ABSTRACT

BACKGROUND: Opioid withdrawal is a key driver of opioid addiction and an obstacle to recovery. However, withdrawal effects on opioid reinforcement and mesolimbic neuroadaptation are understudied, and the role of sex is largely unknown.

METHODS: Male (n = 13) and female (n = 12) rats responded under a fentanyl-versus-food choice procedure during daily 2-hour sessions. In addition to the daily choice sessions, rats were provided extended access to fentanyl during 12-hour self-administration sessions. After 2 weeks of this self-administration regimen, the nucleus accumbens and ventral tegmental area of a subset of rats were subjected to RNA sequencing. In the remaining rats, a third week of this self-administration regimen was conducted, during which methadone effects on fentanyl-versus-food choice were determined.

RESULTS: Before opioid dependence, male and female rats similarly allocated responding between fentanyl and food. Abstinence from extended fentanyl access elicited similar increases in somatic withdrawal signs in both sexes. Despite similar withdrawal signs and extended-access fentanyl intake, opioid withdrawal was accompanied by a maladaptive increase in fentanyl choice in males, but not females. Behavioral sex differences corresponded with a greater number of differentially expressed genes in the nucleus accumbens and ventral tegmental area of opioid-withdrawn females relative to males. Methadone blocked withdrawal-associated increases in fentanyl choice in males but failed to further decrease fentanyl choice in females.

CONCLUSIONS: These results provide foundational evidence of sex-specific neuroadaptations to opioid withdrawal, which may be relevant to the female-specific resilience to withdrawal-associated increases in opioid choice and aid in the identification of novel therapeutic targets.

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Opioid use disorder (OUD) diagnoses increased nearly 500% from 2010 to 2016 in the United States and OUD continues to be a public health crisis (1). In response to this crisis, the United States Food and Drug Administration initiated a dialogue with patients with OUD to identify critical barriers to recovery (2). Patients emphasized that opioid withdrawal elicited similar increases in somatic withdrawal signs in both sexes. Despite similar withdrawal signs and extended-access fentanyl intake, opioid withdrawal was accompanied by a maladaptive increase in fentanyl choice in males, but not females. Behavioral sex differences corresponded with a greater number of differentially expressed genes in the nucleus accumbens and ventral tegmental area of opioid-withdrawn females relative to males. Methadone blocked withdrawal-associated increases in fentanyl choice in males but failed to further decrease fentanyl choice in females.

Opioid use disorder (OUD) diagnoses increased nearly 500% from 2010 to 2016 in the United States and OUD continues to be a public health crisis (1). In response to this crisis, the United States Food and Drug Administration initiated a dialogue with patients with OUD to identify critical barriers to recovery (2). Patients emphasized that opioid withdrawal elicited similar increases in somatic withdrawal signs in both sexes. Despite similar withdrawal signs and extended-access fentanyl intake, opioid withdrawal was accompanied by a maladaptive increase in fentanyl choice in males, but not females. Behavioral sex differences corresponded with a greater number of differentially expressed genes in the nucleus accumbens and ventral tegmental area of opioid-withdrawn females relative to males. Methadone blocked withdrawal-associated increases in fentanyl choice in males but failed to further decrease fentanyl choice in females.

Consistent with these patient reports, opioid withdrawal enhances opioid self-administration in preclinical studies (3–6). Conversely, opioid withdrawal decreases self-administration of non-opioid reinforcers, such as food (3) and electrical brain stimulation (6). These findings suggest that opioid withdrawal can enhance opioid reinforcement not only through direct mechanisms but also indirectly through the simultaneous devaluation of non-opioid reinforcers. Consistent with this hypothesis, opioid withdrawal increases the allocation of operant behavior (i.e., choice) emitted toward opioid injections over concurrently available palatable food in male nonhuman primates (10–14) and rats (15). Choice procedures such as these may be especially useful for studying the mechanisms of opioid withdrawal on both drug and nondrug reinforcement because the maladaptive choice of drugs at the expense of nondrug alternatives is increasingly recognized as a hallmark of substance use disorders (16–19).

The high-efficacy mu opioid receptor (MOR) agonist methadone is the most effective medication for OUD and is administered to patients with a high degree of opioid dependence (20). In contextual agreement with its clinical usage, methadone does not attenuate opioid choice in nondependent laboratory animals (13). However, in opioid-dependent nonhuman primates, methadone and other high-efficacy MOR agonists block withdrawal-associated increases in opioid choice and promote behaviors maintained by alternative reinforcers (10–14,21). Overall, the sensitivity of preclinical
Sex-Specific Effects of Opioid Withdrawal

opiod-versus-food choice procedures to MOR agonist treatments selectively in opioid-withdrawn primates supports the translational utility of these procedures to study the mechanisms of withdrawal-induced behavioral misallocation.

Although sex differences in preclinical opioid abuse-related end points are well documented in non-opioid-dependent subjects, less is known about how opioid withdrawal affects opioid reinforcement between sexes. Recent preclinical rodent studies suggest that opioid withdrawal leads to protracted, sex-specific effects on somatic withdrawal signs, opioid self-administration, and reinstatement [e.g., (22–24)]. However, sex differences in opioid withdrawal effects on opioid-versus-food choice are unexplored. We therefore examined sex differences in spontaneous opioid withdrawal effects on fentanyl-versus-food choice. We also performed RNA sequencing (RNA-seq) in reward-associated brain regions of the nucleus accumbens (NAc) and ventral tegmental area (VTA) of opioid-withdrawn male and female rats as an unbiased and hypothesis-generating approach for evaluating the transcriptional consequences of opioid withdrawal in mesolimbic structures known to be involved in opioid reinforcement (25–29). Our results illustrate a sexually dimorphic behavioral response to fentanyl withdrawal, with increasing fentanyl choice in males (a maladaptive behavioral allocation toward heightened drug use) and decreasing fentanyl choice in females (a proadaptive behavioral allocation toward food consumption). Sex differences in decision making corresponded with a greater number of differentially expressed genes (DEGs) in the NAc and VTA of opioid-withdrawn females relative to males. In addition, methadone administration decreased fentanyl choice in male rats to a similar degree as the spontaneous, withdrawal-associated decrease observed in females. In summary, this work demonstrates that female rats are resilient to the withdrawal-associated increases in fentanyl choice observed in males. Furthermore, the female-specific resilience to increased fentanyl choice may, in part, be the consequence of heightened mesolimbic transcriptional neuroadaptation.

METHODS AND MATERIALS

Overview

In Supplement 1, we describe the animals (33 Sprague Dawley rats: 17 male, 16 female), details of catheterization surgery and maintenance, behavioral procedures, and drugs. Animal maintenance and research were conducted in accordance with the 2011 guidelines for the care and use of laboratory animals and protocols approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Procedure

Self-administration Training. Fentanyl-versus-food choice training: A total of 25 rats (13 male, 12 female) were trained to respond under a fentanyl-versus-food choice procedure as described previously (30) and detailed in Supplement 1.

Briefly, each choice session consisted of five 20-minute response components wherein the available unit fentanyl dose increased by 0.5 log unit increments between each component and the food reinforcer (18 vol/vol vanilla flavor Ensure in tap water; Abbott Laboratories, Chicago, IL) remained constant. Food availability was signaled by illuminating a red stimulus light above the lever, and fentanyl availability was signaled by illuminating a green stimulus light above the right lever (longer flashes signaled availability of larger unit fentanyl doses). During each component, rats could complete up to 10 ratio requirements (i.e., fixed-ratio [FR] 5) between the food- and drug-associated levers. Choice was considered stable when the smallest fentanyl unit dose that maintained at least 80% of completed ratio requirements on the fentanyl-associated lever was within a 0.5 log unit for three consecutive days with no trends (i.e., stability criteria). Stability criteria were not assessed until after at least five choice sessions. Data collected during the final training day served as baseline values for subsequent analyses.

Saline-versus-food choice training: A total of 8 rats (4 male, 4 female) were trained to respond under a saline-versus-food choice procedure. Rats were trained to self-administer 32% vol/vol Ensure under an FR5, 20-second timeout (TO20) schedule using training criteria described in Supplement 1. The Ensure concentration was increased from 18% to 32% to accelerate acquisition. Rats were then provided access to intravenous saline (5-s injection/315 μl). Saline was available under an FR1, TO20 for two sessions and FR5, TO20 for three sessions. Finally, saline and 32% Ensure were made available under identical choice parameters, visual discriminative stimuli, and stability criteria as described above for fentanyl.

Extended-Access Self-administration. Following stability, a regimen of overnight (6:00 PM–6:00 AM) extended access (FR5, TO10 schedule of reinforcement) to fentanyl (3.2 μg/kg/injection) or saline (5-s injection/315 μg) began. Fentanyl or saline availability was signaled by illuminating a green stimulus light above the right lever. Eight hours after each extended-access self-administration session (approximately 1:55 PM), rats were observed for 30 seconds for the presence of nine somatic withdrawal signs (see Supplement 1) and weighed before beginning the daily choice test (2:00 PM). The half-life of intravenous fentanyl has been reported to be <1 hour in male and female rats (31), suggesting that more than 8 half-lives would have elapsed since the last fentanyl exposure. Overnight self-administration tests occurred Sunday to Thursday nights for 2 consecutive weeks (i.e., week 1 and week 2) in all rats, and a subset of rats progressed to a third week (see Methadone Tests below).

Tissue Preparation and RNA Sequencing

A representative sample of the fentanyl-trained rats and saline-trained rats (Figure S1 in Supplement 1) was used in RNA-seq studies. The number of animals used in each experiment is described in the figure legends. In lieu of the final choice session (2:00 PM ±1 hour), rats were rapidly decapitated without anesthesia, brains were sectioned into 1-mm coronal slices using sex-specific brain matrices (Zivic Instruments, Pittsburgh, PA; male: BSRLS001-1; female: BSRLA001-1), and bilateral tissue punches were collected from the NAc (12 gauge; internal diameter, 2.16 mm) and VTA (16 gauge; internal diameter, 1.19 mm). Tissue was frozen on dry ice and stored
Sex-Specific Effects of Opioid Withdrawal

at ~80 °C. Total RNA was extracted with the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA). RNA quality control assays were performed on the TapeStation 4200 (Agilent, Santa Clara, CA), and the RNA integrity number for all samples (±SEM) ranged from 8.0 to 9.2 (mean = 8.7 ± 0.05). Ribosomal RNA depletion and library preparation (Illumina Ribo-Zero) was performed, and RNA-seq was carried out on an Illumina (San Diego, CA) sequencing platform (HiSeq 2500) with a sequencing depth of approximately 22 million reads per sample (mean = ~22 ± 0.03 million). Other overall sample sequencing statistics include the mean quality score (35.54 ± 0.03) and the percent of bases ≥30 (91.94 ± 0.16).

Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the Rattus norvegicus Rnor6.0 reference genome available on Ensembl using the STAR aligner v.2.5.2b. Unique gene hit counts were calculated by using featureCounts from the Subread package v.1.5.2. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between groups of samples was performed. The Wald test was used to generate p values and log2 fold changes. Genes with an adjusted p value < .05 and absolute log2 fold change > 1 were designated as DEGs for each comparison. For pattern identification in union heatmaps, Venn diagrams, and Gene Ontology (GO), genes with an unadjusted p value < .05 and absolute log2 fold change > 1.3 were called as DEGs for each comparison. Heatmaps were generated using Morpheus and GO analysis was performed using GOrilla, running all ontologies (Process, Function, Component) and reporting uncorrected enrichment p values (32).

**Methadone Tests**

A total of 6 male and 5 female fentanyl-trained rats progressed to a third week of extended fentanyl access. Somatic withdrawal signs, weights, and fentanyl-versus-food choice testing continued as in weeks 1 and 2. During each of the 5 testing days (Monday to Friday), rats were administered one of five subcutaneous pretreatments of saline or methadone (0.1, 0.32, 1, 3.2 mg/kg) 20 minutes before fentanyl-versus-food choice tests in a counterbalanced order. Thus, each rat received each dose condition.

**Data Analysis**

Behavioral data were analyzed using Prism 8 (GraphPad, La Jolla, CA) and Geisser-Greenhouse corrections for sphericity when appropriate. Choice-related dependent measures were analyzed by two-way analysis of variance or a mixed-effects analysis in instances of missing data. Other dependent measures include injections earned per 12 hours and bodyweight. These dependent measures were analyzed within each sex by one-way analysis of variance and between sexes by two-way analysis of variance. Withdrawal signs were analyzed by nonparametric Friedman tests. Significant (p < .05) interactions were followed by Dunnett, Sidak, or Dunn post hoc tests as appropriate. See Table S1 in Supplement 1 for a complete reporting of statistical results.

**RESULTS**

**Fentanyl-Versus-Food Choice in Nondependent Rats**

At baseline, liquid food was almost exclusively chosen when no fentanyl or small unit doses (0.32 and 1 μg/kg/injection) were available (dashed lines; Figures 1D and 2A, B). As the fentanyl dose increased, behavior was reallocated toward the fentanyl lever and the largest unit doses of fentanyl (3.2 and 10 μg/kg/injection) maintained near-exclusive choice. In addition, choices per component decreased as a function of increasing fentanyl doses (dashed lines, Figures 1E and 2C, D). No baseline sex differences were detected for either choice-related measure.

**Withdrawal From Overnight Fentanyl Self-administration Produces Opposing, Sex-Specific Effects on Fentanyl-Versus-Food Choice**

The number of fentanyl injections increased (i.e., escalated) across 2 weeks of extended access (Figure 1A) (male: F1,8,21.6 = 17.04, p < .0001; female: F2,4,26.8 = 9.7, p = .0004). Over this same time, somatic withdrawal signs increased (Figure 1B) (male: Friedman statistic = 66.4, p < .0001; female: Friedman statistic = 59.0, p < .0001) and body weights decreased (Figure 1C) (male: F2,0,23.7 = 94.8, p < .0001; female: F2,6,28.1 = 40.8, p < .0001). Other than greater bodyweight decreases in males (sex × time interaction: F9,207 = 4.8, p < .0001), no other sex differences were detected for these dependent measures.

Opioid withdrawal resulted in sex-dependent effects on fentanyl-versus-food choice. Whereas percent fentanyl choice was similar between sexes at baseline (Figure 1D) and during the first week of extended fentanyl access (Figure 1F), fentanyl choice was significantly lower in females relative to males during the second week of extended fentanyl access (Figure 1H) (main effect of sex: F1,23 = 7.6, p = .01). Males completed fewer choices per component during both weeks of extended fentanyl access (Figure 1G, I).

Analyses of withdrawal effects within each sex revealed opposing effects on fentanyl choice. Whereas choice of the smaller unit doses of fentanyl progressively increased across weeks 1 and 2 in male rats (Figure 2A) (interaction: F2,6,48.4 = 10.6, p < .0001), choice of the largest unit dose of fentanyl significantly decreased across weeks 1 and 2 in female rats (Figure 2B) (interaction: F2,1,44.8 = 5.6, p = .0009). Opioid withdrawal decreased the number of choices completed per component irrespective of sex, as depicted in Figure 2C (male: interaction: F2,3,92.0 = 15.4, p < .0001) and Figure 2D (female: interaction: F2,3,8,42.3 = 11.1, p < .0001). The effects of withdrawal on the number of choices completed for each reinforcer type are depicted in Figure S5 in Supplement 1.

**Mesolimbic Transcriptional Consequences of Opioid Withdrawal**

Sex-matched rats subjected to a saline-versus-food choice paradigm were used as reference tissue to generate DEGs in
fentanyl-withdrawn rats. In transcriptional analyses from the NAc, females demonstrated a far greater diversity and degree of up- and downregulated transcripts than males (Figure 3A, B). By reducing statistical thresholds to enable broad pattern recognition and organizing the female transcripts from down- to upregulated in a union heatmap, very little concordance in the pattern of direction and degree of transcript expression was observed across males and females (Figure 3C). However, approximately 36% of the male-identified transcripts were also affected in females, which represented significant overlap in both up- and downregulated DEG lists (Fisher’s exact test two-sided p value < .05) (Figure 3D, E). Finally, we explored the broad GO terms enriched in sex-specific up- and downregulated transcript lists.
In male-specific upregulated transcripts, the top GO terms were enriched for the biological processes of protein kinase activity and signal transduction (Figure 3F). The top GO terms in female-specific upregulated transcripts were enriched in the cytosol and linked to the functions of oxygen transport and pre-microRNA binding (Figure 3G). No significantly enriched GO terms were identified in the male downregulated transcripts. The female-specific downregulated transcripts revealed top GO terms linked to the neuronal component, specifically the glutamatergic synapse, as well as cellular energetics and protein phosphorylation (Figure 3H).

Similar results were observed within the VTA. Again, we observed that females experienced more transcripts regulated to a greater degree than males (Figure 4A, B), minimal concordance in the identity and degree of regulation-specific transcripts across sexes (Figure 4C), and a significant overlap in which transcripts are up- or downregulated across males and females (Fisher’s exact test two-sided p value < .05) (Figure 4D, E). In sex-specific GO analysis, we identified that neuronal projection- and enzymatic activity-related terms were enriched in the male upregulated transcripts (Figure 4F). Similar to the NAc, female upregulated transcripts were linked to oxygen carrier activity (Figure 4G). Relatively fewer GO terms reached significance for male and female downregulated transcripts, with males associated with lipid processing (Figure 4H) and females associated with regulation of cellular secretion (Figure 4I).

To account for potential DEGs driven by baseline differences across sexes, we compared sequencing data for male saline-versus-food choice rats versus female saline-versus-food choice rats for both the NAc and VTA (Figure S2 in Supplement 1). A total of 8 significant baseline DEGs stratified by sex were identified in the NAc and 10 were identified in the VTA. Of these baseline-affected DEGs, five NAc (Tmpress11f, Cox7c, A1Y72581.19, Psma5, and AABR07058976.1) and one VTA (Tph2) were also identified as significant DEGs in female fentanyl-versus-food choice NAc and VTA, respectively (Figures 3B and 4B), indicating that their expression in these datasets is potentially driven by baseline sex differences and not a consequence of our choice procedure. None of the baseline-affected DEGs were identified in the male lists. While these six DEGs are likely driven by baseline differences in our saline groups, the remainder of identified DEGs appear to be the sex-specific result of our fentanyl exposure regimen.

Acute Methadone Decreases Fentanyl-Versus-Food Choice in Opioid-Withdrawn Male but Not Female Rats

A subset of rats were subjected to a third week of extended fentanyl self-administration and choice testing (Figure 5A, B), and subcutaneous saline or methadone injections preceded each choice session. In male rats, 3.2 mg/kg methadone decreased percent fentanyl choice relative to saline (Figure 5C) ($t_5 = 2.9, p = .03$) and increased the number of choices completed per component (Figure 5E) (interaction: $F_{1,9,0.3} = 53.5, p < .0001$). Methadone-induced decreases in fentanyl choice did not correspond with decreased somatic withdrawal signs (Figure S3E in Supplement 1). In female rats, 3.2 mg/kg methadone did not significantly alter percent fentanyl choice (Figure 5D), the number of choices completed per component (Figure 5F), or somatic withdrawal signs (Figure S3F in Supplement 1). A total of 8 significant baseline DEGs stratified by sex were identified in the NAc and 10 were identified in the VTA. Of these baseline-affected DEGs, five NAc (Tmpress11f, Cox7c, A1Y72581.19, Psma5, and AABR07058976.1) and one VTA (Tph2) were also identified as significant DEGs in female fentanyl-versus-food choice NAc and VTA, respectively (Figures 3B and 4B), indicating that their expression in these datasets is potentially driven by baseline sex differences and not a consequence of our choice procedure. None of the baseline-affected DEGs were identified in the male lists. While these six DEGs are likely driven by baseline differences in our saline groups, the remainder of identified DEGs appear to be the sex-specific result of our fentanyl exposure regimen.
Figure 3. Female and male rats experience distinct transcriptional activity within the nucleus accumbens following repeated fentanyl withdrawal. Volcano plots depicting the DEGs from microdissected nucleus accumbens identified in (A) male ($n = 4$) and (B) female ($n = 4$) rats. DEGs were normalized to sex-matched rats subjected to saline-vs.-food protocol. DEG cutoffs for volcano plots are Wald test-adjusted $p$ value $< .05$ and absolute log$_2$ fold change $>1$. (C) Union heatmap comparing female transcripts organized from downregulated to upregulated (top) to male transcripts of the same identity (bottom).
Sex-Specific Effects of Opioid Withdrawal

Supplement 1). The effectiveness of smaller methadone doses is shown in Figure S3 in Supplement 1.

DISCUSSION

Here, we identify sexually dimorphic decision-making strategies in an opioid-versus-food choice context following spontaneous opioid withdrawal. Whereas opioid withdrawal increased choice of fentanyl over a food alternative in males, fentanyl choice significantly decreased in opioid-withdrawn female rats. The female-specific capacity to spontaneously allocate behavior toward a proadapative food reinforcer while undergoing withdrawal mirrors the effects of methadone on opioid choice in opioid-withdrawn males within this procedure and suggests that the neural substrates of the female-specific behavior allocation within the context of drug choice may aid in the identification of novel therapeutic targets for OUD. To this end, we performed RNA-seq profiling of mesolimbic brain areas, revealing a greater diversity and degree of transcript expression in the NAc and VTA of females relative to males. These results provide foundational evidence of sex-specific neuroadaptations to repeated opioid withdrawal, which may be relevant to the female-specific resilience to withdrawal-associated increases in opioid choice.

Non-opioid–dependent male and female rats similarly allocated choice between fentanyl injections and a concurrently available food reinforcer (Figure 1D). In addition, extended-access fentanyl intake and the spontaneous expression of somatic withdrawal signs were similar between sexes (Figure 1A, B). However, opioid withdrawal unmasked behavioral sex differences (Figure 1H). In male rats, spontaneous opioid withdrawal coincided with a robust increase in choice of the smaller unit doses of fentanyl (Figure 2A), consistent with decades of preclinical studies using male opioid-withdrawn rats and nonhuman primates (10–15). Male rats also significantly increased choice for fentanyl over food in the first component, even when fentanyl was unavailable. This may reflect an increase in drug-seeking behavior or a loss of stimulus control. Moreover, this observation was consistent with previous findings in male monkeys using a similar opioid choice procedure (13,14). Conversely, opioid withdrawal coincided with a significant decrease in choice of the largest unit dose of fentanyl in females (Figure 2B). Consistent with these results, a recent study reported rates of oxycodone self-administration to be decreased in female, but not male, rats during protracted opioid withdrawal (23). Collectively, these findings illustrate an adaptive response to opioid withdrawal in female rats, which promotes choice of a nondrug alternative over continued opioid self-administration.

By performing unbiased transcriptional profiling of mesolimbic brain regions, we aimed to identify the brain region–specific molecular correlates of this sex-divergent phenotype. Surprisingly, we observed a far greater number of DEGs in the NAc and VTA of females than in the corresponding male brain regions. The female NAc possessed the greatest number of significantly regulated transcripts, hinting that the aggregate consequence of transcriptional regulation in this brain region may largely contribute to the observed female-specific behavior to allocate effort toward non-opioid reinforcers while in withdrawal, possibly through altered reward processing. Furthermore, while we observed a significant overlap in transcript identities affected across males and females in either brain region (Figures 3D, E and 4D, E), the pattern of individual transcript expression was dissimilar across males and females (Figures 3C and 4C), suggesting that the male and female mesolimbic neuroadaptations in response to opioid withdrawal were considerably distinct.

Male and female transcriptional adaptations in the NAc and VTA may predominantly occur within neuronal cell types, as revealed by our GO analysis enriched for component terms associated with the neuron and neuron projection component (Figures 3F, H and 4F). In the NAc, male upregulated transcripts were linked to an increase in protein kinase activity (Figure 3F), whereas female downregulated transcripts were enriched for kinase binding GO terms (Figure 3H). This raises the interesting possibility that NAc protein kinase function was broadly increased in males yet decreased in females, which would have divergent consequences on NAc function, and may partially explain the observed sex-specific behaviors. Moreover, within females, the top GO terms were nearly identical for upregulated transcripts across the NAc (Figure 3G) and VTA (Figure 4G). These were enriched with GO terms relating to hemoglobin-mediated oxygen transport, driven by some of the most strongly upregulated genes in both brain areas: Hba-a2, LOC689064, Hbb, and Hba-a3. Similar GO terms were not observed for female-specific downregulated transcripts across the NAc (Figure 3H) and VTA (Figure 4I). Finally, an interesting observation was that many of these downregulated female transcripts within the NAc were related to neuronal dendrite development, glutamatergic synaptic neurotransmission, and cellular energetics (Figure 3H), whereas downregulated female transcripts within the VTA were related to the GO functions of secretion (Figure 4I). This hints that the net consequence of female-specific transcriptional downregulation converges on diminished glutamatergic pre- and postsynaptic neurotransmission within the NAc as well as diminished neurotransmitter release emanating from the VTA. It is possible, therefore, that the observed female-specific transcriptional dynamics were an adaptive, active response to opioid withdrawal that consequently resulted in an altered function of NAc cell types, granting females the capacity to allocate behavior toward proadapative reinforcers while in opioid withdrawal. Future research will determine the relationship between these transcriptional responses, cellular function, and opioid choice behavior.

Methadone decreased fentanyl choice in opioid-withdrawn male rats (Figure 5C). The results in male rats are consistent with previous studies in male nonhuman primates showing that MOR agonists reverse opioid withdrawal–associated increases in opioid choice (10–14,21). Furthermore, these results are in
Sex-Specific Effects of Opioid Withdrawal

Figure 4. Female and male rats experience distinct transcriptional activity within the ventral tegmental area following repeated fentanyl withdrawal. Volcano plots depicting the DEGs from microdissected ventral tegmental area identified in (A) male (n = 3) and (B) female (n = 3) rats. DEGs were normalized to sex-matched rats subjected to saline-vs.-food protocol. DEG cutoffs for volcano plots are Wald test–adjusted p value < .05 and absolute log2 fold change >1.
line with the clinical benefits of methadone, which include not only decreasing illicit opioid use (20,33,34) but also increasing quality of life through engagement with non-opioid reinforcers, such as social relationships and leisure activities (35,36). Interestingly, the methadone-induced decrease in fentanyl choice in male rats was similar to the spontaneous decrease in fentanyl choice observed in opioid-withdrawn female rats. An intriguing future direction is whether chronic administration of efficacious OUD pharmacotherapies (e.g., methadone) to opioid-dependent male rats induces similar mesolimbic transcriptional regulation as observed in untreated, opioid-withdrawn female rats.

The failure of methadone to decrease fentanyl choice in female rats is seemingly inconsistent with the clinical literature, as methadone is often reported to be similarly effective in promoting abstinence from illicit opioids in men and women ([37–39], but see [40–43]). The lack of methadone effects in opioid-withdrawn female rats is most likely attributable to lower fentanyl choice in the absence of methadone in females relative to males (Figure 5B), providing a smaller window to detect methadone-induced decreases in fentanyl choice in female rats. In light of this, it is important to restrict our conclusions to our rodent model, because the degree to which these behaviors and neuroadaptations occur in human populations with OUD remains be determined. Overall, these data contribute to emerging preclinical data showing sex-specific effects of methadone in opioid-withdrawn rats (44).

This study extended the study of opioid withdrawal effects on opioid choice to include sex as a biological variable. Opioid withdrawal robustly increased opioid choice in male subjects, which aligns with the prominent role of opioid withdrawal in maintaining maladaptive opioid use in patients with OUD (2). Our finding of decreased opioid choice in female rats does not appear to have an obvious clinical

Figure 5. Acute methadone decreases fentanyl choice in male, but not female, opioid-withdrawn rats. Effects of acute methadone treatment on fentanyl choice in male (n = 6) and female (n = 5) rats during week 3 of overnight (6:00 PM–6:00 AM) fentanyl self-administration. Top abscissas: Experimental day. Middle and bottom abscissas: Unit dose of fentanyl. Top ordinate: Number of fentanyl injections (3.2-μg/kg unit dose) during each 12-hour session in (A) male and (B) female rats. Middle ordinate: Percentage of completed ratio requirements on the fentanyl-associated lever in (C) male and (D) female rats. Bottom ordinate: Number of choices completed per component in (E) male and (F) female rats. Points represent mean ± SEM. *Denotes significant difference at a unit fentanyl dose relative to day 1 in panel (A). +Denotes a main effect of time in panel (A). *Denotes difference from saline within a sex in panels (B, C). Significance is defined as p < .05. See Table S1 in Supplement 1 for statistics relevant to each panel.
correlate, because women are clearly negatively affected by OUD (45). However, our findings in female rats may reflect species-specific responses to opioid withdrawal. Nonetheless, female rats may serve as a useful model organism to interrogate the neurobiology underlying a resilient phenotype to withdrawal-associated increases in opioid choice. Here, we used RNA-seq to begin exploring neurobiological distinctions between male and female opioid-withdrawn rats, with the ultimate goal of identifying targetable substrates to diminish the ability of opioid withdrawal to bias decision-making processes toward increased opioid use at the expense of non-opioid alternatives.

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The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

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