impact of the estrous cycle on opioid-seeking behavior. This study
 58 sought to delineate the behavioral and affective signatures associated with craving, and to
 59 examine the influence of the female estrous cycle on craving. Male and female rats underwent
 60 10 days of intravenous opioid self-administration. Separate cohorts of control rats self-
 61 administered oral sucrose, a natural non-drug reward. Cue-induced seeking tests were
 62 conducted after one or thirty days of forced abstinence. These sessions were recorded and
 63 scored for overall locomotion, instances of sniffing, grooming, or hyperactivity. Ultrasonic
 64 vocalizations were also recorded to determine affective profiles that accompany opioid seeking.
 65 Although active lever presses and overall locomotion increased unanimously over extended
 66 abstinence from heroin and sucrose, a sex and reinforcer-specific behavioral and affective
 67 signature of craving emerged. Furthermore, though the female estrous cycle did not affect
 68 taking or seeking, it appears to influence more granular behaviors.

70 **Significance statement**

71
 72 Craving was recently added as a diagnostic criterion for substance use disorders. Positive and
 73 negative affect may contribute to relapse, though this domain is not fully understood in the
 74 context of cue-induced drug seeking in rodents. To enhance the definition of craving and to
 75 address potential sex differences, we recorded behaviors and ultrasonic vocalizations during
 76 early and late abstinence cue-induced relapse tests in male and female rats. The behavioral
 77 signatures associated with craving were sex- and reinforcer-specific. In females, the estrous
 78 cycle selectively influenced locomotion and hyperactivity during reinforcer seeking. These
 79 findings contribute to a better behavioral definition of craving, which lays the foundation to better
 80 understand the neural mechanisms underlying craving and drug seeking behaviors.

82 **Introduction**

83 Craving is an important hallmark of substance use disorders that was recently added as
 84 a diagnostic criterion in the DSM. Re-exposure to drug-paired cues often triggers craving,
 85 defined as a compulsion to use the drug. Craving intensifies for several weeks after drug use
 86 cessation in humans (Gawin and Kleber, 1986; O'Brien et al., 1992) and rodent models of
 87 addiction, referred to as "incubation of craving" (Grimm et al., 2001; Pickens et al., 2011).
 88 Incubation of craving is a beneficial and translationally relevant model for studying relapse
 89 because 1) it occurs in both humans and rodents, and 2) in rodents, it mirrors the condition in

which drug use is discontinued and then individuals reintegrate into a familiar environment after some time away (hospitalization, incarceration, in-patient rehabilitation, etc.). Drug-related stimuli can gain salience over time and induce sensitization, known as incentive sensitization (Berridge and Robinson, 2016). One prominent construct in the addiction field is that incentive sensitization contributes to chronic, compulsive drug seeking, as well as other documented stereotyped behaviors during cue re-exposure (Tindell et al., 2005). This form of reward-related plasticity can last years after cessation of drug use (Paulson et al., 1991; Carr et al., 2020). Thus, incentive sensitization, which posits that drug-paired cues gain salience, likely contributes to incubation of craving where cue re-exposure elicits drug seeking even after prolonged abstinence. Importantly, these phenomena are thought to lead to high relapse rates.

Drug craving is subjective in humans and difficult to model in rodents. In preclinical self-administration paradigms, craving is often measured using the number of responses on a previously reward-associated lever when the reinforcer is not available. However, lever pressing does not capture other potentially important and more nuanced behaviors associated with drug seeking. This study built upon a wealth of previous behavioral sensitization studies and incorporated some of the drug-induced stereotypical behaviors in establishing a behavioral signature of opioid craving. Behavioral sensitization refers to documented, drug-induced, predictable (stereotyped) responses that emerge in both humans (Sax and Strakowski, 2001) and rodents (Steketee and Kalivas, 2011), and is not to be confused with incentive sensitization – a theoretical concept that stimuli can induce drug-seeking behavior. These specific stereotyped movements are predictive of drug-induced behavioral and neural plasticity and constitute the foundation of the behavioral signature defined in this study.

Craving for the natural reward sucrose also increases over extended abstinence and was used as a comparison to tease out opioid-specific profiles that accompany cue-induced reinforcer seeking. Sucrose is an important control group because it acts on many of the same neural pathways as drugs of abuse, and yet it is not the intended target of treatments aimed to

116 reduce craving. Given that incubation of craving happens for many drugs of abuse as well as
117 non-drug reinforcers like sucrose, this indicates that, in general, the brain is intensifying the cue-
118 reinforcer association over extended abstinence, regardless of reinforcer. Changes that are
119 consistent across reinforcers suggest overlap in the pathways that drive this process, some of
120 which can be adaptive (as is the case for remembering cues that are predictive of positive
121 natural rewards). However, by isolating drug-specific changes, it is possible to disentangle the
122 pathways that specifically drive maladaptive drug-cue association intensification throughout
123 abstinence. These are promising targets for treating opioid craving without affecting the natural
124 reward system.

125 Positive and negative affective states are thought to contribute to relapse potential
126 (McKay et al., 1996; Rubin et al., 1996; Witkiewitz and Bowen, 2010; Becker and Koob, 2016)
127 and clinical models of relapse include affective state (Witkiewitz and Marlatt, 2004). However,
128 this domain is not often considered in preclinical models of reinstatement or cue-induced
129 incubation of craving, particularly for opioids. Rodent affective states can be measured using
130 ultrasonic vocalizations (USVs). Low-frequency 22-kHz USVs (18-33 kHz) correspond to
131 negative-valence affective states and are emitted in situations of fear or anxiety. High-frequency
132 50-kHz USVs (38-90 kHz) correspond to positive-valence affective states and are emitted in
133 response to rewarding situations such as social play and drug or food self-administration
134 (Barker et al., 2015). Ultimately, USVs can elucidate affective components of opioid craving in
135 rats.

136 There are no sex differences in incubation of heroin craving, as measured by active
137 lever presses (Venniro et al., 2019); however, it is possible that males and females differ in
138 terms of more granular addiction-related behaviors. For example, females show enhanced
139 locomotor sensitization in response to experimenter-delivered methamphetamine (Robinson,
140 1984) and cocaine (Carr et al., 2020). Females also show greater incentive sensitization
141 following intermittent access cocaine self-administration (Kawa and Robinson, 2019a). Limited

142 studies have examined the role of biological sex in USV production. Female High Alcohol
143 Drinking (HAD-1) rats emit more 50-kHz USVs during ethanol access compared to males (Mittal
144 et al., 2019). It is unknown whether there is a sex-specific behavioral or affective signature
145 associated with sucrose or opioid craving that persists into protracted abstinence.

146 Another important consideration that may influence some of these behaviors is the
147 female estrous cycle. The estrous cycle affects addiction-like behaviors including cocaine CPP,
148 and incubation of cocaine craving (Paris et al., 2014; Calipari et al., 2017; Nicolas et al., 2019).
149 However, results related to whether the estrous cycle impacts opioid-related behaviors are
150 conflicting. Furthermore, few studies have examined the potential role of the estrous cycle on
151 sucrose self-administration or stereotyped behaviors. We address these gaps in knowledge by
152 including males and females and by tracking the estrous cycle throughout in our assessments of
153 intake, craving, and other addiction-related behaviors.

154 The overarching goal of this work was to delineate and fine-tune the more subtle
155 changes in behavior and affective state that accompany increased drug seeking in response to
156 cue re-exposure. We aimed to identify behaviors that accompany craving generally. Another
157 objective of this study was to identify drug-specific behavioral correlates of craving that do not
158 generalize to natural rewards. This method provides important insight into characteristics of
159 “normal” (non-pathological) craving behaviors versus those that specifically accompany heroin
160 craving. Furthermore, sucrose comparisons can aid in differentiating the mechanisms of cue-
161 reward associations and memory consolidation broadly from those that govern opioid-paired
162 cue-induced craving. These findings can 1) improve the preclinical model of opioid
163 craving/relapse-like behavior and 2) lay the groundwork for identification of circuits
164 governing opioid-cue reactivity. Males and females were included to address potential sex
165 differences. All measurements were tracked across the estrous cycle with the goal of identifying
166 whether fluctuations in gonadal hormones affect self-administration, craving, and/or any of the

167 more nuanced behaviors. These findings contribute to a better behavioral definition of craving,
 168 and aid in identifying opioid-specific neural circuits involved in craving.

169 **Methods**

170

171 Animals

172

173 For all experiments, male (n = 51) and female (n = 66) Long-Evans rats were bred in
 174 house. All animals used in the studies were 60-180 days old and were pair-housed. Rats were
 175 housed in a reverse light-dark cycle colony room with lights off at 9:00AM and were handled
 176 daily for 2-5 minutes each for at least five days prior to the start of any behavioral procedure or
 177 test. Experiments were conducted in the dark phase. Animals had *ad libitum* access to water
 178 and standard laboratory chow throughout the experiment. All animal procedures were
 179 performed in accordance with the [Author University] animal care committee's regulations.

180 Heroin self-administration, forced abstinence, and relapse tests

181 Rats used for intravenous heroin self-administration were anesthetized using an
 182 intraperitoneal injection of 80 mg/kg ketamine and 12 mg/kg xylazine prior to surgery. An
 183 indwelling silastic catheter was threaded subcutaneously over the shoulder blade, inserted in
 184 the jugular vein, and sutured in place. The catheter routed to a mesh back mount platform
 185 (Strategic Applications Inc., Lake Villa, IL), which was sutured below the skin between the
 186 shoulder blades. Catheters were flushed daily with 0.2 mL of timentin (0.93 mg/mL) dissolved in
 187 heparinized saline to prevent clogging. Following the 7-day recovery period, rats were placed in
 188 operant chambers and allowed to lever press on a fixed ratio 1 (FR1) schedule for heroin
 189 infusions (0.10 mg/kg/infusion over 5 seconds). Responses on the "active lever" resulted in one
 190 infusion, accompanied by a 5-second light cue and a subsequent 20-second timeout period
 191 during which the house light was off and lever presses were recorded but had no contingent
 192 drug infusion. Sessions began at the start of the animals' dark cycle (9:00
 193 AM). Heroin self-administration sessions followed an intermittent access paradigm, which
 194 included three hours of "drug-available" time, followed by a 15-minute window of "no-drug

195 available". This procedure repeated three times per session, resulting in a total of nine hours of
 196 drug availability, and was performed daily on 10 consecutive days. Heroin self-administering
 197 rats were tested behaviorally for signs of craving during early (Day 1) and late (Day 30)
 198 abstinence using a within-subjects design. Within-subjects relapse tests ("cue tests") began at
 199 the start of the animals' dark cycle and were 30 minutes long under extinction conditions,
 200 meaning that active lever presses resulted in cue light presentation, but no contingent reward.
 201 Throughout abstinence (one or 30 days), rats were housed in their home cage, were not re-
 202 exposed to the self-administration chambers, and were handled regularly.

203 Sucrose self-administration, forced abstinence, and relapse tests

204 Separate cohorts of male and female rats self-administered sucrose. This natural, non-
 205 drug reward acts as an important control group in establishing baseline behaviors that change
 206 over 30 days of forced abstinence. Unsurgurized drug-naïve rats responded on the "active lever"
 207 for one sucrose pellet (FR1 schedule) that was accompanied by a 5-second light cue, and a
 208 subsequent 20-second timeout period as previously described. Sucrose self-administration
 209 began at the start of the animals' dark cycle and occurred for two hours daily on 10 consecutive
 210 days. At the end of one or 30 days of forced abstinence (as described above), male and female
 211 rats were tested for sucrose seeking for one hour.

212 Recording relapse tests and scoring for stereotyped behaviors

213 Each cue test was video recorded for off-line scoring by investigators blind to condition.
 214 Videos were recorded in 5-minute increments via SD card dashcams (Pruveo, Blaine, WA)
 215 attached to the top of each sound-attenuating chamber to provide an aerial view of the operant
 216 box floor. Raw footage was then edited together using Adobe Premiere Pro (Adobe Inc., San
 217 Jose, CA) to create one continuous video.

218 Scored behaviors included horizontal locomotion and stereotypies. These were tracked
 219 using an online counter and timer. To measure horizontal locomotion, a two by two grid was
 220 superimposed over the floor of the operant chamber displayed on the computer screen in the

221 recording. The grid was measured to ensure that it was approximately 15 cm by 15 cm and that
 222 each quadrant was of equal size. Scorers then counted each “beam break”, or instance in which
 223 the rat moved from one quadrant of the chamber to another by crossing one of the grid lines
 224 with its head and shoulders. Time spent grooming was quantified by the amount of time an
 225 animal spent licking, pawing, or scratching itself. Time spent sniffing was quantified by the
 226 amount of time that an animal’s nose was in contact with the floor or walls of the operant box,
 227 either while stationary, or while mobile. The number of hops or darts across the operant
 228 chamber quantified instances of hyperactivity.

229 Audio recording and scoring ultrasonic vocalizations (USVs)

230 Ultrasonic vocalizations (USVs) were collected from a subset of rats during cue tests.
 231 Ultrasonic microphones from Dodotronic (Ultramic 200K) were placed atop the self-
 232 administration chamber and positioned inside a foam noodle to secure them in an upright
 233 position. Microphones were plugged into HP computers and USV recordings were obtained
 234 using Raven Pro 1.5 beta (Ithaca, NY) at a 192,000-Hz sampling rate in 10-minute segments.
 235 WAVE files were run through DeepSqueak, a Matlab-based USV detection program.

236 The Short Rat Call neural network was used to detect 50-kHz USVs. Calls were
 237 accepted or rejected based on if they met frequency criteria (38-90 kHz) and duration criteria (5
 238 ms or longer). Calls were differentiated from background noise by visual confirmation of a clear
 239 shape on the spectrogram. The Long Rat Call Network was used to detect 22-kHz USVs within
 240 the range of 18-33-kHz. Trained scorers counted the number of USVs during the duration of the
 241 cue test. If needed, calls were verified via playback in DeepSqueak. Calls in the 18–33-kHz
 242 range were termed “22-kHz USVs” and were interpreted as a negative-valence affective state.
 243 Calls in the 38–90-kHz range were termed “50-kHz USVs” and were interpreted as a positive-
 244 valence affective state (Barker et al., 2015).

245 Gonadal hormone measures

246 Gonadal hormone measures were analyzed in a subset of females. Throughout self-
 247 administration, vaginal lavage was conducted daily to collect loose vaginal cells. Lavage data
 248 was collected for a minimum of ten consecutive days prior to, as well as the day of each cue
 249 test. Vaginal cytology was conducted in order to determine the concentration and volume of
 250 three main cell types associated with distinct phases of the female estrous cycle: diestrus, as
 251 indicated by an abundance of sparse leukocytes, proestrus, as indicated by nucleated epithelial
 252 nuclei, and estrus, as indicated by dense, overlapping cornified cells. For the purposes of these
 253 experiments, the cycle was split into estrus (proestrus and estrus) and non-estrus (metestrus
 254 and diestrus) phases.

255 To determine the effect of the female estrous cycle on sucrose and/or heroin self-
 256 administration, the average number of pellets/infusions earned was calculated and compared
 257 across all female rats in estrus versus non-estrus during each day of self-administration. During
 258 the early and late abstinence cue tests, the number of active lever presses was averaged and
 259 compared for female rats in estrus versus those in non-estrus. Overall locomotion, sniffing,
 260 grooming, and hyperactivity were compared for each rat individually and grouped according to
 261 whether a given rat was in estrus or non-estrus on the day of the seeking test. Infusions/pellets
 262 earned were used as a proxy for opioid/sucrose consumption, whereas active lever presses
 263 during the cue test were used to interpret craving.

264 Statistical Analysis

265 Three-way ANOVAs were used to compare active lever responses during the Day 1 or
 266 Day 30 cue test with abstinence condition (Day 1 vs Day 30), sex, and reinforcer (sucrose vs
 267 heroin) as between-subjects factors. Three-way ANOVAs were used for overall locomotion,
 268 sniffing, grooming, hyperactivity, and 50-kHz ultrasonic vocalization analyses with sex,
 269 reinforcer, and abstinence condition as between-subjects factors. In all cases, if a significant
 270 interaction was found after performing ANOVAs, post-hoc comparisons were made using
 271 Bonferroni's multiple comparisons test.

272 For comparisons of heroin and sucrose taking across phases of the estrous cycle, mixed
 273 model ANOVAs were used to compare infusions/pellets earned during self-administration.
 274 Session (self-administration day 1 vs day 2 vs day 3 etc.) was used as the within-subjects
 275 factor, and condition (estrus vs non-estrus) was used as the between-subjects factor. Two-way
 276 repeated-measures ANOVAs were used to compare active lever presses during the early (Day
 277 1) and late (Day 30) abstinence cue tests. Abstinence condition (abstinence Day 1 vs
 278 abstinence Day 30) and estrous cycle phase (estrus vs non-estrus) were used as between-
 279 subjects factors. Again, if a significant interaction was found after performing ANOVAs, post-hoc
 280 comparisons were made using Sidak's or Bonferroni's multiple comparisons test. For
 281 comparisons of locomotion, sniffing, grooming, and hyperactivity in estrus versus non-estrus
 282 females in early and late abstinence, a two-way ANOVA was used. Abstinence day and estrous
 283 cycle phase were both between-subjects factors. Sidak's and Bonferroni's multiple comparisons
 284 tests were used if an interaction was detected.

285 **Results**

286 Incubation of sucrose and heroin craving are associated with reinforcer-specific behavioral 287 profiles

289 Male and female rats were allowed to intravenously self-administer (IVSA) heroin
 290 (0.10mg/kg/infusion) or self-administer sucrose pellets for 10 days, and were then assigned to
 291 either one (Day 1) or 30 days (Day 30) of forced abstinence. At the end of each respective
 292 abstinence time point, rats were tested for drug- or sucrose-seeking behavior. (**Figure 1A**). A
 293 camera was mounted to the ceiling of the sound-attenuating chamber and an ultrasonic
 294 microphone was positioned in the front right corner atop the Plexiglass ceiling of the operant
 295 chamber to score behaviors and record ultrasonic vocalizations (USVs), respectively (**Figure**
 296 **1B**). Active lever responses increased from abstinence Day 1 to abstinence Day 30 in heroin-
 297 and sucrose-treated males and females, indicating incubation of craving (**Figure 1C; additional**
 298 **statistics in Table 1**; day [$F_{1,232}=52.74$, $p<.0001$]). Overall locomotion, as measured by the total

300 number of times a rat crossed one of the superimposed beams bisecting the operant chamber
 301 in the video recording, increased over 30 days of abstinence from both heroin and sucrose in
 302 both sexes (**Figure 1D; additional statistics in Table 1**; day [$F_{1,232}=100.6$, $p<.0001$]).
 303 Grooming decreased in heroin males and females, and was unchanged in sucrose males and
 304 females after 30 days of abstinence (**Figure 1E; additional statistics in Table 1**; interaction
 305 [$F_{1,232}=4.318$, $p=.0388$]; Day 1 male heroin vs Day 30 male heroin, $p=.0138$; Day 1 male sucrose
 306 vs Day 30 male sucrose, $p>.9999$; Day 1 female heroin vs Day 30 female heroin, $p<.0001$; Day
 307 1 female sucrose vs Day 30 female sucrose, $p=.1848$, Bonferroni's multiple comparisons test).
 308 Sniffing was unchanged in all conditions, with the exception of an increase in sucrose-exposed
 309 females (**Figure 1F; additional statistics in Table 1**; interaction [$F_{1,232}=4.304$, $p=.0391$]; Day 1
 310 male heroin vs Day 30 male heroin, $p>.9999$; Day 1 male sucrose vs Day 30 male sucrose,
 311 $p=.1316$; Day 1 female heroin vs Day 30 female heroin, $p=.1848$; Day 1 female sucrose vs Day
 312 30 female sucrose, $p<.0001$, Bonferroni's multiple comparisons test). Hyperactivity, as
 313 measured by hops and darts across the cage, increased over 30 days of abstinence from
 314 heroin, but not sucrose in males and females (**Figure 1G; additional statistics in Table 1**;
 315 interaction [$F_{1,232}=12.11$, $p=.0006$]; Day 1 male heroin vs Day 30 male heroin, $p=.0050$; Day 1
 316 male sucrose vs Day 30 male sucrose, $p>.9999$; Day 1 female heroin vs Day 30 female heroin,
 317 $p<.0001$; Day 1 female sucrose vs Day 30 female sucrose, $p=.7376$, Bonferroni's multiple
 318 comparisons test).

Figure 1. Incubation of craving is associated with sex- and reinforcer-specific behavioral signatures. A. Experimental timeline including 10 days of intravenous heroin or oral sucrose self-administration, followed by a recorded cue test after one or 30 days of forced abstinence. B. Cameras were mounted on the ceiling of the sound-attenuating chamber to record movement and behaviors during the cue test. An ultrasonic microphone was positioned flush against the Plexiglas top of the operant box to record ultrasonic vocalizations. C. Sucrose- and heroin-exposed males and females showed incubation of craving, as indicated by an increase in active levers after 30 days of abstinence compared to one day of abstinence. Post-hoc comparisons revealed that sucrose females responded significantly more than heroin females and sucrose males in early abstinence (day [$F_{1,232}=52.74$, $p<.0001$]; Day 1 female sucrose vs Day 1 female heroin, $p=.0003$; Day 1 female sucrose vs Day 1 male sucrose, $p=.0402$, Bonferroni's multiple comparisons test). D. Overall locomotion, as measured by total beam breaks increased in heroin- and sucrose-exposed males and females over 30 days of abstinence (day [$F_{1,232}=100.6$, $p<.0001$]). E. Grooming decreased in heroin-exposed rats and was unchanged in sucrose-exposed rats of both sexes after 30 days of abstinence (interaction [$F_{1,232}=4.318$, $p=.0388$]; Day 1 male heroin vs Day 30 male heroin, $p=.0138$; Day 1 male sucrose vs Day 30 male sucrose, $p>.9999$; Day 1 female heroin vs Day 30 female heroin, $p<.0001$; Day 1 female sucrose vs Day 30 female sucrose, $p=.1848$, Bonferroni's multiple comparisons test). F. Sniffing was unchanged in all conditions, with the exception of an increase in sucrose-exposed females. Post-hoc comparisons revealed that sucrose males engaged in more sniffing than heroin males in late abstinence, and more sniffing than sucrose females in early abstinence (interaction [$F_{1,232}=4.304$, $p=.0391$]; Day 1 male heroin vs Day 30 male heroin, $p>.9999$; Day 1 male sucrose vs Day 30 male sucrose, $p=.1316$; Day 1 female heroin vs Day 30 female heroin, $p<.0001$; Day 1 female sucrose vs Day 30 female sucrose, $p=.1848$, Bonferroni's multiple comparisons test). G. Hyperactivity, as measured by hops and darts across the cage, increased over 30 days of abstinence from heroin, but not sucrose in males and females (interaction [$F_{1,232}=12.11$, $p=.0006$]; Day 1 male heroin vs Day 30 male heroin, $p=.0050$; Day 1 male sucrose vs Day 30 male sucrose, $p>.9999$; Day 1 female heroin vs Day 30 female heroin, $p<.0001$; Day 1 female sucrose vs Day 30 female sucrose, $p=.7376$, Bonferroni's multiple comparisons test). Additional statistics are listed in Table 1. Data show mean $\pm$ SEM; * $p<.05$.

320

321

322 Heroin-exposed male rats show an increase in 50-kHz USVs after 30 days of forced abstinence

323

324 22-kHz USVs are unchanged in heroin-exposed males and females (data not shown).

325 50-kHz USVs increased only in heroin-exposed male rats and were unchanged in all other

326 groups after 30 days of abstinence (**Figure 2; additional statistics in Table 1**; interaction

327 [$F_{1,51}=60.12$, $p<.0001$]; Day 1 heroin male 50-kHz calls vs Day 30 heroin male 50-kHz calls,

328 $p<.0001$; Day 1 sucrose male 50-kHz calls vs Day 30 sucrose male 50-kHz calls, $p>.9999$; Day

329 1 heroin female 50-kHz calls vs Day 30 heroin female 50-kHz calls, $p>.9999$; Day 1 sucrose

330 female 50-kHz calls vs Day 30 sucrose female 50-kHz calls, $p>.9999$, Bonferroni's multiple

331 comparisons test). Very few 22-kHz calls were observed during any of the cue tests. Many were

332 short in length and captured in conjunction with a series of 50-kHz calls. Compared to 50-kHz

333 calls, 22-kHz calls were very infrequent and did not change from early to late abstinence in
 334 either sex for either reinforcer.
 335

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 338

Figure 2. Incubation of heroin craving is associated with an increase in 50-kHz ultrasonic vocalizations (USVs) in males only (interaction [$F_{1,51}=60.12$, $p<.0001$]; Day 1 heroin male 50-kHz calls vs Day 30 heroin male 50-kHz calls, $p<.0001$; Day 1 sucrose male 50-kHz calls vs Day 30 sucrose male 50-kHz calls, $p>.9999$; Day 1 heroin female 50-kHz calls vs Day 30 heroin female 50-kHz calls, $p>.9999$; Day 1 sucrose female 50-kHz calls vs Day 30 sucrose female 50-kHz calls, $p>.9999$, Bonferroni's multiple comparisons test). Additional statistics are listed in Table 1. Data show mean $\pm$ SEM; * $p<.05$.

339
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 341

342 Estrous cycle does not impact heroin or sucrose self-administration nor incubation of craving

343
 344

Over ten days of self-administration females took increasing amounts of heroin (**Figure 3A**; time, [$F_{9,541}=3.821$, $p=.0001$], interaction [$F_{27,541}=4.405$, $p<.0001$]). There was no difference in heroin taking between females in estrus versus those in non-estrus (**Figure 3A**; estrus, [$F_{3,148}=.5299$, $p=.6624$]). Female rats experienced incubation of heroin craving following 30 days of abstinence (**Figure 3B**; time, [$F_{1,51}=18.02$, $p<.0001$]); however, craving was not affected by phase of the estrous cycle (**Figure 3B**; estrus, [$F_{1,51}=.0881$, $p=.7677$]; interaction,

350 [$F_{1,51}=1.989, p=.1645$]). Similarly, increasing amounts of sucrose were taken over 10 days of
 351 self-administration (**Figure 3C**; time, [$F_{9,760}=66.68, p<.0001$]), and there were no differences
 352 between females in estrus versus those in non-estrus (**Figure 3C**; estrus, [$F_{1,201}=.2342,$
 353 $p=.6290$]; interaction, [$F_{9,760}=.8406, p=.5787$]). Additionally, females experienced incubation of
 354 craving for the natural reward sucrose (**Figure 3D**; time [$F_{1,63}=12.57, p=.0007$]), and there was
 355 no effect of estrous cycle phase on sucrose craving (**Figure 3D**; estrus, [$F_{1,63}=.2057, p=0.6517$];
 356 interaction, [$F_{1,63}=.7248, p=.6517$]).
 357

Figure 3. The effect of the female estrous cycle phase (estrus versus non-estrus) on heroin and sucrose self-administration and craving. A. Female heroin-treated rats earned more infusions over 10 days of intravenous self-administration regardless of estrous cycle phase (time, [$F_{9,541}=3.821, p=.0001$], estrus, [$F_{3,148}=5.299, p=.0624$]; interaction, [$F_{27,541}=4.405, p<.0001$]). B. Females demonstrated incubation of heroin craving, as indicated by an increase in active lever responses during a 30-minute cue test following 30 days of abstinence, and there were no differences in active lever responses between estrus and non-estrus females at either abstinence time point (time, [$F_{1,51}=18.02, p<.0001$], estrus, [$F_{1,51}=0.0881, p=.7677$]; interaction, [$F_{1,51}=1.989, p=.1645$]). C. Female sucrose-treated rats earned more sucrose pellets over 10 days of oral self-administration regardless of estrous cycle phase (time, [$F_{9,760}=66.68, p<.0001$], estrus, [$F_{1,201}=0.2342, p=.6290$]; interaction, [$F_{9,760}=8.406, p=.5787$]). D. Females demonstrated incubation of sucrose craving, as indicated by an increase in active lever responses during a 1-hour cue test following 30 days of abstinence, and there were no differences in active lever responses between estrus and non-estrus females at either abstinence time point (time, [$F_{1,63}=12.75, p=.0007$], estrus, [$F_{1,63}=0.2057, p=.6517$]; interaction, [$F_{1,63}=0.7248, p=.6517$]). Data show mean $\pm$ SEM; * $p<.05$.

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362 Locomotion is the only behavior enhanced during estrus females after 30 days of abstinence
 363 from heroin compared to non-estrus

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365 Total beams breaks increased from Day 1 to Day 30 of abstinence in heroin-treated

366 females (**Figure 4A**; time [$F_{1,45}=33.58, p<.0001$]). Estrus females showed higher locomotion than

367 non-estrus females (**Figure 4A**; estrus, [$F_{1,45}=6.151, p=.0169$]; interaction,

368 [$F_{1,45}=0.5535, p=.4608$]). Heroin females experienced a decrease in grooming over 30 days of

369 abstinence that was not affected by phase of the estrous cycle (**Figure 4B**; time,

370 [$F_{1,45}=18.30, p=.0001$]; estrus, [$F_{1,45}=0.0093, p=.9235$]; interaction, [$F_{1,45}=1.762, p=.1911$]). Sniffing

371 increased over 30 days of abstinence from heroin regardless of phase of the estrous cycle

372 (**Figure 4C**; time, [$F_{1,45}=9.245, p=.0039$]; estrus, [$F_{1,45}=1.018, p=.3183$]; interaction,

373 [$F_{1,45}=0.0221, p=.8826$]). Interestingly, the increase in hyperactivity over 30 days of abstinence

374 from heroin was not affected by the phase of the estrous cycle (**Figure 4D**; time,

375 [$F_{1,45}=15.36, p=.0003$]; estrus, [$F_{1,45}=0.0558, p=.8144$]; interaction, [$F_{1,45}=0.2112, p=.6481$]).

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Figure 4. The effect of the female estrous cycle phase (estrus versus non-estrus) on addiction-related behaviors following one or 30 days of abstinence from heroin. A. Total beam breaks increased over 30 days of abstinence, and were enhanced in estrus females compared to non-estrus females (time [$F_{1,45}=33.58, p<.0001$]; estrus, [$F_{1,45}=6.151, p=.0169$]; interaction, [$F_{1,45}=0.5535, p=.4608$]). B. Grooming decreased over 30 days of abstinence regardless of estrous cycle phase (time, [$F_{1,45}=18.30, p=.0001$]; estrus, [$F_{1,45}=0.0093, p=.9235$]; interaction, [$F_{1,45}=1.762, p=.1911$]). C. Sniffing increased over 30 days of abstinence regardless of estrous cycle phase (time, [$F_{1,45}=9.245, p=.0039$]; estrus, [$F_{1,45}=1.018, p=.3183$]; interaction, [$F_{1,45}=0.0221, p=.8826$]). D. Hyperactivity increased over 30 days of abstinence and there was no effect of estrous cycle phase (time, [$F_{1,45}=15.36, p=.0003$]; estrus, [$F_{1,45}=0.0558, p=.8144$]; interaction, [$F_{1,45}=0.2112, p=.6481$]). Data show mean $\pm$ SEM; * $p<.05$.

378

379

380 Locomotion and hyperactivity are enhanced in estrus females after 30 days of abstinence from
 381 sucrose

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383 As with heroin-treated females, overall locomotion, as measured by total beam breaks

384 increased over 30 days of abstinence from sucrose (**Figure 5A**; time, [$F_{1,86}=54.91, p<.0001$]).

385 Again, locomotion was enhanced in estrus females compared to non-estrus females (**Figure**

386 **5A**; estrus, [$F_{1,86}=6.386, p=.0133$]; interaction, [$F_{1,86}=.0115, p=.9150$]). Grooming increased over

387 30 days of abstinence from sucrose (**Figure 5B**; time [$F_{1,86}=4.194, p=.0436$]), but was not
 388 affected by phase of the estrous cycle (**Figure 5B**; estrus, [$F_{1,86}=1.091, p=.2993$]; interaction,
 389 [$F_{1,86}=1.974, p=.11636$]). Similarly, sniffing increased over 30 days of abstinence from sucrose
 390 (**Figure 5C**; time [$F_{1,86}=23.98, p<.0001$]), but was not affected by phase of the estrous cycle
 391 (**Figure 5C**; estrus, [$F_{1,86}=0.003, p=.9568$]; interaction, [$F_{1,86}=.064, p=.8006$]). Hyperactivity was
 392 enhanced in estrus females at Day 30 of abstinence, but was not affected by estrous cycle
 393 phase in early abstinence (**Figure 5D**; time, [$F_{1,86}=3.923, p=.0508$]; estrus, [$F_{1,86}=5.459, p=.0218$];
 394 interaction, [$F_{1,86}=1.767, p=.1872$]; Day 1 estrus vs non-estrus, $p>.9999$; Day 30 estrus vs non-
 395 estrus, $p=.0479$, Bonferroni's multiple comparisons test).

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Figure 5. The effect of the female estrous cycle phase (estrus versus non-estrus) on addiction-related behaviors following one or 30 days of abstinence from sucrose. A. Total beam breaks increased over 30 days of abstinence, and were enhanced in estrus females compared to non-estrus females (time, [$F_{1,86}=54.91, p<.0001$]; estrus, [$F_{1,86}=6.386, p=.0133$]; interaction, [$F_{1,86}=0.0115, p=.9150$]). B. Grooming increased over 30 days of abstinence regardless of estrous cycle phase (time [$F_{1,86}=4.194, p=.0436$]; estrus, [$F_{1,86}=1.091, p=.2993$]; interaction, [$F_{1,86}=1.974, p=.11636$]). C. Sniffing increased over 30 days of abstinence regardless of estrous cycle phase (time [$F_{1,86}=23.98, p<0.0001$]; estrus, [$F_{1,86}=0.003, p=.9568$]; interaction, [$F_{1,86}=0.064, p=.8006$]). D. Hyperactivity was enhanced at Day 30 in estrus females, and there was no effect of estrous cycle on hyperactivity at Day 1 (time, [$F_{1,86}=3.923, p=.0508$]; estrus, [$F_{1,86}=5.459, p=.0218$]; interaction, [$F_{1,86}=1.767, p=.1872$]; Day 1 estrus vs non-estrus, $p>0.9999$; Day 30 estrus vs non-estrus, $p=0.0479$, Bonferroni's multiple comparisons test). Data show mean $\pm$ SEM; * $p<.05$.

Discussion

This study aimed to refine a more robust picture of behavioral and affective states that accompany opioid seeking after extended abstinence, when craving is high. After chronic self-administration of either intravenous heroin or the natural reward sucrose, rats underwent an abstinence paradigm that is known to induce craving after 30 days (Lu et al., 2004). Sucrose is a natural reward that acts on many of the same brain regions as drugs of abuse and leads to enhanced craving. Thus, it was important to establish behaviors that change in response to extended abstinence and cue re-exposure generally, and subsequently to differentiate opioid-specific behavioral changes. To that end, we explored overall locomotion, sniffing, grooming, hyperactivity, ultrasonic vocalizations (USVs), and estrous cycle phase. Results indicate a reinforcer-specific behavioral profile that emerges after 30 days of forced abstinence in heroin-exposed rats. Interestingly, total beam breaks increased over 30 days of abstinence regardless of sex and reinforcer, indicating that increased craving is associated with a general increase in locomotion. Heroin exposure resulted in a unique behavioral signature, including a decrease in grooming and an increase in hyperactivity that was seen in both sexes but did not generalize to the natural reward sucrose. Sniffing showed a sex- and reinforcer-specific effect, such that it increased in sucrose-treated females over 30 days of abstinence, and was unchanged in all other groups. Heroin-exposed males made significantly more 50-kHz USVs during the late

424 abstinence seeking test when craving was high, which was not seen in females, nor sucrose-
 425 exposed rats of either sex. Estrous cycle phase did not influence self-administration nor craving
 426 for either reinforcer. However, locomotion and/or hyperactivity were enhanced in estrous
 427 females in a reinforcer-specific manner. These results underscore the notion that opioids cause
 428 long-lasting neural adaptations, which take effect and persist throughout abstinence in order to
 429 prime the system for increased craving and compulsive drug seeking when re-exposed to drug-
 430 paired cues. These changes are characterized by decreased grooming and increased
 431 hyperactivity, which represent an opioid-specific behavioral profile of craving in response to cue
 432 re-exposure. An increase in positive-valence affective state is thought to drive this drug-seeking
 433 behavioral signature, though this may be sex-specific. The mechanism for explaining the
 434 different behavioral profiles in heroin- versus sucrose-exposed rats is undetermined and outside
 435 the scope of this report. Ultimately, the goal is that these descriptive data lay the foundation for
 436 further exploration of such a mechanism. Taken together, these results are promising in terms
 437 of developing treatments tailored to opioid craving and associated behaviors without interrupting
 438 the natural reward system.

439 **Increase in lever pressing is associated with increased locomotion in males and females,**
 440 **regardless of reinforcer**

441 Re-exposure to reward-paired cues not only induces an increase in seeking behavior,
 442 but also a general increase in overall locomotion, as measured by beam breaks. There are
 443 similarities between changes in locomotion/stereotypies and incubation of craving, such that
 444 both persist after long periods of abstinence (Paulson et al., 1991; Pickens et al., 2011;
 445 Madangopal et al., 2019). For example, locomotion increases during a cue-induced
 446 reinstatement test following chronic sucrose self-administration and 30 days of abstinence
 447 (Grimm et al., 2007; Aoyama et al., 2014; Harkness et al., 2016), though in some of these
 448 studies, rats had undergone extinction training prior to reinstatement. Following exposure to
 449 experimenter-delivered morphine, a priming injection stimulated increased locomotor
 450

451 sensitization after three and nine weeks of abstinence (Vanderschuren et al., 1997). Others
 452 have shown that this persists for up to three months of abstinence (Powell and Holtzman, 2001).
 453 Though these studies (and the bulk of studies examining sensitization) focus on changes with
 454 opioids on board, one report found that overall locomotion is depressed 24 hours into
 455 abstinence without re-exposure to heroin. Although it is unclear whether this change reached
 456 significance, locomotion appears to increase following 14 days of abstinence, again without a
 457 priming injection (Leri et al., 2003). All of these aforementioned conclusions were based on
 458 male subjects only. This study corroborates these findings and extends them to females.

459 Locomotion and reinstatement are mediated by overlapping neurotransmitter
 460 mechanisms in the ventral tegmental area and accumbens, whereby activation of glutamate
 461 receptors on accumbal dopamine terminals leads to the increase in dopamine that drives
 462 increased locomotion and motivation (Wu et al., 1993; Wise, 2004). In fact, reducing locomotion
 463 and seeking sometimes cannot be dissociated. For example, dopamine D1 receptor antagonist
 464 SCH 23390 attenuates overall locomotion and sucrose seeking, which are increased after 30
 465 days of abstinence without extinction training (Grimm et al., 2011). Additionally, treatments that
 466 reduce incubation of cocaine craving also reduce overall locomotion after 30+ days of forced
 467 abstinence (Murray et al., 2021). Thus, there is an overlapping system that contributes to
 468 behavioral sensitization with drugs on board, cue-induced relapse-like behavior, and
 469 locomotion. Here, we show that these drug-induced neural adaptations can persist and be
 470 triggered by cue re-exposure even after prolonged abstinence.

471 **In both sexes, chronic heroin exposure and extended abstinence results in an opioid-**
 472 **specific behavioral profile**

473 Although craving and overall locomotion increase over 30 days of abstinence regardless
 474 of sex and reinforcer, other behaviors, like grooming and hyperactivity change uniquely
 475 following exposure to heroin versus sucrose. Most of the research on incubation of craving has
 476 utilized responses on a previously reward-paired lever to assess craving, regardless of
 477

reinforcer. Here, we show that other behaviors, such as grooming and hyperactivity, may be better suited for quantifying *opioid* craving. One important limitation of this study is that certain self-administration parameters (session duration and continuous versus intermittent access) were inconsistent across reinforcers, which possibly contributes to the behavioral differences seen here. Though a mechanism for driving these behavioral changes is undetermined, these unique behavioral profiles are significant and relevant because they 1) underscore the wealth of data indicating that increased active lever presses occur in males and females upon cue re-exposure after prolonged abstinence, while 2) adding more nuanced and robust measurements of opioid craving specifically. These data can help improve preclinical models of craving/relapse-like behavior while also serving as a powerful foundation for additional mechanistic exploration.

The decrease in grooming and increase in hyperactivity that are associated with peak heroin craving may represent a hyperactive or compulsive behavioral profile similar to compulsive drug seeking. Noradrenergic hyperactivity can lead to hyperarousal, trouble focusing, and overactivity (Yamamoto et al., 2014). Treatments that reduce noradrenergic outflow have been shown to reduce cue-induced opioid craving (Sinha et al., 2007). Thus, one interpretation is that cue-induced heroin craving in late abstinence is reflective of a hyperactive state, a preoccupation with drug seeking, and a decrease in self-directed behaviors, such as grooming. Grooming and hyperactivity are associated with cocaine- and opioid-induced behavioral sensitization (Becker et al., 2001; Zhan et al., 2015), though not many studies have examined them in the context of cue-induced craving without re-exposure to the drug itself. This study lays the groundwork for further exploration of potential mechanisms underlying the behavioral profile associated with opioid-paired cue re-exposure.

It is interesting that many of the behaviors – incubation of craving, increased locomotion, and the heroin-specific decrease in grooming, and increase in hyperactivity– were consistently altered in both sexes. The only recorded behavior that was differentially affected in males and

504 females was the sucrose-specific increase in sniffing seen in females, but not males. Notably,
 505 though sucrose-exposed females respond more than sucrose-exposed males during early
 506 abstinence, there were no observed sex differences in heroin seeking at either time point. This
 507 is consistent with other reports that males and females experience similar increases in opioid-
 508 seeking behavior after extended abstinence (Venniro et al., 2019). Compared to males, females
 509 show enhanced incentive sensitization to cocaine following intermittent self-administration
 510 (Kawa and Robinson, 2019b), as well as behavioral sensitization to experimenter-delivered
 511 amphetamine (Van Haaren and Meyer, 1991). In some cases, behaviors like grooming are
 512 uniquely affected in only females and unchanged in males when morphine is on board (Zhan et
 513 al., 2015). Here, the magnitude of change in stereotypies and locomotion are similar in males
 514 and females, with the exception of sniffing in sucrose-exposed females. A possible explanation
 515 is that males and females react differently when opioids are present, but that over time, the
 516 opioid- and cue-induced behavioral changes become more comparable across sexes. This
 517 would help explain why males and females show a similar increase in cue-induced opioid
 518 craving over 30 days of abstinence (Venniro et al., 2019). Overall, this is promising in terms of
 519 designing treatments administered throughout abstinence that are likely to be effective in both
 520 sexes at reducing craving and other behaviors associated with compulsive drug seeking.

521 **Extended abstinence from heroin is associated with an increase in 50-kHz ultrasonic**
 522 **vocalizations in males, but not females**

523
 524 Another sex-specific change was reflected in 50-kHz ultrasonic vocalizations (USVs).
 525 USVs were recorded during seeking tests to determine whether there is an affective component
 526 of drug/sucrose seeking in early versus late abstinence, when craving is high and other heroin-
 527 induced behavioral changes emerge. Audio recording of craving tests on Day 1 and Day 30 of
 528 abstinence revealed no change in 22-kHz USVs in either sex, and an increase in 50-kHz USVs
 529 in males, but not females. 22-kHz and 50-kHz USVs were unchanged in sucrose-exposed
 530 males and females. The lack of 22-kHz USVs is inconsistent with previous reports that when a

531 conditioned cue is no longer predictive of a reward, rats will emit 22-kHz calls (Coffey et al.,
 532 2013). Here, there were few 22-kHz calls during any of the cue tests for either sex/reinforcer.
 533 The lack of 22-kHz USVs is consistent with the idea that somatic withdrawal symptoms are not
 534 driving craving behavior. Although rats may experience withdrawal-like behavior in early
 535 abstinence following chronic opioid self-administration (Gipson et al., 2020; Seaman Jr and
 536 Collins, 2021), withdrawal symptoms subside within the first few days of abstinence (Welsch et
 537 al., 2020). Yet, craving continues to intensify for several weeks. As such, withdrawal signs and
 538 craving follow opposite trajectories and one is unlikely to affect the other. Interestingly,
 539 protracted forced abstinence from cocaine (30 or 60 days) does not lead to a difference in 22-
 540 kHz nor 50-kHz calls during a contextual reinstatement test compared to calls made during self-
 541 administration (Barker et al., 2014), though in this study, USVs were not collected during an
 542 early abstinence seeking test. Here, 50-kHz, but not 22-kHz USVs change across abstinence in
 543 a sex- and reinforcer-specific manner.

544 Preclinical studies reveal that there are relatively fewer sex differences associated with
 545 opioid-related behaviors, including relapse (Nicolas et al., 2021). Here, males and females show
 546 similar patterns of relapse-like behavior, and yet females do not display an increase in 50-kHz
 547 USVs during the late abstinence relapse test when craving is high. It is likely that males emit 50-
 548 kHz USVs as an anticipatory behavior during re-exposure to opioid-paired cues. This is
 549 consistent with studies demonstrating that males report elevated moods prior to and during
 550 cocaine and alcohol relapse events (McKay et al., 1996; Rubin et al., 1996). Other studies also
 551 interpret 50-kHz USVs as positive-valence affective states in anticipation of social and drug
 552 rewards (Barfield et al., 1979; Ma et al., 2010). In fact, cues predictive of electrical stimulation of
 553 reward-related brain regions elicit increased 50-kHz calls in males (Burgdorf et al., 2000).
 554 Conversely, women are more prone to anxiety-, stress-, and depression-driven cocaine relapse
 555 (reviewed in McKay et al., 1996; Rubin et al., 1996; Becker and Koob, 2016). Women report a
 556 greater increase in sadness and decrease in joy in response to opioid-paired imagery and

paraphernalia compared to men (Yu et al., 2007). As with many observed sex differences, further research is needed to delineate why other behavioral end points are similar at abstinence Day 30, and yet there is a sex difference in 50-kHz USVs. In other studies examining USVs, females are often not included or not compared to males directly. One possibility is that females are more prone to stress-induced relapse. Re-exposure to drug-paired cues in the absence of drug delivery is likely to evoke mild stress (Schultz et al., 1997), and stress-induced reinstatement does increase over time (Sinha et al., 2011). However, it can be argued that the cue test is not as stressful as the stimuli used to precipitate stress-induced seeking, such as a foot shock. Therefore it is likely not sufficient to induce 22-kHz calls nor 50-kHz calls in females, which would be consistent with a positive affect-induced relapse event. To that point, if a stress-primed reinstatement model was used in place of the cue-induced model here, females may show a greater number of USVs as they are potentially more sensitive to stress-induced relapse.

In males, the increase in 50-kHz positive-valence affective calls, increase in locomotion, and increase in hyperactivity that accompany increased drug seeking may reflect an overactive or “manic”-like state. The simultaneous decrease in grooming may reflect a decrease in self-directed behaviors that is generally consistent with a compulsive drug seeking profile. This should be interpreted with caution so as not to over-anthropomorphize these measures. The intention is simply to characterize the behavioral profiles in a way that may lead to further exploration of underlying mechanisms. For example, these changes are potentially caused by dopamine D1 receptor activation in the nucleus accumbens shell, given that dopamine D1 receptors are linked to initiating anticipatory 50-kHz calls (Simola and Brudzynski, 2018), and D1 receptor antagonism reduces these calls (Buck et al., 2014). Of course, the dopamine system is also extensively linked to drug reward (Wise and Bozarth, 1985), motivation to seek rewards (reviewed in Volkow et al., 2017), as well as pathological manic states when there is an excess of dopamine (Ashok et al., 2017). Moreover, methamphetamine-induced 50-kHz calls

are attenuated by anti-mania drugs and have, in the past, been used to quantify mania-like behavior in methamphetamine-treated rats (Wöhr, 2021). Additional studies are needed to ascertain the exact underlying mechanisms that connect increased locomotion, hyperactivity, and 50-kHz calls to decreased grooming and increased heroin seeking; however, their coexistence could represent a state of hyperarousal and compulsive drug seeking possibly mediated by the dopamine system.

Females take increasing amounts of heroin and sucrose, and experience incubation of craving regardless of estrous cycle phase

Overall, this study shows few sex differences in terms of opioid-related behaviors. It is possible that sex differences will emerge when females are tested in a specific phase of the estrous cycle, and this effect may be obscured given the large sample sizes and estrous cycle variability across samples. Though there are many published reports indicating that the female estrous cycle impacts stimulant- and nicotine-related behaviors (Lynch, 2008; Feltenstein et al., 2009; Lynch et al., 2019; Nicolas et al., 2019), the results are equivocal when it comes to opioids, and there is very little available data concerning natural rewards like sucrose. Here, the female rats were freely cycling and estrous cycle was tracked throughout self-administration, as well as on days that they were tested for craving. There were a similar and ample number of females in non-estrus versus estrus on any given cue test day. Females self-administered increasing amounts of heroin and sucrose over the ten days of self-administration, and there were no differences in consumption between female rats in estrus versus those in non-estrus. All female rats experienced an increase in heroin- and sucrose-seeking behavior over 30 days of protracted abstinence, and there was no difference in lever pressing between estrus and non-estrus females at Day 1 nor Day 30. Thus, the increase in heroin and sucrose seeking cannot be attributed to a specific phase of the estrous cycle, nor is any one phase of the estrous cycle overrepresented here.

609 Some data indicate that female rats self-administer up to 70% less heroin during the
610 proestrus phase of their cycle (Lacy et al., 2016), and that ovariectomized (OVX) females
611 treated with estradiol take a greater number of heroin infusions compared to vehicle-treated
612 OVX females (Roth et al., 2002). However, these studies were either quite short (i.e. four hours
613 in the case of the Lacy study), or a lower dose of heroin was used (i.e. 0.0075 mg/kg as in the
614 case of the Roth study). In freely cycling females treated with vehicle and permitted to self-
615 administer heroin over an extended-access 12-hour session, there was no effect of estrous
616 cycle on heroin taking (Roth et al., 2002). Other studies that utilized four three-hour daily
617 sessions and a range of doses report no differences in heroin taking nor progressive ratio
618 breakpoint between OVX females, OVX females treated with estradiol, and males (Stewart et
619 al., 1996; Sedki et al., 2015). Though results regarding the role of gonadal hormones on opioid
620 self-administration are conflicting across published reports, results from this study are more
621 consistent with other extended-access paradigms.

622 One recent study found that rats self-administered similar amounts of sucrose
623 regardless of estrous cycle phase (Schmidt et al., 2021). In this article, rats were also trained on
624 heroin self-administration prior to sucrose self-administration, and there was an observed
625 decrease in heroin taking during proestrus. However, heroin self-administration and sucrose
626 self-administration sessions were both two hours in duration, and a lower heroin dose of 0.0075
627 mg/kg was used. Thus, the sucrose self-administration paradigm used was similar to the one
628 used here, whereas the heroin self-administration paradigm was not. Ours is the first study that
629 the authors are aware of to track estrous cycle phase alongside incubation of sucrose craving. It
630 has been reported that there are no sex differences in sucrose seeking after 1, 21, 60, 120, and
631 200 days of abstinence (estrous cycle was not tracked in this study) (Madangopal et al., 2019).
632 Sucrose-related findings are extremely limited. Yet, results so far seem to be consistent with the
633 finding that sucrose self-administration is not dependent on fluctuations in gonadal hormones.

634 **Locomotion is the only behavior enhanced in estrus females after 30 days of abstinence**
 635 **from heroin, whereas locomotion and hyperactivity are enhanced in estrus females after**
 636 **30 days of abstinence from sucrose**

637 Locomotion, sniffing, grooming, and hyperactivity were also assessed in the context of
 638 the estrous cycle. Estrus females showed increased locomotion following extended abstinence
 639 from both reinforcers. Sniffing and grooming were affected similarly in estrus versus non-estrus
 640 females at both abstinence time points for both reinforcers. Interestingly, although hyperactivity
 641 did not change across protracted abstinence in sucrose-exposed females, it was higher in
 642 estrus females at Day 30. Hyperactivity increased across over time in heroin-exposed females,
 643 but was not affected by estrous cycle phase. As with data regarding the effects of the estrous
 644 cycle on incubation of craving, there was a sizeable and comparable number of estrus and non-
 645 estrus females in any given group. Thus, the fact that males and females showed largely similar
 646 behavioral profiles in early and late abstinence is unlikely to be attributable to females being
 647 tested in a specific estrous cycle phase.

649 The overall increase in locomotion seen in estrus females builds on previous results
 650 indicating a general increase in activity in female rats in estrus (Giles et al., 2010) by
 651 demonstrating that this occurs reliably even after treatment with either sucrose or heroin. Other
 652 studies examining stimulant-induced behavioral changes show that estrus females
 653 demonstrated reduced cocaine- and amphetamine-induced locomotion and increased
 654 amphetamine-induced stereotypies than those in non-estrus (Becker and Cha, 1989; Quiñones-
 655 Jenab et al., 1999). This is opposite from what was observed here in response to cues only:
 656 estrus females show an increase in locomotion, and no differences in other stereotyped
 657 behaviors. Interestingly, freely cycling females show cocaine-induced locomotor sensitization in
 658 response to cocaine challenge that is not seen in OVX females; however, OVX females treated
 659 with estradiol showed enhanced stereotyped behaviors and OVX females treated with
 660 progesterone showed reduced stereotyped behaviors (Souza et al., 2014). In these
 661 experiments, cocaine was on board. Here, we build on these previous results by demonstrating

662 that, not only is overall locomotion increased following extended abstinence, it appears to be
 663 further enhanced in estrus females. Moreover, the increase in hyperactivity associated with
 664 heroin exposure is not attributable to the female estrous cycle since only sucrose-exposed
 665 estrus females show enhanced hyperactivity.

666 **Conclusions**

667 Cue-induced incubation of craving occurred similarly across 30 days of extended
 668 abstinence, regardless of sex and reinforcer. This increase in active lever pressing was
 669 associated with an increase in locomotion. Analysis of more granular behaviors revealed a drug-
 670 specific behavioral profile. Specifically, heroin exposure and extended abstinence were
 671 associated with a decrease in grooming and an increase in hyperactivity in both males and
 672 females that did not generalize to sucrose-exposed rats. Whereas increased responding on a
 673 previously reward-paired lever is suitable for assessing craving broadly (as has been the
 674 standard in the field), grooming and hyperactivity should be considered in future quantifications
 675 of opioid craving specifically. Therein, these data help to refine preclinical models of opioid
 676 relapse-like behavior. Heroin craving was also associated with an increase in 50-kHz USVs in
 677 males, but not females. This indicates that males may experience a greater degree of
 678 anticipation and/or positive-valence affective states during opioid relapse events. Analysis of the
 679 female estrous cycle revealed that heroin and sucrose self-administration increase across ten
 680 days and are not different between estrus and non-estrus females. Similarly, all female rats
 681 showed enhanced craving after protracted abstinence, and there were no differences between
 682 estrus and non-estrus females in neither early nor late abstinence. Examination of addiction-
 683 related behaviors in the context of the estrous cycle revealed that female rats in estrus showed
 684 enhanced locomotion, regardless of whether they self-administered sucrose or heroin.
 685 Interestingly, although hyperactivity was one of the heroin-specific changes, it was also
 686 enhanced in estrus females, though only those that had self-administered sucrose. Taken
 687 together, these results are a promising foundation for identifying neural mechanisms and
 688

689 behavioral signals associated with opioid craving in males and females. Additionally, they
690 suggest that potential treatments may not need to be quite as sensitive to fluctuations in
691 gonadal hormones in order to be effective.
692

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Figure 1. Incubation of craving is associated with sex- and reinforcer-specific behavioral signatures. A. Experimental timeline including 10 days of intravenous heroin or oral sucrose self-administration, followed by a recorded cue test after one or 30 days of forced abstinence. B. Cameras were mounted on the ceiling of the sound-attenuating chamber to record movement and behaviors during the cue test. An ultrasonic microphone was positioned flush against the Plexiglas top of the operant box to record ultrasonic vocalizations. C. Sucrose- and heroin-exposed males and females showed incubation of craving, as indicated by an increase in active levers after 30 days of abstinence compared to one day of abstinence. Post-hoc comparisons revealed that sucrose females responded significantly more than heroin females and sucrose males in early abstinence (day [$F_{1,232}=52.74$, $p<.0001$]; Day 1 female sucrose vs Day 1 female heroin, $p=.0003$; Day 1 female sucrose vs Day 1 male sucrose, $p=.0402$, Bonferroni's multiple comparisons test). D. Overall locomotion, as measured by total beam breaks increased in heroin- and sucrose-exposed males and females over 30 days of abstinence (day [$F_{1,232}=100.6$, $p<.0001$]). E. Grooming decreased in heroin-exposed rats and was unchanged in sucrose-exposed rats of both sexes after 30 days of abstinence (interaction [$F_{1,232}=4.318$, $p=.0388$]; Day 1 male heroin vs Day 30 male heroin, $p=.0138$; Day 1 male sucrose vs Day 30 male sucrose, $p>.9999$; Day 1 female heroin vs Day 30 female heroin, $p<.0001$; Day 1 female sucrose vs Day 30 female sucrose, $p=.1848$, Bonferroni's multiple comparisons test). F. Sniffing was unchanged in all conditions, with the exception of an increase in sucrose-exposed females. Post-hoc comparisons revealed that sucrose males engaged in more sniffing than heroin males in late abstinence, and more sniffing than sucrose females in early abstinence (interaction [$F_{1,232}=4.304$, $p=.0391$]; Day 1 male heroin vs Day 30 male heroin, $p>.9999$; Day 1 male sucrose vs Day 30 male sucrose, $p=.1316$; Day 1 female heroin vs Day 30 female heroin; Day 1 female sucrose vs Day 30 female sucrose, $p<.0001$, $p=.1848$, Bonferroni's multiple comparisons test). G. Hyperactivity, as measured by hops and darts across the cage, increased over 30 days of abstinence from heroin, but not sucrose in males and females (interaction [$F_{1,232}=12.11$, $p=.0006$]; Day 1 male heroin vs Day 30 male heroin, $p=.0050$; Day 1 male sucrose vs Day 30 male sucrose, $p>.9999$; Day 1 female heroin vs Day 30 female heroin, $p<.0001$; Day 1 female sucrose vs Day 30 female sucrose, $p=.7376$, Bonferroni's multiple comparisons test). Additional statistics are listed in Table 1. Data show mean $\pm$ SEM; * $p<.05$.

Figure 2. Incubation of heroin craving is associated with an increase in 50-kHz ultrasonic vocalizations (USVs) in males only (interaction [$F_{1,51}=60.12$, $p<.0001$]; Day 1 heroin male 50-kHz calls vs Day 30 heroin male 50-kHz calls, $p<.0001$; Day 1 sucrose male 50-kHz calls vs Day 30 sucrose male 50-kHz calls, $p>.9999$; Day 1 heroin female 50-kHz calls vs Day 30 heroin female 50-kHz calls, $p>.9999$; Day 1 sucrose female 50-kHz calls vs Day 30 sucrose female 50-kHz calls, $p>.9999$, Bonferroni's multiple comparisons test). Additional statistics are listed in Table 1. Data show mean $\pm$ SEM; * $p<.05$.

Table 1. Additional statistics associated with results from Figure 1 and Figure 2.

Figure 3. The effect of the female estrous cycle phase (estrus versus non-estrus) on heroin and sucrose self-administration and craving. A. Female heroin-treated rats earned more infusions over 10 days of intravenous self-administration regardless of estrous cycle phase (time, [$F_{9,541}=3.821$, $p=.0001$], estrus, [$F_{3,148}=5.299$, $p=.6624$]; interaction, [$F_{27,541}=4.405$, $p<.0001$]). B. Females demonstrated incubation of heroin craving, as indicated by an increase in active lever responses during a 30-minute cue test following 30 days of abstinence, and there were no differences in active lever responses between estrus and non-estrus females at either abstinence time point (time, [$F_{1,51}=18.02$, $p<.0001$], estrus, [$F_{1,51}=0.0881$, $p=.7677$]; interaction, [$F_{1,51}=1.989$, $p=.1645$]). C. Female sucrose-treated rats earned more sucrose pellets over 10

934 days of oral self-administration regardless of estrous cycle phase (time, [$F_{9,760}=66.68, p<.0001$],
 935 estrus, [$F_{1,201}=.2342, p=.6290$]; interaction, [$F_{9,760}=.8406, p=.5787$]). D. Females demonstrated
 936 incubation of sucrose craving, as indicated by an increase in active lever responses during a 1-
 937 hour cue test following 30 days of abstinence, and there were no differences in active lever
 938 responses between estrus and non-estrus females at either abstinence time point (time,
 939 [$F_{1,63}=12.75, p=.0007$], estrus, [$F_{1,63}=.2057, p=.6517$]; interaction, [$F_{1,63}=.7248, p=.6517$]). Data
 940 show mean $\pm$ SEM; * $p<.05$.

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 942 **Figure 4.** The effect of the female estrous cycle phase (estrus versus non-estrus) on addiction-
 943 related behaviors following one or 30 days of abstinence from heroin. A. Total beam breaks
 944 increased over 30 days of abstinence, and were enhanced in estrus females compared to non-
 945 estrus females (time [$F_{1,45}=33.58, p<.0001$]; estrus, [$F_{1,45}=6.151, p=.0169$]; interaction,
 946 [$F_{1,45}=0.5535, p=.4608$]). B. Grooming decreased over 30 days of abstinence regardless of
 947 estrous cycle phase (time, [$F_{1,45}=18.30, p=.0001$]; estrus, [$F_{1,45}=0.0093, p=.9235$]; interaction,
 948 [$F_{1,45}=1.762, p=.1911$]). C. Sniffing increased over 30 days of abstinence regardless of estrous
 949 cycle phase (time, [$F_{1,45}=9.245, p=.0039$]; estrus, [$F_{1,45}=1.018, p=.3183$]; interaction,
 950 [$F_{1,45}=0.0221, p=.8826$]). D. Hyperactivity increased over 30 days of abstinence and there was
 951 no effect of estrous cycle phase (time, [$F_{1,45}=15.36, p=.0003$]; estrus, [$F_{1,45}=0.0558, p=.8144$];
 952 interaction, [$F_{1,45}=0.2112, p=.6481$]). Data show mean $\pm$ SEM; * $p<.05$.

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 954 **Figure 5.** The effect of the female estrous cycle phase (estrus versus non-estrus) on addiction-
 955 related behaviors following one or 30 days of abstinence from sucrose. A. Total beam breaks
 956 increased over 30 days of abstinence, and were enhanced in estrus females compared to non-
 957 estrus females (time, [$F_{1,86}=54.91, p<.0001$]; estrus, [$F_{1,86}=6.386, p=.0133$]; interaction,
 958 [$F_{1,86}=0.0115, p=.9150$]). B. Grooming increased over 30 days of abstinence regardless of
 959 estrous cycle phase (time [$F_{1,86}=4.194, p=.0436$]; estrus, [$F_{1,86}=1.091, p=.2993$]; interaction,
 960 [$F_{1,86}=1.974, p=.11636$]). C. Sniffing increased over 30 days of abstinence regardless of estrous
 961 cycle phase (time [$F_{1,86}=23.98, p<.0001$]; estrus, [$F_{1,86}=0.003, p=.9568$]; interaction,
 962 [$F_{1,86}=0.064, p=.8006$]). D. Hyperactivity was enhanced at Day 30 in estrus females, and there
 963 was no effect of estrous cycle on hyperactivity at Day 1 (time, [$F_{1,86}=3.923, p=.0508$]; estrus,
 964 [$F_{1,86}=5.459, p=.0218$]; interaction, [$F_{1,86}=1.767, p=.1872$]; Day 1 estrus vs non-estrus, $p>0.9999$;
 965 Day 30 estrus vs non-estrus, $p=0.0479$, Bonferroni's multiple comparisons test). Data show
 966 mean $\pm$ SEM; * $p<.05$.

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Figure	Factors	F values	p-values
Figure 1C	Day	$F(1, 232) = 52.74$	$P < 0.0001$
	Sex	$F(1, 232) = 9.825$	$P = 0.0019$
	Reinforcer	$F(1, 232) = 2.824$	$P = 0.0942$
	Day x Sex	$F(1, 232) = 0.6877$	$P = 0.4078$
	Day x Reinforcer	$F(1, 232) = 3.340$	$P = 0.0689$
	Sex x Reinforcer	$F(1, 232) = 3.139$	$P = 0.0777$
	Day x Sex x Reinforcer	$F(1, 232) = 4.549$	$P = 0.0340$
Figure 1D	Day	$F(1, 232) = 100.6$	$P < 0.0001$
	Sex	$F(1, 232) = 2.124$	$P = 0.1464$
	Reinforcer	$F(1, 232) = 1.397$	$P = 0.2384$
	Day x Sex	$F(1, 232) = 3.380$	$P = 0.0673$
	Day x Reinforcer	$F(1, 232) = 0.2113$	$P = 0.6461$
	Sex x Reinforcer	$F(1, 232) = 0.02831$	$P = 0.8665$
	Day x Sex x Reinforcer	$F(1, 232) = 0.2019$	$P = 0.6536$
Figure 1E	Day	$F(1, 232) = 10.78$	$P = 0.0012$
	Sex	$F(1, 232) = 0.2969$	$P = 0.5864$
	Reinforcer	$F(1, 232) = 6.620$	$P = 0.0107$
	Day x Sex	$F(1, 232) = 0.1863$	$P = 0.6664$
	Day x Reinforcer	$F(1, 232) = 20.91$	$P < 0.0001$
	Sex x Reinforcer	$F(1, 232) = 0.8497$	$P = 0.3576$
	Day x Sex x Reinforcer	$F(1, 232) = 4.318$	$P = 0.0388$
Figure 1F	Day	$F(1, 232) = 23.20$	$P < 0.0001$
	Sex	$F(1, 232) = 6.569$	$P = 0.0110$
	Reinforcer	$F(1, 232) = 16.82$	$P < 0.0001$
	Day x Sex	$F(1, 232) = 1.523$	$P = 0.2184$
	Day x Reinforcer	$F(1, 232) = 2.408$	$P = 0.1221$
	Sex x Reinforcer	$F(1, 232) = 4.304$	$P = 0.0391$
	Day x Sex x Reinforcer	$F(1, 232) = 0.001682$	$P = 0.9673$
Figure 1G	Day	$F(1, 231) = 24.49$	$P < 0.0001$
	Sex	$F(1, 231) = 2.717$	$P = 0.1007$
	Reinforcer	$F(1, 231) = 16.05$	$P < 0.0001$
	Day x Sex	$F(1, 231) = 2.268$	$P = 0.1334$
	Day x Reinforcer	$F(1, 231) = 12.11$	$P = 0.0006$
	Sex x Reinforcer	$F(1, 231) = 0.8109$	$P = 0.3688$
	Day x Sex x Reinforcer	$F(1, 231) = 1.068$	$P = 0.3025$
Figure 2	Day	$F(1, 51) = 65.73$	$P < 0.0001$
	Sex	$F(1, 51) = 84.83$	$P < 0.0001$
	Reinforcer	$F(1, 51) = 69.82$	$P < 0.0001$
	Day x Sex	$F(1, 51) = 67.04$	$P < 0.0001$
	Day x Reinforcer	$F(1, 51) = 56.88$	$P < 0.0001$
	Sex x Reinforcer	$F(1, 51) = 68.35$	$P < 0.0001$
	Day x Sex x Reinforcer	$F(1, 51) = 60.12$	$P < 0.0001$