Priority Communication

Biological Psychiatry

Cocaine Self-administration Alters Transcriptome-wide Responses in the Brain's Reward Circuitry

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ABSTRACT

BACKGROUND: Global changes in gene expression underlying circuit and behavioral dysregulation associated with cocaine addiction remain incompletely understood. Here, we show how a history of cocaine self-administration (SA) reprograms transcriptome-wide responses throughout the brain's reward circuitry at baseline and in response to context and/or cocaine re-exposure after prolonged withdrawal (WD).

METHODS: We assigned male mice to one of six groups: saline/cocaine SA + 24-hour WD or saline/cocaine SA + 30-day WD + an acute saline/cocaine challenge within the previous drug-paired context. RNA sequencing was conducted on six interconnected brain reward regions. Using pattern analysis of gene expression and factor analysis of behavior, we identified genes that are strongly associated with addiction-related behaviors and uniquely altered by a history of cocaine SA. We then identified potential upstream regulators of these genes.

RESULTS: We focused on three patterns of gene expression that reflect responses to 1) acute cocaine, 2) context re-exposure, and 3) drug + context re-exposure. These patterns revealed region-specific regulation of gene expression. Further analysis revealed that each of these gene expression patterns correlated with an addiction index—a composite score of several addiction-like behaviors during cocaine SA—in a region-specific manner. Cyclic adenosine monophosphate response element binding protein and nuclear receptor families were identified as key upstream regulators of genes associated with such behaviors.

CONCLUSIONS: This comprehensive picture of transcriptome-wide regulation in the brain's reward circuitry by cocaine SA and prolonged WD provides new insight into the molecular basis of cocaine addiction, which will guide future studies of the key molecular pathways involved.

Keywords: Basolateral amygdala, Dorsal striatum, Gene expression, Nucleus accumbens, Prefrontal cortex, RNA sequencing, Ventral hippocampus

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Addiction arises from genetic and environmental factors, which determine individual responses to initial and repeated drug exposure at the molecular, cellular, and circuit levels (1). A key feature of addiction is the ability for drug or drug-associated cues to trigger relapse, even after periods of prolonged abstinence (2). It is hypothesized that susceptibility to relapse depends on long-term neuroadaptations within the brain's reward circuitry (3–5).

Behavioral responses to cocaine self-administration (SA) after withdrawal (WD) and re-exposure to drug or contextual cues are well characterized in rodent models. However, the underlying molecular mechanisms remain elusive. Most studies investigating transcriptional changes associated with long-term WD followed by cocaine/context re-exposure have focused on candidate genes within one or two brain regions. These studies have found that long-term WD from cocaine SA is associated with changes in growth factors and their signaling cascades (6–9), neurotransmitter and neuropeptide systems (10,11), and immediate early genes (10,12).

The few studies investigating transcriptome-wide changes after short-term WD from cocaine SA (13,14), or long-term WD but without re-exposure (15), focused primarily on the nucleus accumbens (NAc), ventral tegmental area (VTA) (14), or prefrontal cortex (PFC) (13,15). No study has characterized transcriptome-wide changes across multiple interconnected brain reward regions. Furthermore, no transcriptomic study has compared multiple stages of WD plus drug/context re-exposure while leveraging individual variability to identify

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genes that are associated with addiction-related behaviors transcriptome-wide.

Here, we performed RNA sequencing (RNA-seq) on six reward-related brain regions in mice with a history of saline or cocaine SA. We profiled the transcriptome in these regions after short- and long-term WD with drug/context re-exposure. We hypothesized that a history of cocaine SA reprograms the transcriptome, resulting in priming or desensitization of molecular targets upon re-exposure to drug-related context ± cocaine.

METHODS AND MATERIALS

See Supplement 1 for detailed methods.

Experimental Animals

Male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee at Mount Sinai.

RNA Sequencing

Brain regions were dissected rapidly and frozen on dry ice. RNA extraction, library preparation, and RNA-seq were conducted as described (16–18). Multiple targets were validated by quantitative polymerase chain reaction using TaqMan assays (Figure S1 in Supplement 1) (Thermo Fisher, Foster City, CA).

Statistics and Bioinformatics

Behavior. Behaviors were analyzed using analysis of variance or Kruskal-Wallis test depending on homozygosity of variance. All analyses were conducted using SPSS statistical software version 24 (IBM Corp., Armonk, NY).

Transcriptomic Analysis. Pairwise differential expression comparisons were performed as reported (16,17) using Voom-Limma (19); a significance threshold of fold change >1.15 and nominal $p < .05$ were applied.

Factor Analysis and Linear Modeling. Factor analysis was used to reduce the dimensions of interdependent behavioral variables. The transformed behavioral data were then used as continuous covariates to predict gene expression in linear models. A composite addiction index (AI) of 3 factors (Figure S3 and S4 in Supplement 1) most closely associated with SA behaviors was calculated (Supplemental Methods in Supplement 1). Regression analysis was conducted using Voom-Limma to determine AI associations with gene expression (19).

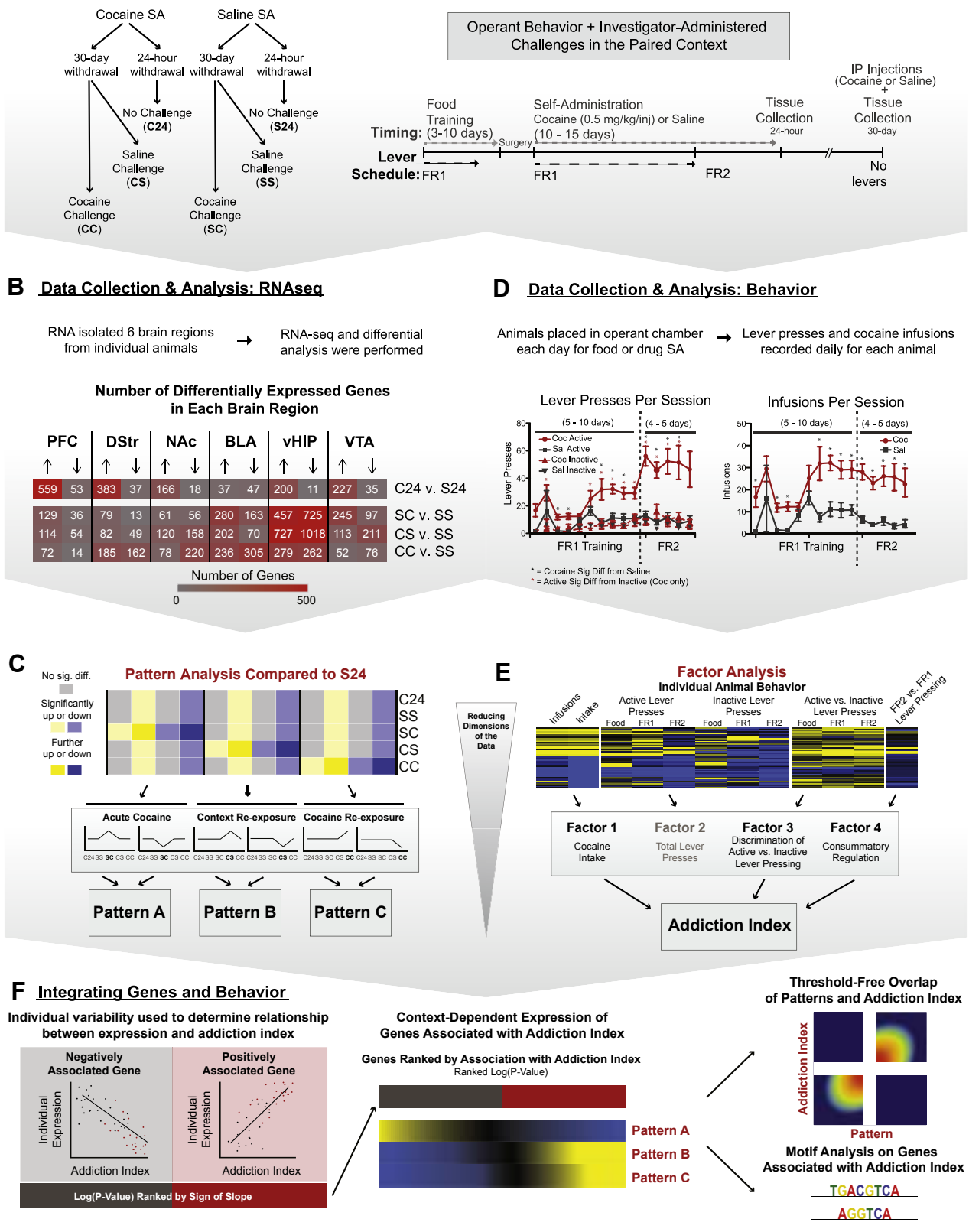
All other bioinformatic analyses were conducted as reported (16–18,20,21).

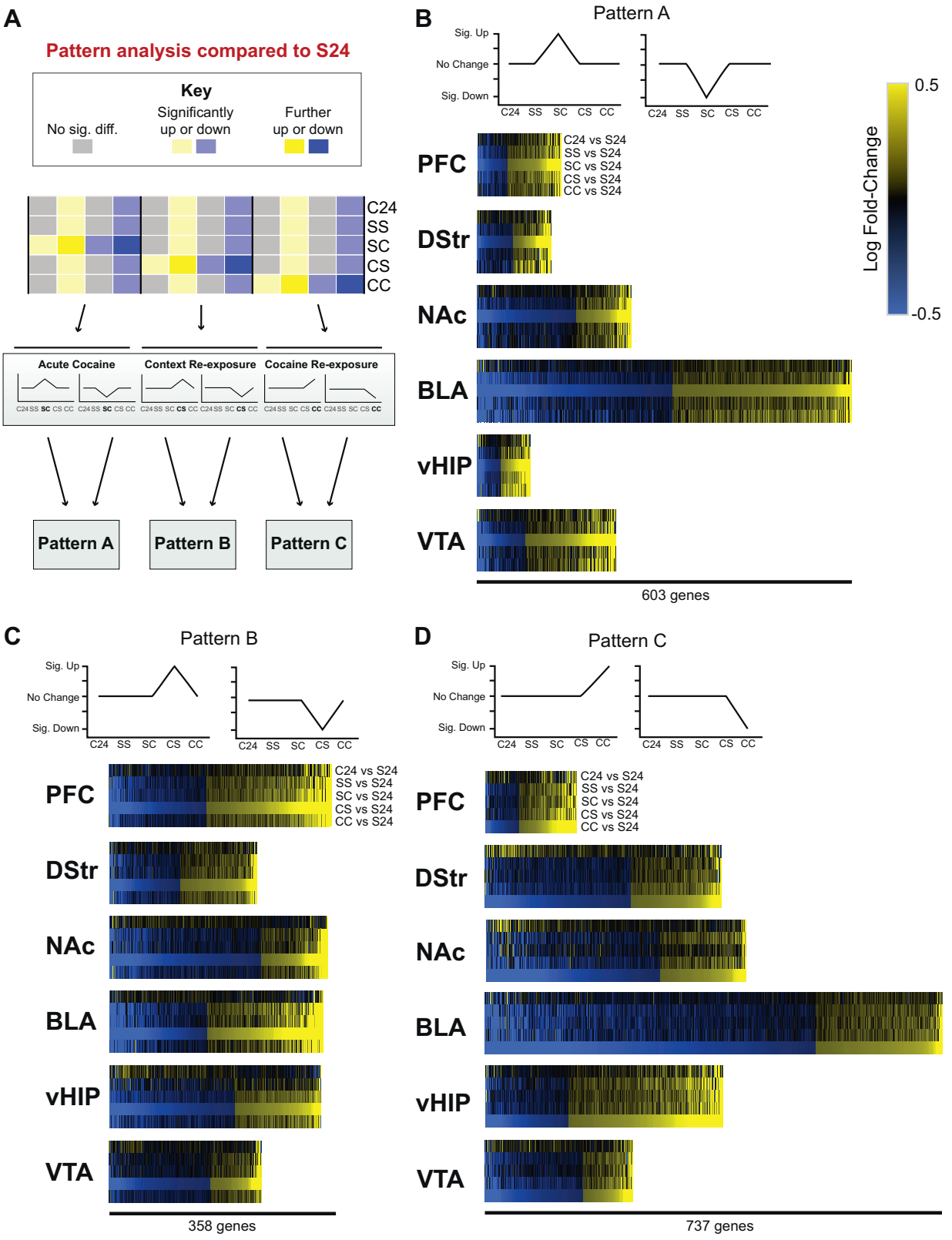
RESULTS

Cocaine SA Behavior

Figure 1 provides an outline of experimental procedures, which are explained in detail in Supplemental Methods in Supplement 1. To determine how a history of cocaine SA influences circuit-wide transcriptomes, RNA-seq was performed on the PFC, dorsal striatum (DStr), NAc, basolateral amygdala (BLA), ventral hippocampus (vHIP), and VTA, obtained from the following six groups of male mice (Figure 1A): saline SA + 24-hour WD (S24) (n = 5–8), cocaine SA + 24-hour WD (C24) (n = 5–8), saline SA + 30-day WD + saline re-exposure (SS) (n = 5–8), saline SA + 30-day WD + cocaine exposure (SC) (n = 5–8), cocaine SA + 30-day WD + saline exposure (CS) (n = 3–7), and cocaine SA + 30-day WD + cocaine re-exposure

Figure 1. Outline of experimental approach and bioinformatic analyses. **(A)** Experimental design and summary of groups. Mice were food trained followed by 5–10 days of fixed-ratio 1 (FR1) scheduling and 4–5 days of fixed-ratio 2 (FR2). One group was euthanized 24 hours after its last self-administration (SA) session while another cohort of animals was group housed in their home cage for 30 days. After withdrawal (WD), animals were given an injection (inj) of saline (Sal) or cocaine (Coc) and re-exposed to their original SA chamber for 1 hour and euthanized immediately. **(B)** Data collection and RNA sequencing (RNA-seq) data analysis. RNA-seq was performed on microdissections of six reward-associated brain regions. Differential expression analysis was performed to identify differentially expressed genes compared with their control group (saline SA + 24-hour WD [S24] or saline SA + 30-day WD + saline re-exposure [SS]). Number of differentially expressed genes per brain region are indicated (red = greatest, gray = least). **(C)** In an effort to identify genes that were uniquely altered by cocaine re-exposure, we used pattern analysis and compared all groups to the same baseline (S24). Three patterns were investigated: pattern A: genes uniquely altered by an initial dose of cocaine (saline SA + 30-day WD + cocaine exposure [SC]) (1 hour postinjection); pattern B: genes uniquely altered by re-exposure to cocaine-paired context (cocaine SA + 30-day WD + saline exposure [CS]); and pattern C: genes uniquely altered by cocaine re-exposure (cocaine SA + 30-day WD + cocaine re-exposure [CC]) (1 hour postinjection). **(D)** Data collection and analysis of cocaine SA behavioral data. Because all animals (saline included) underwent varying numbers of SA trials at FR1, behavioral data were aligned to the day each animal transitioned onto an FR2 schedule (i.e., the last day on FR1). Therefore, data for days 5–10 of FR1, but not days 1–4, include a majority of the animals in the study. In SA animals, cocaine (red) acted as a reinforcer as shown by increased active lever (solid line) vs. inactive lever (dotted line) responding on day 3 of FR1 (indicated by red asterisk). This did not occur for saline animals (black). Cocaine SA animals began pressing the active lever significantly more than saline (indicated by black asterisk) beginning on day 6 of FR1, which continued throughout FR2. Cocaine SA animals (red) received more infusions than their saline counterparts (black) and maintained the same number of infusions after switching to an FR2 schedule, indicating that cocaine was reinforcing lever pressing in these mice. **(E)** We generated an addiction index using exploratory factor analysis to reduce the multidimensional behavioral data to factors associated with components of cocaine SA behavior. We then combined the 3 factors most strongly associated with an addicted-like phenotype to differentiate between individual animals with high performance across multiple behavioral endpoints. **(F)** Integration of genes and behaviors to identify transcripts important for the addicted-like phenotype. Enrichment testing reveals transcripts regulated across multiple brain regions. In silico analysis of potential upstream regulators of the enriched genes. Rank–rank hypergeometric overlap was used to determine if gene expression patterns are associated with the addiction index within a brain region. Behavioral data were analyzed using Kruskal-Wallis test followed by Mann-Whitney nonparametric test; *$p < .05$; data are presented as mean ± SEM. BLA, basolateral amygdala; C24, cocaine self-administration + 24-hour withdrawal; DStr, dorsal striatum; IP, intraperitoneal; NAc, nucleus accumbens; PFC, prefrontal cortex; Sig Diff, significant difference; vHIP, ventral hippocampus; VTA, ventral hippocampus.


A Experimental Design
Cocaine SA
Saline SA
30-day withdrawal
24-hour withdrawal
No Challenge (C24)
No Challenge (S24)
Saline Challenge (CS)
Saline Challenge (SS)
Cocaine Challenge (CC)
Cocaine Challenge (SC)
Operant Behavior + Investigator-Administered Challenges in the Paired Context
IP Injections (Cocaine or Saline) + Tissue Collection 30-day
Timing:
Food Training (3-10 days)
Surgery
Self-Administration Cocaine (0.5 mg/kg/inj) or Saline (10 - 15 days)
Tissue Collection 24-hour
Lever Schedule:
FR1
FR1
FR2
No levers
B Data Collection & Analysis: RNAseq
RNA isolated 6 brain regions from individual animals
RNA-seq and differential analysis were performed
Number of Differentially Expressed Genes in Each Brain Region
PFC
DStr
NAc
BLA
vHIP
VTA
559 53 383 37 166 18 37 47 200 11 227 35 C24 v. S24
129 36 79 13 61 56 280 163 457 725 245 97 SC v. SS
114 54 82 49 120 158 202 70 727 1018 113 211 CS v. SS
72 14 185 162 78 220 236 305 279 262 52 76 CC v. SS
Number of Genes
0
500
D Data Collection & Analysis: Behavior
Animals placed in operant chamber each day for food or drug SA
Lever presses and cocaine infusions recorded daily for each animal
Lever Presses Per Session
Infusions Per Session
(5 - 10 days)
(4 - 5 days)
Coc Active
Sal Active
Coc Inactive
Sal Inactive
Coc
Sal
Lever Presses
Infusions
FR1 Training
FR2
* = Cocaine Sig Diff from Saline
* = Active Sig Diff from Inactive (Coc only)
C
No sig. diff.
Significantly up or down
Further up or down
Pattern Analysis Compared to S24
C24
SS
SC
CS
CC
Acute Cocaine
Context Re-exposure
Cocaine Re-exposure
Pattern A
Pattern B
Pattern C
E
Factor Analysis
Individual Animal Behavior
Reducing Dimensions of the Data
Infusions
Intake
Active Lever Presses
Inactive Lever Presses
Active vs. Inactive Lever Presses
FR2 vs. FR1 Lever Pressing
Food
FR1
FR2
Factor 1
Cocaine Intake
Factor 2
Total Lever Presses
Factor 3
Discrimination of Active vs. Inactive Lever Pressing
Factor 4
Consummatory Regulation
Addiction Index
F Integrating Genes and Behavior
Individual variability used to determine relationship between expression and addiction index
Negatively Associated Gene
Positively Associated Gene
Individual Expression
Addiction Index
Log(P-Value) Ranked by Sign of Slope
Context-Dependent Expression of Genes Associated with Addiction Index
Genes Ranked by Association with Addiction Index
Ranked Log(P-Value)
Pattern A
Pattern B
Pattern C
Threshold-Free Overlap of Patterns and Addiction Index
Addiction Index
Pattern
Motif Analysis on Genes Associated with Addiction Index
TGACGTCA
AGGTCA


A
Pattern analysis compared to S24
Key
No sig. diff.
Significantly up or down
Further up or down
C24
SS
SC
CS
CC
Acute Cocaine
Context Re-exposure
Cocaine Re-exposure
Pattern A
Pattern B
Pattern C
B
Pattern A
Sig. Up
No Change
Sig. Down
C24 SS SC CS CC
PFC
DStr
NAc
BLA
vHIP
VTA
C24 vs S24
SS vs S24
SC vs S24
CS vs S24
CC vs S24
Log Fold-Change
0.5
-0.5
603 genes
C
Pattern B
358 genes
D
Pattern C
737 genes

(CC) (n = 5–7). Supplemental Methods in Supplement 1 provides a complete breakdown of sample size by brain region.

Gene Up- and Downregulation as a Function of History of Cocaine SA and Drug Re-exposure

Previous work demonstrates that repeated, noncontingent cocaine injections cause gene "priming" or "desensitization" in NAc upon cocaine re-exposure after prolonged WD (22,23). We therefore used RNA-seq to investigate this phenomenon genome-wide and analyze transcriptomic changes throughout the reward circuitry in response to drug re-exposure after cocaine SA. Baseline transcriptional effects of cocaine SA were established by differential gene expression profiling in each brain region. Figure 1B shows pairwise comparisons of each cocaine treatment group with their saline control counterparts (C24 vs. S24; SC, CS, and CC vs. SS) and numbers of differentially expressed genes (DEGs) ($p < .05$; fold change >15%) in each brain region (Table S1 in Supplement 2).

To focus on genes that were uniquely altered following context/drug re-exposure after WD, we compared all groups with the same baseline (S24) (Figures 1C and 2A; detailed description of pattern identification in Supplemental Methods in Supplement 1). Figure 2B–D shows heatmaps of DEG patterns within each brain region for all comparisons (C24, SS, SC, CS, and CC vs. S24). This approach revealed two key findings: 1) most DEGs change in the same direction across all re-exposure paradigms (SS—CC) and 2) the magnitude of change for these transcripts was significantly different depending on the animals' history of cocaine SA and re-exposure (Figure 2B–D).

We focused on three patterns associated with drug use: first-ever exposure to cocaine (SC, pattern A) (Figure 2B), re-exposure to cocaine-paired context (CS, pattern B) (Figure 2C), and re-exposure to cocaine-paired context + cocaine (CC, pattern C) (Figure 2D). Each pattern includes genes that were both differentially expressed from S24 ($p < .05$; fold change >15%) and distinct from all other groups. Table S2 in Supplement 2 provides complete gene lists for each pattern. Figure 3A–C shows the number of up- and downregulated DEGs in each pattern, with a cell-type analysis of DEGs shown in Table S7 in Supplement 2.

One challenge in devising treatments for addiction is that many genes show different, sometimes opposite, regulation across brain regions. It was therefore of interest to identify specific transcripts that show similar directional changes across brain regions. Fisher's exact tests to compare overlap of DEGs associated with patterns A–C (Figure 3E, F; Table S3 in Supplement 2) revealed significant overlap of upregulated genes across brain regions in patterns A–C and identified two transcripts that are upregulated across a majority of brain regions in pattern A (*Atp5j2* and *Sox18*). In pattern C, overlap of seven downregulated genes occurred in the DStr, NAc, and BLA, two of which were also downregulated in the VTA (*Lmtk3* and *Map4k2*). All genes with fold change >15% from each pattern were validated by quantitative polymerase chain reaction in three brain regions (Figure 3G–I; Figure S1 in Supplement 1). Therefore, we used a fold-change cutoff of 15% for all comparisons.

Predicted Upstream Regulators Have Unique Gene Targets Based on Cocaine SA History and Re-exposure Across Brain Regions

We hypothesized that these pattern-associated genes may have common upstream regulators across brain regions, which could serve as potential targets for therapeutic intervention. Exploration of upstream regulators was conducted using Ingenuity Pathway Analysis, version 01-12 (Qiagen, Fredrick, MD) for each brain region and each pattern. Comparison analysis was conducted to identify upstream regulators shared across brain regions (Figure 3J–L). Only those upstream regulators with an activation Z score >2 and $p < .01$ in at least one brain region were included.

Seven molecules (cyclic adenosine monophosphate response element binding protein 1 [CREB1], epidermal growth factor, transforming growth factor-β1, cyclic adenosine monophosphate response element modulator, vascular endothelial growth factor, hepatocyte nuclear factor 4-α [HNF4A], and transcription factor 7-like 2) were predicted as upstream regulators in pattern C and at least one other pattern. Notably, CREB1 was a predicted upstream regulator across all three patterns (highlighted in red, Figure 3J–L). CREB1 was the top upstream regulator in patterns A and C and a predicted upstream regulator of genes in the PFC, NAc, and BLA for patterns A, B, and C (Figure 3J–L). CREB1 is activated by initial cocaine exposure and is critical for synaptic plasticity involved in cocaine reward (24,25). Therefore, the prediction that CREB1 is an upstream regulator of genes responding to an acute dose of cocaine in all brain regions (pattern A) validates our pattern identification methodology (Figure 3J). It should be noted that each gene list is unique for a pattern within a brain region. Therefore, the finding that CREB1 is a predicted upstream regulator in all three patterns in the PFC, NAc, and BLA suggests that a history of cocaine SA with drug/context re-exposure results in different targets for CREB1 in these regions. Transforming growth factor-β1, cyclic adenosine monophosphate response element modulator, endothelial growth factor, and vascular endothelial growth factor were

Figure 2. Gene expression patterns associated with cocaine exposure. **(A)** To reduce the dimensions of our RNA sequencing data and identify genes that were uniquely changed by a specific exposure paradigm, we used pattern analysis to categorize genes into patterns of expression when compared with the same S24 baseline. Categorization of genes affected uniquely by **(B)** an initial dose of cocaine (pattern A), **(C)** re-exposure to the cocaine-paired context after 30-day withdrawal from cocaine self-administration (pattern B), and **(D)** re-exposure to cocaine in the cocaine-paired context after 30-day withdrawal from cocaine self-administration (pattern C). Heatmaps show that, for all brain regions, expression of genes categorized in each pattern is, by definition, most pronounced in the comparison that represents that pattern (e.g., pattern A is most pronounced in saline self-administration + 30-day withdrawal + cocaine exposure [SC] vs. saline self-administration + 24-hour withdrawal [S24] when compared with other groups). BLA, basolateral amygdala; C24, cocaine self-administration + 24-hour withdrawal; CC, cocaine self-administration + 30-day withdrawal + cocaine re-exposure; CS, cocaine self-administration + 30-day withdrawal + saline exposure; DStr, dorsal striatum; NAc, nucleus accumbens; PFC, prefrontal cortex; Sig. diff., significant differences; SS, saline self-administration + 30-day withdrawal + saline re-exposure; vHIP, ventral hippocampus; VTA, ventral tegmental area.

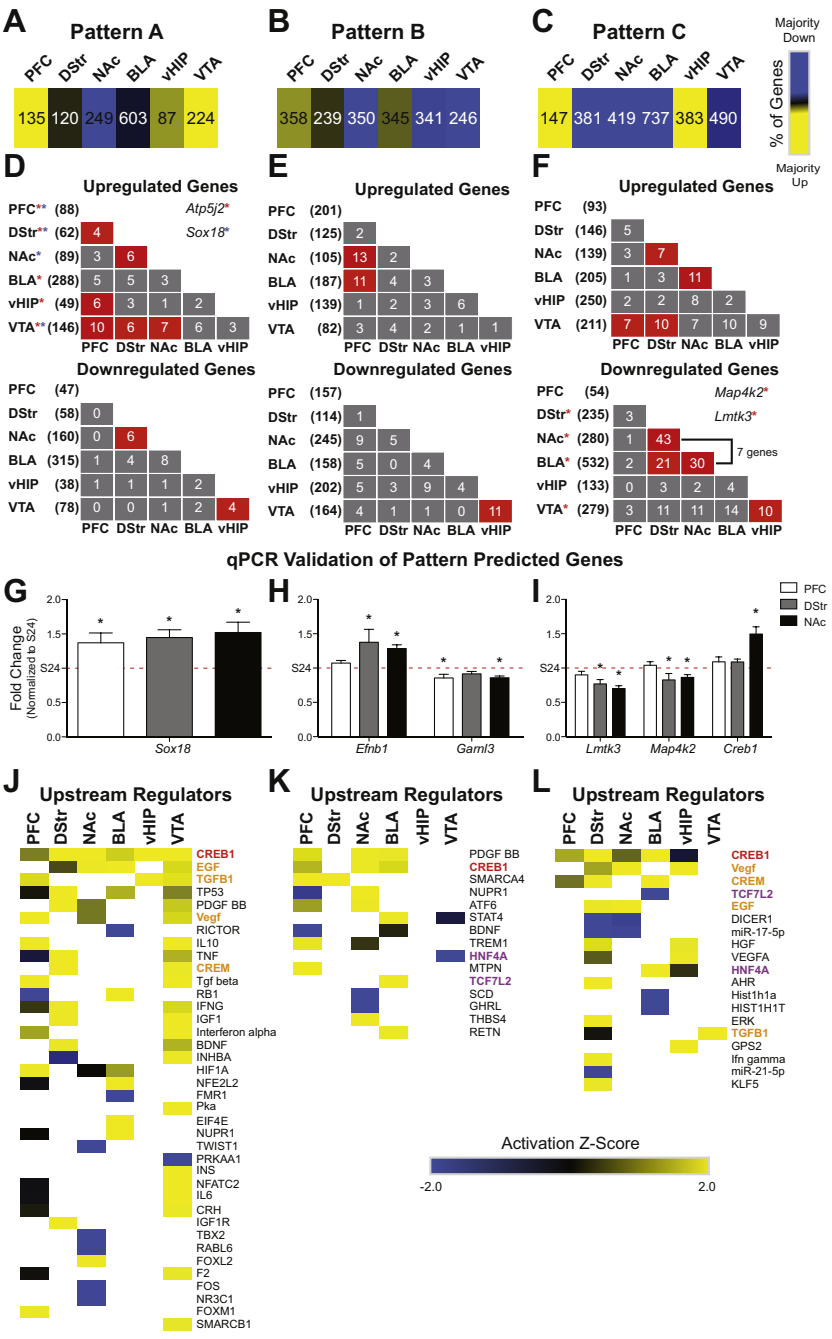


Figure 3. Gene expression patterns associated with cocaine exposure reveal circuit-wide transcriptional changes and upstream regulators. **(A–C)** Number and percentage of genes up- and downregulated (yellow = >60% up; blue = >60% down) in each brain region for each of the three patterns defined in Figure 2. **(D–F)** Overlap across brain regions of upregulated (top) and downregulated (bottom) genes, color coded for significance. The total number of regulated genes in each region is shown in parentheses. Examples of transcripts up- or downregulated across more than two brain regions are listed in the insets. **(G–I)** Patterns were validated using quantitative polymerase chain reaction (qPCR) on technical replicates. Patterns were validated for eight transcripts across three brain regions. Representative transcripts from each pattern are presented. Fold changes of at least 15% in the RNA sequencing data were validated using qPCR across all patterns analyzed, supporting use of this fold change in all analyses. **(J–L)** Upstream regulator analysis was conducted across brain regions for each pattern. Five upstream regulators were consistently predicted to regulate genes across brain regions: cyclic adenosine monophosphate response element binding protein 1 (CREB1) (highlighted in red) is a predicted upstream regulator of all patterns. Regulators overlapping between patterns A and C are highlighted in orange and are likely indicative of those important for regulating the response to acute cocaine exposure independent of a history of cocaine self-administration. Regulators overlapping between patterns B and C are highlighted in purple and are likely indicative of those important for regulating the response to a cocaine-paired context after a history of cocaine self-administration. Activation *Z* scores in heatmaps: positive (yellow) = over-representation of targets activated by regulator; negative (blue) = overrepresentation of targets repressed by regulator; no direction (black) = no significant enrichment of activated versus repressed targets; white = not a predicted upstream regulator. *$p < .05$; blue and red asterisks = transcripts overlap across multiple brain regions. BLA, basolateral amygdala; DStr, dorsal striatum; NAc, nucleus accumbens; PFC, prefrontal cortex; vHIP, ventral hippocampus; VTA, ventral tegmental area.

predicted upstream regulators of patterns associated with an acute dose of cocaine, with or without a history of cocaine SA (patterns A and C) (highlighted in orange, Figure 3J and L). Finally, HNF4A, a nuclear receptor, and transcription factor 7-like 2 were predicted upstream regulators in patterns associated with cocaine SA + WD (patterns B and C) (highlighted in purple, Figure 3K, L). Molecular pathway analysis also identified biological processes associated with the three patterns (Figure S2 in Supplement 1).

Association of Gene Expression Regulation With Behavioral Features of Cocaine SA

We next studied whether individual differences in cocaine SA behavior contributed to the regulation of gene expression observed across brain regions and gene expression patterns. We used exploratory factor analysis to reduce multidimensional behavioral data to factors associated with interrelated variables (Figures 1E and 4A; Figure S3 in Supplement 1).

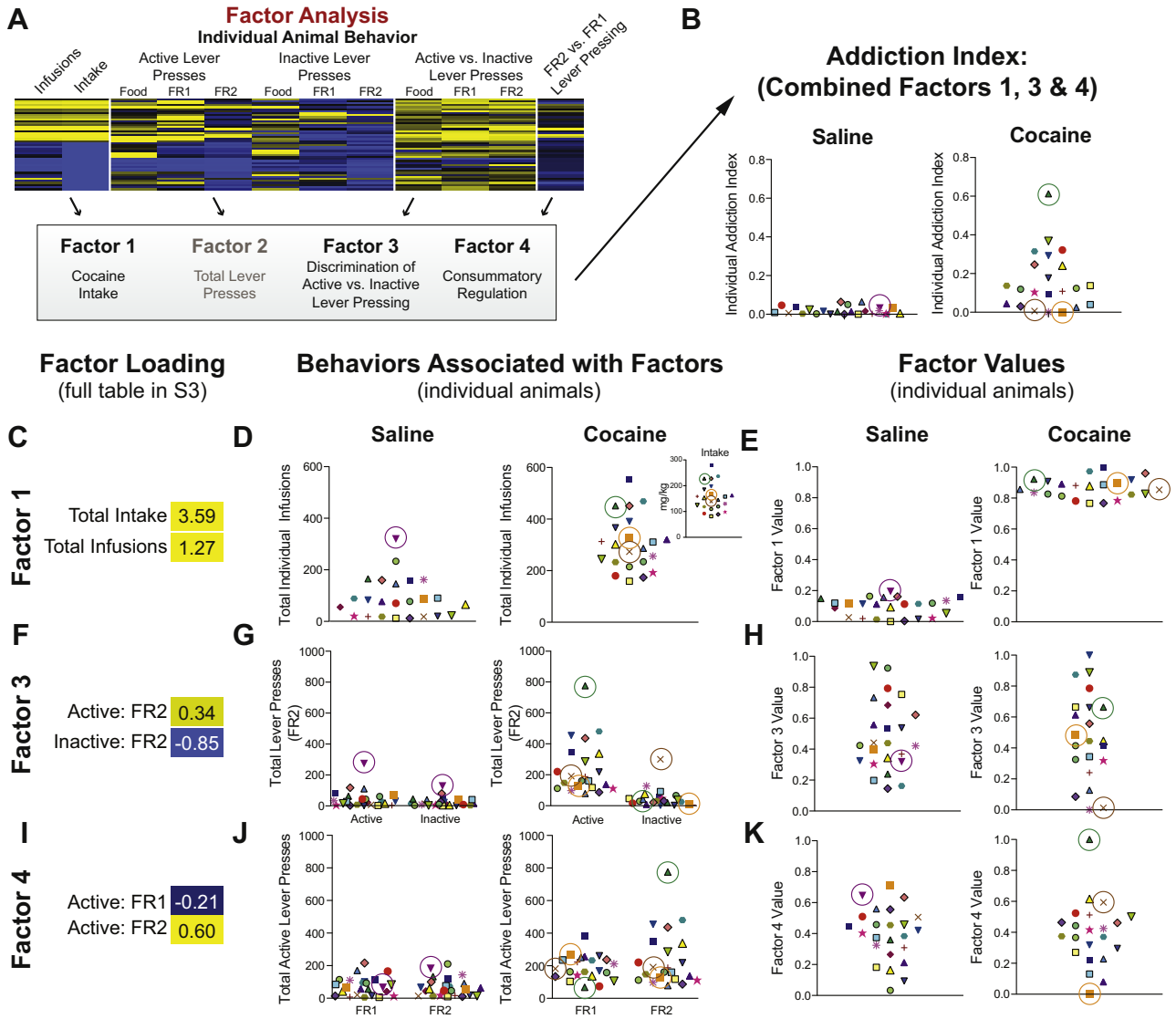


Figure 4. Generation of an addiction index (AI) for individual animals. **(A, B)** Exploratory factor analysis on multiple behavioral endpoints reduced multidimensional behavioral data to eight factors. A composite score, or AI, of those factors most strongly associated with behaviors reflective of an addicted-like phenotype was generated using the individual transformed data for factors 1, 3, and 4. **(C–K)** Data for individual animals for each behavior and each factor are presented. Each animal is represented by the same unique shape and color. **(C, F, I)** Factor loading, or associations, of factors 1, 3, and 4 with self-administration behaviors (yellow = positive; blue = negative) are presented. **(D, G, J)** Individual data presented for the behaviors associated with each factor. **(D)** Factor 1 is associated with intake and infusions, **(G)** factor 3 is positively associated with active lever and negatively associated with inactive lever under a fixed-ratio 2 schedule, and **(J)** factor 4 is positively associated with fixed-ratio 2 lever presses and negatively associated with lever pressing on a fixed-ratio 1 schedule. **(E, H, K)** Individual transformed data for **(E)** factor 1, **(H)** factor 3, and **(K)** factor 4. The product of these values was calculated to generate an AI for each individual. An animal must display high performance on all three factors (upright green triangle) to have a high AI. By contrast, if an animal performs poorly on one of the behaviors (tan X or yellow square) its AI is lower.

We identified 3 factors that are associated with SA behaviors and reflect important components of addiction: factor 1, cocaine intake and infusion; factor 3, discrimination between active and inactive levers; and factor 4, consummatory regulation (altered intake between fixed-ratio 1 and fixed-ratio 2) (Figures 1E and 4A).

To simplify these measures of addiction-related behaviors, we calculated a composite score, or AI, for each animal (Figure 4B; Supplemental Methods in Supplement 1). Individual data are presented for each factor (behavior: Figure 4D, G, J; factor values: Figure 4E, H, K). If an animal scored high on all three factors (e.g., upright green triangle in the cocaine SA group), it has a high AI. However, if an animal scored low on one factor (e.g., tan X does not discriminate between active and inactive levers and yellow square does not increase lever pressing when moved to fixed-ratio 2) their AI is lower. Factor 2 was not included in the AI because it represents differences in total lever pressing (Figure S4 in

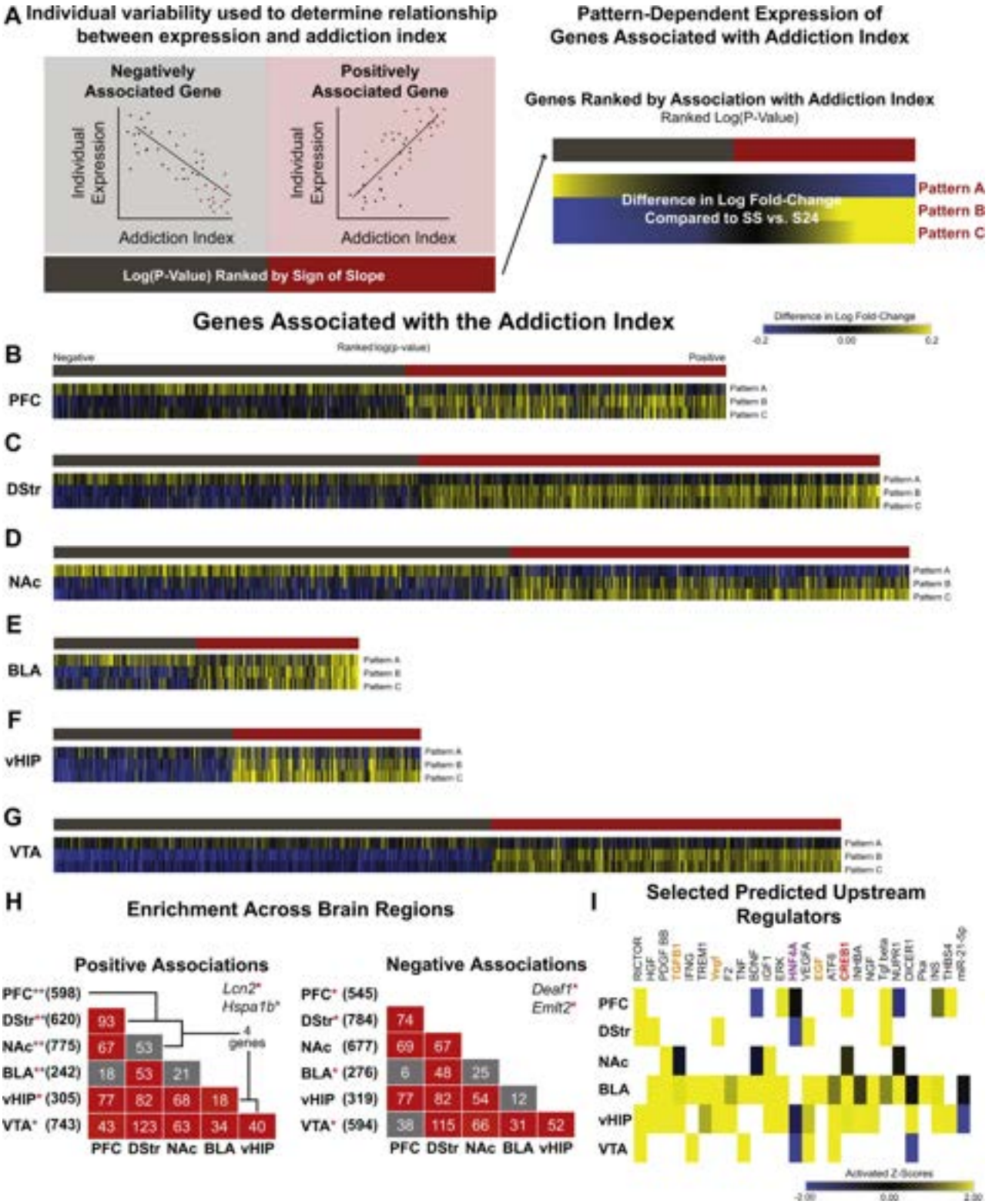


Figure 5. Genes associated with the addiction index (AI) are reprogrammed by cocaine self-administration to be responsive to drug- or cocaine-paired context. **(A)** Linear modeling was used to identify genes associated with the AI within each brain region. Only genes with a slope of at least ±15% and a nominal $p < .05$ were investigated. Similar to the gene expression patterns (Figure 2), we observed that directional changes in expression were similar across all re-exposure comparisons (saline self-administration + 30-day withdrawal + saline re-exposure [SS], saline self-administration + 30-day withdrawal +

Supplement 1), a behavior more reflective of locomotor activity and not SA per se. Use of this factor analysis and calculated AI scores illustrates their utility in identifying key components of complex behavioral datasets and in discriminating between baseline individual differences in behavior and those driven specifically by cocaine SA.

We used linear modeling to identify genes associated with AI scores (Figures 1F and 5A; Table S4 in Supplement 2) to test the hypothesis that individual differences in SA behavior are associated with transcriptional regulation. We noted that the direction of expression changes in genes associated with AI scores were similar across all four 30-day WD groups (Figure S5 in Supplement 1). Because we observed changes in magnitude but not direction in genes categorized as patterns, we hypothesized that a similar effect would be observed in genes associated with AI scores. We calculated the magnitude change by subtracting the log fold change in expression of the SS versus S24 groups from all other comparisons (SC, CS, and CC vs. S24) (Figure 5B). This allowed us to adjust for gene expression differences observed between the two saline control groups. For example, if a gene is further downregulated after cocaine re-exposure, it has a negative value (blue). However, if the downregulation is blunted in comparison with that of the SS control animals, it has a positive value (yellow). Heatmaps of genes significantly associated with AI scores are displayed ($p < .05$, $|\text{slope}| > 0.2$) ranked by $-\log(p \text{ value})$ and sign of slope (red = positive association; gray = negative association).

The heatmaps reveal that a history of cocaine SA (patterns B and C) augments the transcriptional response observed in the SS groups of those genes positively and negatively associated with AI in all six brain regions (Figure 5C–H). The same is not true after an animal's first dose of cocaine. Notably, in the NAc, the transcriptional response of genes associated with AI is attenuated when compared with the SS group (Figure 5E). These data suggest that one dose of cocaine has little impact on genes associated with addiction-related behaviors.

We next used Fisher's exact tests to identify specific transcripts positively or negatively associated with AI across brain regions (Figure 5H). More transcripts overlapped across brain regions in our pairwise comparisons than in the patterns. Notably, genes encoding activator protein 1 transcription factors, including *Fos, Fosb*, and *Fosl2*, were associated with AI in the BLA, vHIP, and NAc. This is consistent with prior work implicating activator protein 1 as an important transcriptional mediator of drug action (25). Genes associated with AI were enriched for neuronal-specific transcripts in all regions (Table S7 in Supplement 2). Six transcripts (*Hspb1*, *Dnajc3*, *Mpdz*, *Tmem252*, *Lcn2*, and *Hspa1b*) were positively associated across five brain regions. Notably, lipocalin 2 (*Lcn2*) was associated with AI all regions except the VTA, where there was a trend (slope = 1.84; $p = .07$), suggesting that *Lcn2* may be a potential novel therapeutic target for addiction.

Upstream regulator analysis identified 192 molecules predicted to regulate genes associated with AI (Figure 5J; Table S5 in Supplement 2). Rapamycin-insensitive companion of mammalian target of rapamycin was the top-predicted regulator in the PFC, DStr, vHIP, and VTA, and CREB1 (highlighted in red) was a predicted upstream regulator of genes in the PFC, NAc, BLA, and vHIP. Finally, HNF4A was a predicted upstream regulator in four of six brain regions. Notably, CREB1 and HNF4A were both predicted in cocaine SA + WD patterns (patterns B and C).

Transcriptome-wide Expression Profiles Dependent on a History of Cocaine SA and Re-exposure Reflect Region-Specific Roles in Addiction-Related Behaviors

To determine if genes associated with AI overlap with genes changed in the condition defining each pattern of gene expression, we used rank–rank hypergeometric overlap (RRHO) analysis, which compares large datasets in a threshold-free manner (16,21,26,27) (Figures 1F and 6). In each brain region, there was significant overlap of genes up- and downregulated in patterns B and C—patterns related to cocaine SA—and genes positively and negatively associated with AI, respectively. This finding is supported by Fisher's exact tests on filtered lists (left) showing significant overlap of up- and downregulated genes in pattern B in all brain regions except the NAc. In contrast, overlap between pattern A—associated with initial, acute cocaine exposure—and AI was absent or far weaker. This is similar to SS versus S24 comparisons (Figure S7 in Supplement 1) in all brain regions except vHIP, where AI overlaps strongly with pattern A (Figure 6E). Additionally, each region showed some pattern-specific associations with the AI (Figure 6). Notably, the NAc displayed strong associations with pattern C (Figure 6C) only and the BLA showed the strongest associations with pattern B (Figure 6D).

Motif Analysis Reveals Nuclear Receptors as Important Regulators of Transcription After a History of Cocaine SA

We conducted HOMER (http://homer.ucsd.edu/homer/) motif analysis on genes associated with AI and categorized as either pattern B or C for each brain region (Figures 1F and 7A;

cocaine exposure [SC], cocaine self-administration + 30-day withdrawal + saline exposure [CS], and cocaine self-administration + 30-day withdrawal + cocaine re-exposure [CC] vs. saline self-administration + 24-hour withdrawal [S24]). Genes that were negatively associated with AI (gray bar) were downregulated and genes positively associated with AI (red bar) were upregulated (Table S4 in Supplement 2). **(B–G)** Heatmaps were transformed to indicate change in expression from SS control animals. Blue indicates fold change in the negative direction from SS vs. S24; yellow indicates fold change in the positive direction from SS vs. S24. Cocaine self-administration programs those transcripts associated with AI to be hyper-response to context either with or without drug. **(H)** Overlap of genes positively (left) or negatively (right) associated with AI across brain regions, color-coded for significance. Total number of genes in each brain region listed in parentheses and total number of genes overlapping between regions indicated in corresponding boxes. There is significant overlap of genes associated with the AI across most brain regions. **(I)** Upstream regulator analysis reveals similar putative transcriptional regulators in genes associated with AI as those associated with specific gene expression patterns. Colors correspond to regulators overlapping in multiple patterns (see Figure 3). Activation *Z* scores: positive (yellow) indicates overrepresentation of targets activated by regulator; negative (blue) indicates overrepresentation of targets repressed by regulator; no direction (black) indicates no significant enrichment of activated or repressed targets; white indicates not a predicted upstream regulator. BLA, basolateral amygdala; DStr, dorsal striatum; NAc, nucleus accumbens; PFC, prefrontal cortex; vHIP, ventral hippocampus; VTA, ventral tegmental area.

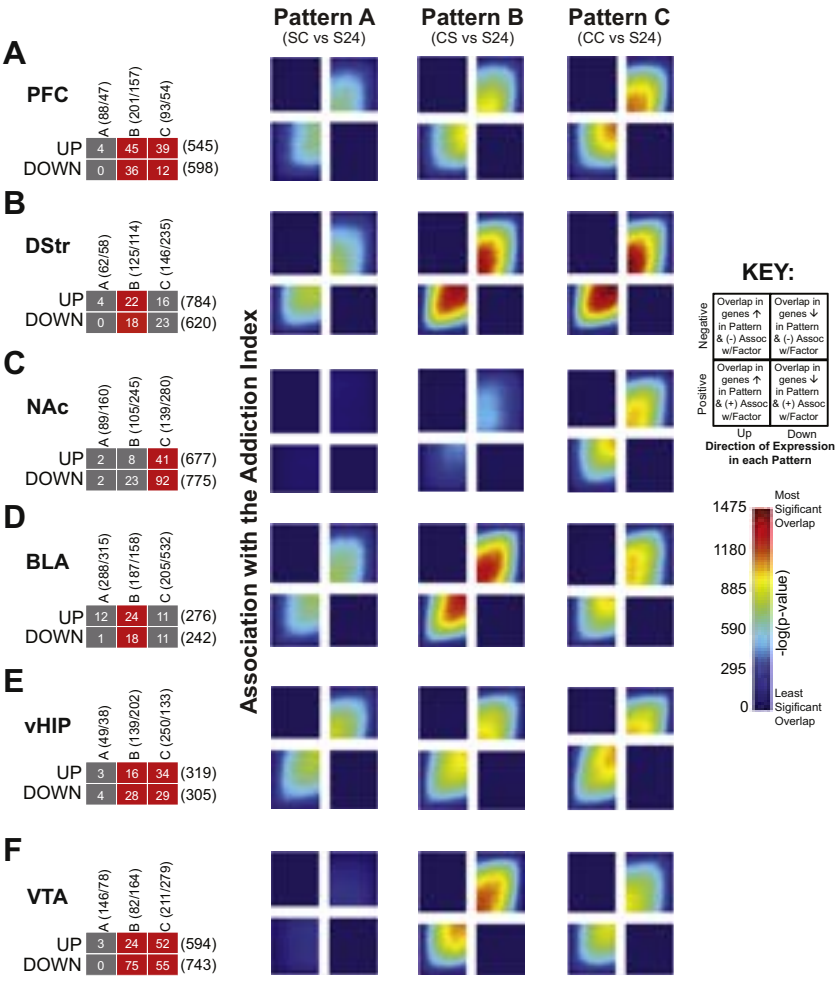


Figure 6. Overlap of transcriptional profiles related to the addiction index (AI) and gene expression patterns reveals which pattern contributes most to AI. **(A–F)** Overlap of genes positively or negatively associated with AI and also up- or downregulated within each gene expression pattern within the gene lists filtered for significance (Fisher's exact test; left) or transcriptome-wide expression profiles (rank–rank hypergeometric overlap plots; right). Overlap of genes associated with AI are specific to brain regions. For example, significant overlap of up- and downregulated genes across patterns B and C with AI are observed in the prefrontal cortex (PFC) and ventral tegmental area (VTA). The ventral hippocampus (vHIP), basolateral amygdala (BLA), and dorsal striatum (DStr) are enriched in genes in pattern B and the nucleus accumbens (NAc) only shows enrichment of genes in pattern C. Rank–rank hypergeometric overlap plots to the right of each panel reveal significance of overlap between region-specific transcriptional profiles associated with AI for patterns A–C. A key for these plots is shown to the right. CC, cocaine self-administration + 30-day withdrawal + cocaine re-exposure; CS, cocaine self-administration + 30-day withdrawal + saline exposure; S24, saline self-administration + 24-hour withdrawal; SC, saline self-administration + 30-day withdrawal + cocaine exposure.

Table S6 in Supplement 2). We found enrichment of several putative transcription factor binding sites implicated previously in reward-associated behaviors (SMAD, E2F, CREB, early growth response protein, and activator protein 1 families) across multiple brain regions (7,8,28–33). Interestingly, the nuclear receptor (NR) family was predicted in every brain region. HNF4A (NR2A1) was a predicted regulator in patterns associated with a history of cocaine SA (patterns B and C) (Figure 3J–L) and genes associated with AI (Figure 5I). NRs have recently been identified as critical for CREB-regulated learning and memory in the hippocampus (34) and important for aspects of cocaine SA in the NAc (35). This, in combination with the prediction of CREB as an upstream regulator across all three patterns and AI, raised the hypothesis that NRs may influence CREB transcriptional regulation in a context-dependent manner throughout the brain.

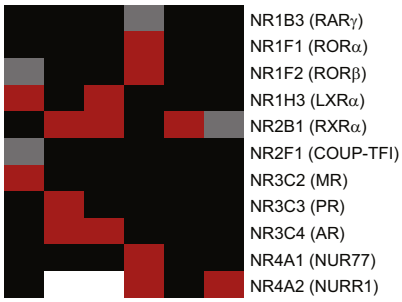
Because NR family members are associated with AI across all brain regions and show region-specific alterations in expression (Figure 7B), we considered the possibility that the region-specific association of NRs with AI, coupled with known regulation of CREB activity and binding, could influence the magnitude of expression of addiction-related genes after a history of cocaine SA. We used in silico analysis to test the hypothesis that CREB and NRs could potentially interact to influence expression in a context-specific manner. We identified proximally located CREB and NR binding sites (MatInspector; Genomatix, Munich, Germany) in a representative gene, *Lcn2*, that was positively associated with AI across multiple brain regions (Figure 7C). Hypothetical transcription factor binding states in each brain region are presented based on region-specific NR expression, association with AI, and known binding data from the MatInspector database (Figure 7C, D). This illustrates the concept that different NRs could influence CREB-induced transcriptional regulation in a region-specific manner. For example, there are two regions in the promoter of *Lcn2* where CREB and NR binding motifs occur within 50 bp of each other. In the NAc and VTA, different NRs are expressed and/or associated with AI. Thus, two putative binding states are represented: 1) in the NAc, NR2B1 binds near CREB in the more distal binding zone, while both NR3C4 and NRC3C bind near CREB in the more proximal binding zone; 2) in the VTA, because NR2B1 is negatively associated with AI, it is not available to bind, while NR4A2 is positively associated and available (Figure 7C). Figure 7C serves to illustrate just one hypothetical mechanism by which the same upstream regulator (e.g., CREB) can have different downstream effects across brain regions and

A Transcription Factor Motif Enrichment in Genes Associated with the Addiction Index

	PFC	DStr	NAc	BLA	vHIP	VTA
Pattern B	E2F3 EGR1 EGR2 **NR1A2** **NR2E3** SMAD3 SMAD4	ATF1 CREB1 CREB3L2 E2F3 EGR1 **NR2F2** **NR3B3**	EGR1 EGR2 **NR2A1** **NR4A2** **NR5A2** SMAD4 TBOX:SMAD	EGR1 EGR2 **NR2E1** **NR2E3** **NR2F2** **NR3B1** SMAD3	ATF1 CREM E2F4 FOS::JUN **NR2E3** SMAD3 SMAD4	CREB3L2 E2F FOS::JUN **NR2F2** **NR4A1**
Pattern C	ATF1 E2F3 **NR2F2** **NR2F6** **NR3B1** TBOX::SMAD	ATF1 CREB1 E2F1 E2F3 FOS::JUN **NR2E3** **NR3B1** **NR4A1** **NR5A1**	ATF4 E2F4 EGR1 **NR2A1** **NR2E3** **NR3B1**	CREB1 E2F1 E2F2 EGR1 FOS::JUN	**NR3C1** **NR3C2** **NR2A1** **NR2F2** **NR4A1** **NR Class**	CREB1 E2F EGR1 EGR2 FOS

(Nuclear Receptors)

B Nuclear Receptors Associated with the Addiction Index



C

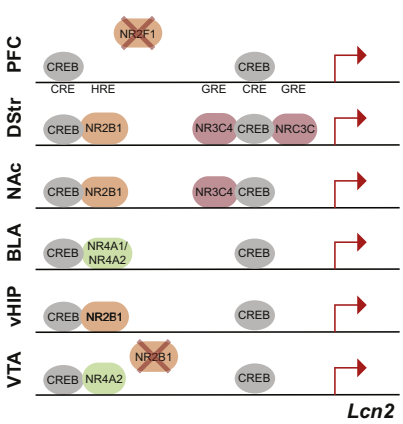


Figure 7. Motif analysis reveals putative role for nuclear receptors (NRs) in controlling region-specific cocaine-induced gene expression. **(A)** HOMER motif analysis was conducted on genes defined as either pattern B or C and significantly associated with the addiction index (AI) (lists from Figure 6 enrichment tests). Table of putative transcription factor families whose motifs were enriched in at least four of six brain regions. Members of the NR family were predicted upstream regulators in all brain regions and were pattern specific. **(B)** NR family members are positively (red) and negatively (gray) associated with the AI in a region-specific manner. Black indicates no association and white indicates no detectable expression. Only NRs with a significant association in at least one brain region are displayed. **(C)** Hypothetical model of transcriptional coregulation by cyclic adenosine monophosphate response element binding protein (CREB) and NRs in a gene positively associated with AI across all brain regions (ventral tegmental area [VTA] indicates trend). In silico analysis of transcription factor binding sites, identified using MatInspector, indicate motifs in close proximity to each other (<50 bp), and binding data from the MatInspector database indicate binding of specific NRs within the *Lcn2* promoter. Based on our AI data, we extrapolated possible region-specific binding states that could be regulating the transcriptional response to drug or context re-exposure. Color indicates subfamily of NRs: orange indicates NR2 subfamily; pink indicates NR3 subfamily; green indicates NR4 subfamily. An X indicates a negative association with AI. BLA, basolateral amygdala; DStr, dorsal striatum; NAc, nucleus accumbens; PFC, prefrontal cortex; vHIP, ventral hippocampus.

behavioral histories. Furthermore, this analysis serves as an example of how our extensive datasets can be used moving forward.

DISCUSSION

These data provide the first unbiased assessment of gene regulation across various time points of cocaine SA—short- and long-term WD— and two different re-exposure paradigms in six interconnected brain reward regions. While prior studies have investigated transcriptional responses to cocaine re-exposure after SA (6–12), these have not done so transcriptome-wide across a range of brain regions. Furthermore, this study is particularly powerful as we used individual variability to identify transcripts associated with aspects of cocaine SA behavior. We leveraged two statistical approaches (pattern identification and factor analysis) to characterize novel gene expression patterns throughout the reward circuitry that are sensitive to drug re-exposure after prolonged WD from cocaine SA.

Traditional methods of analyzing RNA-seq data have focused on pairwise comparisons to identify DEGs when compared with a single control group. Our dataset contained two control groups, so pairwise comparison using each condition's control group (S24 and SS) could not uncover all transcriptional differences. Therefore, we utilized a novel approach to identify patterns of expression that reflect differences from both baselines and identified transcripts that were uniquely altered by either context re-exposure alone or context + drug re-exposure. This revealed that many genes associated with long-term WD and re-exposure were altered in magnitude but not direction. Pattern identification therefore allowed us to detect genes that were uniquely altered by acute cocaine (pattern A), cocaine-paired context (pattern B), or context + cocaine re-exposure (pattern C) independent of baseline changes. Furthermore, each gene was only characterized as one pattern per brain region, thus revealing those genes associated uniquely with context- and/or drug-induced relapse.

This pattern identification analysis revealed individual transcripts that are regulated across multiple brain regions and may serve as therapeutic targets for addiction. For example, in pattern C, two protein kinases (*Lmtk3* and *Map4k2*) are downregulated in the DStr, NAc, BLA, and VTA. Knockout of *Lmtk3* increases locomotor activity and dopamine turnover in striatum (36). Both are involved in actin cytoskeletal remodeling (37,38) and *Map4k2* has been linked to inflammatory responses (39), two key processes in synaptic plasticity (6,40). Similarly, transcripts were identified that were associated with AI across multiple brain regions (Figure 5H). Notably, *Lcn2* was positively associated with AI across all 6 brain regions (VTA = trend). LCN2 forms a complex with matrix metalloproteinase 9 and protects it from degradation, thus prolonging its activity (41). Matrix metalloproteinase 9 activity has been shown to be critical for cue- and cocaine-

induced reinstatement (42). These transcripts provide valuable information regarding biological processes important for cocaine addiction, and serve as potential brain-wide therapeutic targets.

One key finding of the pattern analysis came from upstream regulator analysis, which showed that many predicted transcriptional regulators were consistent across patterns and brain regions (Figure 3J–L). This is significant because each gene list is unique for a pattern within a brain region, suggesting that the targets of these predicted regulators change depending on cocaine history and re-exposure paradigm. This provides a potential mechanism for our hypothesis that a history of cocaine SA primes the reward circuitry at the transcriptional level to respond to context/drug re-exposure.

We identified CREB1 as a predicted upstream regulator in patterns A, B, and C in the PFC, BLA, and NAc—brain regions implicated in cue-induced reinstatement (43–45). CREB1 has long been implicated in addiction-related phenomena (24,25,46) and is critical for synaptic plasticity and reward learning. Prediction of CREB1 as a regulator of expression in all brain regions upon initial exposure to cocaine validates our pattern identification methodology.

Individual differences in SA behavioral responses correlate with gene expression changes following WD. To date, those correlations have been restricted to drug-taking animals without including saline control animals, and none have been performed transcriptome-wide (47,48). Two limitations of previous analyses are 1) false positives/negatives due to constraints in statistical analysis of small sample sizes typical of RNA-seq experiments and 2) the inability to use all available SA behavioral data in correlation analysis (e.g., saline control animals cannot be correlated with intake). To understand how individual differences in cocaine SA behavior may influence the transcriptional landscape after long-term WD and re-exposure, we used factor analysis to generate a composite AI that incorporates variability in SA behaviors associated with addiction-like outcomes and discriminates between saline and cocaine animals (Figure 4). This allowed us to use the saline control animals in our linear model to account for baseline differences in behavior and substantially increased our sample size, reducing the likelihood of false discovery.

The greater transcriptional response in patterns B and C drive association with the AI in a region-specific manner (Figure 5B–G). This is further reflected in the RRHO analyses (Figure 6). Thus, context is exceptionally important for the transcriptional component of relapse, and the response appears to be region specific. RRHOs highlight which pattern of gene expression contributes to AI in each brain region, thus showing which pattern most reflects addiction-related behaviors. Together, our data suggest that transcriptional reprogramming occurs during long-term WD and is associated with the degree of the addictive phenotype.

The high degree of overlap of transcripts associated with AI across brain regions (Figure 5I) suggests once again that there is a suite of transcripts throughout the reward circuitry being targeted by similar upstream regulators. As in the patterns, CREB1 was a predicted upstream regulator in the PFC, NAc, BLA, and vHIP of genes associated with AI (Figure 5J). HNF4A was also a predicted upstream regulator of genes associated with AI and was one of two upstream regulators (transcription factor 7-like 2) predicted for both patterns B and C. HNF4A is implicated in epigenetic mechanisms (49–52) and dendritic spine morphology (51). While expression of *Hnf4a* was not detected in our sequencing data, other NRs were. Additionally, many NRs share a consensus sequence and compete for DNA binding (53).

Based on this knowledge, we used HOMER de novo motif analysis to identify putative transcription factor binding sites across genes in patterns B or C that were also associated with AI. Strikingly, NRs were present in every brain region in a similar pattern-specific manner as seen by RRHO. Furthermore, CREB1 and other CREB family members were predicted in all brain regions. Thus, we posit that NRs may influence transcriptional regulation by CREB proteins in response to drug/context re-exposure in a region-specific manner.

Using novel analytic approaches followed by upstream-regulator, motif and other in silico analyses, we present here candidate genes and transcriptional regulators that may serve as therapeutics for addiction-related disorders. While CREB and NRs are highlighted for follow-up, this serves as just one example for how this vast dataset can be mined in future studies. To conclude, our datasets provide a highly unique resource of transcriptional regulation throughout the brain's reward circuitry and across cocaine SA, WD, and re-exposure. The transcriptional reprogramming that occurs offers valuable information regarding gene expression correlating with high performance on a highly ethologically relevant model of addiction. Thus, this work provides an increasingly complete understanding of the molecular basis of cocaine addiction and allows us to work toward individualized therapeutics.

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