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Transcriptomics in the nucleus accumbens shell reveal sex- and reinforcer-specific signatures associated with morphine and sucrose craving

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Incubation of craving is a well-documented phenomenon referring to the intensification of drug craving over extended abstinence. The neural adaptations that occur during forced abstinence following chronic drug taking have been a topic of intense study. However, little is known about the transcriptomic changes occurring throughout this window of time. To define gene expression changes associated with morphine consumption and extended abstinence, male and female rats underwent 10 days of morphine self-administration. Separate drug-naive rats self-administered sucrose in order to compare opioid-induced changes from those associated with natural, non-drug rewards. After one or 30 days of forced abstinence, rats were tested for craving, or nucleus accumbens shell tissue was dissected for RNA sequencing. Morphine consumption was predictive of drug seeking after extended (30 days) but not brief (1 day) abstinence in both sexes. Extended abstinence was also associated with robust sex- and reinforcer-specific changes in gene expression, suggesting sex differences underlying incubation of morphine and sucrose seeking respectively. Importantly, these changes in gene expression occurred without re-exposure to drug-paired cues, indicating that chronic morphine causes long-lasting changes in gene expression that prime the system for increased craving. These findings lay the groundwork for identifying specific therapeutic targets for curbing opioid craving without impacting the natural reward system in males and females.

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INTRODUCTION

Relapse rates for substance use disorder are nearly 85% within the first year of sobriety [1]. Cues associated with previous drug use—such as environment, friends, and paraphernalia—trigger intense cravings to use [2, 3], which often precipitates relapse. Cue-induced craving intensifies over the course of abstinence, which is a phenomenon known as "incubation of drug craving". This has been documented in both rodent models [4, 5], as well as clinical populations [6–9]. Cue-precipitated seeking of the natural reward sucrose also intensifies following a period of abstinence in rats [10]. Identifying the molecular underpinnings that contribute to increased craving represents a promising avenue to improve therapeutics for substance use disorders. Importantly, disentangling the mechanisms underlying sucrose seeking from those involved in drug craving is critical for delineating opioid-specific therapeutic targets that are unlikely to affect the natural reward system.

Males and females experience some differences in the progression, susceptibility, and intensity of drug addiction and relapse [11, 12]. However, many of these findings are derived from cocaine use disorder research. Sex differences in opioid craving are far more equivocal, and in some cases, non-existent [13]. Some studies report higher opioid relapse rates in women during early [14], but not protracted abstinence [15]. In preclinical models, sex differences are not observed in incubation of opioid craving [13, 16–18]. Males and females also show similar fentanyl self-administration and cue-induced relapse behavior following 14 days of voluntary abstinence [19]. The goal of this study was to compare the mechanisms underlying incubation of opioid and sucrose craving in males and females to delineate sex- and reinforcer-specific molecular correlates of cue-induced craving.

Changes in gene expression within the nucleus accumbens core accompany cue-induced reinstatement of heroin seeking [20]. The two sub-regions of the nucleus accumbens are functionally dissociable, in that the core is critical for learning reward-cue pairings, and the shell is important for enacting cue-elicited responses [21, 22]. Changes in gene expression produced by heroin self-administration are distinct when comparing the shell and core regions of the nucleus accumbens [23]. The current studies focused on the accumbens shell, which is part of a critical circuit involved in contextual reinstatement of heroin seeking [24], and potentially involved in cue-induced cocaine and alcohol seeking, although pharmacological inactivation studies provide differing results [25, 26]. The $mGluR_{2/3}$ agonist LY379268 [27], and D_1-receptor antagonist SCH 23390 [28] in the shell also reduce contextual reinstatement of heroin seeking after extinction. Others have explored neural adaptations following re-exposure to drug-associated cues. However, cue re-exposure itself can induce gene

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expression changes [29]. Much less is known about transcriptomic alterations following drug exposure and abstinence without re-exposure to the drug-paired cues, which could prime the nucleus accumbens shell to respond when re-exposed to such cues. A better understanding of these mechanisms is critical for isolating the processes that contribute to craving. These data can inform potential treatments that mitigate enhanced craving prior to an individual re-integrating into their previous environment.

METHODS AND MATERIALS

Subjects

For all experiments, 60–180-day-old male ($n = 192$) and female ($n = 156$) Long-Evans rats were bred in house and pair-housed after weaning. There was counterbalancing within each cohort and each group so that all cohorts included equal representation of age within the range used in the study. Rats were handled daily for at least 5 days prior to the start of any behavioral procedure or test. All rats had ad libitum access to water and standard laboratory chow. The Institutional Animal Care and Use Committee of Temple University approved all animal care and experiments.

Drugs

Morphine sulfate was gifted by the NIDA drug supply and was dissolved in sterile 0.9% saline.

Catheter implantation surgery

Rats were anesthetized and the jugular vein was catheterized according to previous methods [30]. Catheters were flushed daily with 0.2 mL of timentin (0.93 mg/mL) dissolved in heparinized saline to prevent clogging.

Morphine self-administration, forced abstinence, and relapse tests

During morphine self-administration experiments, rats were pair-housed on a regular 12-h light/dark cycle. All morphine self-administration and tissue collection occurred during the dark phase. During self-administration, the rats were in the operant chambers with access to food and water from 9:00 P.M. to 9:00 A.M. (12-h self-administration sessions during the dark cycle). A small number of rats had to be euthanized due to complications stemming from surgery or other health concerns. Out of 207 morphine-treated rats, 25 were singly housed for the duration of the study. None of the morphine-treated rats used for RNA sequencing were singly housed. Rats self-administered morphine (0.75 mg/kg/infusion over 5 s) on a fixed ratio 1 (FR1) schedule. A 5-s cue light was activated during infusion delivery, followed by a 20-s timeout during which responses were recorded, but no drug was delivered. There was a maximum of 75 infusions per session. An "inactive lever" with no programmed consequence was also included in all studies. Control rats from these cohorts underwent identical procedures, but only had access to intravenous saline. For each comparison presented in the manuscript, cohorts included equal representation of subjects for each of the groups and ages. This design precluded the possibility of "cohort"/"batch" effects for all sex- and reinforcer-specific comparisons. Moreover, every cohort of rats included subjects that were processed for behavioral endpoints (drug seeking) and subjects that were dedicated to tissue collection. Tissue processing for library preparations and RNA sequencing was also done side-by-side with equal representation for each of the groups (i.e. controls, Day 1 of abstinence, and Day 30 of abstinence). Differentially expressed genes (DEGs) were identified using comparisons of groups that were run, processed, and sequenced together. For comparisons across groups that were not run together (i.e. male vs. female or morphine vs. sucrose), only the list of DEGs and/or enrichment terms were compared. Several cohorts of rats were run to collect sufficient tissue and behavioral data for each comparison. Each of these cohorts contained comparable number of rats in each of the groups included in the comparison. Following self-administration, rats from each cohort were randomly sub-divided into those that were sacrificed on abstinence Day 1 versus those that were sacrificed on abstinence Day 30. Regardless of condition or sex, all rats underwent either 24 h or 30 days of forced abstinence in a between-subjects design. During the abstinence period, rats were housed with ad libitum access to food and water, but had no access to the self-administration chambers and were handled regularly. At the end of each abstinence time point (24 h = Morphine Day 1/D1; 24 h = saline; 30 days = Morphine Day 30/D30), a subset of rats were tested for morphine seeking during 1-h "cue tests". Therein, responses on the previously drug-paired active lever resulted in illumination of the cue light, but no infusion of morphine.

Sucrose experiments

Separate (drug-naive) male and female rats were used for oral sucrose self-administration. Rats were housed in reverse light/dark cycle colony rooms with lights on from 9:00 P.M. to 9:00 A.M. All self-administration and tissue collection occurred during the dark phase. Self-administration experiments took place from 9:00 A.M. to 11:00 A.M. (2-h self-administration sessions in the dark cycle). Out of 141 sucrose-exposed rats; seven were singly housed at some point during the study. The same operant chambers and FR1 schedule were used; active lever presses resulted in one sucrose pellet paired with a 5-s cue light. There was a maximum of 90 sucrose pellets per session. For each comparison presented in the manuscript; the subjects were run in cohorts that included equal representation of subjects in each of the groups. Control ("cue only") rats were run on identical conditions but no sucrose pellets were delivered. All rats had ad libitum access to water and standard laboratory chow throughout the experiment. At the end of one or 30 days of forced abstinence ("Sucrose Day 1/D1" and "Sucrose Day 30/D30"), male and female rats were tested for sucrose seeking for 1 h starting at 9:00 A.M. (dark phase). Lever presses resulted in cue presentation but no sucrose was available during the tests.

Tissue collection and RNA sequencing

Rats used for RNA sequencing were never re-exposed to the drug- or sucrose-taking context after completing self-administration. Tissue collection occurred during the dark phase (between 9:00 A.M. and 11:00 A.M.) either 24 h or 30 days after the final self-administration session. Tissue from saline and cue only control rats was collected on abstinence Day 1 only. Immediately following decapitation, fresh brains were sectioned on a 1 mm brain block. Nucleus accumbens shell tissue was quickly micro dissected using a 2 mm puncher, flash-frozen on dry ice, and stored at −80 C. Each group contained 7–11 biological replicates. All samples prepared for RNA extraction contained bilateral nucleus accumbens shell (NAcSh) from one rat. Total RNA was isolated using QIAzol lysis reagent and purified using the RNeasy micro kit (Qiagen). RNA quality was assessed via Nanodrop (260/280 nm and 260/230 nm). RNA quantity was measured using the Qubit™ RNA HS Assay kit (Invitrogen, USA). All RNA samples used for sequencing had an RNA integrity number >8. Library preparation was performed using the NEBNext Ultra II Directional RNA Library Prep Kit (New England BioLabs). Samples were sequenced by Genewiz (South Plainfield, NJ) according to sex and condition (i.e. all male heroin-exposed samples together, all female heroin-exposed samples together, etc.). No comparisons were made across sequencing runs in order to help mitigate variability across mRNA extraction and subsequent library preps. The criteria to define DEGs was a 50% change in the expression ($|\log 2$ Fold change$| > 0.58$) and an adjusted p-value < 0.1. R statistical software (version 4.0.1) was used for downstream analysis and visualization of the RNA sequencing analysis output [31–33], including, but not limited to drawing heat maps and Venn diagrams. Pathways (from KEGG) and gene ontology terms (biological process, molecular function and cell compartment) by adjusted p-value < 0.1 were selected for further analysis. To compare gene expression agnostically, the Rank–Rank Hypergeometric Overlap (RRHO) analysis was used. This algorithm compares two gene lists ranked by the p-value of differential expression observed in two experiments and estimates the number of overlapping genes. Subsequently, a heat map is created showing the strength and pattern of correlation between two expression profiles [34]. RRHO2 library in R [34] was used to compare early and late abstinence DEGs in males and females for overlap in upregulated and downregulated DEGs, respectively. Only the resulting list of genes from each group was compared across groups.

Correlation analysis

Pearson correlations between total morphine or sucrose consumption during self-administration and number of active lever presses during the Day 1 or Day 30 cue test were calculated using ggscatter code from ggpubr library (ver 0.4.0) in R (ver 4.0.3).

Data analysis

GraphPad Prism (version 9.0.1) was used for ANOVAs. Mixed model ANOVAs were used to compare infusions/pellets during self-

administration. Session was used as the within-subject factor, and condition (saline or "cue only" control vs. abstinence Day 1 vs. abstinence Day 30) were used as between-subject factors. Two-way repeated-measures ANOVAs were used to compare active lever responses during the Day 1 or Day 30 cue test. Lever (active vs. inactive) was used as the within-subject factor, and abstinence condition (Day 1 vs. Day 30) was used as the between-subjects factor. In all cases, if a significant interaction was found post-hoc comparisons were made using Bonferroni's multiple comparisons test. If data were not spherical, the Geisser–Greenhouse correction was applied.

RESULTS

Incubation of morphine craving is accompanied by sex-specific changes in gene expression in the nucleus accumbens shell

Following 10 days of intravenous self-administration (IVSA), rats underwent either one or 30 days of forced abstinence. Rats were then tested for craving, or tissue was collected from the nucleus accumbens shell and processed using RNA sequencing (Fig. 1A). Over 10 days of IVSA, male rats earned more morphine infusions compared to saline-treated rats (Fig. 1B; condition, [$F_{2,126} = 17.82$, $p < 0.0001$], time, [$F_{9,1134} = 1.450$, $p = 0.1619$, Geisser–Greenhouse epsilon = 0.2752]; interaction, [$F_{18,1134} = 7.567$, $p < 0.0001$]). Rats that underwent 1 or 30 days of abstinence earned a similar number of morphine infusions (Fig. 1B; Saline vs. Morphine Day 1, $p = 0.0003$; Saline vs. Morphine Day 30, $p < 0.0001$; Morphine Day 1 vs. Morphine Day 30, $p = 0.1313$). During morphine seeking tests, active, but not inactive lever presses were higher on Day 30 compared to Day 1 (Fig. 1C; condition, [$F_{1,21} = 10.57$, $p = 0.0038$], lever [$F_{1,21} = 37.67$, $p < 0.0001$]; interaction, [$F_{1,21} = 9.165$, $p = 0.0064$]; active lever presses, $p = 0.0001$; inactive lever presses, $p = 0.7160$), indicating that incubation of morphine seeking occurred in male rats.

Female rats underwent the same regimen. The number of morphine infusions increased over 10 days of self-administration, and females self-administered more morphine than saline controls (Fig. 1D; condition, [$F_{2,75} = 10.52$, $p < 0.0001$], time, [$F_{9,675} = 4.027$, $p < 0.0001$, Geisser–Greenhouse epsilon = 0.3368]; interaction, [$F_{18,675} = 5.531$,$p < 0.0001$]). There were no differences in morphine taking between rats that eventually underwent one or 30 days of abstinence (Fig. 1D; Saline vs. Morphine Day 1, $p = 0.0012$; Saline vs. Morphine Day 30, $p = 0.0003$; Morphine Day 1 vs. Morphine Day 30, $p = 0.9290$). Female rats also exhibited incubation of drug seeking after 30 days (Fig. 1E; condition, [$F_{1,10} = 7.325$, $p = 0.0221$], lever [$F_{1,10} = 7.008$, $p = 0.0244$]; interaction, [$F_{1,10} = 1.853$, $p = 0.2033$]; active lever presses, $p = 0.0163$; inactive lever presses, $p = 0.5256$). Females earned more morphine infusions than males overall and both sexes showed in an increase in the number of infusions earned over the 10 days of morphine self-administration (sex [$F_{1,140} = 7.117$, $p = 0.0085$]; time [$F_{2.509,351.2} = 17.29$, $p < 0.0001$]; interaction [$F_{9,1260} = 1.694$, $p = 0.0857$]). Morphine seeking increased on Day 30 compared to Day 1 of abstinence similarly for males and females (sex [$F_{1,31} = 2.441$, $p = 0.1283$]; Day [$F_{1,31} = 15.43$, $p = 0.0004$]; interaction [$F_{1,31} = 1.499$, $p = 0.2300$]).

Rats used for tissue collection and RNA sequencing did not undergo cue tests. However, all rats from which tissue was collected were part of cohorts in which morphine seeking was tested and shown to increase over extended abstinence (Fig. 1A). RNA sequencing of the accumbens shell revealed sex-specific changes in gene expression following forced abstinence. Genes that were unchanged between control saline-treated rats and Day 1 of abstinence from morphine, but showed a large change in expression after 30 days of abstinence were of particular interest. These "incubation-induced" changes in gene expression—those that occur over prolonged abstinence—mirror the increase in craving behavior that co-occurs over the same period.

In males, 315 DEGs were identified after 30 days of abstinence compared to one day of abstinence (Morphine D1 vs. Morphine D30); 89 DEGs were identified comparing one day of abstinence to controls (saline vs. Morphine D1); and 173 DEGs were identified comparing 30 days of abstinence to controls (saline vs. Morphine D30) (Fig. 2A; Supplementary Table 1). In females, 79 Morphine D1 vs. Morphine D30 DEGs, 155 saline vs. Morphine D1 DEGs, and 155 saline vs. Morphine D30 DEGs were identified (Fig. 2B; Supplementary Table 1). Only 22 overlapping DEGs were identified between males and females when comparing the saline and Morphine D30 groups (Fig. 2C; Supplementary Table 1), indicating sex-specific gene expression changes following extended forced abstinence from morphine self-administration. Heatmaps sorted by fold change comparing saline to Morphine D30 DEGs revealed sex-specific, incubation-induced patterns of gene expression (Fig. 2D, E). In both sexes, the majority of DEGs comparing 30 days of abstinence from morphine to saline were unchanged in the comparison of one day of abstinence to saline, mirroring the pattern of craving-like behavior.

RRHO analyses were used to compare the overlap in overall gene expression patterns in males and females between saline controls and morphine-exposed rats after one (Fig. 2F) or 30 (Fig. 2G) days of abstinence. RRHO analyses compared genes across sex that show similar (top right and bottom left quadrants) or opposite patterns (top left and bottom right quadrants) of expression following abstinence. Warm colors ("hot spots") indicate significant overlap in the direction of the change, whereas cooler colors represent minimal overlap in expression. At day one of abstinence from morphine compared to saline (Fig. 2F) males and females showed similar patterns of upregulated (hot spots in bottom left quadrant) and downregulated (hot spots in top right genes. At Day 30 (Fig. 2G), there was some overlap between males and females for genes downregulated by extended abstinence (upper right quadrant) but substantially less overlap for genes upregulated (cool colors in bottom left quadrant). Interestingly, many genes showed a sex-specific change (highlighted in bottom right and top left quadrants), showing upregulation in one sex but downregulation in the other.

Gene Ontology KEGG pathway enrichment analysis of DEGs identified biological pathways altered by abstinence from morphine self-administration comparing Day 30 of abstinence to saline. ShinyGo analysis was used to identify predicted transcription factors. In males, 28 KEGG pathways (Fig. 2H) and six transcription factors (Fig. 2I) were identified. In females, 12 KEGG pathways (Fig. 2J) and 13 transcription factors (Fig. 2K) were identified. Interestingly, only four common KEGG pathways were identified in both males and females (Fig. 2L). However, the majority of predicted transcription factors were common to both males and females (Fig. 2M).

Incubation of sucrose craving is accompanied by reinforcer- and sex-specific changes in gene expression

In order to examine incubation of craving for a non-drug reward, separate cohorts of male and female rats self-administered oral sucrose for 10 days; controls received only cues and no sucrose. Importantly, rats had free access to food and water throughout the studies. The number of earned sucrose pellets increased over 10 days of self-administration and was higher than "cue only" controls (Fig. 3A; condition, [$F_{2,60} = 180.1$, $p < 0.0001$], time, [$F_{2.678,160.7} = 56.10$, $p < 0.0001$, Geisser–Greenhouse epsilon = 0.2975]; interaction, [$F_{18,540} = 12.65$, $p < 0.0001$]). There were no differences in the number of pellets earned between rats that eventually underwent one compared to 30 days of abstinence (Fig. 3A; Control vs. Sucrose Day 1, $p < 0.0001$; Control vs. Sucrose Day 30, $p < 0.0001$; Sucrose Day 1 vs. Sucrose Day 30, $p = 0.9921$). During the cue tests, active, but not inactive lever presses, were higher on Day 30 of abstinence from sucrose compared to Day 1

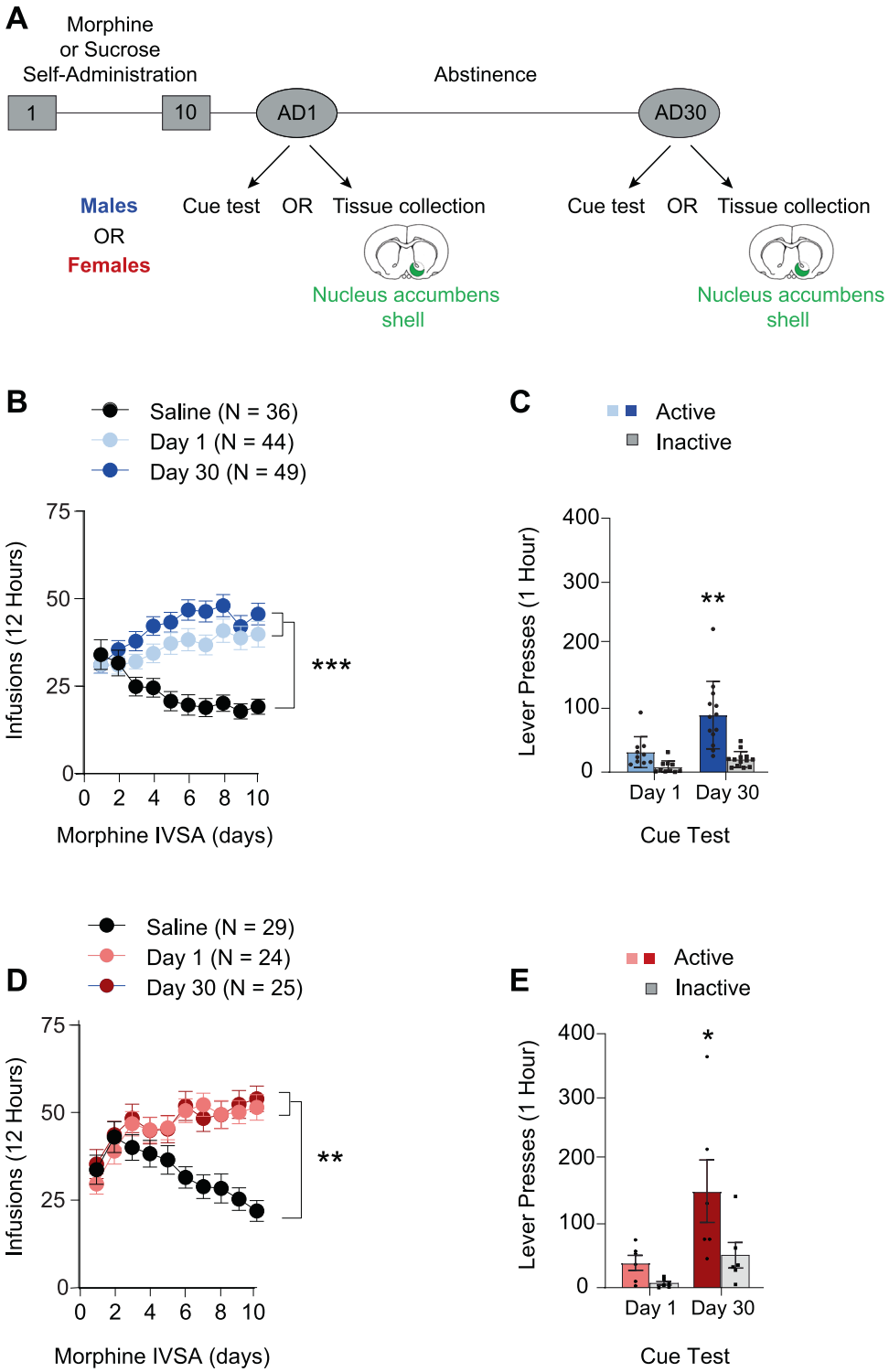
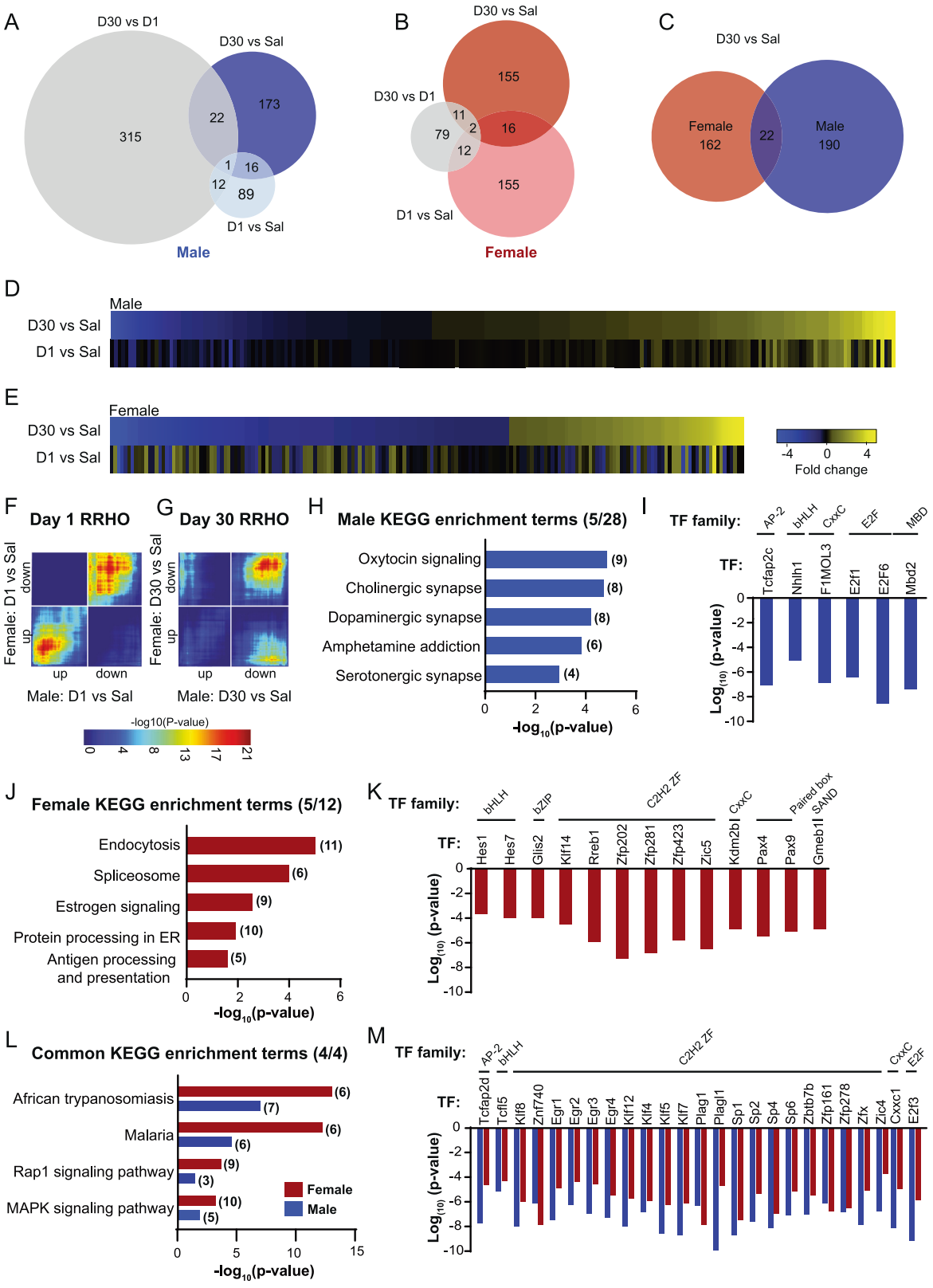


Fig. 1 Self-administration and incubation of morphine craving behavior in male and female rats. A Experimental timeline including 10 days of morphine or saline intravenous self-administration (IVSA), followed by either 1 or 30 days of forced abstinence. Separate cohorts self-administered oral sucrose with the same experimental timeline. **B** Male morphine-treated rats earned more infusions over 10 days of IVSA, whereas controls earned fewer infusions of saline. Morphine infusions did not differ between rats that eventually underwent one or 30 days of abstinence. **C** Males demonstrated incubation of morphine craving, as indicated by an increase in active, but not inactive lever presses during a 1-h cue test following 30 days of abstinence. **D** Female morphine-treated rats earned more infusions over 10 days of IVSA, whereas controls earned fewer infusions of saline. Infusions did not differ between rats that eventually underwent one or 30 days of abstinence. **E** Female rats also demonstrated incubation of morphine craving, as shown by an increase in active, but not inactive lever presses after 30 days of abstinence. Data show mean ± SEM; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.



for male rats (Fig. 3B; condition, [$F_{1,6} = 30.02$, $p = 0.0015$], lever [$F_{1,6} = 27.34$, $p = 0.0020$]; interaction, [$F_{1,6} = 16.73$, $p = 0.0064$]; active lever presses, $p = 0.0010$; inactive lever presses, $p = 0.7281$). RNA sequencing of the accumbens shell revealed robust changes in gene expression associated with 30 days of abstinence from sucrose in males (Fig. 3C, D).

Sucrose-treated female rats took increasing amounts of sucrose over 10 days of self-administration and earned more sucrose than "cue only" controls (Fig. 3E; condition, [$F_{2,75} = 103.7$, $p < 0.0001$], time, [$F_{2.261,169.6} = 41.69$, $p < 0.0001$, Geisser–Greenhouse epsilon = 0.2512]; interaction, [$F_{18,675} = 8.168$, $p < 0.0001$]). Active lever presses were higher on Day 30 of abstinence from sucrose

Fig. 2 Transcriptomics associated with incubation of morphine craving in male and female rats. A RNA-sequencing was performed on nucleus accumbens shell tissue from cue test-naive rats after either one (D1) or 30 (D30) days of forced abstinence from morphine self-administration or saline self-administration (Sal). Venn diagram shows the number of differentially expressed genes (DEGs) across conditions (Sal = Saline controls, D1 = rats that underwent one day of forced abstinence, D30 = rats that underwent 30 days of forced abstinence) in males. **B** Venn diagram shows the number of DEGs across conditions in females. **C** Venn diagram shows the number of D30 vs. Sal DEGs between males and females. **D** Heatmap sorted by fold change of D30 vs. saline DEGs compared to D1 vs. Sal DEGs in males. **E** Heatmap sorted by fold change of D30 vs. Sal DEGs compared to D1 vs. Sal DEGs in females. **F** Rank rank hypergeometric overlap analysis comparing gene expression patterns between D1 vs. Sal in males and females. **G** Rank rank hypergeometric overlap analysis comparing gene expression patterns between D30 vs. Sal in males and females. **H** Top five (out of 28 total) KEGG enrichment terms identified using D30 vs. Sal DEGs in males. The number of genes identified in each term is identified in parentheses to the right of the bar graph. **I** Transcription factors predicted by ShinyGo to regulate D30 vs. Sal DEGs in males. **J** Top five (out of 12 total) KEGG enrichment terms identified using D30 vs. Sal DEGs in females. The number of genes identified in each term is identified in parentheses to the right of the bar graph. **K** Transcription factors predicted by ShinyGo to regulate D30 vs. Sal DEGs in females. **L** Common KEGG enrichment terms identified using D30 vs. Sal DEGs in both males and females. The number of genes identified in each term is identified in parentheses to the right of the bar graph. **M** Transcription factors predicted by ShinyGo to regulate D30 vs. Sal DEGs in both males and females.

compared to Day 1 for females (Fig. 3F; condition, [$F_{1,32} = 26.68$, $p < 0.0001$], lever [$F_{1,32} = 67.16$, $p < 0.0001$]; interaction, [$F_{1,32} = 13.52$, $p = 0.0009$]; active lever presses, $p < 0.0001$; inactive lever presses, $p = 0.6007$) indicating that incubation of cue-induced sucrose craving occurred in both sexes. The total number of sucrose pellets earned increased over the 10 days of self-administration and was comparable in males and females (sex [$F_{1,100} = 0.5802$, $p = 0.4480$]; time [$F_{9,900} = 123.2$, $p < 0.0001$]; interaction [$F_{9,900} = 1.244$, $p = 0.2642$]). Sucrose seeking increased on Day 30 compared to Day 1 of forced abstinence similarly for males and females (sex [$F_{1,22} = 0.1087$, $p = 0.7448$]; Day [$F_{1,22} = 31.32$, $p < 0.0001$]; interaction [$F_{1,22} = 0.0147$, $p = 0.9046$]).

Females also showed abstinence-specific patterns in gene expression (Fig. 3G, H). Comparison of the 230 DEGs between controls and the Sucrose Day 30 abstinence group revealed only 10 overlapping genes between males and females (Fig. 3I; Supplementary Table 2). KEGG pathway analyses also showed that there are no common KEGG pathways for sucrose-treated males and females (Supplementary Fig. 1), suggesting a sex-specific mechanism. When comparing controls to 30 days of abstinence from either morphine or sucrose in males, the resulting DEGs were largely unique to the reinforcer: 93 sucrose-specific DEGs, 202 morphine-specific DEGs, and only 10 DEGs that overlapped across reinforcers (Fig. 3J). Similarly, females had 121 sucrose-specific DEGs, 178 morphine-specific DEGs, and 6 common DEGs between morphine and sucrose (Fig. 3K). Furthermore, KEGG pathway analyses revealed morphine- and sucrose-specific terms (Supplementary Table 3 and Supplementary Table 4).

The amount of morphine consumed is positively correlated with drug seeking in late abstinence

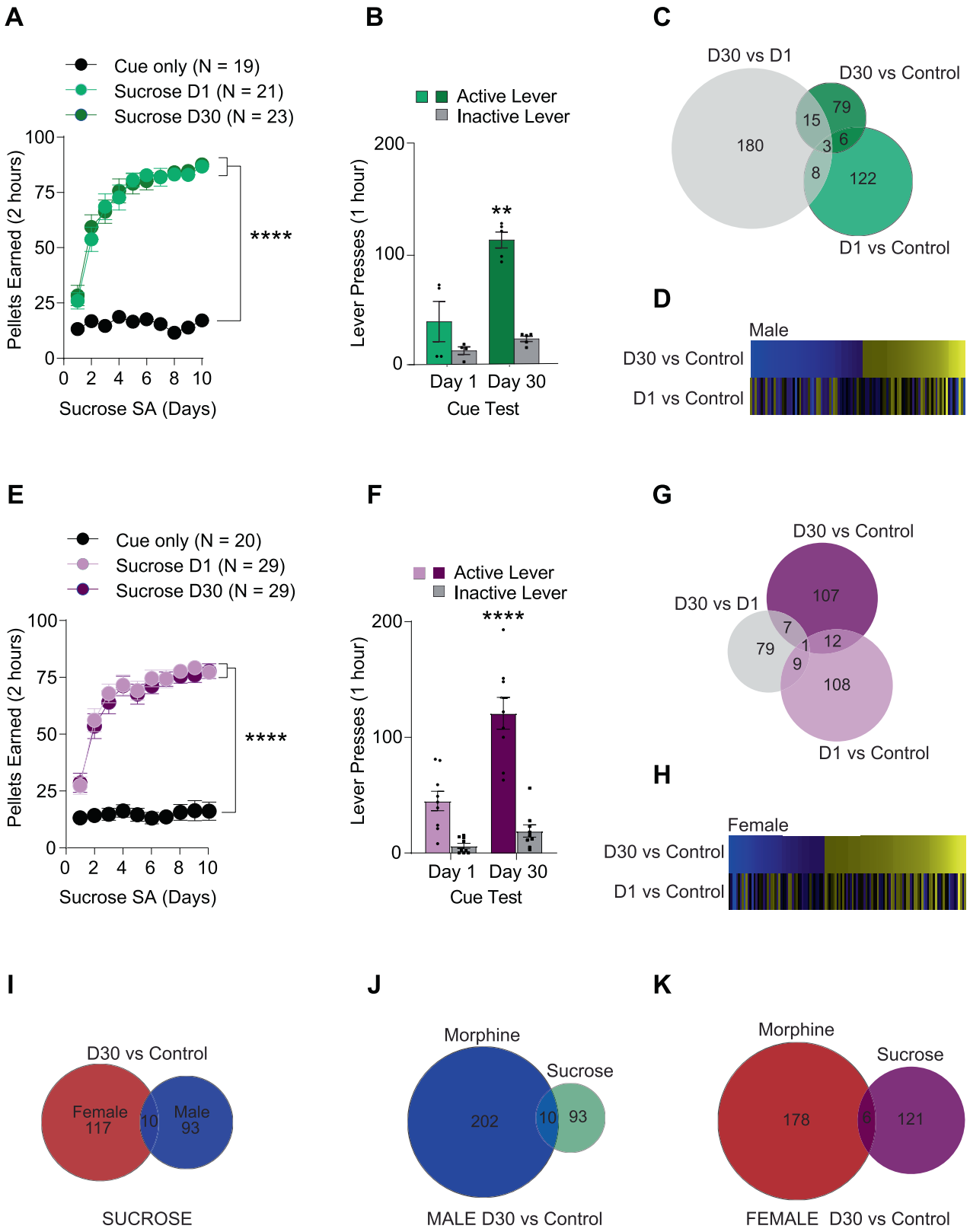
Pearson correlations comparing total consumption during self-administration and reward-seeking behavior during early or late abstinence revealed that morphine and sucrose exposure impact craving at opposite time points. In males, total morphine infusions earned during IVSA was positively correlated with active lever presses at Day 30 of forced abstinence (Fig. 4A; Day 30, [$R = 0.7$, $p = 0.0018$) but there was no correlation between total consumption and morphine seeking at Day 1 (Fig. 4A; Day 1, [$R = -0.27$, $p = 0.45$]). Females showed a similar pattern, with total morphine infusions correlating positively to morphine seeking only in late abstinence (Fig. 4B; Day 1, [$R = 0.52$, $p = 0.19$], Day 30, [$R = 0.85$, $p = 0.016$]). This effect was reversed in sucrose self-administering rats. In males, total sucrose pellets earned was positively correlated with sucrose seeking during the Day 1, but not Day 30 cue test (Fig. 4C; Day 1, [$R = 0.51$, $p = 0.0015$], Day 30, [$R = -0.11$, $p = 0.46$]). The same pattern was observed in sucrose self-administering females (Fig. 4D; Day 1, [$R = 0.26$, $p = 0.017$], Day 30, [$R = 0.073$, $p = 0.6$]).

DISCUSSION

Opioid consumption produces long-lasting changes in gene expression in the brain, some of which persist after extended abstinence [35–37]. However, whether these molecular changes mediate the intensification of drug seeking remains an open question and little is known about how these mechanisms differ across biological sex. Our results indicate that, although incubation of craving occurred similarly in both sexes, the transcriptomic signature associated with extended abstinence and craving was sex- and reinforcer-specific in the nucleus accumbens shell. In comparison to saline controls, hundreds of genes were unchanged in early abstinence and became either upregulated or downregulated over the course of prolonged abstinence from morphine ("incubation-induced"). These genes are of particular interest, given that their expression patterns mirror the change in drug-seeking behavior observed after extended abstinence. The saline and "cue only" control groups served as a baseline to determine the direction of observed changes, which cannot be determined by comparing abstinence Day 1 to abstinence Day 30 groups. One notable limitation of this study is that tissue from the control groups was only collected on Day 1 of abstinence. Hence, the possibility that some of the transcriptomic changes are caused by the passage of time alone cannot be completely excluded. "Incubation-induced genes" are potential targets for novel treatment development and drug discovery to mitigate craving. Interfering with the development of some of these changes and/or aiming to reverse them after extended abstinence has the potential to remedy the root of craving behavior, rather than treating the symptoms that emerge during extended abstinence. One critical first step in this endeavor will be to functionally validate the role of some of the identified transcripts in mediating the intensification of drug craving over time.

Incubation of morphine craving is associated with sex-specific alterations

The overwhelming majority of research examining opioid-induced changes in gene expression has been conducted in males. Of the 374 DEGs associated with extended abstinence from morphine, only 22 overlapped between males and females. It should be noted that transcriptomic changes are likely sex- and region-specific throughout the reward circuitry [38, 39] and further studies are needed to test this possibility. Opioid exposure induces changes in the expression and phosphorylation states of transcription factors ERK, CREB, and ΔFosB, as well as plasticity-related genes and nuclear receptors [36, 40–43]. These plasticity-related changes persist after the cessation of exposure and therefore provide likely mechanisms for stable behaviors such as incubation of craving [36, 44, 45]. Many of these documented changes were observed only in males that were re-exposed to the drug-paired cues [20, 35], which itself induces changes in gene



expression [46–48]. Here, we used cue-naive rats to characterize the transcriptomic changes that occur throughout abstinence independently of re-exposure to drug-paired cues. In all cohorts of rats, a subset of rats were re-exposed to cues to ensure that enhanced drug seeking occurred for that particular cohort. Therefore, we are confident that the RNA sequencing results are representative of neural adaptations occurring in rats displaying incubation of morphine craving.

Morphine dependence produced by chronic non-contingent injections elicits long-lasting changes in gene expression of

Fig. 3 Transcriptomics associated with incubation of sucrose craving in male and female rats. A Males self-administering sucrose earned increasing numbers of pellets over 10 days of self-administration and earned more sucrose pellets than cue presentations in controls. **B** Male rats demonstrated incubation of sucrose craving, as indicated by an increase in active, but not inactive lever responses during a 1-h cue test. **C** Tissue was collected from the nucleus accumbens shell of cue test-naive male rats after either one or 30 days of forced abstinence and processed for RNA sequencing. Venn diagram shows the number of differentially expressed genes across conditions (Control = cue only controls, D1 = rats that underwent one day of forced abstinence, D30 = rats that underwent 30 days of forced abstinence). **D** Heatmap sorted by fold change of D30 vs. Control DEGs compared to D1 vs. Control DEGs in males. **E** Females self-administering sucrose earned increasing numbers of pellets over 10 days of self-administration and earned more sucrose pellets than cue presentations in controls. **F** Females showed incubation of sucrose craving. **G** Venn diagram shows the number of differentially expressed genes across in females. **H** Heatmap sorted by fold change of D30 vs. Control DEGs compared to D1 vs. Control DEGs in females. **I** Venn diagram shows the number of differentially expressed genes across males and females in the sucrose Day 30 vs. Control category. **J** Morphine vs. sucrose DEGs Venn diagram compares DEGs between male morphine 30 days of abstinence from morphine vs. Control (D30 vs. Sal) alongside male 30 days of abstinence from sucrose vs. Control (D30 vs. Control). **K** Morphine vs. sucrose DEGs Venn diagram compares DEGs between female 30 days of abstinence from morphine vs. control (D30 vs. Sal) alongside female 30 days of abstinence from sucrose vs. Control (D30 vs. Control). Data show mean ± SEM; $^{****}p < 0.0001$, $^{**}p < 0.01$.

molecules involved in GPCR and neurotrophic signaling, as well as GABA and glutamate neurotransmission in multiple brain regions of the reward pathway [37, 43, 49, 50]. We found many DEGs that fall in the aforementioned categories in our male data sets. *Gria2* and *Gria3*, which encode AMPA receptor subunits, were upregulated in males during late abstinence and in females during early abstinence, respectively, with no changes in the expression of NMDA or kainate receptor subunits in either sex. Genes related to GABA-B receptor function, including *Gabbr1*, were upregulated in both sexes at Day 30, and expression of this gene was also lower in females at the early abstinence time point. Consistent with previous reports [37], neurotrophic *Ntrk1* expression was unchanged in males in late abstinence. In sharp contrast, *Ntrk1* was upregulated in females at Day 30. Incubation of oxycodone craving is associated with access- and region-specific changes in the expression of fibroblast growth factors following a relapse test at abstinence Day 30. *Fgf1*, *Fgf2, Fgfr1, Fgfr2*, and *Fgfr4* are unchanged in the nucleus accumbens [51]. We corroborate that all of these genes are unchanged in late abstinence for both sexes. Protein kinase C beta (PRKCB), which helps regulate CREB signaling, is downregulated in male human heroin users [52]. A single nucleotide polymorphism in *Prkcb* is also associated with alcohol cue reactivity in men and women [53]. Here, *Prkcb* expression was unchanged in male rodents exposed to morphine, but was drastically reduced at Day 1 and increased at Day 30 of abstinence in female rats. These findings add to a growing list of studies that identified sex-specific transcriptional changes following exposure to cocaine [54] and fentanyl [55]. Interestingly, others have found that females show greater drug-induced transcriptional changes in the nucleus accumbens compared to males [55]. Here, females had a greater number of DEGs in early abstinence compared to males, though there was more overlap in expression between sexes in early compared to late abstinence. Another important consideration is that females consumed more morphine overall than males during 10 days of chronic access, which may also have contributed to the distinct signatures observed in each sex. This sex difference in morphine intake is consistent with other reports that females experience a faster acquisition of opioid self-administration [56, 57], maintain more self-administered morphine and heroin (though these findings are dose-dependent), and exhibit higher progressive ratio breakpoints for morphine and heroin compared to males [58–61]. It should be noted that others fail to observe sex differences and/or observed greater intake in males under different access conditions [62]. Sex differences in opioid self-administration are inconsistent, and it is highly likely that dose, drug, and route of administration play critical roles [63]. However, our results that males and females show similar opioid craving following extended abstinence is consistent with other reports [13, 16–18].

KEGG pathway enrichment analyses revealed minimal overlap between males and females that underwent prolonged abstinence from morphine. In morphine-exposed rats, male-specific pathways included cholinergic and dopaminergic synapses, and amphetamine addiction. Genes within these pathways are associated with alcohol cue reactivity, long-term heroin exposure in humans, and chronic amphetamine exposure in rodents [52, 53]. Female-specific pathways included spliceosome and protein processing in the endoplasmic reticulum. Enrichment of the spliceosome pathway may indicate differential involvement of RNA polymerase II and epigenetic regulation via alternative splicing [64]. Genes within these pathways include *Pcbp1*, which is implicated in post-transcriptional regulation of mu opioid receptor mRNA [65], and several heat shock proteins, which have been broadly implicated in opioid exposure, withdrawal, and behavioral sensitization [66, 67]. Hsp70 Binding Protein 1 (*Hspbp1*) is downregulated in females, but not males after 30 days of abstinence from morphine. *Hspa1b*, which encodes HSP70-2, is upregulated in females, but not males in late abstinence. Others have found that escalating doses of morphine over a 10-day treatment period induces changes in *Hsp70, Hsp27, Hsp40*, and *Hsp105*, though this was seen in the frontal cortex after experimenter-delivered morphine, and was only studied in males [49].

There were 25 overlapping predicted transcription factors between morphine-exposed males and females. Only six male- and 13 female-specific transcription factors were identified. Male-specific transcription factors include TCFAP2C, which is involved in hippocampal glutamatergic neurogenesis [68], and E2F1, which is altered by nicotine and alcohol exposure [69, 70]. Female-specific transcription factors include HES1 and HES7, which are linked to synaptic plasticity, alcohol exposure, and cannabis dependence [71–74]. KLF4—predicted to contribute in both sexes here—is a transcription factor in the toll-like receptor 4 pathway involved in incubation of heroin craving [75]. It is possible that sex-specific transcription factors regulate the expression of sex-specific target genes, which ultimately affect craving behavior similarly in males and females.

Immediate early genes (IEGs) are thought to participate in drug-induced neuroplasticity and have been shown consistently to respond to many drugs of abuse, including heroin and cocaine [20, 23, 35, 49, 76–87]. Several IEGs, including EGR1, EGR2 and EGR3 were identified as potential upstream transcription factors; however, we did not observed changes in the expression of *Fos/c-fos* or *JunB/c-jun*, which are changed in the nucleus accumbens of male rats that have been tested for incubation of craving after 30 days of abstinence from long-access oxycodone self-administration [51]. We found *Egr1* and *Egr2* to be unchanged in the shell of males and females at either point of abstinence. Our results are consistent with previous studies for *Egr1*, the expression of which does not change after 1 or 14 days of abstinence from self-administered heroin [78]. However, *Egr2* expression is altered in the accumbens during early abstinence from experimenter-delivered morphine [37] or self-administered heroin [78]. *Egr2* is also upregulated in the accumbens after

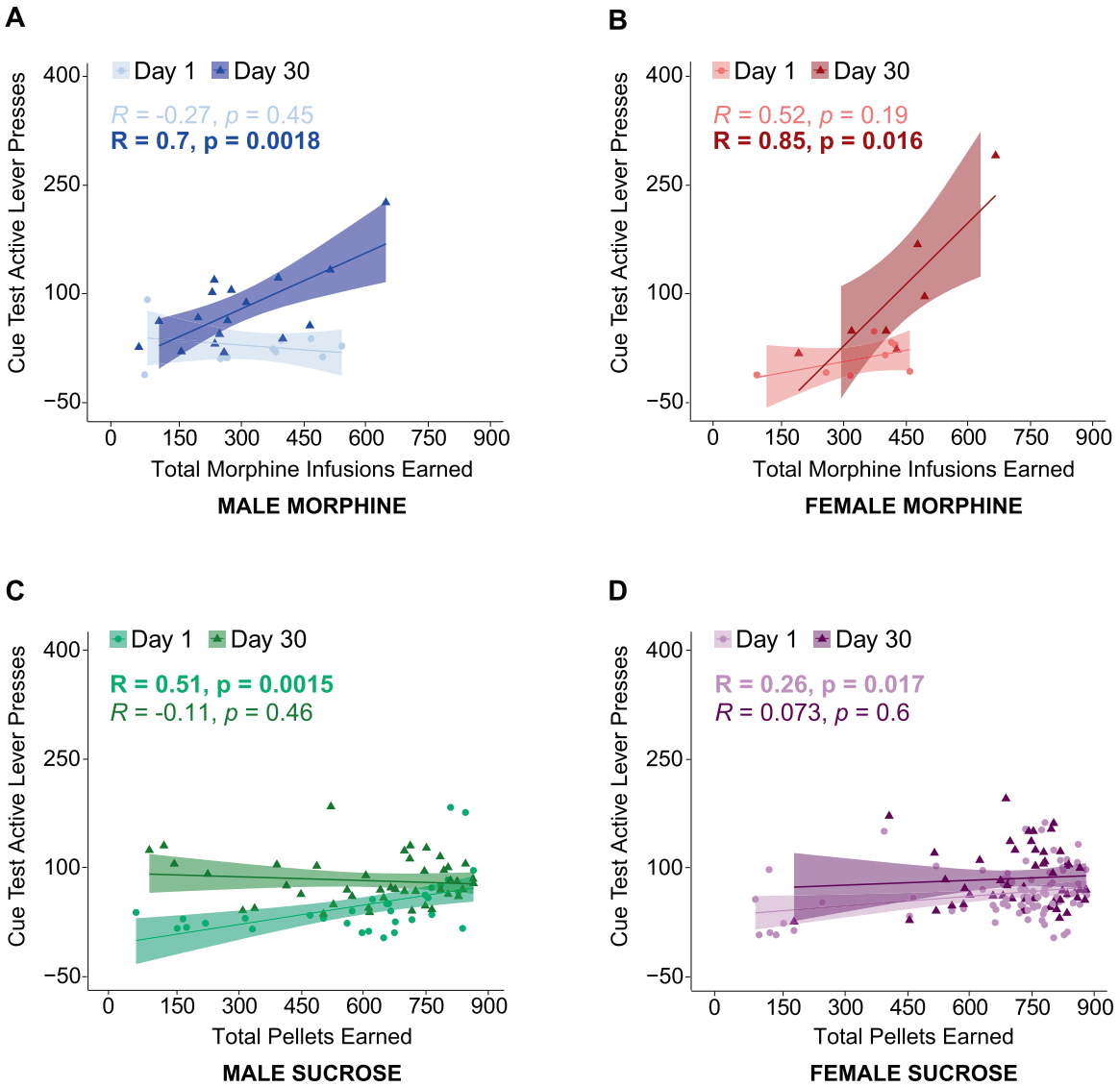


Fig. 4 Morphine consumption predicts drug seeking in late abstinence for males and females. A Total morphine infusions earned by males during intravenous self-administration showed a significant positive correlation with morphine seeking, as measured by active lever presses, during the Day 30 cue test, but not Day 1 cue test. **B** Total morphine infusions earned by females during intravenous self-administration showed a significant positive correlation with morphine seeking during the Day 30 cue test, but not Day 1 cue test. **C** Total sucrose pellets earned by males during self-administration showed a significant positive correlation with sucrose seeking during the Day 1, but not Day 30 cue test. **D** Total sucrose pellets earned by females during self-administration showed a significant positive correlation with sucrose seeking during the Day 1, but not Day 30 cue test.

14 days of abstinence, but only in rats that received yolked heroin infusions [78], and shows a dramatic downregulation after three weeks of abstinence in rats that received experimenter-delivered morphine [37]. The present results reinforce the idea that re-exposure to cues, the route and regimen of exposure, as well as biological sex are all critical considerations in establishing the molecular correlates of drug seeking and drug craving behaviors [20, 37, 78, 88].

Another important consideration are the well-researched sexually dimorphic aspects of the reward circuitry, which are established by organizational sex differences in reward-related neurotransmission and anatomy [89]. As it relates to drug abuse, males and females express different dopaminergic neuron density [90] and cocaine-induced dopamine release [91]. Thus drug-induced sex-specific transcriptional changes [54] may be partly explained by evolutionarily conserved sex differences that support divergent reproductive strategies and needs based on biological sex [92]. In fact, drug addiction is likely one of many sexually convergent behaviors influenced by sex-specific molecular underpinnings.

Changes in gene expression after prolonged abstinence are reinforcer-specific

Craving for sucrose, a non-drug reward, also increases over time [10, 93–97]. Given that natural rewards produce similar effects in the mesocorticolimbic reward pathway [98, 99], and induce similar changes in transcription factors, such as ΔFosB [36, 100], a comparison of the molecular changes associated with incubation of opioid versus sucrose craving can parse opioid-specific mechanisms that minimally affect the natural reward system. Sucrose-treated rats demonstrated fewer changes in gene expression overall; however, similar to morphine, robust alterations were observed over 30 days of abstinence from sucrose. Although the incubation-induced patterns are similar across

reinforcers, the genes themselves show minimal overlap. In males, only ten genes were changed consistently following extended abstinence from sucrose or morphine; in females, only six genes were found to overlap. KEGG enrichment terms were also unique to sucrose versus morphine. This is consistent with other findings that examined changes in gene expression following opioid or sucrose self-administration, extinction, and re-exposure to drug-paired cues, and found that very few alterations generalized across reinforcers [3, 20, 78, 101]. Although others have compared changes in gene expression in sucrose- versus opioid-exposed rats [20], this study builds on previous findings by examining transcriptional changes in controls, as well as opioid- and sucrose-exposed rats of both sexes at different abstinence time points. This provides a robust understanding of the patterns and time course of gene expression changes. These data strongly suggest that incubation of morphine and sucrose craving are driven by distinct mechanisms.

Total intake is correlated with reward seeking

Total morphine consumption was correlated with drug-seeking behavior in late abstinence. This effect was reversed in rats that self-administered sucrose, such that sucrose consumption was correlated with sucrose-seeking behavior in early abstinence. The finding that increased morphine exposure is linked to higher levels of relapse-like behavior is in line with previous findings that long access self-administration is necessary for incubation of oxycodone, methamphetamine, and cocaine craving [102–104]. Here, all rats were permitted extended access to intravenous morphine; however, those that self-administered higher amounts exhibited enhanced incubation of craving, thus indicating that chronic exposure is necessary for relapse-like behavior. Conversely, extended access is not required for incubation of sucrose craving, given that sucrose seeking is similar after 30 days of abstinence, regardless of whether rats underwent 2- or 6-h access daily self-administration sessions [94]. It is possible that heightened opioid exposure acts uniquely to induce robust transcriptomic changes that affect drug-seeking behavior on a longer-term scale. The finding that morphine, but not sucrose consumption was linked to reward seeking in late abstinence is further evidence that natural rewards act via distinct mechanisms and do not require prolonged exposure to induce craving.

CONCLUSIONS

Male and female rats exhibited cue-induced incubation of craving following 10 days of self-administration and 30 days of abstinence from either morphine, or the non-drug reward sucrose. Morphine consumption was correlated with drug-seeking behavior after extended, but not protracted abstinence in both sexes, whereas sucrose exposure was correlated with seeking behavior in early abstinence only. RNA sequencing of the nucleus accumbens shell of cue test-naive rats showed robust changes in gene expression that emerged over prolonged abstinence. Comparison of transcriptomic alterations in males and females indicated that although the increase in drug-seeking behavior is similar across sexes, the underlying genes and pathways are dissociable by biological sex. Additionally, although abstinence from both morphine and sucrose showed incubation-induced changes in gene expression, the genes and pathways were reinforcer-specific. Overall, these results are promising for developing treatments that target opioid craving without interfering with the natural reward system. Additionally, they provide insight into potentially sex-specific treatment options that will improve efficacy in both men and women.

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AUTHOR CONTRIBUTIONS

HLM, CCB and MEW designed the studies. HLM, CCB, RK, DRP, ARB, ASE, ABT and SHD contributed to data collection and data analysis. HLM, CCB and MEW drafted the manuscript. All authors edited and approved manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

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