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Genetic Loci Influencing Cue-Reactivity in Heterogeneous Stock Rats

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ABSTRACT

Addiction vulnerability is associated with the tendency to attribute incentive salience to reward predictive cues. Both addiction and the attribution of incentive salience are influenced by environmental and genetic factors. To characterize the genetic contributions to incentive salience attribution, we performed a genome-wide association study (GWAS) in a cohort of 1596 heterogeneous stock (HS) rats. Rats underwent a Pavlovian conditioned approach task that characterized the responses to food-associated stimuli ("cues"). Responses ranged from cue-directed "sign-tracking" behavior to food-cup directed "goal-tracking" behavior (12 measures, SNP heritability: 0.051–0.215). Next, rats performed novel operant responses for unrewarded presentations of the cue using the conditioned reinforcement procedure. GWAS identified 14 quantitative trait loci (QTLs) for 11 of the 12 traits across both tasks. Interval sizes of these QTLs varied widely. Seven traits shared a QTL on chromosome 1 that contained a few genes (e.g., *Tenm4*, *Mir708*) that have been associated with substance use disorders and other psychiatric disorders in humans. Other candidate genes (e.g., *Wnt11*, *Pak1*) in this region had coding variants and expression-QTLs in mesocorticolimbic regions of the brain. We also conducted a Phenome-Wide Association Study (PheWAS) on addiction-related behaviors in HS rats and found that the QTL on chromosome 1 was also associated with nicotine self-administration in a separate cohort of HS rats. These results provide a starting point for the molecular genetic dissection of incentive motivational processes and provide further support for a relationship between the attribution of incentive salience and drug abuse-related traits.

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1 | Introduction

Addiction vulnerability is influenced by genetic and environmental factors. These factors are thought to include differences in cognitive and motivated behaviors, such as the tendency to attribute incentive value to reward cues [1–3], novelty seeking [4–6], locomotor response to novelty [7–9], and impulsivity [10–13]. Thus, a major avenue for understanding the genetics of addiction vulnerability is to delineate the genetic basis of these addiction-related traits.

Sensitivity to reward-paired stimuli is a particularly important addiction-related trait [14, 15] because incentive cues can instigate the craving and drug motivation that lead to relapse [16–19]. In rats, the incentive value of cues can be measured using a Pavlovian conditioned approach (PavCA) procedure, which measures individuals' conditioned responses to a food-predictive reward cue. Some rats ("sign-trackers"; ST) show a strong tendency to approach and interact with cues that have become associated with a food reward, whereas others ("goal-trackers"; GT) instead approach and interact with the food-delivery location [20–23]. Thus, in sign-trackers, cues acquire incentive salience, as indicated by the extent to which cues elicit approach and become reinforcing [14]. Sign-trackers also show heightened responses to cocaine cues [1], are sensitive to the ability of cocaine cues to support drug-taking [24], and motivate cocaine and nicotine seeking [2, 3, 25]. Sign-tracking is therefore an easily observed measure of cue-responsivity that predicts the effects of cues on several addiction-related traits [26].

Although there is substantial variability in the tendency to sign- or goal-track within outbred rat populations [27–29], few studies have examined the genetic basis for variation in the tendency to attribute incentive salience to reward cues [27, 30]. To address this knowledge gap, we conducted a genome-wide association study (GWAS) of PavCA to determine the genetic underpinnings of sign- and goal-tracking in a large population ($n = 1596$) of heterogeneous stock rats (HS) [31, 32]. HS rats were selected because of their high genotypic and phenotypic variability, as well as the many complementary resources available for this population [33, 34]. We have used data from this same cohort of HS rats previously to compare sign- and goal-tracking to other drug-associated traits, including responses to cocaine and cocaine cues [29].

2 | Materials and Methods

2.1 | Subjects

Subjects were NMcwi:HS rats (RRID:RGD_2314009; formerly known as N:NIH; N:NIH-HS; hereafter referred to as HS) that were shipped to the University at Buffalo from the laboratory of Dr. Leah Solberg Woods at the Medical College of Wisconsin. HS rats were originally established at the NIH by interbreeding eight inbred strains (ACI/N, BN/SsN, BUF/N, F344/N, M520/N, MR/N, WKY/N, and WN/N [31, 34]). To preserve genetic diversity, HS rats have been maintained by various laboratories, using dozens of breeding pairs per generation, in conjunction with various breeding schemes that were designed to minimize inbreeding. Wherever possible, no more than one male and one female per rat litter were used in this study. This limited the use of closely related individuals, thereby increasing statistical power in GWAS studies. This study used $n = 1596$ HS rats from generations 71–88. Several rats were dropped from analysis (range: $n = 1$–9) due to data collection error for some measures.

Rats were shipped at approximately 33 days of age to the University at Buffalo, where they underwent 14 days of quarantine before being sent to the Clinical Research Institute on Addictions (CRIA) at the University at Buffalo. Rats of the same sex were pair-housed in plastic cages (42.5 × 22.5 × 19.25 cm) containing sawdust bedding (Aspen Shavings) in a temperature-controlled vivarium (22°C ± 1°C) with continuous access to water and food (Harlan Teklad Laboratory Diet #8604, Harlan Inc., Indianapolis, IN, USA). No environmental enrichment was provided. Rats underwent behavioral testing at the CRIA before being transferred to the University at Buffalo's North Campus by laboratory animal facility staff (25-min by car). Traits measured during testing at the CRIA are being prepared for a separate publication and include tests for locomotor activity, light reinforcement, choice reaction time task, patch-depletion foraging test, and social reinforcement (Described in: [35]). Rats were acclimatized for a minimum of 7 days following transfer to North Campus, during which time they were handled daily. Rats were maintained on a reverse light/dark cycle at both CRIA and the University at Buffalo (lights off at 7:30 am) and were tested a minimum of 1-h following the onset of the dark cycle. PavCA testing began on average at PND162, range 140–204 in 16 batches, with each batch containing 7 groups of between 6 and 16 subjects per group. All studies were conducted according to the National Research Council (2003) "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" and approved by the University at Buffalo Institutional Animal Care and Use Committees.

2.2 | Procedure and Apparatus

Pavlovian conditioned approach (PavCA). Rats were tested in 16 modular testing chambers (20.5 × 24.1 cm floor area, 29.2 cm high; MED-Associates Inc., St. Albans, VT). These chambers were housed in custom-built enclosures to attenuate external light and sound, and were outfitted with fans that provided ventilation and background noise (A&B Display Systems, Bay City, MI). During PavCA, rats learned the association between the presentation of a ~45 mg banana-flavored food pellet and a conditioned stimulus (CS) (a backlit lever-CS) over 5 sessions. Prior to testing, rats were exposed to the flavored food pellets in their home cage for 2 days (~25 pellets per day; Bio-Serv, Flemington, NJ, #F0059). Next, rats underwent a single day of food cup training, which included a 5-min chamber habituation before receiving 25 pellets delivered into an infrared photobeam-equipped food cup, or "magazine" on a variable interval (VI)-30 s (1–60 s range) schedule. Details about the testing apparatus and equipment can be found on Open Behavior (https://edspace.american.edu/openbehavior/project/pavca/).

Rats then received five daily conditioning sessions in which they received 25 lever-food pairings, with each food pellet

delivery preceded by the presentation of a retractable backlit lever for 8 s. During testing, chambers were illuminated by a red light (27 cm high) on the back wall of the testing apparatus, and retractable levers were situated on either the left or right side of the food cup (2 cm length, 6 cm above floor). Lever presses and head entries into the food cup had no programmed consequences. Each lever-food pellet trial was separated on a VI-90 schedule (30–150 s range) such that sessions lasted an average of 37.5 min. A summary of the testing procedure is available on protocols.io (https://doi.org/10.17504/protocols.io.x54v9yjx4g3e/v1; [36]).

Conditioned reinforcement (CRf). CRf was conducted the day after the final Pavlovian conditioning session and was used to assess the effectiveness of the lever-CS to reinforce a new instrumental response. Testing was conducted in the same chamber as PavCA, although the devices inside the chamber were organized differently. Specifically, the retractable lever was moved to the center of the instrument panel, and the food cup was removed entirely. On either side of the lever were two nose poke ports with head-entry detectors. Each of the two nose poke ports was assigned as either active or inactive. Entries into the active port resulted in a 3-s delivery of the lever-CS. Responses in the inactive port had no programmed consequences. Sessions lasted 40 min. All data for PavCA and CRf were collected using the Med-PC IV software package (version 4.2, build 56).

2.3 | Behavioral Measures

We examined lever- and food-cup directed behavior during PavCA sessions. Approach to the lever was operationalized by incidental lever deflections (i.e., lever contacts) whereas approach to the food cup was operationalized as food cup entries (food-cup contacts) during each of the 25 trials. Food cup entries during the inter-trial interval period were also recorded. For each trial, the latency to deflect the lever or enter the food cup was also recorded. Previously, we have used these measures to calculate a general tendency to engage with the lever ("sign-tracking") or food cup ("goal-tracking") by calculating the PavCA index [23]. The index contains several calculated measures: (1) The probability differential of contact with the lever versus food cup during each CS period (average probability of a lever contact on a given CS trial − average probability of a food-cup contact on a given CS trial), (2) the response bias directed towards either the lever or the food cup ([# lever contacts − # food-cup contacts]/[# lever + # food-cup contacts]), and finally (3) a latency score across trials to initiate contact with either the lever or food cup (food-cup latency − lever latency/8). The PavCA index was computed by averaging these three measures, yielding a value from −1 to 1, with −1 reflecting an exclusive tendency to goal-tracking and 1 reflecting an exclusive tendency to sign-track.

For CRf the primary measures were total active and inactive responses, total earned lever reinforcers, total lever deflections, and an incentive value index ([responses in active port − responses in inactive port]/lever contacts). We chose to separately examine total lever deflections and lever deflections corrected for total responses because we have previously shown that these measures are more strongly correlated to the PavCA index [28, 29].

2.4 | Selection of Measures

We focused on a battery of 12 measures that reflected key terminal (i.e., session 5) indicators of sign- and goal-tracking during PavCA (shown in Table 1a) and CRf (shown in Table 1b). Descriptions of each measure and SNP heritability estimates (discussed later) are also shown in Table 1. We have shown previously that this set of 12 behaviors is stable by the end of conditioning and most directly related to the sign- and goal-tracking phenotypes [29].

2.5 | Tissue Collection and Genotyping

Upon completion of behavioral testing, spleens were collected from each rat and then sent to the University of California San Diego for genotyping [37, 38]. This genotyping produced 3,400,759 single-nucleotide polymorphisms (SNPs) with an estimated error rate of less than 1%. All coordinates are based on the Rnor_6.0 assembly (Accession number GCA_000001895.4) of the rat genome. The sex chromosomes (X and Y) and mitochondria were not genotyped.

2.6 | Statistical Analysis

2.6.1 | Phenotypic and Genetic Correlations, Heritability Estimates

To address the non-normal distribution of several of the traits (phenotypic distributions are available online at the UC San Diego Library Digital Collections at https://doi.org/10.6075/J0MW2HG7; [39]), and to remove potential sex differences, each trait was quantile-normalized separately for males and females. The quantile normalization procedure randomly breaks "ties" such that when two or more individuals have identical values, they are assigned different values. Other covariates, including age, batch number, and testing apparatus, were examined for each trait (available in Supporting Information S1), and regression was used to correct for covariate effects if they explained more than 2% of the variance. Age did not explain more than 2% of the variance. However, two batches covaried with lever presses during CRf (5.0% and 2.02%, respectively) and the resulting residuals were quantile-normalized again before being used for GWAS. The Spearman test was used for phenotypic correlations. SNP heritability estimates were obtained using the REML method, and genetic correlations between traits were computed through bivariate GREML analysis, both performed with GCTA [40, 41].

2.6.2 | Genome-Wide Association Analysis

To perform GWAS, we used a linear mixed model, as implemented in GCTA [40, 41], using all SNP genotypes to create a genetic relatedness matrices (GRM) which accounted for the complex familial relationships that are characteristic of

TABLE 1 | Measures of sign- and goal-tracking, and accompanying SNP heritability estimates. Several measures of goal- and sign-tracking behavior were collected during (a) the Pavlovian conditioned approach (PavCA) task and (b) the conditioned reinforcement (CRf) task. (a) The highest SNP heritability estimates tended to reflect measures of sign-tracking at the end of training relative to measures of goal-tracking. (b) Similarly, conditioned reinforcement also showed modest SNP heritability, with the most heritable traits reflecting measures that directly assess lever-directed sign-tracking behavior (lever presses and overall incentive value index). All heritability estimates were significantly different from zero.

Trait	Measure description	SNP heritability	SE
Table 1a: PavCA measures (Day 5)			
Sign-tracking			
Lever CS contacts	Number of lever CS deflections	0.209	0.035
Lever CS latency	Latency to deflect lever CS	0.215	0.035
Lever CS probability	Probability of a lever CS deflection	0.186	0.034
Goal-tracking			
Food-cup entries	Number of food cup entries during lever CS	0.114	0.03
Food-cup latency	Latency to enter food cup during lever CS	0.111	0.03
Food-cup probability	Probability of a food cup entry during lever CS	0.107	0.029
Overall			
Response bias	Corrected total food-cup and lever CS responses	0.203	0.034
Index	General tendency to engage in sign- and goal-tracking	0.153	0.032
Non-specific			
Food-cup ITI entries	Total food cup entries during the inter-trial-interval	0.142	0.031
Table 1b: Conditioned reinforcement measures			
Sign-tracking			
Lever presses	Total lever deflections following reinforcement	0.22	0.035
Incentive value index	(Responses in active port—responses in inactive port)/lever contacts	0.19	0.034
Active—inactive ratio	Responses in active port/responses in inactive port	0.051	0.025

laboratory populations like the HS rats. We used the Leave One Chromosome Out (LOCO) method to avoid proximal contamination [42, 43]. Using permutation for a genome-wide alpha of 5%, the significance threshold was $-\log(p) > 5.95$, and for a genome-wide alpha of 10%, it was $-\log(p) > 5.67$. Because all traits were quantile normalized, a single permutation analysis could be used for all traits and the same threshold could be used [44]. Quantitative trait loci (QTLs) were identified by scanning each chromosome for SNPs that exceeded the permutation-derived threshold. To avoid spurious results, we required that each QTL be supported by at least one additional SNP within 0.5 Mb that had a p-value within $2 - \log_{10}(p)$ units. To detect multiple significant loci on the same chromosome, we initially selected the most significant SNP on a given chromosome. We then used that SNP as a covariate and performed a second scan of the same chromosome to determine whether there was a second significant and conditionally independent QTL on the same chromosome. If necessary, we would have continued to repeat this process until no further significant QTLs were detected on the chromosome in question. This procedure was performed for each autosome. For simplicity and ease of illustration, these conditional analyses are shown as Manhattan plots, which depict the initial scan prior to any conditional analysis.

3 | Results

Multiple measures of sign- and goal-tracking were collected across the five sessions of conditioning. We focused on the final session of conditioning (session 5), which most directly reflects the stable sign- and goal-tracking phenotype. For CRf, we focused on three key measures of the reinforcing value of the lever. The full GWAS results are available in Supporting Information S1 and as an interactive .html file at https://doi.org/10.6075/J0MW2HG7 [39]. Tables presented in the manuscript are available in Supporting Information S2. Note that the term "magazine" is used to refer to the food cup in the Supporting Information.

There was substantial variability in tendency to sign- and goal-track during PavCA in both males and females. The tendency to sign-track was strongly associated with the subsequent reinforcing value of the lever during CRf ($r^2 = 0.39$ and 0.48 for males and females, respectively) as described previously [29]. The behavioral analyses of these two tasks are described in detail in King et al. [29] and so for brevity, we do not present these data here. Selected measures from the two tasks, described below, were used to examine genetic loci associated with tendency to attribute incentive salience to reward cues (i.e., sign-track).

3.1 | Genetic Correlations

The genetic correlation analysis of PavCA and CRf measures indicated significant shared genetic influence on a pair of behaviors [40]. To examine the genetic relatedness among PavCA and CRf, phenotypic and genetic correlations (r_g) for the set of behavioral measures were computed (Figure 1). Notably, two measures reflecting the attribution of incentive salience to the reward cue, "PavCA: Lever Contacts" and "CRf: Incentive Value Index", were highly genetically correlated ($r_g = 0.954$), suggesting a shared genetic basis. Some measures had inverse phenotypic relationships, such as between similar the sign-tracking measures "PavCA: Lever CS Latency". As a result, Lever CS latency has a strongly negative genetic correlation with "PavCA: Lever Presses" ($r_g = -0.849$). The "PavCA: Terminal Index" and related measures also exhibit strong positive correlations, underscoring their genetic relatedness. However, general activity measures like "PavCA: Food-Cup ITI" show weaker correlations, highlighting distinct genetic influences on other behaviors. Overall, the results reveal a shared genetic architecture underlying behavioral measures reflecting sign-tracking.

3.2 | PavCA and CRf Show Modest Heritability

Next, we examined SNP heritability, which was generally moderate for PavCA and CRf measures. Heritability estimates for sign- and goal-tracking traits during PavCA ranging from 0.215 ± 0.04 (latency to lever CS contact) to 0.107 ± 0.02 (probability of food-cup entry) (Table 1a). Heritability estimates are shown clustered by sign- and goal-tracking measures, with the strongest heritabilities reflecting measures related to terminal sign-tracking. CRf heritability also showed

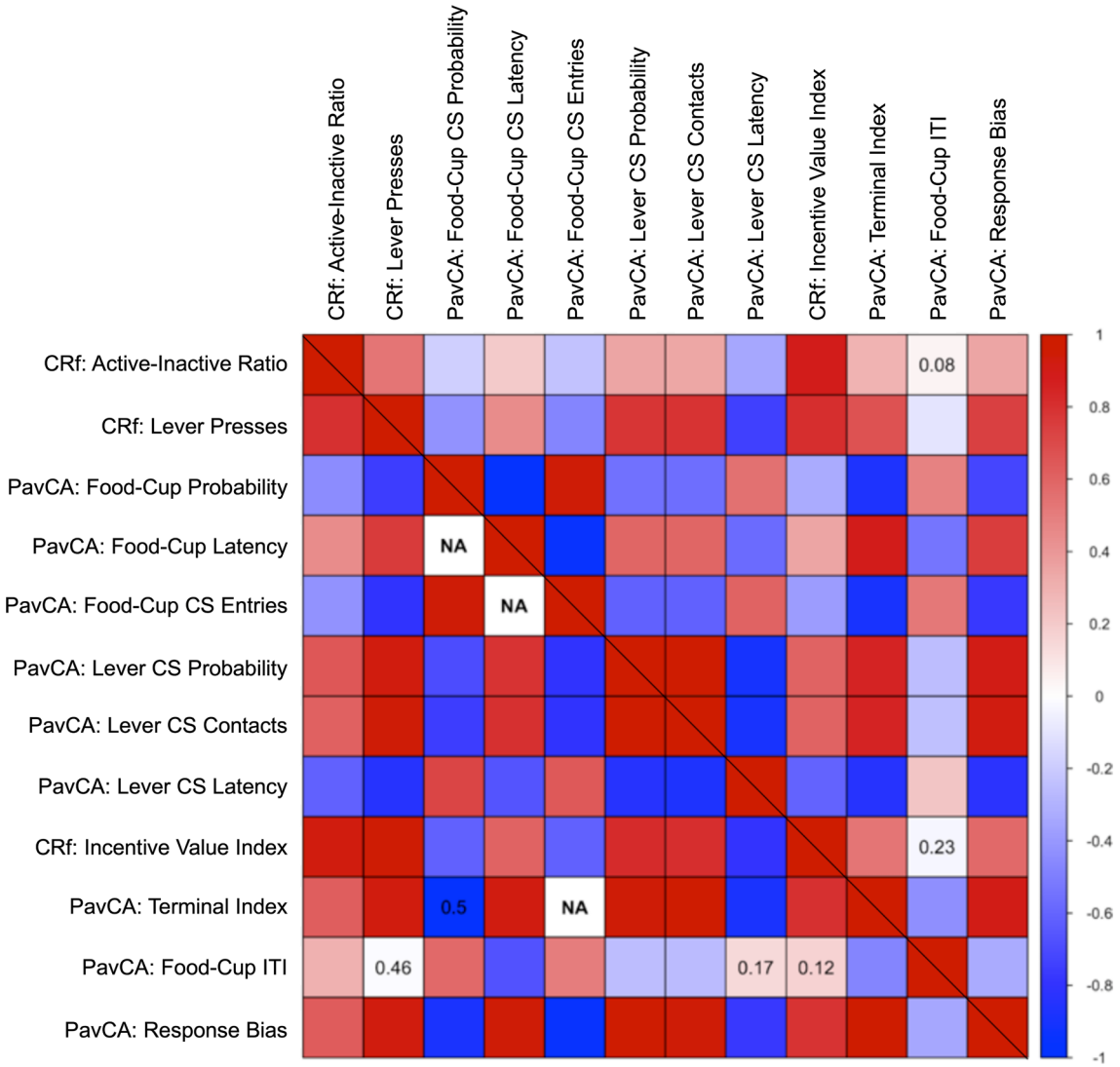


FIGURE 1 | Phenotypic and genetic correlations for key sign- and goal-tracking measures. Phenotypic correlations between day 5 measures are shown in the top-right triangle, and genotypic correlations are shown on the lower-left triangle. Phenotypic and genetic correlations were computed using the Spearman test and bivariate GREML analysis, respectively. Red and blue squares reflect positive and negative correlations, respectively. The strongest genetic correlations were between sign- and goal-tracking measures, with weaker correlations occurring with inter-trial interval food-cup entries. All correlations were significant ($p < 0.05$) except where p-values are numerically indicated. 'NA' values denote genetic correlation pairs that were excluded due to non-invertible variance–covariance matrices, likely reflecting multicollinearity or insufficient variation.

similarly modest values, with the highest relating to traits most directly reflecting lever-directed sign-tracking behavior during CRf (Table 1b). Additionally, on day 1, goal-tracking heritability was higher than sign-tracking (Supporting Information S2). The heritability of sign-tracking increased across sessions, with the highest observed on the terminal Day 5 sessions. All SNP heritability estimates were significantly greater than zero.

3.3 | Identification of Multiple GWAS Hits

We next performed a GWAS to identify specific genetic loci that were significantly associated with the tendency to sign- and goal-track. At least one QTL was identified for 11 of the 12 measures. Some measures were associated with more than one QTL, and some QTLs were associated with more than one trait, such that a total of 6 unique QTLs were identified for the 12 measures (Table 2a). Two of the three CRf QTLs overlapped with PavCA QTLs (Table 2b) suggesting pleiotropy among these theoretically related traits.

The most notable example of pleiotropy was found on chromosome 1 (Table 2a,b) with several loci associated with two or more measures. Three additional loci on chromosomes 4 and 18 were identified for both PavCA and CRf. The number of genes identified in the various QTLs ranged from 2 to 113 (full list of identified QTLs and Manhattan plots reported in Supporting Information S1).

In order to determine whether our measures of incentive salience attribution could be reduced into simpler dimensions, we used Principal Components Analysis. For each of the resulting three components, we conducted a GWAS and found that the first component yielded three QTLs identical to those identified using GWAS for our primary set of measures (Supporting Information S1). None of the other components yielded any significant QTLs, and taken together, they suggest that the different measures used in this GWAS likely similarly cluster as a single component driven by incentive salience.

The chromosomal locations for identified regions of interest are shown below as a porcupine plot (Figure 2). Traits related to sign-tracking showed generally similar patterns, with overlap of the identified loci occurring on chromosomes 1, 4, and 18. These similar results partially reflect the high correlations among measures (Figure 1). Specifically, measures of sign-tracking during PavCA (response bias, lever latency, lever contacts) and CRf (incentive value index, lever presses) overlapped at each of these three regions.

3.4 | Sex Differences

In order to evaluate whether the pattern of QTLs across traits was sex-dependent, we conducted an exploratory analysis on sex differences separately in males and females. We first report the sex-specific p-values for each pooled GWAS QTL using each region's top SNP (Table 2a,b). We identified sex-specific QTLs for 13 measures in males (8) and females (5) (Supporting Information S2). Nine QTLs that were observed in the pooled analysis were also significant in the male-only or female-only GWAS. In addition, we identified 4 QTLs that were unique to the sex-specific GWAS on chromosomes 4 and 5 (Supporting Information S2). The sex-specific GWAS results are available in Supporting Information S1 and as an interactive .html file containing LocusZoom plots at https://doi.org/10.6075/J0MW2HG7 [39]. Significance values comparing each sex to the pooled GWAS results are also available at [39].

3.5 | Candidate Gene Identification

The number of genes within each QTL varied from 2 to 113. We used several criteria to narrow down the list of candidate genes. For regions that contained multiple genes, we examined coding variants predicted to have moderate to high impact on protein function. We also examined genes for which there were heritable expression differences (expressions QTLs; eQTLs; see [45]) in the central nervous system (CNS) or that had functional relevance from the literature (i.e., also identified in human GWAS on psychiatric traits).

Figure 3 shows two representative regional association ("LocusZoom") plots for QTLs on chromosomes 1 and 18. Additional LocusZoom plots are provided in [39]. One QTL that contained four genes was identified for four behavioral measures on chromosome 1 (Figure 3A). Two of these genes, *Tenm4* and *Mir708*, are functionally linked. *Tenm4* expression is regulated by *Mir708* and has been previously identified as a candidate genetic component for psychiatric disorders [46]. A nearby QTL on chromosome 1 contained over 100 genes (some of which are discussed later). The shown QTL identified on chromosome 18 for seven different measures (Figure 3B) contained nine genes (4 shown: *Socs6, Rttn, Cd226, Dok6* plus five others: *Pclaf-ps2*, PCNA clamp associated factor, pseudogene 2; *Chn3*, chimerin 3; *LOC689116*, *Ncbp2*, nuclear cap binding protein subunit 2); *LOC100362807. Snapc5-ps1*, snRNA-activating protein complex subunit 5, pseudogene 1. Two of these genes code for proteins that are involved in functionally regulating tyrosine kinase expression (SOCS6; [47]) and binding to tropomyosin-related kinase receptors, influencing nervous system development (Dok6; [48]). Table 2 provides a numerical summary of each region's gene set size; detailed information can be found in Supporting Information S1 and in [39] with information such as strain distribution patterns (SDPs), LocusZoom plots, and the full list of genes for each interval.

To identify candidate genes in larger gene-rich regions, we looked for coding variants with potentially damaging effects on protein coding. For example, on chromosome 1, a 4.6 MB region was identified with 103 genes. A total of 11 genes with moderate coding variants were identified across the entire set of QTLs (*Usp35, Alg8, Tsku, Serpinh1, Atg16l2, Art2b, LOC102549471, Chrna10, Nup98, Shq1, Numa1*). A subset of these genes (Table 3a) were highly associated ($r^2 > 0.9$) with the observed trait. We examined candidate genes using the PubMed and GWAS catalog mining tool GeneCup [49] to probe for results from previous omics and gene-function studies.

TABLE 2 | QTLs identified for PavCA and CRf. The top SNPs and associated QTLs across the 12 traits examined in this GWAS are shown. QTLs for (a) PavCA and (b) CRf are sorted by the chromosomal location of the top SNP. Regions are arranged by chromosome. $-\log(P)$ are indicated in italics for males and females separately. Significant sex associations are shown in bold (using a genome-wide alpha of <0.05 ($-\log_{10}(p)>5.95$)). Top SNPs during PavCA largely reflected sign-tracking measures with QTLs identified on chromosomes 1, 4, and 18. Top SNPs during CRf also included overlapping regions of chromosomes 1 and 18 for lever-directed behavior. Identified QTLs varied in the number of genes contained in that interval, in some cases reflecting gene rich areas with as many as 113 genes. *Mir708*, microRNA 708; *Tenm4*, teneurin transmembrane protein 4; *Pdzd11-ps1*, PDZ domain containing 11, pseudogene 1; *Rpl23a-ps7*, ribosomal protein L23A, pseudogene 7. All coordinates are based on the Rnor_6.0 assembly of the rat genome.

chr	Trait	Position	topSNP $-\log_{10}P$	Alpha	Male $-\log_{10}P$	Female $-\log_{10}P$	Start (Bp)	Stop (Bp)	Size (Mb)	Allele frequency	Genes in interval
Table 2a: Summary of PavCA QTLs											
1	Lever CS latency	chr1:159701681	8.08	5%	***7.42***	*1.70*	159,695,583	161,481,604	1,786,021	0.83	*Tenm4,Pdzd11-ps1,Mir708,Rpl23a-ps7*
	Lever CS contacts	chr1:160053871	8.40	5%	***7.81***	*2.16*	159,695,583	161,481,604	1,786,021	0.83	*Tenm4,Pdzd11-ps1,Mir708,Rpl23a-ps7*
	Response bias	chr1:160204141	9.22	5%	***7.64***	*2.70*	159,695,583	161,625,992	1,930,409	0.80	*Tenm4,Pdzd11-ps1,Mir708,Rpl23a-ps7*
	Index	chr1:160273674	7.78	5%	***7.08***	*1.73*	159,695,583	161,572,301	1,876,718	0.81	*Tenm4,Pdzd11-ps1,Mir708,Rpl23a-ps7*
	Food-cup entries	chr1:160448253	6.79	5%	*3.33*	*4.32*	160,162,160	162,853,810	2,691,650	0.66	23
	Lever CS probability	chr1:166162182	7.87	5%	*3.53*	*5.22*	162,971,035	167,605,025	4,633,990	0.60	103
	Food-cup probability	chr1:167160047	5.69	10%	*4.34*	*2.06*	166,089,239	167,353,015	1,263,776	0.71	34
4	Response bias	chr4:69793795	6.56	5%	*3.33*	*4.08*	69,665,977	70,194,375	528,398	0.66	*Rpl30-ps10,Rpl4-ps2*
	Lever CS contacts	chr4:69796826	6.59	5%	*3.34*	*4.09*	69,665,977	70,194,375	528,398	0.65	*Rpl30-ps10,Rpl4-ps2*
	Lever CS probability	chr4:69796826	6.41	5%	*3.13*	*4.19*	69,665,977	70,194,375	528,398	0.65	*Rpl30-ps10,Rpl4-ps2*
	Index	chr4:133998243	6.01	5%	*3.41*	*3.27*	132,854,325	135,764,373	2,910,048	0.64	12
15	Intertrial interval (ITI) food-cup entries	chr15:74224438	6.51	5%	*5.05*	*2.90*	72,572,640	75,275,341	2,702,701	0.41	8

(Continues)

TABLE 2 | (Continued)

chr	Trait	Position	topSNP $-\log_{10}P$	Alpha	Male $-\log_{10}P$	Female $-\log_{10}P$	Start (Bp)	Stop (Bp)	Size (Mb)	Allele frequency	Genes in interval
18	Index	chr18:85843693	7.12	5%	*5.34*	*3.02*	85,838,595	86,468,878	630,283	0.93	9
	Food-cup entries	chr18:85843693	6.05	5%	*4.25*	*2.70*	85,838,595	86,468,878	630,283	0.93	9
	Response bias	chr18:85843693	5.90	10%	*5.40*	*2.06*	85,838,595	86,468,878	630,283	0.93	9
	Lever CS latency	chr18:86488158	5.85	10%	*3.77*	*2.33*	85,838,695	86,488,158	649,463	0.91	9
	Lever CS contacts	chr18:86488158	6.25	5%	*4.44*	*2.32*	85,838,695	86,488,158	649,463	0.91	9
	Lever CS probability	chr18:86488158	6.39	5%	*4.54*	*2.41*	85,838,695	86,488,158	649,463	0.91	9
Table 2b: Summary of CRF QTLs											
1	Lever presses	chr1:163339560	9.53	5%	*4.63*	*5.24*	162,477,196	167,605,025	5,127,829	0.56	113
	Incentive value index	chr1:166077595	7.20	5%	*3.72*	*4.24*	162,971,035	167,605,025	4,633,990	0.58	103
2	Active-inactive ratio	chr2:248891968	5.87	10%	*1.42*	*5.72*	248,891,968	248,969,222	77,254	0.29	0
4	Incentive value index	chr4:135061594	6.19	5%	*3.27*	*3.81*	133,337,808	135,764,373	2,426,565	0.35	8
	Lever presses	chr4:135061594	5.99	5%	*3.23*	*3.85*	133,337,808	135,764,373	2,426,565	0.35	8
18	Lever presses	chr18:85854393	5.75	10%	*4.42*	*2.20*	85,838,695	86,488,158	649,463	0.96	9

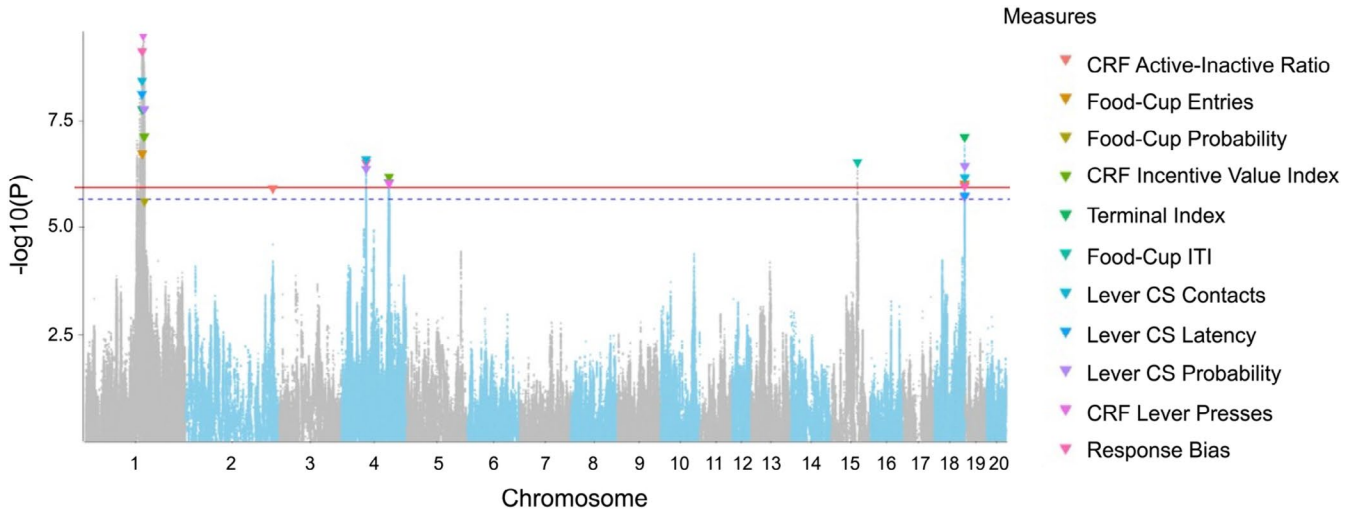


FIGURE 2 | Porcupine plot for selected PavCA and CRf measures. Combined Manhattan plots from the genome-wide association study (GWAS) data for 11 traits with significant SNPs for sign- and goal-tracking. Chromosomal distribution of all p values ($-\log_{10} p$ values) is shown, and top SNPs are indicated by colored triangles. The cutoff for genome-wide alpha of <0.05 ($-\log_{10}(p) > 5.95$) is shown as a solid red line, and alpha <0.10 ($-\log_{10}(p) > 5.67$) is shown as a dotted blue line. p values are indicated in italics for males and females separately. The largest cluster of SNPs was located on chromosome 1. For PavCA sign-tracking traits, more than one top SNP was identified in overlapping regions on chromosomes 1, 4, and 18. CRf lever-directed behavior also showed QTL overlap with similar regions of chromosome 1 and 18, relative to PavCA.

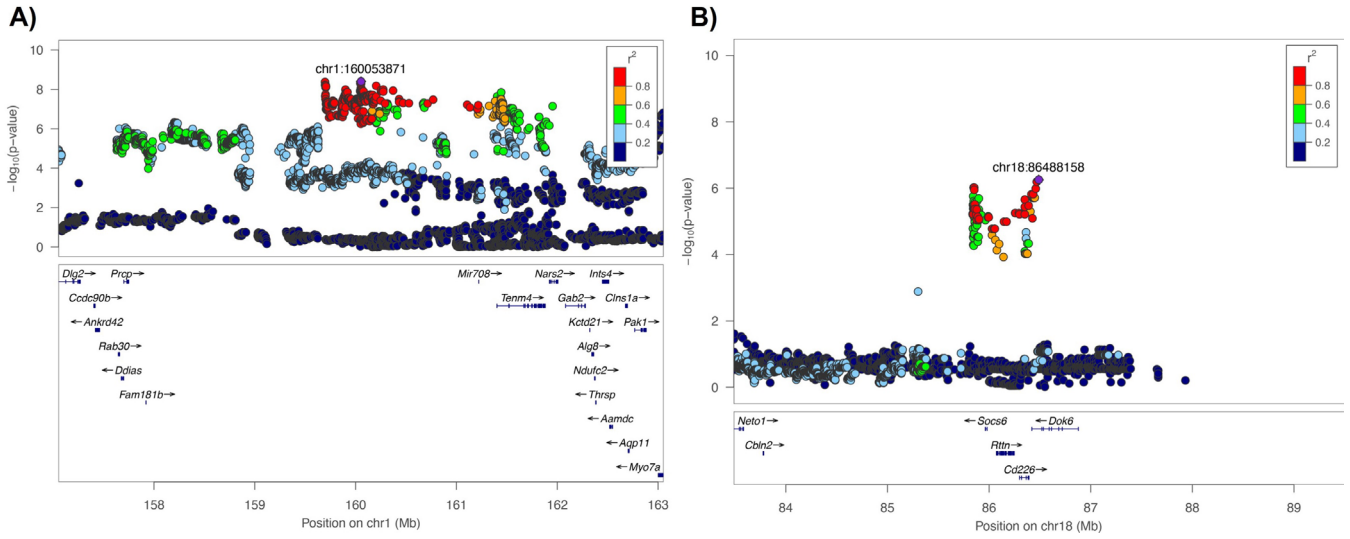


FIGURE 3 | LocusZoom regional association plots of two top QTLs identified on chromosomes 1 and 18. The x-axis shows chromosomal position. The single-nucleotide polymorphism (SNP) with the lowest p-value is shown and labeled in purple ("top-SNP"). Color of dots indicates the degree of linkage disequilibrium (LD) of other SNPs relative to the top-SNP. The bottom half of each panel shows the genes in a particular region as annotated by the reference sequence. Panel (A) shows genes contained in the QTL identified by the top SNP, two of which are named genes with known interactions (*Tenm4, Mir708*). Eight measures were associated with a QTL on chromosome 18 (B), which contains four genes (*Socs6*, suppressor of cytokine signaling 6; *Rttn*, rotatin; *Chn3*, chimerin 3; *Dok6*, docking protein 6).

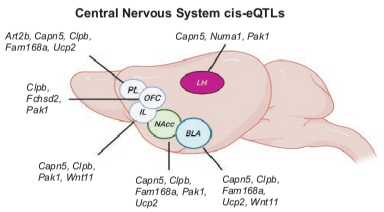
3.6 | eQTLs

In addition to coding polymorphisms, we considered eQTLs, which are loci that confer heritable differences in gene expression. The eQTL data we used are available at www.ratGTEx.org [45]. When a behavioral QTL and an eQTL are located near each other and are in strong linkage disequilibrium, it is possible that the behavioral differences (the behavioral QTL) are caused by the expression differences (the eQTL). In addition to liver, eye, and adipose tissue gene expression, we identified CNS cis-eQTLs in the infralimbic cortex (IL), prelimbic cortex (PL), orbitofrontal cortex (OFC), nucleus accumbens (NAcc), basolateral amygdala (BLA), and lateral habenula (LHb) that were colocalized with the QTLs for PavCA and CRf. Many cis-eQTLs were in strong linkage disequilibrium ($r^2 > 0.9$) with more than one behavioral trait. These brain regions were selected due to their functional relevance across various addiction-related behavioral traits and because a recent study identified numerous eQTLs in these intervals [45]. There were 66 genes with cis-eQTLs identified in this dataset, but for brevity, only the most relevant ones are shown in Table 3c. In addition to eQTLs, we also include splice-QTLs (sQTLs) in which heritable differences

TABLE 3 | Coding variant impact chart and cis-eQTLs for candidate genes. (a) Coding variants with moderate impact were mostly found to be located on chromosome 1, including those influencing sign-tracking traits. All genes contained missense coding substitutions. Traits with coding variants highly associated (r^2) with the top SNP or functional relevance are shown. (b) Eight genes with coding variants also had heritable differences in expression (cis-eQTLs) in various tissues; many based on expression in the liver. (c) Other functionally relevant genes highly correlated with measures of sign-tracking had cis-eQTLs throughout the central nervous system. Summarized cis-eQTLs in the brain are shown in the top right of the figure (created with BioRender.com). *Art2b*, ADP-ribosyltransferase 2b; *Chrna10*, cholinergic receptor, nicotinic, alpha polypeptide 10; *Tsku,* tsukushi, small leucine-rich proteoglycan; *Alg8*, alpha-1,3-glucosyltransferase; *Atg16l2*, autophagy related 16-like 2; *Numa1*, nuclear mitotic apparatus protein 1; *Nup98*, nucleoporin 98 and 96 precursor; *Capn5*, calpain 5; *Clpb*, caseinolytic mitochondrial matrix peptidase chaperone subunit B; *Clpb*, ClpB family mitochondrial disaggregase; *Fchsd2*, FCH and double SH3 domains 2; *Pak1*, p21 (RAC1) activated kinase 1; *Fam168a*, family with sequence similarity 168, member A; *Ucp2*, uncoupling protein 2; *Wnt11*, Wnt family member 1; IL, infralimbic cortex; PL, prelimbic cortex; OFC, orbitofrontal cortex; NAcc, nucleus accumbens; BLA, basolateral amygdala; LH, lateral habenula.

Table 3a: Selected moderate-impact coding missense variants for PavCA and CRF

Gene	QTL	SNP position	Amino acid change	r^2 with top trait topsnp	Trait in top r^2
		chr1: 166670064	*p.Thr29Lys*	0.996	Food-cup probability
		chr1: 166670064	*p.Thr48Lys*	0.996	Food-cup probability
		chr1: 166669539	*p.Arg204His*	0.952	Lever CS probability
		chr1: 166669539	*p.Arg223His*	0.952	lever CS probability
		chr1: 166667732	*p.Ala250Thr*	0.951	Lever CS probability
Art2b	chr1	*chr1: 166667732*	*p.Ala269Thr*	0.951	Lever CS probability
		chr1: 166669842	*p.Ala103Gly*	0.948	Lever CS probability
		chr1: 166669842	*p.Ala122Gly*	0.948	Lever CS probability
		chr1: 166669908	*p.Arg100Met*	0.948	Lever CS probability
		chr1: 166669908	*p.Arg81Met*	0.948	Lever CS probability
		chr1: 166670639	*p.Leu12His*	0.948	Lever CS probability
Chrna10	chr1	*chr1: 167208630*	*p.Arg235His*	1.000	Food-cup probability
Tsku	chr1	*chr1: 163319672*	*p.Thr14Ile*	0.999	Lever CS contacts



Gene	Trait with top r^2	# of traits	Location	Trait top SNP	eQTL top SNP	pval_nominal_ threshold	r^2 with trait topsnp
Table 3b: Brain and liver cis-eQTLs containing coding variants							
Atg16l2	Terminal index	3	Adipose	chr1:167184389	chr1:167157386	0.005	**1.000**
Art2b	Terminal index	3	PL	chr1:167184389	chr1:166995654	0.002	**0.991**
Numa1	Lever CS latency	3	LHb, Liver	chr1:166576706	chr1:166624073	0.004	**0.996**
Nup98	Lever CS probability	7	Liver	chr1:166162182	chr1:166634347	0.004	**0.951**
Alg8	Lever CS probability	1	Liver, Adipose	chr1:163564216	chr1:163224414	0.004	**0.914**

(Continues)

TABLE 3 | (Continued)

Gene	Trait with top r^2	# of traits	Location	Trait top SNP	eQTL top SNP	pval_nominal_ threshold	r^2 with trait topsnp
Table 3c: Selected central nervous system cis-eQTLs with functional relevance							
Capn5	CRF lever presses	8	NAcc, IL, PL, BLA, LHb, Liver	chr1:163339560	chr1:163381418	0.001	**1.000**
Clpb	Food-cup probability	8	PL, PL2, BLA, OFC, IL, NAcc, Adipose	chr1:167160047	chr1:167156903	0.001	**1.000**
Fchsd2	CRF incentive value index	7	OFC	chr1:166077595	chr1:166019073	0.002	**0.998**
Pak1	CRF lever presses	8	OFC, IL, NAcc, LHb, Liver	chr1:163339560	chr1:163038579	0.002	**0.997**
Ucp2	Lever CS contacts	7	PL2, NAcc, BLA, Eye, Liver	chr1:166077595	chr1:166021653	0.000	**0.997**
Fam168a	Lever CS contacts	7	NAcc2, BLA, PL	chr1:166077595	chr1:165861976	0.002	**0.995**
Wnt11	Terminal index	7	IL, BLA	chr1:163214915	chr1:163761081	0.001	**0.900**

in transcript isoforms are associated with QTL (contained in Supporting Information S1).

Some genes with coding variants (Table 3a) may be of greater interest to our behavioral traits because they are expressed in the CNS, such as *Tsku* ([50]; discussed below). Each of the genes with coding variants, including *Art2b, Tsku*, and the peripheral nicotinic receptor gene *Chrna10* [51] is also notable given their high association with the top-SNP in that QTL, which is consistent with them being the putatively causal SNPs. *Alg8*, which has been previously reported in anomics study on depression in smokers [52], contained multiple coding variant SNP as well as a cis-eQTL in the liver (Table 3b). The complete list of genes with both a coding variant and a cis-eQTL is shown in Table 3b. Among these, two are involved in cellular regulation mechanisms, including *Nup98* [53] and *Atg16l2* [54]. Five coding variant genes reflected cis-eQTLs identified in the liver.

Many genes with cis-eQTLs did not contain coding variants, although for many of these eQTLs, the top eQTL SNP was in high linkage disequilibrium (LD as measured by r^2) with the SNP most strongly implicated in the PavCA or CRf behaviors (Table 3c). For brevity, only those eQTLs with an r^2 above 0.9 are shown (the full set of eQTLs is available in the Supporting Information S1). However, a set of 7 genes (*Capn5, Clpb, Fchsd2, Pak1, Fam168a, Ucp2, Wnt11*) was identified in multiple brain areas across multiple indices of sign- and goal-tracking traits. This set of genes serves a variety of functions (discussed below). Additionally, *Tenm4*, one of the few genes with better-characterized psychiatric relevance [46], was identified as a differentially expressed eQTL in the prelimbic cortex RatGTEx, although there were no cis-eQTLs identified in this GWAS.

3.7 | Phenome-Wide Association Study (PheWAS) Analysis

In GWAS, many individual SNPs are tested for their association with a single phenotype, whereas PheWAS tests the association of a single SNP with many traits [55], which is referred to as the "phenome" [56]. This approach is useful because PheWAS can identify SNPs that influence many traits (pleiotropy; [57]).

We examined whether the genetic loci associated with the attribution of incentive salience were also associated with drug conditioning and other addiction-related behaviors collected in HS rats (public data available from this project at genenetwork.org). Here, each 3 MB window surrounding a top SNP for each identified QTL was tested for its association with a separate set of behavioral traits collected at each of three HS rat testing centers (University at Buffalo, University of Michigan, University of Tennessee Health Science Center) (Table 4). These traits included socially acquired nicotine self-administration [58], PavCA in a separate cohort of HS rats [28], sequential patch depletion [35, 59], locomotor response to novelty, and reaction time [35]. Table 4 shows key measures for each behavioral task, selected based on the relevance to the trait being measured. A full list of PheWAS results for each task are available in the Supporting Information S1.

TABLE 4 | Phenome-Wide Association with drug conditioning and addiction related traits in HS rats. PheWAS tables show the association between addiction-related traits in other tests ("PheWAS trait") and measures from tests reported here in the GWAS for PavCA ("PavCA trait in top r^2"). The chromosomal location for the "PheWAS trait topSNP" is shown in association with the location of the "PavCA Trait topSNP," and the strength of the association is reported as r^2. In many cases, more than one PavCA QTL yielded a PheWAS association. The total number of PavCA measures in each QTL, and which PavCA categories those measures reflect, are shown in the rightward columns. (a) PheWAS results are shown for two tasks measuring drug response to nicotine and cocaine. (b) A separate cohort of rats that underwent an identical PavCA procedure at the University of Michigan were tested for PheWAS with the same measures for incentive salience described in this GWAS. (c) PheWAS results for multiple measures of behavioral regulation collected at the Clinical and Research Institute on Addictions (Buffalo, NY).

PheWAS trait	PheWAS trait topSNP	PavCA trait in top r^2	PavCA trait topSNP	r^2	$-\log_{10}P$	dprime	# Measures	PavCA behavioral categories
Table 4a: Between-center PheWAS traits (drug response)								
Nicotine self-administration								
Acquisition of nicotine self-administration (Day 1–3 responses)	chr1:160071477	Lever CS contacts	chr1:160053871	1	4.052	1	5	ST, overall, GT
Acquisition of nicotine self-administration (Day 1 responses)	chr1:160845434	Food-cup entries	chr1:160448253	0.844	4.295	0.993	6	GT, ST, CRF, overall
Table 4b: Between-center PheWAS traits (PavCA and CRF)								
Pavlovian conditioned approach (Michigan)								
Terminal food-cup entries (GT)	chr18:85843691	Food-cup entries	chr18:85843693	0.943	4.229	1	7	GT, overall, CRF, ST
Terminal food-cup probability (GT)	chr18:85843691	Food-cup entries	chr18:85843693	0.943	4.278	1	7	GT, overall, CRF, ST
Conditioned reinforcement (Michigan)								
Incentive value index (CRF)	chr18:86146075	Lever CS contacts	chr18:86488158	1	6.666	1	7	ST, GT, CRF
Lever presses (CRF)	chr18:85859393	CRF lever presses	chr18:85854393	1	7.491	1	7	ST, GT, CRF, overall
Table 4c: Within-center PheWAS traits (Behavioral regulation)								
Patch-depletion foraging test								
Water consumption rate (12 s delay)	chr4:135452263	CRF incentive value index	chr4:135061594	0.962	4.493	0.988	3	CRF, overall
Locomotor response to novelty								
Response to novelty (Rearing)	chr18:86363830	Food-cup entries	chr18:85843693	0.752	5.954	0.995	7	GT, ST, CRF, overall
Reaction time task								
Change in premature responses per opportunity across time	chr4:69757343	response bias	chr4:69793795	0.757	4.115	0.986	3	Overall, ST

We found that loci associated with the attribution of incentive salience overlapped with those associated with measures of drug response (Table 4a). As part of this process, we used unpublished data from a socially acquired adolescent nicotine self-administration protocol as described previously [58] where rats engaged in operant licking for infusions of nicotine. We examined two major features of nicotine-directed behavior: the acquisition of nicotine self-administration on day 1 and accross days 1–3, and reinstatement to nicotine seeking following extinction (terminal cue responses) as a measure of relapse. The strongest association ($r^2 > 0.84$) was identified for initial responding for nicotine on chromosome 1. By comparison, the association with reinstatement to nicotine seeking on chromosome 1 (non-shown) was quite weak (r^2: 0.29–0.39).

In addition to drug response, we used PheWAS to test the association between measures of sign- and goal-tracking with a separate (currently unpublished) cohort of HS rats that underwent an identical PavCA and CRf procedure at the University of Michigan [28] (Table 4b). PheWAS yielded strong associations (r^2: 0.73–0.93) for measures of goal-tracking during PavCA in the Michigan cohort, identifying overlapping regions on chromosomes 2 and 18. The association with measures of sign-tracking during CRf was particularly strong ($r^2 = 1$) on chromosome 18, suggesting that PheWAS can validate chromosomal regions using independently phenotyped cohorts.

Finally, PheWAS was used to determine if genetic loci identified for PavCA overlapped with other (currently unpublished) measures of behavioral regulation (Table 4c) [35]. The QTL on chromosome 18, which was associated with food-cup CS entries, also influenced the locomotor (rearing) response to novelty ($r^2 = 0.75$). However, more complex behaviors, including foraging and impulse control, yielded PheWAS associations that varied widely depending on the specific task and measure. For example, during a patch-depletion foraging test [59], water-depleted rats consumed water in one of two "patches" in which the amount of water available at a particular patch depletes over time. Switching patches is an adaptive response to patch depletion that varies between subjects [60], but patch switching results in one of several experimenter-imposed delays. The rate of patch switching and consumption can therefore be used as a measure of foraging under different conditions. The Incentive value index QTL on chromosome 4 was also associated with the rate at which rats maximized water consumption when the experimenter-imposed delay was high (12 s) ($r^2 = 0.96$). There were no associations when the imposed delay was shorter (not shown). This PheWAS association is therefore dependent on task performance, specifically when the task is made most difficult by imposing a longer patch switching delay (12 s).

4 | Discussion

This study is the first to use a large population of HS rats to identify genetic loci associated with the tendency to attribute incentive salience to reward cues, as measured by sign-tracking and CRf. Measures of sign-tracking were moderately heritable and strongly phenotypically and genetically correlated, suggesting common loci underlying individual variability in these measures. Among the GWAS loci identified, there were multiple candidate genes, including previously identified SUD genes, as well as genes not previously associated with SUD. Both coding variation and eQTLs offer possible molecular mechanisms for these QTLs. Further, the identified chromosomal regions were significantly associated with other behavioral traits, including nicotine self-administration, underscoring the importance of incentive salience for understanding substance-abuse traits. These results also demonstrate the utility of HS rats for the genetic mapping of complex behavioral traits. Some of the candidate genes identified are particularly promising targets with known functional or psychiatric relevance.

HS rats are valulable for genetic mapping of small regions; in some cases, these regions contain a small number of genes. However, some loci were gene-rich regions, making it more challenging to identify the underlying candidate genes more difficult. Thus, we examined candidate genes in these QTLs by using several strategies: (1) Identifying genes with coding variants that are predicted to have moderate or large impacts on protein function, (2) identifying genes with corresponding eQTLs in relevant brain regions, and (3) identifying genes previously associated with psychiatric functions in other -omics studies, particularly human GWASs. Thus, we highlight and discuss several of these genes in addition to presenting the report containing the full dataset of genes.

4.1 | Sex Differences

We have previously shown that females exhibit greater incentive salience attribution than males [29], and here present data that suggest there is both a shared genetic basis between sexes, as well as sex-specific QTLs. For example, unique sex-specific QTLs were identified for three measures in females (chr4:21846682) and one measure in males (chr5:107716241). Interestingly, although QTLs were identified on chromosome 1 in both males and females separately, the region containing Tenm4 (chr1:159919116) was strongly significant in males and not in females (Table 2a,b). This is an important example of how the genetic basis of behavior may be sex-dependent. Larger population sizes will be necessary to accurately determine these differences with sufficient power. Ongoing investigations from our group and others will be crucial for understanding the differential genetic basis of addiction vulnerability between sexes, leveraging large sample sizes.

4.2 | Tenm4 and Mir708

One especially promising gene candidate within a chromosome 1 QTL is *Tenm4*. Teneurins are surface-bound transmembrane glycoproteins conserved across species [61, 62] and are located in synapses with multiple functions, including cell adhesion [63]. *Tenm4*, which is expressed in the CNS [64], is involved in functions such as axon guidance [65] and is associated with disorders such as schizophrenia [66]. Teneurin-4 interacts with proteins involved in postsynaptic density function, which may be related to the pleiotropic effects of *Tenm4* in multiple psychiatric disorders [46].

Interestingly, the teneurins are well situated to affect complex behavior through regulation of corticotropin-releasing hormone (CRH)-mediated stress effects [67–69] via cleavable teneurin C-terminal associated signaling peptides (TCAPs) [61, 62, 70] which work as extracellular soluble signaling proteins. The TCAPs (1–4) correspond to the teneurin 1–4 genes, and as such, TCAP-4 is an interesting signaling peptide for future functional studies. In one earlier study, TCAP-1, which produces an anxiolytic effect [71], reduced stress-induced reinstatement to cocaine seeking behavior [72]. Although no behavioral studies have examined the role of TCAP-4, our data suggest that it may be a promising target for future research.

Tenm4 is located in the same QTL as *Mir708*. In humans, *MIR708* is a microRNA contained in an intron of the protein-coding gene, or mirtron, [73] for *TENM4*. Similar to *Tenm4*, *Mir708* is expressed in the brain and is differentially expressed across mesocorticolimbic circuitry in mice [74]. Previous work suggests that dopamine and subcortical neural circuits are important in the attribution of incentive salience [75–77], and therefore, these two genes may work in concert to regulate forebrain function.

4.3 | Genes With Coding Polymorphisms

Several genes within QTLs contained coding variants that were predicted to have significant impacts on gene function. Many of these genes are expressed in the brain, which is consistent with them having a role in complex behavioral traits such as cue-responsivity. For example, *Tsku* is a member of the small leucine-rich proteoglycans (SLRPs) family [78] and has established functions in the CNS where it is crucial for commissure development [50] and has recently been characterized for roles in hippocampal neuronal development [79]. Other genes have been previously identified in human -omics studies. *ALG8*, for example, has been identified in human GWAS for estimated incidence of depression in smokers based on the HADS depression subscale and antidepressant use [52]. *Chrna10* is another notable candidate, which codes for the nicotinic receptor α10 subunit and is expressed in the ear [80, 81] and peripheral sympathetic nervous system [51]. *CHRNA10* has been previously associated with the subjective response to nicotine [82] and nicotine dependence [83].

Other genes with coding polymorphisms and cis-eQTLs are novel candidate targets, such as *Serpinh1, Atg16l2, Art2b*, and *Nup98*. We are not aware of any prior evidence implicating them in behavioral or psychiatric traits, suggesting that they could represent the discovery of novel targets. Two of these genes are involved in cellular regulation. For example, *Nup98* regulates the transport of proteins into the cell nucleus [53] and *Atg16l2* regulates cellular autophagy [54]. Among the coding variant-containing genes, *Tsku*, *Chrna10*, and *Alg8*, which have been previously implicated in CNS function, development, and psychiatric relevance, are compelling targets for their roles in complex behavior.

4.4 | Expression-QTL Genes

Using cis-eQTL analysis, we identified a group of eight genes that were differentially expressed in regions of the brain, some of which have yet to be reported in the literature in relation to behavioral and nervous system function. To further investigate genes with eQTLs that colocalized with behavioral QTLs, we used GeneCup [49] to retrieve psychiatrically and mechanistically relevant pre-existing literature. Genes with coding variants that also had cis-eQTLs were largely identified in the liver and adipose tissue, despite many of these genes being expressed in the brain (e.g., *Art2b, Nup98, Alg8*) (Table 3b). The tissue sample size was larger for liver and adipose tissues ($n = 411$) [84] which likely heightened the detection threshold for these genes. Here, we focus on genes with cis-eQTLs in the brain.

Capn5 is an attractive candidate gene given its strong association with multiple PavCA traits and five cis-eQTLs throughout the forebrain (NAcc, IL, PL, BLA, LHb). Experimental data suggest that *Capn5* may be broadly involved in CNS function and expressed throughout the brain, including granule cells of the hippocampus, cerebellum [85], and piriform cortex [86] where it functions enzymatically as a calcium-dependent protease [87]. Calpains have been implicated in neurodegenerative disorders [88, 89] but *Capn5* does not have well-characterized pathology-related mechanisms in the nervous system outside of the retina [90]. The potential of these genes as genetic targets for the regulation of behavior and cue-responsiveness will benefit from additional experimental and -omics studies that examine functional and behavioral relevance to the various domains of psychological function.

Pak1 is also notable for its strong association with four PavCA measures and four cis-eQTLs throughout the brain. Pak1 (p21 RAC1-activated kinase 1) is a kinase active in the CNS and an effector of the family of Rac1 and Cdc42 GTPases, regulating neuronal morphology and synapses [91], axon migration and synaptic plasticity [92], and dendrite initiation [93]. Further, PAK1 has been implicated in neuropsychiatric disorders including schizophrenia [94, 95] depression [96], and neurodegenerative diseases [97]. It is likely that *Pak1* is pleiotropic and broadly regulates complex behaviors.

Ucp2 is another candidate influencing the response to food cues, given its expression in the NAcc and PL, strong association with three measures of PavCA, and previously established roles in diet and food response. *Ucp2* (Uncoupling protein 2) codes for an uncoupling protein in the mitochondria that reduces ATP production, with a role in energy balance [98]. *UCP2* is polymorphic in humans, which results in differential mRNA expression and association with obesity risk [99]. In the CNS, UCP2 negatively regulates glucose-sensing in melanin-concentrating hormone-expressing neurons in the lateral hypothalamus [100], a key region in the regulation of appetite. Ghrelin-induced activation of neuropeptide Y and agouti-related peptide neurons in the arcuate nucleus, another key pathway in the instigation of feeding behavior, is dependent on UCP2 [101]. This suggests that genetic variants involved in energy regulation and appetite may extend to alter individual differences in responses to cues associated with food delivery. UCP2 is likely involved in other processes as well, including the regulation of anxiety-like behavior in mice [102]. Two regions in which *Ucp2* is differentially expressed, the PL and NAcc, are both functionally relevant for sign-tracking. Both regions are engaged in sign-trackers

following the presentation of an incentive cue [77] specifically activating glutamatergic signaling during sign-tracking [103]. The differential expression of *Ucp2* in these regions raises the possibility that heritable differences in the anticipatory response to palatable food are regulated by this gene system in critical circuits for incentive salience attribution.

We found a cis-eQTL for *Wnt11* expression in the IL and BLA that colocalized with three behavioral QTLs, including lever presses during CRf. *Wnt11* has been previously implicated in acetylcholine and nicotinic receptor function. *Wnt* signaling is involved in nervous system development and may be involved in major psychiatric diseases such as schizophrenia and bipolar disorder [104]. Interestingly, although *Wnt11* expression has been shown to enhance acetylcholine nicotinic receptor clustering in neuromuscular junctions [105] it has not been identified in the CNS, so its relevance to the behaviors we examined is uncertain. Forebrain acetylcholine plays a role in sign- and goal-tracking [106], in that attentional top-down deficits in sign-trackers relative to goal-trackers appear to reflect attenuated cholinergic functioning in the basal forebrain [106, 107] involving choline transporter systems [108]. Further, work from our lab and others demonstrates that nicotinic receptor agonism facilitates sign-tracking [3, 109–111], raising the intriguing possibility that central *Wnt11* may be involved in regulating the sign-tracking phenotype via CNS acetylcholine modulation. Interestingly, *Tenm4* loss of function in mice further impairs Wnt protein signaling [112] suggesting that these candidate genes we identified in our GWAS may interact to affect CNS function and behavior.

Finally, other cis-eQTLs identified genes in the CNS such as *Fchsd2* and *Fam168a* that were in high linkage disequilibrium with multiple other traits. However, the function of these genes is limited by the lack of experimental behavioral studies using preclinical models examining function. *Fchsd2* and *Fam168a* have been identified as possible loci in human GWAS for nicotine dependence (Fchsd2; [113]) and smoking initiation (Fam168a; [114]). The lack of attention to these genes may make them attractive novel candidates for their role in complex behavior.

4.5 | Phenome-Wide Associations for Drug Response and Behavioral Regulation

We conducted a PheWAS to determine whether the genetic loci associated with the attribution of incentive salience in our study were also associated with other behavioral traits collected in other HS rat cohorts. Strikingly, a region identified on chromosome 1 (160 Mb) was strongly associated with the acquisition of nicotine self-administration on the initial day of drug-taking ($r^2 = 0.84$) and the initial three sessions ($r^2 = 1$) [58], suggesting that genetic loci on chromosome 1 are pleiotropic in that they may be involved in both incentive salience and the initial response to nicotine. Ongoing studies will be important for determining which loci are causal for specific behaviors. We have previously shown that sign-trackers show heightened cue-induced reinstatement to nicotine-seeking in Sprague–Dawley rats [3], although the association on chromosome 1 with measures of reinstatement to nicotine-seeking was modest (not shown; r^2: 0.29–0.39). Although there are likely many loci underlying the relationship between these two traits, these data suggest that this region of chromosome 1 contains variants related to nicotine response. It is notable that several of the genes identified here have been previously associated with features of smoking dependence in humans. We are currently conducting a separate GWAS in these HS rats for the genetic basis of socially acquired adolescent nicotine self-administration, and future data will better identify the overlapping regions influencing nicotine self-administration and incentive salience attribution.

In addition to nicotine self-administration, we conducted PheWAS on a separate cocaine contextual conditioning task, where rats receive repeated injections of cocaine in a designated cocaine "context" [28]. In contrast to nicotine, we found no associations with measures of cocaine sensitization (locomotor activation and head-waving). We have shown previously that sign- and goal-trackers do not differ in locomotor response to a modest (10 mg/kg i.p.) dose of cocaine, although sign-trackers show heightened unconditioned ultrasonic vocalizations (USVs) [115]. It is therefore perhaps not surprising that PheWAS yielded associations with nicotine response relative to cocaine, suggesting a different genetic relationship between sign-tracking and these two drug categories. However, cocaine self-administration involves processes other than locomotor sensitization. For example, sign-trackers are more sensitive to the presence of cocaine cues during drug taking [2] and the motivational properties of cocaine [24]. We are currently testing a cohort of HS rats under several models of cocaine self-administration, including intermittent access [116, 117] and long-access self-administration [118], and ongoing work will determine whether the shared genetic basis of sign-tracking with cocaine responses depends on the model of cocaine conditioning.

Genetic loci identified for incentive salience attribution should theoretically be similar among separate cohorts of HS rats, even if the cohorts were tested at different locations, ages, and had different histories of behavioral testing. To test this, we used PheWAS to determine whether significant loci identified in our cohort at the University at Buffalo for PavCA would be associated with PavCA in another large cohort phenotyped at the University of Michigan ($n = 1583$). The genetic correlations between both the Buffalo and Michigan cohorts were high (not shown). Notably, we found that a region reflected by a QTL on chromosome 18 (chr18: 85843691) that strongly influenced measures of goal-tracking and CRf (r^2: 0.943–1) at both locations, although surprisingly, there were no PheWAS findings from the Michigan dataset for the chromosome 1 locus. GWAS and PheWAS are therefore useful together for identifying loci across separate testing cohorts.

4.6 | Limitations and Future Directions

This study has several limitations. One caveat is that we have presented limited sex-specific GWAS results due to power constraints. The tendency to sign-track is higher in females, a pattern that we also observed in this cohort of rats [29] and others [28, 119, 120]. To address this, we separately quantile normalized males and females before pooling them, which allows us to avoid mean differences in tendency to sign- or goal-track across the different measures. One of the major limitations of this study is the insufficient sample size to thoroughly examine the genetic

basis of sex differences. We plan to address this by conducting a meta-analysis in an additional cohort of HS rats and a large cohort of Sprague–Dawley rats. This will expand our target PavCA population in the future and will allow us to probe for sex-specific QTLs and gene-sex interactions.

A second limitation of this study is that the HS rats undergoing PavCA were not behaviorally naïve but had instead undergone a battery of behavioral regulation tests. As a result, the effect of certain genes on complex behavior may be influenced by age and testing history. To address differences in ages, we examined our major traits of interest using age at the start of testing as a continuous predictor for our primary measures [29]. There were some significant main effects or interactions with age at the start of testing for several of these measures (food cup entries, entry probability, food cup latency, PavCA index, earned reinforcers during CRf) but the effect sizes were minimal ($\eta 2 < 0.005$). To address this, we are examining an additional cohort of HS rats tested at the University of Michigan that underwent PavCA prior to any behavioral testing; those analyses will be included in a future publication. Genetic correlations (r_g) between the Buffalo and Michigan cohorts are strong (> 0.9) for terminal measures of PavCA, and by pooling these data, we will be able to disentangle testing- and age-related genetic effects.

The genetic basis underlying distinct phases of learning across time may be an important consideration underlying complex behavior. In this study, heritability of measures changes across sessions, and goal-tracking likely yields the stronger heritability in the initial session of the task when all subjects must first learn the lever-CS and food reward association. The heritability of sign-tracking becomes stronger following the initial associative learning and emergence of the lever-directed behavior, which likely coincides with multiple neurobiological correlates, including dopaminergic activation [21] and ventral pallidum signaling [121]. Continuing work into the genetics underlying the basis of learning over time will help characterize these features of incentive salience learning.

These data provide a framework for identifying causal genetic loci and complex gene-network interactions underlying behavior. For example, future research will experimentally assess the gene-behavior relationship using functional manipulation studies. We are developing a pipeline to manipulate the expression of candidate genes (e.g., *Tenm4*) using CRISPR-mediated modulation of gene expression and other molecular biology methods. We are also using our GWAS data to develop polygenic risk scores that incorporate many SNPs and have been successful in predicting cue-reactivity in HS rats. This technique, which we call RATTACA [122], is a more comprehensive model of genetic risk and enables the study of gene networks and biological pathways underlying cue-reactivity. Finally, future translational work will use network-based approaches to intergrate our GWAS data with human studies to identify common biological networks that underlie SUD-relevant traits across species [123].

5 | Conclusion

This study, using a large population of HS rats, identified multiple genes and loci associated with the attribution of incentive salience to reward cues. Many of these genes are expressed in the CNS or have prior associations with psychiatric GWASs, making them strong candidates for experimental follow-up. Unlike traditional GWAS and -omics studies focused on neuropsychiatric disorders, these loci may influence behavioral endophenotypes along a normal continuum of functioning in HS rats. This work supports the use of HS rats for mapping of complex traits and provides candidate genes for additional studies on behavioral regulation.

Dual Publication Statement: In addition, the HS rats analyzed in this submission from this University at Buffalo cohort were also part of a separate publication [29]. In this previous publication, rats were behaviorally characterized during Pavlovian Conditioned Approach and then compared with two measures of cocaine sensitivity. None of the behavioral data from that publication appear directly in this submission; instead, this behavioral data is used as the basis for the Genome-Wide Association Study presented here. The primary findings, results, and conclusions presented in this paper address a different scientific question than those presented in [29]. An earlier iteration of this manuscript is available as a preprint for this online at BioRxiv.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The genotype data are available in the UC San Diego Digital Library Collection under the following citation: Palmer, Abraham A. (2023). "Heterogeneous Stock (HS) Rat Genotypes, Version 1. In Genotype Data from: NIDA Center for GWAS in Outbred Rats." UC San Diego Library Digital Collections at https://doi.org/10.6075/J0028RR4.

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Supporting Information

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