Evidence suggests that single housing in rats acts as a chronic stressor, raising the possibilities that it contributes to measures of heroin craving and that pair housing ameliorates such measures. This study aimed to determine whether pair housing after heroin self-administration reduces the incubation of craving, extinction, and reinstatement of heroin seeking. Single-housed female and male Sprague-Dawley rats underwent daily 6-hr heroin self-administration, wherein active lever presses produced a heroin infusion paired with light/tone cues. One day after self-administration, rats underwent a baseline cued-seeking test wherein active lever presses only produced light/tone cues. Immediately following this cued-seeking test, rats were either pair-housed with weight- and sex-matched naïve rat or remained single-housed for the rest of the study. For 14 days, rats remained in their homecages, after which they underwent a cued-seeking test to assess the incubation of craving compared to their baseline test. Rats then underwent extinction sessions followed by cue-induced and heroin-primed reinstatements. The findings reveal that pair-housed rats did not differ from single-housed rats in terms of the incubation of craving, extinction, or reinstatement of heroin seeking. Additionally, the results did not reveal any evidence of sex-based differences in the study. The present work indicates that pair housing during the forced abstinence period does not alter measures of heroin craving-seeking. These findings suggest that the chronic stress of single housing specifically during forced abstinence does not contribute to the degree of such measures.

**Keywords:** heroin seeking, incubation of craving, heroin self-administration, pair housing, social housing
heroin, and have decreased drug-primed reinstatement of heroin seeking compared to isolated rats without environmental enrichment (Imperio et al., 2018). However, such studies do not isolate the social housing factor from the other enrichment factors.

Nonetheless, most opioid self-administration studies use single-housed rats, raising the possibility that isolation-induced stress contributes to commonly used measures of drug seeking. The increase in drug craving that happens over time during forced abstinence, known as the incubation of craving, occurs across drugs of abuse in humans and is readily observed in rats, allowing for increased translational relevance for manipulations that decrease drug craving (Venniro et al., 2016). In these experiments, rats typically remain isolated in their home cages after their last self-administration session for multiple weeks before cue-driven craving tests are given. Thus, isolation-induced stress may contribute to incubation of opioid craving and, conversely, pair-housing rats after self-administration (i.e., during the incubation period) may reduce such craving. Other work indicates that social housing as a component of environmental enrichment given during the period of forced abstinence reduces measures of craving for natural rewards (Grimm et al., 2008) and cocaine (Thiel et al., 2012). Nonetheless, whether social housing following self-administration and during forced abstinence reduces the incubation of opioid craving is unknown.

To address this issue, single-housed female and male rats were trained to self-administer heroin during daily 6 hr sessions. Following a baseline cued-seeking test, rats were pair-housed with a naïve rat of the same sex or remained single-housed for the rest of the study. Incubation of heroin craving was tested 14 days after self-administration. To determine whether such housing affected other measures of heroin seeking, rats then underwent extinction training and cued and heroin-primed reinstatement tests. Our results indicate that pair housing after self-administration did not reduce the incubation of craving in females or males and did not alter the extinction or reinstatement of heroin seeking.

Method

Subjects

Female and male Sprague-Dawley rats (200–225 g and 225–250 g, respectively at the time of first surgery; Envigo; n = 31) were used for this study. Of the total rats, 10 of them did not undergo any of the procedures described below and only served as the pair-housing partner. Sample sizes were determined based on previous work (Müller Ewald et al., 2019), and we report all data exclusions (if any), manipulations, and all measures in the study. All rats were initially single-housed in a temperature-controlled environment under a 12 hr light/dark cycle (lights on a 07:00) and allowed to acclimate to the vivarium at least 2 days before surgery. All procedures followed the National Institutes of Health (NIH) guidelines for care of laboratory animals and were approved by the University of Iowa Institutional Animal Care and Use Committee. This study’s design and its analysis were not preregistered.

Surgery

Those rats that were to self-administer heroin (n = 21) were anesthetized with 5% isoflurane and maintained at 2–3% isoflurane. Meloxicam (2 mg/kg, s.c.) was administered as an analgesic before surgery, as well as 24 hr after surgery. Rats also received sterile saline (3 ml, s.c.) after surgery for rehydration. Rats received catheter implants, wherein a rounded tip jugular vein catheter (SAI Infusion Technologies) was inserted 3.5 cm into the right jugular vein and sutured to the vein. Suture beads reinforced the tubing at the suture point. The opposite end of the catheter was externalized between the shoulder blades and connected to a harness with a 22-gauge guide cannula (PlasticsOne, Inc.), which was used for heroin delivery. Catheters were flushed 6 days per week with 0.1 ml of heparinized saline and glycerol to ensure catheter patency.

Heroin Self-Administration

Self-administration training sessions were carried out 6 days per week in standard operant conditioning chambers, housed within sound-attenuating chambers (Med Associates, Fairfax, VT) and equipped with a central reward magazine flanked by two retractable levers. Cue lights were located directly above the levers, and a 4,500 Hz Sonalert pure tone generator module was positioned above the right lever. A 6 W house light on the opposite wall of the operant chamber was illuminated throughout the training sessions. Heroin (kindly provided by the National Institute on Drug Abuse) was dissolved in 0.9% sterile saline. A dose of 0.15 mg/kg infusion heroin was used for the first 2 days of heroin self-administration, followed by 0.067 mg/kg/infusion heroin for all following days of self-administration. A lever press on the active (right) lever resulted in a 50 μL heroin infusion and a 5 s presentation of light and tone cues. A 20 s timeout period followed each lever press, during which additional active lever presses were recorded but had no scheduled consequence. Rats self-administered heroin for at least 12 days with >10 infusions on 10 of the days and >15 infusions on each of the final 3 days.

Incubation of Heroin Craving After Single or Pair Housing

After the above criteria were met and 1 day after the last self-administration session, rats underwent a 30 min cued-seeking test in which an active lever press resulted in light/tone cue presentation, but no heroin infusion. This test served as the baseline measure for incubation of craving (Test Day 1). Following the cued-seeking test, rats remained in their homecages for 14 days. Two groups characterized this phase. Some rats remained single-housed whereas others were pair-housed with a naïve rat. Each naïve rat had been previously single-housed, was weight-matched to its new cage mate, and was monitored for 1 hr upon pairing for good socialization (i.e., no signs of aggression or fighting). Once paired, rats remained pair-housed until the completion of the experiment.

Following 14 days in the homecages, rats were returned to the operant chamber for a 1 hr cued-seeking test (Test Day 14). Again, an active lever press resulted in light/tone cues but no heroin infusion. The first 30 min of this seeking test was used to assess incubation of heroin craving compared to the Test Day 1 baseline.

Extinction and Reinstatement of Heroin Seeking Tests

One day after the last cued-seeking test, rats began daily 3 hr extinction sessions, in which a lever press had no consequence.
Rats continued extinction for at least 7 days until three consecutive days had <20 active lever presses. Rats then underwent 3 hr cue-induced and heroin-primed reinstatements in a counterbalanced manner, with at least 3 days of extinction with <20 lever presses between each reinstatement. For cue-induced reinstatement, an active lever press resulted in light/tone cues. For heroin-primed reinstatement, rats received a small priming injection of heroin (0.25 mg/kg, s.c.) immediately prior to the reinstatement session, and active lever presses had no consequence.

### Statistical Analysis

All self-administration data were analyzed using a two-way repeated measures analysis of variance (ANOVA) with day as the within-subject variable and housing (single vs. pair) as the between-subject variable. For examining incubation of craving, active lever presses from Test Day 1 and the first 30 min of Test Day 14 were compared. To determine whether there were any important differences across the time course of active lever presses on Test Day 14, lever pressing was divided into four 15-min bins and a two-way repeated measures ANOVA was used, with bin as a within-subject factor and housing as a between-subject factor. For examining active lever presses during extinction, a two-way repeated measures ANOVA was used to assess active lever presses, infusions, and mg/kg heroin across self-administration for female versus male rats. A similar pattern of heroin self-administration was observed with females and males separately (Figure 1e–g). Table 1 shows the statistical analyses for all three figures, split by sex, and likewise confirms that males and females show the same pattern of statistical results across the analyses. Collapsing across housing, a two-way repeated measures ANOVA was used to assess active lever presses, infusions, and mg/kg heroin across self-administration for female versus male rats. Analysis of active lever presses revealed no main effect of day ($F_{1,70.70} = 0.64, p = 0.62$), a trend toward a main effect of sex ($F_{1,19} = 3.08, p = .10$), and a trend toward an interaction between day and sex ($F_{11, 209} = 1.69, p = .08$). The trends appear to reflect the nonsignificant increase in active lever presses in male rats relative to the more stable active lever presses in females across the 12 days, though the males displayed noticeably greater variability in their lever presses. Analysis of infusions revealed a main effect of day ($F_{4,2.2, 80.27} = 3.91, p < .01$), a trend toward a main effect of sex ($F_{1, 19} = 3.33, p = .08$), and no interaction between day and sex ($F_{11, 209} = 0.99, p = .46$). The trend toward a main effect of sex reflects slightly higher infusions taken by males compared to females, though this does not appear to lead to differences in body-weight-adjusted heroin intake. Indeed, analysis of mg/kg heroin revealed a main effect of day ($F_{4,1.15, 78.90} = 3.80, p < .01$), no main effect of sex ($F_{1, 19} = 1.35, p = .26$), and no interaction between day and sex ($F_{11, 209} = 0.85, p = .59$). Together, these results suggest that female and male rats did not significantly differ in heroin-taking measures, though males had a nonsignificant increase in active lever presses compared to females.

Figure 2 shows the results of pair versus single housing on the incubation of craving. The two-way repeated measures ANOVA for active lever presses on Test Day 1 and 14 (Figure 2a) revealed a main effect of test day ($F_{1, 19} = 18.75, p < .001$), no main effect of housing ($F_{1, 19} = 0.20, p = .67$), and no interaction between housing and test day ($F_{1, 19} = 0.002, p = .96$; Figure 2a). To confirm the incubation of craving in both groups, post hoc analyses revealed that both groups had significantly more lever presses on Test Day 14 compared to Test Day 1 ($p < .05$ in both cases). The two-way repeated measures ANOVA for active lever presses during Test Day 14 across 15 min bins (Figure 2b) revealed a main effect of bin ($F_{2,06.39} = 8.08, p < .01$), no main effect of housing ($F_{1, 19} = 0.06, p = .81$; Figure 2b), and an interaction between bin and housing ($F_{3, 57} = 2.86, p = .05$). Post hoc analyses revealed no difference between housing group at any of the four timepoints (15 min, $p = .84$; 30 min, $p = .20$; 45 min, $p = .95$; 60 min, $p = .88$). The same pattern of active lever pressing during Test Day 14 was observed with both females and males (Figure 2c; Table 1). These results suggest that pair housing during the incubation period did not affect the incubation of heroin craving.

Figure 3 shows the active lever presses during extinction and cue-induced and heroin-primed reinstatement for the pair- versus single-housed rats. The two-way repeated measures ANOVA of active lever presses during extinction (Figure 3a) revealed a main effect of day ($F_{2,03.38} = 43.36, p < .0001$), no main effect of housing ($F_{1, 19} = 0.17, p = .68$), and no interaction between day and housing ($F_{6, 114} = 0.35, p = .91$). A similar pattern of extinction lever pressing was...
observed in females and males separately (Figure 3b; Table 1). The two-way repeated measures ANOVA of active lever presses during cue-induced reinstatement (Figure 3c) revealed a main effect of day ($F_{1,18} = 33.08, p < .0001$), no main effect of housing ($F_{1,18} = 0.43, p = .52$), and no interaction between day and housing ($F_{1,18} = 0.43, p = .52$). To confirm cue-induced reinstatement in both groups, post hoc analyses revealed that active lever presses were significantly higher in both groups during cue-induced reinstatement compared to the extinction baseline ($p < .01$ in both cases). The two-way repeated measures ANOVA of active lever presses during heroin-primed reinstatement (Figure 3d) revealed a main effect of day ($F_{1,19} = 22.32, p = .0001$), no main effect of housing ($F_{1,19} = 0.17, p = .69$), and no interaction between day and housing ($F_{1,19} = 0.22, p = .65$). To confirm heroin-primed reinstatement in both groups, post hoc analyses revealed that active lever presses were significantly higher in both groups during heroin-primed reinstatement test compared to extinction baseline ($p < .05$ in both cases). The same pattern of results for both cue-induced and heroin-primed reinstatement was observed in females and males separately (Figure 3c, d, Table 1). These results reveal no effect of housing on the extinction of heroin seeking and cue-induced or heroin-primed reinstatement of heroin seeking.
PAIR HOUSING DOES NOT ALTER HEROIN CRAVING

Discussion

The present results indicate that pair housing during forced abstinence after heroin self-administration did not alter the incubation of heroin craving, the extinction of heroin seeking, or cue-induced or heroin-primed reinstatement. Moreover, of relevance for understanding the influence of sex on drug-related behavior, the findings did not reveal any statistically significant sex differences in measures of heroin self-administration and craving/seeking or an interaction between sex and single versus pair housing influences on such measures. Overall, the present study suggests that, at least with the procedures used herein, the ability of social housing to attenuate measures of heroin craving and seeking is ineffective.

Single Housing During Forced Abstinence Does Not Increase Measures of Heroin Craving

Prior studies indicate that single housing during forced abstinence, compared to social housing with environmental enrichment, increases

### Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Main effects</th>
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<tr>
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<tr>
<td>Self-administration (infusions)</td>
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<td>Self-administration (mg/kg heroin)</td>
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<tr>
<td>Incubation of craving (Test Day 1 vs. Test Day 14)</td>
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<td>M</td>
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<tr>
<td>Incubation of craving (timecourse of Test Day 14)</td>
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<td>Heroin-primed reinstatement (active lever presses)</td>
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Note. ANOVA = analysis of variance; F = female; M = male. Significant p values are in bold.

*p < .05.

Figure 2

Incubation of Craving

(a) Active lever pressing on Test Day 1 and 14. Mean ± SEM of active lever presses during the 30 min Test Day 1 and from the first 30 min of Test Day 14. Triangles represent individual responses for females (red) and males (blue). (b) Active lever presses during Test Day 14. Mean ± SEM of active lever presses every 15 min during the 1 hr session. (c) Active lever presses during Test Day 14 between females (top, red) and males (bottom, blue). Mean ± SEM is shown with individual data points in the background. SEM = standard error of the mean. See the online article for the color version of this figure.

*p < .05 compared to Test Day 1.
the incubation of sucrose and cocaine craving (Grimm et al., 2008; Thiel et al., 2012), as well as other measures of heroin seeking, such as the motivation to consume heroin and cue-induced heroin seeking (Imperio et al., 2018). However, the present work using only pair housing found no effect of such housing on various measures of heroin seeking, including incubation of craving. For social animals like rats, evidence indicates that prolonged isolation acts as a chronic stressor (Hofford et al., 2018; Mastrogiovanni et al., 2021; Turner et al., 2014), and stress influences drug seeking (Mantsch et al., 2016). Nonetheless, the present findings suggest that chronic social isolation, at least on its own, does not contribute to heroin craving/seeking.

There are two important distinctions between the present work and the findings from Imperio et al. (2018): (a) the presence/absence of environmental enrichment and (b) the timing of the behavioral manipulations. In the study by Imperio et al. (2018), rats were socially housed in conjunction with environmental enrichment, whereas, in the present study, rats did not receive environmental enrichment when pair housed. It is possible that environmental enrichment more effectively alters heroin seeking compared to

![Figure 3: Extinction and Reinstatement](image)

**Note.** (a) Lever pressing throughout extinction. Mean ± SEM of active lever presses for the first 7 days of extinction. (b) Active lever pressing during extinction between females (top, red) and males (bottom, blue). Mean ± SEM is shown with individual data points in the background. (c) Cue-induced reinstatement of heroin seeking. Mean ± SEM of active lever presses from the last 3 days of extinction (extinction baseline) and from the cue-induced reinstatement session. (d) Heroin-primed reinstatement of heroin seeking. Mean ± SEM of active lever presses from the last 3 days of extinction (extinction baseline) and from the heroin-primed reinstatement session. Females, red triangles; Males, blue triangles. SEM = standard error of the mean. See the online article for the color version of this figure.

*p < .05 compared to extinction baseline.
social housing. Indeed, for single-housed rats, there is evidence that environmental enrichment alone reduces cue-induced heroin seeking (Barrera et al., 2021; Galaj et al., 2016; Imperio et al., 2018), prevents heroin-conditioned place preference (Galaj et al., 2016), and decreases compulsive cocaine and heroin seeking (Ewing & Ranaldi, 2018; Peck et al., 2015). Alternatively, social housing may only have effects as an interaction between housing and environmental enrichment.

Moreover, Imperio et al. (2018) kept their rats in their housing conditions beginning with self-administration, whereas the housing manipulations in the present study began during the forced abstinence period (i.e., after self-administration). Thus, it is possible that social housing must be present from self-administration onward for it to influence subsequent drug seeking. Prior studies indicate that single-housed rats, compared to socially housed rats without environmental enrichment, acquire heroin self-administration faster (Bozarth et al., 1989), consume more morphine (Raz & Berger, 2010), and reacquire heroin-conditioned place preference faster (Turner et al., 2014).

Such findings suggest that social housing during self-administration itself protects against some effects of heroin taking, though the neurobiological mechanism for this is unclear. One mechanism may involve oxytocin, as social housing raises oxytocin levels in rats (Faragi et al., 2018). Indeed, evidence suggests that chronic opioid use alters endogenous oxytocin signaling (You et al., 2000; Zanos et al., 2014), and enhancing oxytocin has been shown to reverse drug-induced neuroadaptations underlying tolerance and withdrawal (King et al., 2020) and decrease heroin self-administration (Kovács et al., 1985). Oxytocin also enhances the social buffering of fear, or the ability of a conspecific to attenuate behavioral and autonomic measures of conditioned fear (Kiyokawa et al., 2007, 2014; Morozov & Ito, 2019; Peen et al., 2021). Taken together, these findings raise the possibility that oxytocin-enhancing manipulations, such as the pair housing used in the present study, has limited efficacy following chronic opioid use, but if provided throughout self-administration, acts as a buffer of heroin craving by increasing oxytocin signaling, potentially preventing alterations in endogenous oxytocin signaling that occur from chronic opioid use.

Notably, recent drug self-administration work has used “social choice” procedures in which rats choose to either receive the drug or access to a same-sex partner. Evidence from such studies indicates that “voluntary,” but not forced, abstinence, in which rats choose the social reward over the drug, prevents the incubation of methamphetamine and heroin craving (Venniro et al., 2018, 2019). The present results suggest that the lack of incubation in such models is not due to social interactions alone but reflects an interaction between social reward and volitional control during the abstinence period. Additionally, in those studies, rats were pair-housed prior to beginning self-administration and those former partners served as the social reward. This preexposure to their partner likely increased the overall value of the social reward, which may be critical for the ameliorating effects of social interactions on measures of craving.

Unlike many studies examining pair housing with drug seeking, the present study used a 6-hr “long-access” self-administration procedure. To our knowledge, only one study examining the effects of environmental enrichment on heroin seeking used long-access (≥6 hr) heroin self-administration and found that environmental enrichment during forced abstinence reduces the incubation of heroin craving (Sikora et al., 2018). However, that work did not address the effect of social housing itself, as all rats were housed in triads. Indeed, future work examining the effects of social housing should consider whether greater access to the drug via long-access self-administration overwhelms the effects of social housing to ameliorate different drug-related behaviors.

Notably, the present study observed a significant interaction between housing condition and time during the 1 hr Test Day 14 cued-seeking test (Figure 2b). However, post hoc analyses revealed no significant differences at any individual time point. It is likely that the interaction reflects the inherent bin-by-bin variability that occurs across a 1-hr cued-seeking test rather than a meaningful finding, particularly given that all other measures did not differ based upon housing conditions. Overall, evidence from the present study observed no differences between measures of heroin seeking and incubation of heroin craving for rats that were single-versus pair-housed, suggesting that such measures are not influenced by isolation during forced abstinence. However, it is unclear to what degree methodological details (i.e., heroin dosage, length of self-administration, timing of pair housing, etc.) influence these measures. It is likely that subtle differences in self-administration procedures, including length of session, drug dosage, and total days of self-administration, contribute to measures of drug craving and seeking, potentially obscuring findings from manipulations with a smaller effect size.

Female and Male Rats Self-Administer Heroin Similarly

Although not the focus of the present study, females and males were used in the experiments, and no sex differences in heroin taking, craving, or seeking measures were observed. There were also no apparent interactions between housing condition and sex, though the groups were not fully powered to rule this out. Critically, female and male rats undergoing long-access (6 hr) heroin self-administration did not differ in total mg/kg heroin consumed. Some studies report sex differences in certain measures of opioid taking (Becker & Koob, 2016; Carroll et al., 2001; George et al., 2021; Lynch & Carroll, 1999). For example, George et al. (2021) found that female rats displayed increased responding and intake across heroin doses following 6-hr heroin self-administration. However, not all studies report such differences (D’Ottavio et al., 2022; Lynch & Carroll, 1999). D’Ottavio et al. (2022) found that females had increased incubation of heroin craving compared to males, but only after intermittent access, and not continuous access, heroin self-administration. Nonetheless, a recent comprehensive review surveying the field found that studies examining measures of opioid craving in rodents largely did not reveal sex differences (Nicolas et al., 2022). Taken together with the present findings, it appears that, when sex differences are observed in opioid taking, craving, and/or seeking, they likely depend upon methodological parameters rather than reflecting widespread and robust differences between females and males.

Conclusion

The present findings add to our understanding of psychosocial factors and their ability to influence opioid craving and seeking in rodents. The absence of differences between single- and pair-housed rats indicates that, during forced abstinence from heroin, housing
condition does not contribute to the incubation of heroin craving or other measures of heroin seeking.

References


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