Archival Report

Transcriptional Profile of Exercise-Induced Protection Against Relapse to Cocaine Seeking in a Rat Model

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ABSTRACT

BACKGROUND: Exercise has shown promise as a treatment for cocaine use disorder; however, the mechanism underlying its efficacy has remained elusive.

METHODS: We used a rat model of relapse (cue-induced reinstatement) and exercise (wheel running, 2 hours/day) coupled with RNA sequencing to establish transcriptional profiles associated with the protective effects of exercise (during early withdrawal [days 1–7] or throughout withdrawal [days 1–14]) versus noneffective exercise (during late withdrawal [days 8–14]) against cocaine-seeking and sedentary conditions.

RESULTS: As expected, cue-induced cocaine seeking was highest in the sedentary and late-withdrawal exercise groups; both groups also showed upregulation of a *Grin1*-associated transcript and enrichment of Drd1-Nmdar1 complex and glutamate receptor complex terms. Surprisingly, these glutamate markers were also enriched in the early- and throughout-withdrawal exercise groups, despite lower levels of cocaine seeking. However, a closer examination of the *Grin1*-associated transcript revealed a robust loss of transcripts spanning exons 9 and 10 in the sedentary condition relative to saline controls that was normalized by early- and throughout-withdrawal exercise, but not late-withdrawal exercise, indicating that these exercise conditions may normalize RNA mis-splicing induced by cocaine seeking. Our findings also revealed novel mechanisms by which exercise initiated during early withdrawal may modulate glutamatergic signaling in dorsomedial prefrontal cortex (e.g., via transcripts associated with non-NMDA glutamate receptors or those affecting signaling downstream of NMDA receptors), along with mechanisms outside of glutamatergic signaling such as circadian rhythm regulation and neuronal survival.

CONCLUSIONS: These findings provide a rich resource for future studies aimed at manipulating these molecular networks to better understand how exercise decreases cocaine seeking.

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More than 5.1 million individuals in the United States over the age of 12 reported using cocaine in 2020 and 1.3 million met the criteria for cocaine use disorder (CUD) (1). As the supply of cocaine continues to grow, the number of users and individuals with CUD is projected to increase (2–4). New treatments are critically needed, especially because there is no U.S. Food and Drug Administration–approved treatment for CUD and current strategies result in ~50% relapse (5). Exercise has shown promise as a nonpharmacological intervention for substance use disorders in human and animal studies. In humans, clinical trials of exercise have revealed beneficial health and anticraving effects (6–12), with recent findings showing that drug craving is immediately and persistently attenuated following an acute bout of aerobic exercise in individuals undergoing inpatient treatment for polysubstance use disorder (8). Exercise has also been shown to alleviate depression, reduce measures of anxiety, and enhance cognition (6,13–15), factors known to contribute to drug craving (16,17). Similarly, animal studies show that exercise, such as

voluntary running in a wheel, markedly decreases drug use and drug seeking and, when available during withdrawal following drug self-administration, prevents the incubation of drug craving, a phenomenon that occurs in both humans and animals and is believed to reflect an enhanced vulnerability to relapse (18–30). This antirelapse effect of exercise has been observed for multiple addictive drugs, including cocaine (31–34).

Despite these encouraging findings, the efficacy of exercise in humans has been variable, and while lack of power and compliance issues undoubtedly contribute to the variability (33–43), preclinical results similarly demonstrate that certain exercise regimens are not effective (18,19,28). For example, we showed that exercise (wheel running) during early withdrawal (days 1–7) or throughout withdrawal (days 1–14) prevents the development of high levels of cocaine seeking and persistently protects against cocaine seeking as assessed on withdrawal day 15. In contrast, exercise during late withdrawal (days 8–14) was not effective at reducing cocaine-seeking response on

withdrawal day 15, suggesting that the timing of exercise during withdrawal is more important than the amount or recency of exercise [exercise 2 hours/day (19); exercise 1–6 hours/day (21); exercise 1–24 hours/day (22); see (44,45) for reviews]. It also suggests that effects in humans could be prolonged by initiating exercise during early withdrawal rather than after detoxification, as it typically occurs. A better understanding of the molecular mechanisms that underlie these differential effects of exercise would also help guide the development of exercise in humans with CUD because such information will help define the exercise conditions necessary for achieving an antirelapse effect.

In our previous study (18), we started to isolate the molecular changes associated with effective versus noneffective exercise conditions, focusing on neuroadaptations in the dorsomedial prefrontal cortex (dmPFC), which is known to be critically involved in cocaine seeking and its incubation over withdrawal (46–51). We focused on markers known to be regulated by exercise and/or drug seeking, including genes involved in brain-derived neurotrophic factor (Bdnf exons I, IV, and IX and its receptor, Ntrk2), glutamate (AMPA type subunits 1, 2, and 3: Gria1, Gria2, and Gria3; NMDA type subunit 1: Grin1; metabotropic glutamate receptors mGlu1, mGlu2, and mGlu5: Grm1, Grm2, and Grm5), and dopamine signaling (D$_1$ and D$_2$ receptors: Drd1 and Drd2). mGlu1 and mGlu5 showed the most promise as a potential mechanism underlying the protective effect of exercise against cocaine seeking, given that dmPFC gene expression levels (Grin1 and Grm5) were positively associated with cocaine-seeking responses (or responding during extinction/reinstatement testing). Site-specific activation of mGlu5 receptors during early withdrawal also mimicked the efficacy of exercise. However, blockade of these receptors during early withdrawal did not block the efficacy of exercise, indicating that while dmPFC mGlu5 contribute to the incubation of cocaine seeking, they are not necessary for the efficacy of exercise to reduce cocaine seeking (18). Thus, the mechanism for the efficacy of exercise in reducing cocaine seeking remains elusive.

The purpose of the present study was to use RNA sequencing (RNA-seq) as an unbiased and hypothesis-generating approach to explore transcriptional changes in the dmPFC associated with cocaine seeking and the protective effects of exercise against cocaine seeking. Although several studies have characterized transcriptional changes associated with cocaine self-administration and/or cocaine seeking, most of these studies have used short-access conditions (1–2 hour/day access to the drug), which are best for studying initial vulnerability to drug use [e.g., (52–56), but see (57)]. Notably, to our knowledge, RNA-seq has not been used to assess changes that occur in response to cue-induced cocaine seeking following withdrawal from extended-access drug self-administration (i.e., ≥6-hour/day access to the drug), which is the gold standard for inducing addiction-like features in animals, including enhanced drug seeking/vulnerability to relapse (58). We focused on changes in transcripts rather than genes, given mounting evidence indicating that RNA mis-splicing contributes to numerous human diseases (e.g., Parkinson's disease and dilated cardiomyopathy) (59), with preliminary support for a role in substance use disorders (e.g., alcohol use disorder) (60). Based on our previous findings

in male rats showing that cocaine seeking is attenuated by exercise during early withdrawal (days 1–7) or throughout withdrawal (days 1–14) but not during late withdrawal (days 8–14) (18–19), we predicted that the protective effects of exercise against cocaine seeking would be revealed by isolating transcriptional changes induced by early- and throughout-withdrawal exercise versus late-withdrawal exercise and sedentary conditions (relative to saline).

METHODS AND MATERIALS

Subjects

Adult male Sprague Dawley rats (Charles River) (N = 21) weighing approximately 380 g at the start of the study were used as subjects. These animals were representative subsets of the sedentary (5 of 8), early (4 of 14), throughout (4 of 8), late (4 of 10), and saline (5 of 10) groups we used in our previous study on the impact of early- versus late-withdrawal–initiated exercise on cocaine seeking and associated gene expression changes (as assessed using quantitative polymerase chain reaction) (19). All procedures were approved by the University of Virginia Animal Care and Use Committee and were conducted within the guidelines set by the National Institutes of Health.

Behavioral Procedure

The procedures used for housing, lever pretraining, and surgical implantation of jugular catheters; cocaine self-administration during training and the extended-access period; exercise during withdrawal; and cue-induced cocaine-seeking testing are thoroughly described in our previous study (19) (Figure 1). Briefly, rats were trained to self-administer cocaine (1.5 mg/kg/ infusion) under a fixed-ratio 1 schedule with a maximum of 20 infusions available per day and, once acquired (20 infusions on 2 consecutive days), were given extended (24 hours/day) access to the drug using a discrete trial procedure (2 trials/hour, 1.5 mg/ kg/infusion) for 10 days. Two additional fixed-ratio 1 sessions were run to confirm patency before withdrawal. Then, rats were housed in polycarbonate cages without (sedentary; n = 5) or with 2-hour/day access to a running wheel during early withdrawal (days 1–7; n = 4), late withdrawal (days 8–14; n = 4), or throughout withdrawal (days 1–14; n = 4). Saline controls underwent the same procedures as those described for the sedentary cocaine group (n = 4) except that they previously self-administered saline instead of cocaine. Rats were returned to their operant chambers on day 14 of withdrawal and then underwent relapse testing on withdrawal day 15 using a within-session extinction/cue-induced reinstatement procedure (i.e., a minimum of six 1-hour extinction sessions wherein responding was without consequence followed by a 1-hour reinstatement session in which each response was reinforced with cocaine-associated cues, which included a stimulus light and the sound of the pump).

Tissue Preparation and RNA-Seq

The morning following the test session, anesthetized rats were euthanized by rapid decapitation, and the dmPFC was dissected from 2-mm-thick coronal brain slices based on Paxinos and Watson (61) coordinates (bregma 3.2 mm). Brain tissue
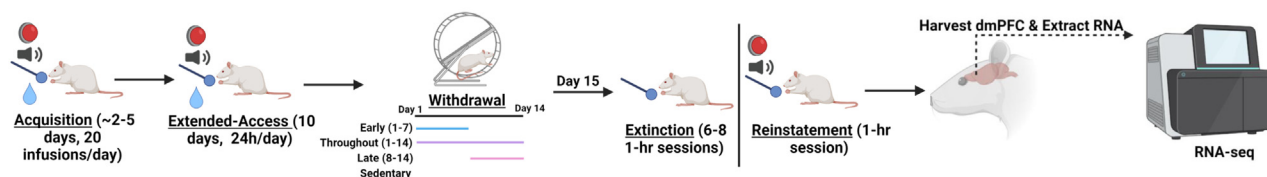
Figure 1. Summary of experimental events. Male rats were trained to self-administer cocaine. Following acquisition of cocaine self-administration (2 consecutive sessions wherein all 20 infusions available were obtained), rats were given 24 hour/day, extended access to cocaine (1.5 mg/kg/infusion) under a discrete trial procedure for 10 sessions. Following the last cocaine self-administration session, rats were housed without (sedentary, $n = 5$) or with access to wheel running (2 hours/day) during early (days 1–7; $n = 4$), late (days 8–14; $n = 4$), or throughout (days 1–14; $n = 4$) a 14-day withdrawal period. Additional rats were given access to saline and housed without access to a wheel during withdrawal ($n = 4$). On day 15 of withdrawal, rats underwent extinction/reinstatement testing. On the morning following this 1-day test session, tissue was collected from the dmPFC and RNA-seq was performed. This graphical illustration was made using BioRender (https://biorender.com/). dmPFC, dorsomedial prefrontal cortex; RNA-seq, RNA sequencing.

was rapidly frozen and stored at $-80\,°C$ until further processing. To generate RNA-seq libraries, total RNA was isolated from each sample using RNeasy Lipid Tissue Mini Kit (Qiagen), and 5 μg of total RNA was processed for ribosomal RNA removal using the Illumina Ribo-Zero Kit. RNA-seq libraries were prepared using the Illumina ScriptSeq v.2 RNA-seq Library Preparation Kit and purified using Beckman Coulter AMPure XP beads. The library quality and quantity were assessed by a 2100 Bioanalyzer (Agilent) prior to sequencing by the University of Virginia Core facility. Datasets were assessed for quality and consistency using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were then aligned to the rat rn6 genome using HiSAT2 specifying –rna-strandness FR (62). SAMtools was used to convert the resulting sequence alignment/map files to binary alignment map format, and transcripts were assembled and quantified with StringTie, using the Ensembl Rattus_norvegicus_Rnor_6.0.85 annotations (63,64). The resulting count tables were then used for identification of differentially expressed transcripts. DESeq2 (version 1.30.1) was used to normalize the raw counts and to perform differential expression analysis (65). Gene set enrichment was performed using g:Profiler (66). The RNA-seq analysis was performed using the tidyverse (version 1.3.1) and dplyr (version 1.0.8) software packages, and the term plots were made using ggplot2 (version 3.3.3). The network maps were made using Cytoscape (version 3.8.2). Volcano plots were created using the EnhancedVolcano (version 1.8.0) R package (67), and the heatmap was produced using the Pheatmap (version 1.0.12) R package (68).

Integrative Genomics Viewer

To visualize transcript changes relative to saline control animals, the merged RNA-seq datasets were loaded on the Integrative Genomics Viewer (IGV) using the rat rn6 genome (69). Differential normalized read counts for each condition were generated by subtracting the merged saline reads. IGV coverage tracks were visualized from bigwig files of paired reads, which join the 2 mates of the paired-end reads, and therefore span the entire range between the 2 mates. Exon numbering was generated by IGV using the National Center for Biotechnology Information RefSeq rat rn6 transcripts.

Data Analysis

We first confirmed that the effects of exercise in these subsets of rats were similar to those observed previously in the larger

sample of sedentary, early, throughout, and late groups (19). Specifically, univariate analysis of variance was used to examine group differences in cocaine seeking during extinction (total responses), and repeated measures analysis of variance was used to examine group differences in cocaine seeking during reinstatement relative to responses during the last extinction session. Repeated measures analysis of variance was also used to verify that cocaine intake was similar between each of the cocaine groups during the extended-access phase and that levels of running were similar between each of the exercise groups during the 7-day exercise period (first 7 days for throughout exercise). Post hoc comparisons were Tukey corrected and based on either one-tailed (for predicted differences; e.g., higher cocaine seeking in sedentary vs. early and throughout exercise) or two-tailed (nonpredicted differences) distributions. The association between the efficacy of exercise to reduce cocaine-seeking responses and transcriptional changes (normalized counts) were examined using Benjamini-Hochberg–corrected Pearson correlations.

RESULTS

Behavioral Results

Extended-Access Cocaine Self-administration and Exercise. As with the larger dataset (19), results from these subsets of rats showed high levels of cocaine self-administration during the 10-day extended-access period with similar levels and patterns of intake between the groups (Figure 2A, B) (group and group by session, ps $> .05$). Intake was the highest in each of the groups during initial versus later sessions (session, $F_{9,117} = 3.22$, $p < .01$; session 1 vs. 10, $p < .05$). Overall levels and patterns of running during withdrawal were also similar between the 3 exercise groups (Figure 2C) (group and group by session, ps $> .05$). Levels of running progressively increased in each of the groups over the 7 exercise sessions (session, $F_{6,54} = 5.18$, $p < .001$; session 1 vs. 7, $p < .01$). Thus, prior to extinction/reinstatement testing, levels and patterns of cocaine intake and exercise were similar between the groups.

Timing of Exercise During Withdrawal on Cue-Induced Cocaine Seeking. Also consistent with the larger dataset (19), cocaine seeking (or responding during
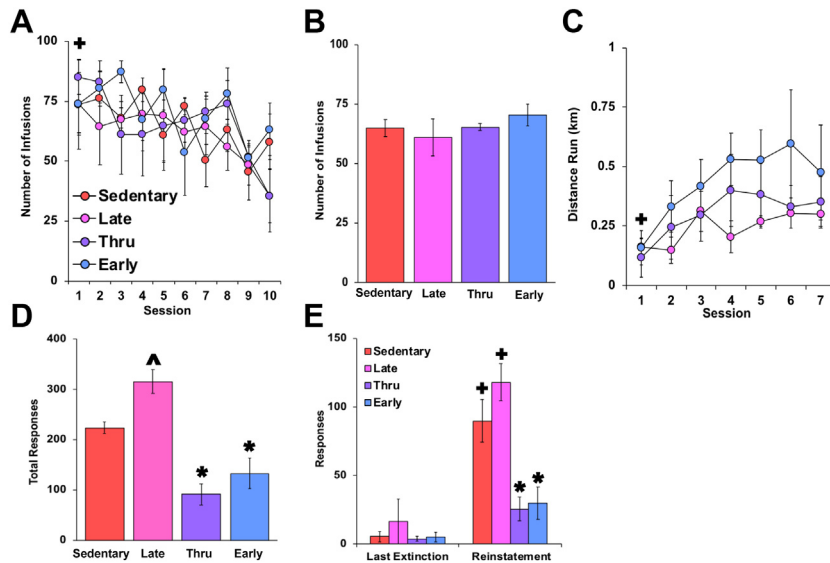
Figure 2. Timing of exercise during withdrawal on cue-induced cocaine-seeking response. **(A)** Mean (±SEM) number of infusions for each of the 10 extended-access sessions, **(B)** cocaine intake averaged across the extended-access period (mg/kg), **(C)** distance run for the first 7 exercise sessions (km), and **(D)** total number of responses made on the lever formerly associated with cocaine during extinction and **(E)** during the last extinction session vs. the reinstatement session for male rats in the sedentary ($n = 5$), early ($n = 4$), throughout ($n = 4$), or late ($n = 4$) exercise conditions. + indicates significantly different from session 10 **(B)**, session 7 **(C)**, and the last extinction session **(E)**. * indicates significantly decreased vs. sedentary and late. ^ indicates significantly increased vs. sedentary. Thru, throughout.

extinction/reinstatement testing) in these subsets of rats was affected by the timing of exercise during withdrawal. For extinction, responses were significantly lower in early and throughout (Figure 2D) (group, $F_{3,17} = 20.30$, $p < .001$) than in both sedentary and late (ps < 0.01) and significantly higher in late than in sedentary ($p < .01$) conditions. Similarly, reinstatement of cocaine seeking in response to cocaine-associated cues was markedly attenuated in early and throughout versus sedentary and late (Figure 2E) (group by session, $F_{3,13} = 10.43$, $p < .001$) conditions. Specifically, while no group differences were observed during the last extinction session ($p > .05$), a significant group effect was observed within the reinstatement session ($F_{3,13} = 11.27$, $p < .001$), and post hoc comparisons revealed significantly lower responses in early and throughout than in sedentary and late ($p < 0.05$) conditions. Further analysis within each group revealed

significantly higher responses during the reinstatement session than during the last extinction session for sedentary and late conditions ($p < .05$), but not for early and throughout conditions ($p > .05$). Thus, as with the larger dataset (19), results from these subsets of rats show that exercise initiated during early withdrawal, but not late withdrawal, reduces cocaine-seeking response.

Molecular Results

Transcriptional Changes Associated With Cue-Induced Cocaine Seeking. The analysis of transcriptional changes in the dmPFC of sedentary versus saline controls revealed 29 upregulated and 37 downregulated transcripts based on adjusted $p = .1$ and $\log_2$(fold change) > 1.3 (Figure 3A). Consistent with our previous findings (18,70),

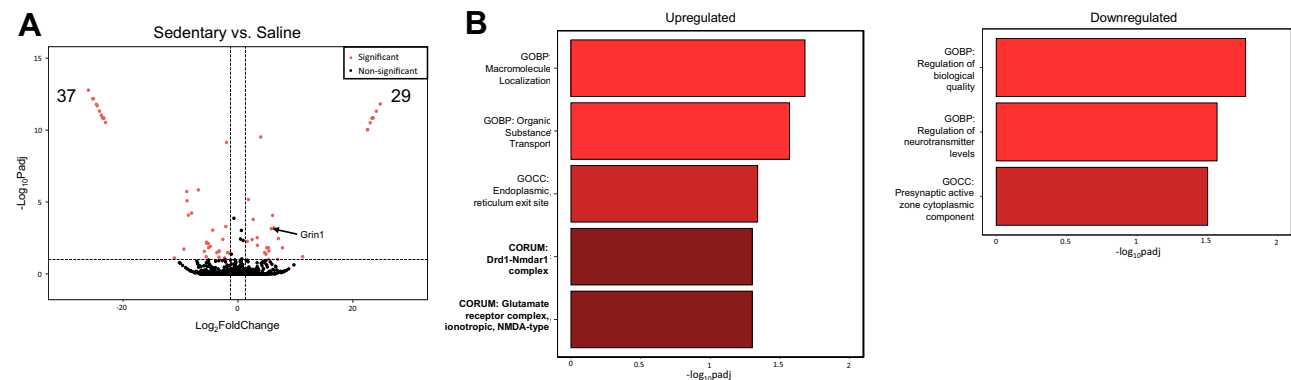**Figure 3.** Cue-induced cocaine-seeking–associated transcriptional changes in the dorsomedial prefrontal cortex. **(A)** Volcano plot depicting the differentially expressed transcripts in the dorsomedial prefrontal cortex of male rats in the sedentary group ($n = 5$) relative to the saline group ($n = 5$), with statistically significant differences depicted in red [$p_{adj} < .1$, $\log_2$(fold change) < 1.3]. **(B)** Bar plots depicting the enriched GO and CORUM terms using the differentially up- and downregulated transcripts in the cocaine vs. saline comparison. Bars with the same shade of red represent terms gathered from the same database. Adjusted p values were calculated using DESeq2. GO, Gene Ontology; GOBP, GO biological process; GOCC, GO cellular component; p_{adj}, adjusted p.

one of the upregulated transcripts was *Grin1* (ENSR-NOT00000044246), which encodes the NR1 subunit of the NMDA receptor. Analysis of the upregulated transcript list using Gene Ontology (GO) and CORUM, the comprehensive resource of mammalian protein complexes (which was manually created using reliable experimental evidence and utilizes the specific genes that contribute to the formation of specific quaternary protein structures) (71) revealed an enrichment of terms related to glutamate receptors, including glutamate receptor complex and Drd1-Nmdar1 complex, and macromolecule localization and organic substrate transport (Figure 3B). Interestingly, the downregulated transcript list revealed terms linked to the regulation of neurotransmission and the presynaptic active zone cytoplasmic component. Thus, cue-induced cocaine seeking appears to be associated with genes related to increased glutamate/NMDA receptor signaling and decreased regulation of presynaptic neurotransmission.

Transcriptional Changes Associated With the Protective Effects of Exercise Against Cocaine Seeking. A similar strategy was used to investigate transcriptional changes associated with cue-induced cocaine seeking (vs. saline) in the dmPFC of rats that had previously exercised during early, throughout, or late withdrawal (Figure 4A). Surprisingly, despite the similarity between early and throughout exercise with regard to cocaine seeking, only 5 upregulated and 8 downregulated gene-associated transcripts overlapped between the groups, and of these, only 2 upregulated transcripts and 6 downregulated transcripts were specific to early and throughout (Figure 4B; Table 1). Notably, the *Grin1* transcript was one of the three overlapping upregulated transcripts common to each of the exercise groups. We also investigated transcripts that were similarly upregulated or downregulated in early and throughout, but not late, relative to sedentary. This analysis produced results similar to
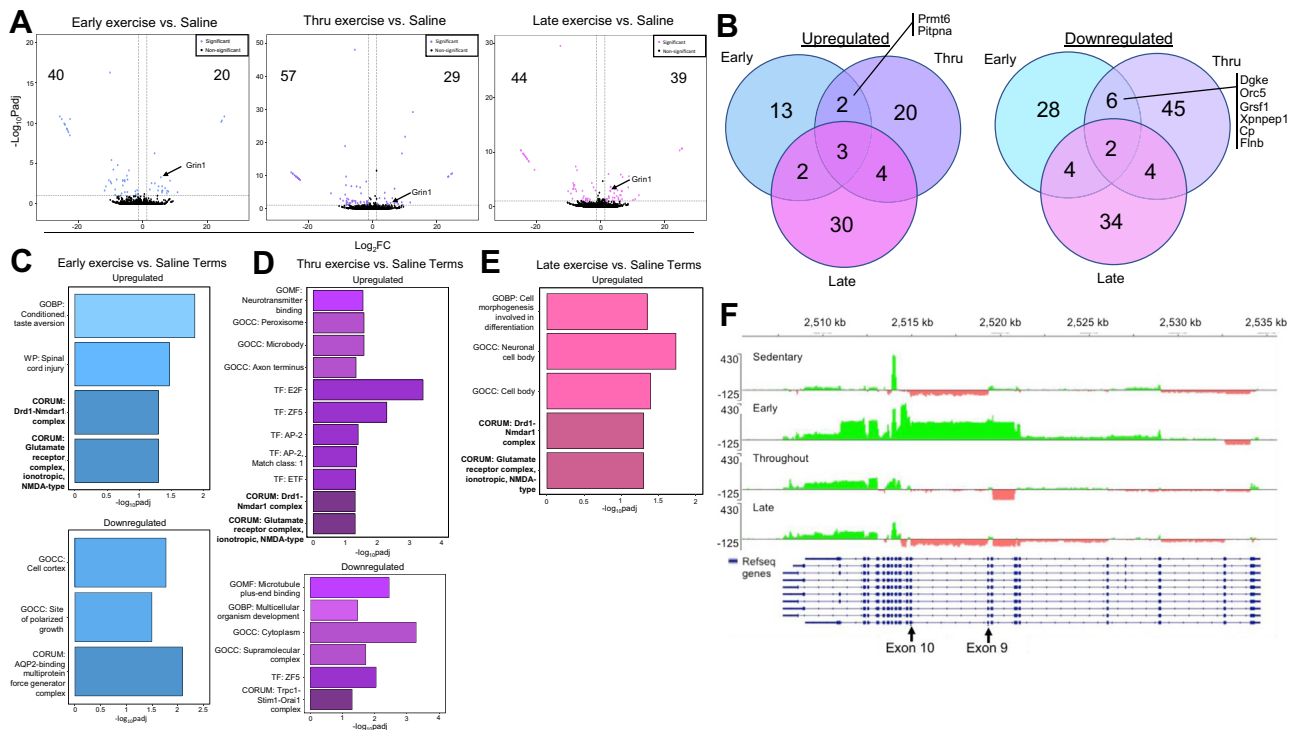

Figure 4. Transcriptional changes associated with the protective effects of exercise against cocaine seeking. **(A)** Volcano plots depicting the differentially expressed transcripts in the dorsomedial prefrontal cortex of male rats in the early (*n* = 4), throughout (*n* = 4), and late (*n* = 4) exercise groups, each relative to the saline group (*n* = 5), with statistically significant transcripts depicted in blue, purple, and pink for the early, throughout, and late comparisons, respectively ($p_{adj} < .1$, $log_2FC < 1.3$). *Grin1* is labeled in all 3 volcano plots. **(B)** Venn diagrams showing the overlap of the significantly up- and downregulated transcripts in the early, thru, and late groups, each compared with the saline group. The uniquely overlapping transcripts between early and throughout exercise are shown. **(C)** Bar plot depicting the enriched GO, WP, and CORUM terms using the significantly up- and downregulated transcripts in the early exercise vs. saline comparison. Bars with the same shade of blue represent terms gathered from the same database. **(D)** Bar plot depicting the enriched GO, TF, and CORUM terms using the significantly up- and downregulated transcripts in the throughout exercise vs. saline comparison. Bars with the same shade of purple represent terms gathered from the same database. **(E)** Bar plot depicting the enriched GO and CORUM terms using the significantly upregulated transcripts in the throughout exercise vs. saline comparison. Bars with the same shade of pink represent terms gathered from the same database. No terms were enriched using the significantly downregulated transcripts. **(F)** Saline-subtracted, normalized RNA sequencing paired-read density for *Grin1* in the sedentary, early, throughout, and late exercise conditions were mapped to chromosome 3 of the rat rn6 genome using the Integrated Genome Viewer. Differential transcripts above saline (green) or below saline (red) are indicated relative to known RefSeq exon transcripts (blue). Adjusted *p* values were calculated with DESeq2 using the Wald test for significance after fitting to a negative binomial linear model and performing the Benjamini-Hochberg procedure to limit false discoveries. GO, Gene Ontology; GOBP, GO biological process; GOCC, GO cellular component; GOMF, GO molecular function; log_2FC, log_2(fold change); p_{adj}, adjusted *p*; TF, transcription factor; thru, throughout; WP, WikiPathways.

Table 1. Gene-Associated Transcripts That Were Similarly Up- or Downregulated in the Early and Throughout, but Not Late, Exercise Groups Relative to Saline Controls

Gene	Transcript
Upregulated	
Pitpna	ENSRNOT00000090554
Prmt6	ENSRNOT00000023079
Downregulated	
Cp	ENSRNOT00000082627
Dgke	ENSRNOT00000076587
Flnb	ENSRNOT00000066546
Grsf1	ENSRNOT00000086571
Orc5	ENSRNOT00000015426
Xpnpep1	ENSRNOT00000033148

those obtained from the analysis relative to saline with only 5 upregulated and 4 downregulated gene-related transcripts unique to early and throughout exercise (Table 2).

To further assess similarities and differences in transcriptional profiles between the sedentary and exercise groups, we performed a GO, Kyoto Encyclopedia of Genes and Genomes (KEGG), CORUM, transcription factor database (TRANSFAC), and WikiPathways analysis on the differentially upregulated and downregulated gene-associated transcripts in each exercise group relative to saline (Figure 4C–E). To our surprise, this analysis revealed that there were 2 shared terms common for each of the exercise groups, glutamate receptor complex and Drd1-Nmdar1 complex. It is notable that these 2 terms were also found in the sedentary versus saline comparison (Figure 4C), suggesting that the efficacy of exercise is not mediated via normalization of glutamate/NMDA receptor levels in the dmPFC (but see IGV analysis below). Another notable finding from this analysis is that throughout exercise was uniquely associated with an upregulation of transcripts related to the transcription factor AP-2 (Figure 4D), which plays an important role in synaptic vesicle endocytosis and NMDA-stimulated AMPA receptor endocytosis (72,73). Finally, given

Table 2. Gene-Associated Transcripts That Were Similarly Up- or Downregulated in the Early and Throughout, but Not Late, Exercise Groups Relative to Sedentary Controls

Gene	Transcript
Upregulated	
Ctbp2	ENSRNOT00000023574
Ten1	ENSRNOT00000086382
Elvol1	ENSRNOT00000092972
Mipep	ENSRNOT00000018845
Ndufa12	ENSRNOT00000089442
Downregulated	
Ten1	ENSRNOT00000083022
Ggnbp2	ENSRNOT00000085728
Ybx3	ENSRNOT00000007427
AABR07030184.3	ENSRNOT00000080772

the relative lack of overlapping transcripts and enrichment terms between the early and throughout exercise groups, it is likely that different mechanisms underlie the protective effects of exercise during early versus throughout withdrawal.

Given our previous work and work from others indicating a role for Grin1/NR1 in cocaine seeking and the efficacy of exercise in reducing cocaine seeking (18,70,74), Grin1 transcription was visualized using IGV. This analysis revealed that the normalized mapped read densities for the sedentary and exercise conditions varied significantly by exon (Figure 4F). For example, there was a robust loss of transcripts spanning exons 9 and 10 in the sedentary group relative to saline that was either countered or normalized with early and throughout, but not late exercise. Given that these effects mirror effects on cocaine seeking, it is possible that early and throughout exercise may normalize the RNA mis-splicing induced by cocaine seeking. Additionally, while throughout exercise normalized expression in these exon regions, early exercise led to an elevation of transcripts from these exon regions, indicating that, as suggested above, the mechanisms by which early- and throughout-withdrawal exercise exert their efficacy are different.

Transcriptional Changes Associated With the Protective Effects of Exercise Throughout Withdrawal Against Cocaine Seeking. Given the relative lack of overlapping transcriptional profiles between the early and throughout exercise groups, we focused on characterizing the transcriptional profile associated with the protective effects of exercise throughout withdrawal cocaine seeking (relative to sedentary). We also compared effects with those observed in late (relative to sedentary) as a negative control for throughout exercise because the last exercise session was the day before the cue-induced cocaine-seeking test for both groups, yet only throughout exercise decreased cocaine seeking. This analysis revealed 28 upregulated gene-associated transcripts unique to throughout exercise (Figure 5A; Table 3) and further analysis of this transcript list using GO, KEGG, and CORUM revealed enrichment of terms related to neuron projection, cell cortex, growth hormone synthesis, secretion, and action; circadian entrainment; gonadotropin-releasing hormone, cortisol, renin, and aldosterone secretion; and neurotransmitter synapses, including serotonergic, dopaminergic, cholinergic, and glutamatergic synapses (Figure 5B). We also confirmed that transcriptional changes for 4 of the gene-associated transcripts unique to throughout exercise (Adcy10, Septin6, Plcb2, and Ank2) corresponded to the efficacy of exercise to reduce cocaine seeking such that higher expression levels (normalized counts) were predictive of significantly lower drug seeking (Figure 5C) ($p < .05$). It is notable that 3 of these transcripts are involved in neuron projection (Ank2, Septin6, and Adcy10).

The same analysis revealed 37 downregulated gene-associated transcripts unique to throughout exercise (Figure 6A; Table 4) as well as an enrichment of terms related to cytoplasm, the Trpc1-Stim1-Orai1 complex, and several transcription factors, including ZF5, E2F, and PLAG1 (Figure 6B). Interestingly, while E2F has been implicated in neuronal migration, it also has been shown to be a positive regulator of neuronal apoptosis (75,76). The normalized transcript counts of several of the transcripts unique to throughout
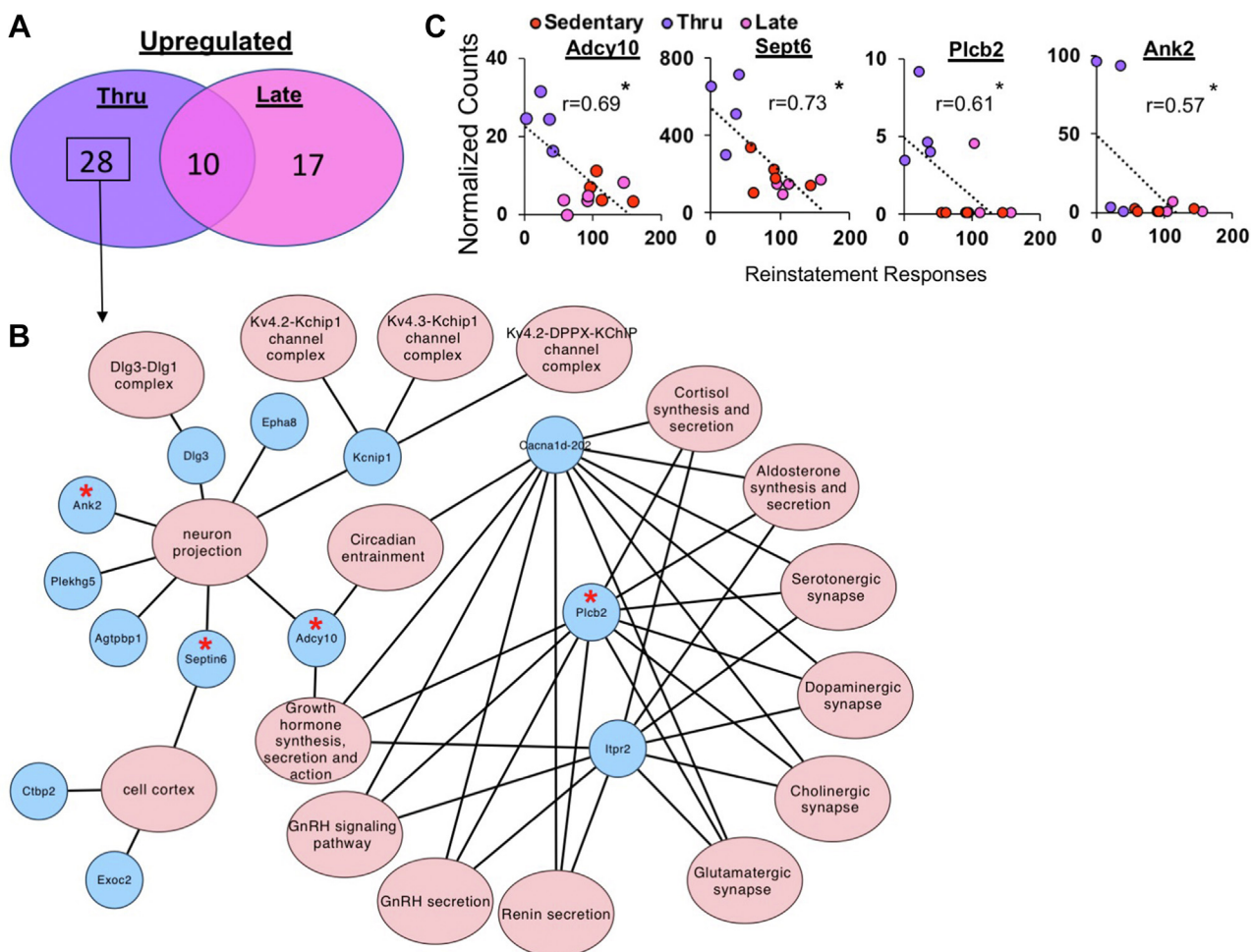
Figure 5. Transcriptional upregulation associated with the protective effects of exercise throughout withdrawal against cocaine seeking. **(A)** Venn diagram depicting the shared significantly upregulated transcripts between the throughout ($n = 4$) vs. sedentary ($n = 5$) and late ($n = 4$) vs. sedentary ($n = 5$) comparisons [adjusted $p < .1$, $\log_2$(fold change) < 1.3]. **(B)** Network map depicting the enriched GO, KEGG, and CORUM terms using the 28 uniquely upregulated transcripts in the throughout vs. sedentary comparison. Terms are in red ovals and genes are in blue circles. Lines connect the genes that contributed to each of the enriched terms. Red stars denote transcripts that had significant behavioral correlations. **(C)** The normalized counts of *Adcy10*, *Septin6*, *Plcb2*, and *Ank2* were negatively associated with cocaine-seeking response as indicated by a significant correlation (*). *$p < .05$. GnRH, gonadotropin-releasing hormone; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; thru, throughout.

exercise also corresponded to the efficacy of exercise. Specifically, reinstatement responses were positively associated with normalized transcript counts for *Exoc3*, *Ggnbp2*, *Syt3*, *Trmt10a*, *Ldb1*, *Pfkb3*, *RGD1564855*, and *Nfxl1* (*ps* $< .05$) (Figure 6C). These transcripts all intersected with at least one of the aforementioned enrichment terms. Thus, throughout exercise may serve to reduce cocaine seeking by promoting neuronal synapses and hormone secretion and preventing neuronal apoptosis.

DISCUSSION

The current study used RNA-seq to identify the transcriptional changes in the dmPFC associated with cue-induced cocaine seeking and its modulation by exercise. As expected (18,19), the sedentary group demonstrated high levels of cocaine-

seeking response, which may be associated with increased glutamate signaling as indicated by the upregulation of a *Grin1*-associated transcript and an enrichment of Drd1-Nmdar1 complex and glutamate receptor complex terms. To our surprise, each of the exercise groups also showed an upregulation of the same *Grin1*-associated transcript and an enrichment of the same glutamate receptor–related terms (i.e., Drd1-Nmdar1 complex and glutamate receptor complex). While on the surface, these findings suggest that the protective effects of exercise against cocaine seeking are not mediated through normalization of glutamatergic signaling, particularly via gene-associated *Grin1* transcripts, a closer examination revealed a robust loss of transcripts spanning exons 9 and 10 in sedentary controls (relative to saline) that was normalized with early and throughout exercise, but not late exercise, suggesting that exercise initiated during early withdrawal

Table 3. Upregulated Gene-Associated Transcripts Induced by Exercise During Throughout, but Not Late, Withdrawal (Relative to Sedentary Controls)

Upregulated Gene	Transcript
AABR07063152.1	ENSRNOT00000089442
Adcy10	ENSRNOT00000082677
Agtpbp1	ENSRNOT00000091370
Ank2	ENSRNOT00000084756
Arid5a	ENSRNOT00000089673
Brpf3	ENSRNOT00000092566
Cacna1d	ENSRNOT00000047737
Ctbp2	ENSRNOT00000023574
Dhrs7b	ENSRNOT00000066250
Dlg3	ENSRNOT00000088114
Elovl1	ENSRNOT00000092972
Epha8	ENSRNOT00000017559
Exoc2	ENSRNOT00000090706
Gba2	ENSRNOT00000022002
Itpr2	ENSRNOT00000040255
Kcnip1	ENSRNOT00000079031
LOC103689920	ENSRNOT00000090459
Lonrf3	ENSRNOT00000017550
Mipep	ENSRNOT00000018845
Nckap5l	ENSRNOT00000081434
Plcb2	ENSRNOT00000078037
Plekhg5	ENSRNOT00000082586
Ppp1r18	ENSRNOT00000082999
Septin6	ENSRNOT00000067942
Ten1	ENSRNOT00000086382
Thop1	ENSRNOT00000082486
Usp19	ENSRNOT00000074772
Wbp11l1	ENSRNOT00000082235

normalizes cocaine cue-induced RNA mis-splicing of *Grin1*. It is also notable that, despite similar protection against cocaine seeking by early and throughout exercise, there was minimal overlap between these groups in their transcriptional profiles. Each of these findings are discussed further below.

As expected, cue-induced cocaine seeking was high following extended-access cocaine self-administration and protracted withdrawal and appears to be associated with increased glutamate signaling in the dmPFC based on our findings showing upregulation of the *Grin-1* associated transcript and an enrichment of terms related to Drd1-Nmdar1 complex and glutamate receptor complex. These findings are consistent with findings in both humans and animals showing a strong association between drug craving/seeking and dmPFC hyperactivity (46–51) as well as work from our group (18,70) and others [e.g., (77–80)], supporting the involvement of NMDA receptors in particular. Thus, targeting overactive glutamatergic signaling in the dmPFC, particularly via NMDA receptors, could serve as a potential intervention for drug craving. Drug side effects would need to be considered when selecting an NMDA receptor antagonist for treatment, but it may be possible given that high doses of methadone, a full μ opioid agonist that also acts as an NMDA receptor antagonist,

are commonly prescribed for use in humans. High doses of methadone have also been shown to significantly reduce cocaine use in patients undergoing methadone maintenance treatment (81).

Surprisingly, a *Grin1*-associated transcript was upregulated in all the exercise groups along with the sedentary controls. While it is possible that this change was induced by extended-access cocaine self-administration independent of cue-induced cocaine seeking (77), given that NR1 has previously been associated with levels of cocaine seeking in rats (18,70) and vulnerability to substance use disorders in humans (82,83), a more likely possibility is that exercise that effectively reduces cocaine seeking/craving does so by normalizing signaling downstream of NR1/NMDA receptor signaling or, as suggested by the IGV analysis, by normalizing mis-splicing at *Grin1*. For example, our findings showing the loss of transcripts spanning exons 9 and 10 in the sedentary condition and normalization by early and throughout, but not late, exercise is potentially significant, considering that these exon regions are largely extracellular and include the first 5 amino acids of the ligated ion channel L-glutamate and glycine binding site. Therefore, splicing out these exons (and loss of the first 5 amino acids) could change the fidelity of NMDA receptor signaling (84). Notably, alternative splicing of 3 other *Grin1* exons (5,21,22) has been shown to affect the pharmacological properties and intracellular binding partners of NMDA receptors (85). This highlights an area for future research that may provide insight on the mechanisms underlying CUD, because these splicing effects are often overlooked in RNA-seq studies and possibly missed by quantitative polymerase chain reaction, depending on the primers selected. It is also notable that the rigor of these findings is demonstrated by the fact that our RNA-seq results with *Grin1* in the sedentary condition are consistent with previous gene expression findings from our group (18,70) and others (77) as well as findings in humans with substance use disorders (82).

Also, to our surprise, despite similar effects with regard to cocaine seeking, there were few transcriptomic similarities between the early and throughout exercise conditions. Of the 146 transcripts that were differentially expressed in the early and throughout exercise groups (relative to saline), only 8 were shared between the two conditions. The functional classification analysis of the differentially expressed transcripts also revealed only 2 overlapping enrichment terms between the early and throughout exercise groups (i.e., Drd1-Nmdar1 complex and glutamate receptor complex), and both terms overlapped with the sedentary and late exercise groups. Together, these findings suggest that the mechanisms underlying the protective effects of exercise against cocaine seeking are different for exercise during early versus throughout withdrawal; however, recency of exercise and methodological limitations, such as the homogeneous analyses of messenger RNA transcripts from a heterogeneous brain region with different cell- and projection-specific functions, may also have contributed to the differences observed. Thus, future research investigating the molecular changes induced by exercise during early, late, and throughout withdrawal (prior to reinstatement testing) or using techniques, such as single-cell RNA-seq, are necessary to examine these possibilities.
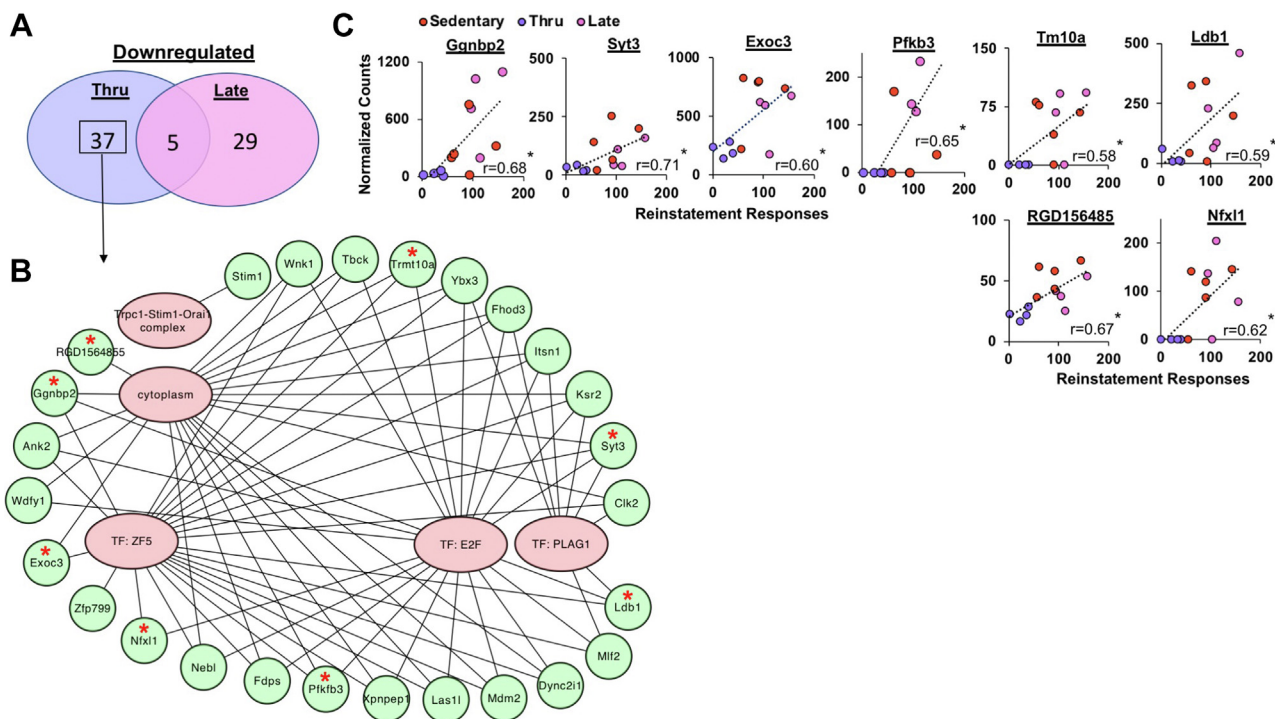
Figure 6. Transcriptional downregulation associated with the protective effects of exercise throughout withdrawal against cocaine seeking. **(A)** Venn diagram depicting the shared significantly downregulated transcripts between the throughout ($n = 4$) vs. sedentary ($n = 5$) and late ($n = 4$) vs. sedentary ($n = 5$) comparisons [adjusted $p < .1$, $\log_2$(fold change) < 1.3]. **(B)** Network map depicting the enriched GO, TF, and CORUM terms using the 37 uniquely upregulated transcripts in the throughout vs. cocaine sedentary comparison. Terms are in red ovals and genes are in green circles. Lines connect the genes that contributed to each of the enriched terms. Red stars denote genes that had significant behavioral correlations. **(C)** The normalized counts of *Ggnbp2*, *Syt3*, *Exoc3*, *Pfkb3*, *Tm10a*, *Ldb1*, *RGD156485*, and *Nfxl1* were negatively associated with cocaine-seeking response as indicated by a significant correlation (*). *$p < .05$. GO, Gene Ontology; TF, transcription factor; thru, throughout.

Our findings also reveal novel potential mechanisms by which exercise mediates its protective effects against cocaine seeking. Specifically, exercise initiated during early withdrawal was associated with the downregulation of transcripts associated with presynaptic active zone cytoplasmic component and regulation of neurotransmitter release. Additionally, the comparison that isolated transcriptional changes specific to exercise throughout withdrawal (relative to sedentary controls) revealed an upregulation of terms associated with neurotransmitter synapses, including glutamatergic synapses. One of the transcript intersections for the glutamatergic synapse enrichment term was *Plcb2*, which is a phosphodiesterase that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to the second messenger inositol 1,4,5-triphosphate (IP3). Interestingly, IP3 is downstream of mGlu5 (86), which we showed previously to be necessary for the incubation of cocaine seeking but not for the efficacy of exercise to decrease cocaine seeking (18). Thus, one possibility that we plan to address in future studies is that exercise exerts its efficacy downstream of mGlu5 via interactions with IP3.

The efficacy of exercise to reduce cocaine seeking was also associated with terms associated with circadian rhythms and neuronal survival. There is a large body of literature showing that circadian rhythms play a critical role in substance use

disorder, including drug seeking/craving (87). Specifically, findings in rats show that circadian genes mutations enhance ethanol consumption and that melatonin treatment reduces motivation for cocaine and drug seeking (88,89). One interesting transcript in this regard is *Adcy10*, which contributed to the enrichment of the circadian entrainment term, was upregulated by throughout exercise (relative to sedentary controls), and was positively correlated with behavior. The downregulation of *E2F*-related transcripts, which contributed to the neuronal survival term, is also of interest, considering that it was also correlated with behavior; it has also been shown to prevent morphine- and heroin-induced apoptosis in cerebellar neurons (90–92). Our RNA-seq findings thus shed light on the potential pivotal role that circadian entrainment and neuronal survival may play in preventing cocaine-seeking response, but, as with the glutamatergic findings, we will need to provide functional confirmation in future studies.

In conclusion, these findings reveal possible glutamatergic mechanisms and other novel candidate mechanisms that may underlie cocaine seeking and the efficacy of exercise to reduce cocaine seeking. We plan to use these findings to guide our future studies, which will focus on determining the functional consequences of these molecular changes. Future studies also are needed to address mechanistic differences between

Table 4. Downregulated Gene-Associated Transcripts Induced by Exercise During Throughout, but Not Late, Withdrawal (Relative to Sedentary Controls)

Downregulated Gene	Transcript
AABR07013729.1	ENSRNOT00000080332
AABR07030184.9	ENSRNOT00000080772
AABR07046765.1	ENSRNOT00000071767
AABR07064415.1	ENSRNOT00000082472
Ank2	ENSRNOT00000015386
Clk2	ENSRNOT00000085817
Coro7	ENSRNOT00000006067
Exoc3	ENSRNOT00000020251
Fdps	ENSRNOT00000080185
Fhod3	ENSRNOT00000041961
Ggnbp2	ENSRNOT00000085728
Igf1	ENSRNOT00000081822
Itsn1	ENSRNOT00000047843
Ksr2	ENSRNOT00000071074
Las1l	ENSRNOT00000016042
Ldb1	ENSRNOT00000040904
LOC100911361	ENSRNOT00000076198
LOC103689920	ENSRNOT00000084136
Mdm2	ENSRNOT00000066767
Mlf2	ENSRNOT00000088622
Nebl	ENSRNOT00000068553
Nfxl1	ENSRNOT00000077080
Pfkfb3	ENSRNOT00000051067
RGD1307947	ENSRNOT00000036141
RGD1564855	ENSRNOT00000079980
Rpl9	ENSRNOT00000076914
Stim1	ENSRNOT00000088370
Syt3	ENSRNOT00000077427
Tbck	ENSRNOT00000083937
Ten1	ENSRNOT00000083022
Trmt10a	ENSRNOT00000014694
Wdfy1	ENSRNOT00000063834
Dync2il	ENSRNOT00000006144
Wnk1	ENSRNOT00000013355
Xpnpep1	ENSRNOT00000033148
Ybx3	ENSRNOT00000007427
Zfp799	ENSRNOT00000087294

early- versus throughout-withdrawal exercise because their protective effects against cocaine seeking are likely mediated via different mechanisms. Finally, these findings suggest RNA mis-splicing as a potential mechanism underlying cocaine seeking and highlight a need for further research on its role in CUD.

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