Genome-wide association study reveals multiple loci for analgesia and heroin taking behavior in outbred rats

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Abstract

The increased prevalence of opioid use disorder (OUD) world-wide has made it imperative to disentangle the biological mechanisms contributing to OUD propensity. OUD shows strong heritability, however genetic variants contributing toward vulnerability remain under explored. In the current study, genome-wide association (GWAS) analysis on over 850 male and female heterogeneous stock rats were performed to identify genes underlying heroin-taking, refraining and seeking behaviors. By using an animal model of OUD, we were able to identify genetic variants associated with distinct behaviors contributing to OUD, an experimental design not easily achieved in human GWAS analysis, and not performed to date for OUD GWAS studies. Our findings show, akin to the human population, several heritable traits related with OUD. We also identified genetic variants associated with heroin consumption, escalation of intake, motivation to obtain heroin, and basal levels of analgesic threshold prior to heroin experience, which was behaviorally unrelated to the heroin behaviors. Several of the genes identified are known biomarkers of other substance use disorders and mediators of neurobiological mechanisms underlying addiction-related behaviors. These findings emphasize the need to assess the role of these genetic variants in OUD, thereby augmenting our understanding of risk factors and neurobiological processes associated with OUD vulnerability.

Introduction

Opioid use disorder (OUD) is characterized by compulsive opioid use and relapse propensity, despite efforts to maintain abstinence. OUD diagnosis has increased substantially in the past decade[1], affecting approximately 16 million people annually in the world[2]. This rise in diagnosis is not constrained to one demographic[1], but is prevalent in individuals across socio-economic class, age and sex[3], making it imperative to better understand the biological mechanisms contributing to OUD vulnerability. Despite the behavioral and diagnostic heterogeneity present in the human population for a substance use disorder (SUD) diagnosis[4, 5], OUD shows a high level of heritability (~50%)[6, 7], suggesting genetic variants contribute to OUD propensity.

Genome-wide association (GWAS) studies are a useful tool for identifying genetic variants present in a population affected with a disorder. GWAS analyses for OUD, though limited, have yielded valuable insight into genetic contributions to diagnostic vulnerability[8-11]. However, human GWAS studies are limited in assessing the relationship between specific behavioral traits that contribute to a diagnosis and genetic interactions. SUD diagnosis is composed of drug-taking, refraining (i.e., withholding from seeking) and seeking (e.g., relapse) behaviors. Understandably, due to a lack of empirical data from study participants, human GWAS studies have largely focused on the terminal end point of a diagnosis for analysis purposes. Using rodent models of addiction, we are not constrained with this caveat, and can quantify specific traits associated with addiction-related behaviors to directly assess the relationship between these metrics and genetic variance. By doing so, we are able to offer a feed-forward approach to better understand genetic variants that may contribute to the different phases of SUD to then be assessed in the human population. This allows for the possible development of individualized OUD treatment options based on the most prevalent behavior traits contributing toward a SUD diagnosis.

In the current study, we assessed several measures of heroin-taking, refraining and seeking behaviors in outbred male and female heterogeneous stock (HS) rats. Additionally, anxiety- and stress-like behaviors, as well as heat-induced analgesia, were assessed prior to and following heroin experience. HS rats are a diverse rat line founded by NIH to capture genetic and behavioral heterogeneity reflective of the human population[12-14]. Compared to inbred rat lines, HS rats exhibit comparatively greater diversity[14], and have been used in several studies assessing addiction-related behaviors[15-19], including those focusing on genetic analyses[20-22], furthering the translational validity of our findings. In this study, we found genetic variants specific to different OUD behavioral traits, many of which have known involvement in neurobiological processes associated with SUD.

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and the Italian Ministry of Health. All testing procedures were compliant with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of Laboratory Animals Care, as well as the European Community Council Directive for Care and Use of Laboratory Animals.

Subjects

Male and female heterogeneous stock rats (HS; NMcwiWFsm:HS, Rat Genome Database number 13673907) bred at Wake Forest University were shipped to the Medical University of South Carolina (MUSC, USA) and the University of Camerino (UCAM, Italy) in batches of 40 (20 females and 20 males/site) at 5 weeks of age. Upon arrival, rats were left undisturbed for 3 weeks in a climate-controlled vivarium room with a standard 12-hr light/dark cycle prior to testing. Rats were pair-housed and received ad libitum access to food and water throughout the duration of testing. Behavioral tests for stress, anxiety and analgesic threshold occurred during the light cycle, whereas all heroin-associated testing occurred during

the dark phase. A total of 1160 animals underwent testing, with the following animals excluded from all analyses: 147 for non-heroin self-administration procedures (sucrose, n=36; saline, n=111), 107 due to death (post-surgical illness, n=84; surgery, n=23); 29 for behavioral data collection technical issues, and 3 for spleen identification issues. Final behavioral and genetic analyses were conducted on 874 rats (MUSC, n=479; UCAM, n=395).

Drugs

Heroin hydrochloride supplied by the National Institute on Drug Abuse (Bethesda, MD) was dissolved in 0.9% sterile saline.

Behavioral testing

The experimental timeline is shown in Figure 1a. Experimental testing occurred as previously described in Kuhn et al., 2022[15]. Extensive behavioral analyses for these animals, including sex and site differences, have been conducted and reported in prior publications. Underlying genetic variants that may be contributing toward these behaviors will be discussed here. Regardless, a brief description of behavioral testing procedures are provided below. Tests assessing stress- and anxiety-like behaviors (elevated-plus maze and open field test), as well as analgesic threshold (tail flick test), were performed prior to and following heroin experience.

Behavioral testing

<u>Elevated-plus maze (EPM):</u> Sessions were conducted in an elevated apparatus (San Diego Instruments; 4 arms at 43.5” long and 4” wide) that consisted of two “closed” arms (12” high walls) and two “open” arms (i.e., not enclosed). Animals were placed in the center of the maze to start and movement was tracked automatically using ANY-maze behavioral tracking software (Stoelting, Wood Dale; version 6.17) over the

course of the 5-min session. A minimum of 85% of the rat's body had to be within an arm for it to be counted. The percent time the animal spent in each arm was calculated.

Open field test (OFT): Animals were placed in the center of a Plexiglas chamber housed within a metal frame (Omnitech Elctronics, Columbus, OH; 16" L x 16" W x 12" H) containing photocells that tracked vertical and horizontal movements. Sessions lasted for 60-min and data was recorded and analyzed for distance and time spent moving using Versamax (Omnitech Electronics, Columbus, OH; version 1.80-0142).

Tail flick test (TF): Testing occurred on a platform with the rat's tail over an infrared light beam equipped with a motion sensor that turned the beam off once the tail was removed, or after 10 seconds (Ugo Basile S.R.L., Gemonio, Italy). TF was comprised of two phases: baseline (1 mg/kg saline injection, s.c.) and test (0.75 mg/kg heroin, s.c.). Injections were administered 15 min prior to testing and the phases were separated by 1 hour. Each test consisted of 4 trials with the location of the tail tested being adjusted by 1 cm each trial to prevent tissue damage. Average latency to remove the tail from the beam was calculated.

Heroin taking, refraining and seeking measures

Prior to training, rats were outfitted with an indwelling jugular catheter and post-operatively administered an antibiotic (Cefazolin, 0.2 mg/kg, s.c.; or enrofloxacin, 1 mg/kg, i.v.) and analgesic (Ketorolac, 2 mg/kg, s.c.; or Meloxicam, 0.5 mg/rat, s.c.). Training occurred to standard behavioral testing chambers (Med Associates, St. Albans, VT) housed within a sound attenuating box outfitted with a ventilation fan. Two levers with a light above each were on one chamber wall, and opposite was a house light and speaker. The house light turned on at the start of all sessions. A fixed ratio 1 schedule of reinforcement was used for heroin self-administration whereupon a press on the active lever resulted in an infusion of heroin (20 µg/kg/100 µl infusion over 3-sec) and presentation of a light/tone cue. The house light turned off for 20-sec at the time of infusion to signal a time out period during which additional active lever presses were

recorded but without consequence. Four training sessions (12-h or 300 infusions earned) occurred per week (Monday-Friday) with one randomized day off per week, totaling 12 sessions. Motivation to take heroin was assessed using a progressive ratio test whereupon the number of lever presses needed to earn an infusion exponentially increased[23], with the maximum number of lever presses exerted to receive an infusion considered the break point. Testing terminated after 12-h or 1-h of no earned infusion. Heroin self-administration training was then re-established for 3 days, followed by a within-session extinction-prime test lasting 6 hours. Testing occurred under extinction conditions (i.e., active lever presses no longer result in heroin infusion or light/tone cue presentation). With two hours left in the session, animals received a heroin prime injection (0.25 mg/kg, s.c.). Animals next underwent 6 daily 2-h extinction training sessions prior to a test for cue-induced reinstatement (2-h), during which presses on the active lever resulted in light/tone cue presentation, but no heroin infusion. Throughout testing, presses on the inactive lever were recorded but without consequence.

Tissue collection and genotyping

Following all behavioral testing, animals were sacrificed and spleen samples immediately collected. Spleens were submerged in 0.5 ml of sterile saline, then frozen and stored at -80°C prior to shipment to University of California San Diego for genome-wide association (GWAS) analysis. Genotyping-by-sequencing was employed as previously described to assess genotypes[24, 25]. A total of 7,955,513 single nucleotide polymorphisms (SNPs) were identified with an error rate <1%. However, prior to GWAS analysis, SNPS with high linkage disequilibrium identified using PLINK[26] with an r^2 cutoff of 0.95 were removed, resulting in the assessment of 5,308,726 SNPs for all analyses.

Statistical analyses

Behavioral testing traits both prior to (time point 1) and following (time point 2) heroin experience were included for analysis, as well as the difference in individual testing measures between the time points.

Traits representative of heroin taking, refraining and seeking measures were included in analysis. Heroin-taking behaviors included: escalation of intake across the entire session (ug/kg; average consumption days 1-3 subtracted from days 10-12; Escalation of intake: Hour 1-12), escalation of intake in the first hour of the session across training (ug/kg; average consumption hour 1 days 1-3 subtracted from hour 1 days 10-12 ; Escalation of intake: Hour 1), total consumption (ug/kg) across training sessions 1-12, and break point achieved during the progressive ratio test. Refraining traits include active lever presses made during the following: the first (Extinction burst) and last (Extinction: Prime) 2 hours of the within-session extinction-heroin prime reinstatement test, the last day of extinction training prior to the test for cued reinstatement (Extinction: Day 6) and the difference in lever presses between the first and last day of extinction training (Extinction de-escalation). Active lever presses made during the tests for heroin prime and cued reinstatement we assessed for seeking measures.

Genetic and phenotypic correlations: Prior to analysis, all data was quantile normalized separately for males and females prior to being combined. Using a linear model, covariates that explained more than 2% of variance in each trait within the data were identified then regressed out, and data was again quantile normalized to maintain a normal distribution. Covariates included sex, age at different testing points, batch number, surgery weight, animal coat color, testing location, and selected testing chambers at both locations. By using this method, behavioral differences between sexes, which were thoroughly examined for this data set in previous publications[15], were accounted for without affecting possible gene x sex interactions. To assess the degree of genetic similarity between two traits (i.e., genetic estimation), Genome-wise Complex Analysis (GCTA) was implemented. Hierarchical analysis using scipy's linkage function with a 70% maximum linkage distance was applied to the genetic correlations to establish clusters. Spearman correlations with Bonferroni test to correct for multiple comparisons were used to assess behavioral trait similarities. SNP heritability, the proportion of genetic variance that contributes toward behavior variance, for behavioral traits was determined using GCTA-GREML analysis.

Quantitative trait loci (QTL) and SNP identification: GCTA software employing a genetic relatedness matrix and the Leave One Chromosome Out method to account for proximal contamination was employed for GWAS analysis[27, 28]. Because all data was quantile normalized, only one significance threshold determined by permutation was necessary[29]. Chromosomes were scanned for the presence of QTLs by assessing if any SNPs surpassed the permutation-derived threshold of $-\log_{10}$ p>5.58, and if there was an additional SNP located within 0.5 Mb with a significance within 2 $-\log_{10}(p)$ units of the first SNP[30]. Independence of QTLs on the same chromosome was established by making the top SNP in the identified QTL a covariate and reapplying GWAS analysis. This process was continued, with the new significant SNPs being added as covariates, until no more QTLs were identified[30]. Linkage disequilibrium for QTLs was detected by identifying markers with a high correlation coefficient ($r^2 \geq 0.6$) with the top marker. SNPEff was used on all SNPs within a QTL for variant annotation[31].

Principle component analysis: To assess if variance in behavioral traits within significant QTLs could be explained by common factors, a principle component analysis was applied to the data. The Kaiser-Meyer-Olkin measure of sampling adequacy (KMO) and Bartlett's test of sphericity were first conducted to ensure PCA analysis could be applied to the data set. Number of components was determined using a minimum threshold of Eigenvalue of 1 and Varimax method with a minimum factor loading (i.e., correlation between trait and component) of 0.3 was used to rotate identified components. Analyses occurred in IBM SPSS version 27.

Results

Behavioral and genetic correlations

Significant behavioral traits revealed relatively weak shared variation overall (Fig 1b, Supplemental Table 1), with the exception of inter-correlated behavioral traits in the OFT, EPM and TF tests. Weak co-variance between several heroin taking, refraining and seeking behaviors was present (Supplemental Table 1),

implying significant heterogeneity present in the factors (e.g., behavioral, genetic or epigenetic) that are guiding these behaviors. However, behavior during the progressive ratio test, cued reinstatement and total heroin consumption were all positively correlated (r^2>0.5), suggesting overlapping behavioral mechanisms guiding these behaviors.

Shared variance in behavioral trait genetic factors were more present, with the identification of four distinct clusters (Fig 1b). Cluster compositions did not align to a great extent with correlated behavioral trends. However, high within cluster interconnectivity for analgesia threshold and anxiety-like behaviors observed in the EPM were present (Fig 1), indicating shared genetic variability, as to be expected. Total distance travelled during both OFTs showed weak genetic co-variance with behavior during cued reinstatement (OFT distance 1, r^2=0.13; OFT distance 2, r^2=0.12; $p<0.001$ for both). A behavioral relationship between these measures is well documented[32], however, for heroin this relationship is only present in male rats[15].

PCA

A total of five significant SNPs on four different chromosomes were identified for four different behavioral traits (Fig 2a). In order to assess whether these traits were potentially related PCA analysis was conducted. A KMO value of 0.60 and $p<0.001$ for Bartlett's test of sphericity verified PCA analysis could be applied to the data set. Two components were identified accounting for a total of 71.55% variance in the data set (Fig 2b). Heroin-taking behaviors were exclusive to component 1 and accounted for 46.24% of variance with traits exhibiting strong loading (Break point: 0.86; Consumption: 0.81; and Escalation of intake: Hour 1-12: 0.67; Fig 2b). Behavior during the first TF baseline test loaded strongly (0.99) onto component 2 and accounted for 25.31% of variance (Fig 2b). These data imply that analgesic threshold prior to heroin experience and heroin consummatory traits are behaviorally unrelated to one another, aligning with the behavioral correlations in Fig 1b.

Heritability estimates

The SNP heritability estimate for each behavioral trait are shown in Table 1. Total heroin consumed across self-administration training showed significant heritability (h^2=0.17), suggesting extent to which an animal will consume heroin is affected by genetic background. Refraining behavior, or the inability to refrain, during the first experience under extinction conditions (Extinction: Burst) also showed significant heritability (h^2=0.098), indicating a genetic influence over aspects of compulsive seeking behavior. Other measures of heroin-taking, refraining or seeking behaviors did not show significant heritability.

Several behavioral measures prior to and following heroin experience showed significant heritability measures. Notably, analgesic threshold under basal conditions is a heritable trait to an extent (TF baseline 1, h^2=0.11), with genetic factors having more impactful influence over behavioral responding following prolonged heroin experience (TF baseline 2, h^2=0.15). Interestingly, analgesic threshold in the presence of heroin is not heritable before heroin experience, but does show heritability following prolonged heroin use (TF test 2, h^2=0.12). Locomotor behavior in an inescapable environment is reliably influenced by genetic factors, regardless of heroin experience (OFT distance 1, h^2=0.14; OFT distance 2, h^2=0.23; OFT time 1, h^2=0.10; OFT time 2, h^2=0.16). This trait has previously shown heritability[20, 21], however, these data suggests an enduring genetic contribution to locomotor behavior regardless of environmental circumstances, such as prolonged drug exposure. Lastly, time spent in the enclosed arm of the EPM, an indicator of increased stress- and anxiety-like behavior, is a heritable trait (EPM closed 1, h^2=0.08) prior to heroin self-administration. In contrast, after heroin experience, genetic contribution to behavior shifts toward mediating less stress- and anxiety-like behavior (EPM open 2, h^2=0.07).

GWAS

The Manhattan plot in Fig 2a reveals that chromosome 19 was pleiotropic as this locus had a significant SNP for both TF baseline 1 and break point. The SNPs are separated by approximately 4 Mb, and do not

overlap with one another. In addition to a significant SNP on chromosome 19, analgesic threshold under baseline conditions prior to heroin experience had another SNP on chromosome 2 (Fig 3a). Twelve genes were identified on chromosome 2 (Fig 3b), and 3 on chromosome 19 (Fig 3c). The top SNP on chromosome 19, as well as several others, are shown on the regional association plot, however, no known genes are present for that region of the chromosome. SNPs encompass both coding and non-coding regions of a chromosome, thus is it possible that these genes are in non-coding regions with unknown identity and functional relevance.

Several genes for SNPs associated with heroin taking were identified (Fig 4-6). A substantial number of genes (n=27) were identified for the SNP on chromosome 10 for escalation of heroin intake across the entire training session (Fig 4a, b). Of the 27 genes identified, eight encode olfactory receptors (*Olr* genes), implying olfactory mechanisms may contribute toward directing the acquisition and maintenance of heroin-taking behavior. Total heroin consumed across training yielded 20 genes on chromosome 11 (Fig 5a, b). A significant SNP for break point achieved during the progressive ratio test was identified on chromosome 19 (Fig 6a). However, akin to this same locus for heroin naïve baseline analgesia, only 3 of these genes are known, which did not include the top SNP (Fig 6b).

Discussion

The economic toll on society for OUD totals over one trillion dollars annually[33]. Furthermore, over 1 million individual seek treatment for OUD every year[34], posing a necessity to better understand the biological traits contributing to OUD vulnerability. Using a rat line emulating human genetic and phenotypic variability[12], we assessed genetic heritability and variants identified by GWAS analysis associated with several OUD-like behaviors, including heroin taking, refraining and seeking traits.

Heritability

There is a significant heritable contribution to OUD in the human population, approximating between 23-54%[6, 7]. However, given the complex and multi-symptomatic nature of OUD[4], assessing heritability of distinct behavioral traits associated with OUD vulnerability can be difficult. In a large sample of HS rats, we identified several traits associated with OUD. Both heroin consumption and initial refraining behavior (extinction burst) exhibited heritability, indicating some measures of consumption and compulsivity are heritable and should be considered when seeking treatment for OUD. However, behaviors following prolonged heroin use and forced abstinence, such as cued reinstatement, did not exhibit heritability. A cascade of neuroplastic adaptations occurs following heroin use that likely mediates these behaviors to a greater extent than genetic heritability[35, 36].

In alignment with work done in humans, we showed using a rodent model that analgesic threshold to painful heat stimuli under baseline conditions is heritable[37]. Following opioid use, both humans[38, 39] and rats[40] demonstrate opioid-induced hyperalgesia in response to a painful stimuli, a finding replicated in these data[15]. While heritability factors associated with hyperalgesia are not known, epigenetic factors modulating this behavior have been identified in a rodent model[41]. Given the inherent behavioral and genetic heterogeneity present in HS rats, and the translational relevance of the findings reported here, HS rats should be ideal for disentangling genetic factors contributing toward opioid-induced analgesia.

Novelty-induced locomotion has been associated with vulnerability in opioid addiction-related behaviors in both rodent models[15, 42-46] and humans[47], and is a heritable behavior[20, 21]. Heritability for this trait is relatively stable and affects behavior both prior to and following heroin, suggesting an enduring genetic contribution to locomotor behaviors that are not greatly affected by factors (e.g., epigenetic changes) associated with prolonged heroin experience.

GWAS

Genetic variants associated with heroin addiction-related behaviors can not only be used as biomarkers of SUD vulnerability, but also to gain better understanding of the neurobiological mechanisms underlying distinct traits associated with OUD. Within the significant SNPs for this study, a many genes were identified, however, only a portion are currently known to have functional relevance to the behavioral traits they are associated with and will be further discussed.

Baseline analgesic threshold prior to heroin experience: Genetic variants on two separate chromosomes were found for analgesic threshold under baseline conditions prior to heroin experience. On chromosome 19, the Ces5A gene was identified. This gene encodes for a protein involved in the hydrolysis of exogenous compounds including cocaine and heroin[48]. Hydrolysis of heroin is necessary for it to be converted into 6-monoacetylmorphine whereupon it is able to cross the blood-brain barrier and act at μ-opioid receptors to exert its main effects[49]. On chromosome 2, two genes that operate in tandem with one another were also associated with pre-heroin experience baseline analgesia. SELENOP1 encodes for a selenoprotein that is secreted by the liver and acts to deliver selenium, a trace element necessary for proper brain function[50], via the bloodstream to regions including the brain. Selenium has been implicated in mediating dopamine release and transmission within the substantia nigra[51, 52] and the nucleus accumbens[53]. The presence of SELENOP1 on nucleus accumbens dopamine terminals is proposed to attenuate dopamine release into the synaptic cleft via direct mediation of the dopamine 2 autoreceptors, protecting against neurotoxic effects of drugs of abuse as evidenced for methamphetamine[53]. Ccdc152, another gene on chromosome 2, is encoded within the SELENOP1 gene, and acts to attenuate SELENOP1 levels via post-translational modification[54]. Though the periaqueductal gray is the predominant brain nuclei processing pain signaling[55], striatal dopamine signaling has recently been implicated in affecting pain processing and perception[56, 57]. The association between SELENOP1 and Ccdec152 to pre-heroin baseline analgesia infers a further contributory role of dopamine signaling to pain processing, and the possible role of dopamine-based therapies for pain treatment.

Escalation of heroin intake across the entire session: Escalation of heroin intake was associated with Adamts2, a biomarker for heavy tobacco smoking[58]. Adamts2 is as an extracellular matrix proteinase[59] localized within the mesolimbic and mesocortical regions within the brain[60], and is upregulated in the periphery in response to dopamine[60]. Overexpression of Adamts2 is associated with schizophrenia[60], however, a downregulation of this gene as a consequence of inhibition of dopamine D2 receptors was observed following long-term morphine exposure and withdrawal[61]. Low levels of dopamine D2 receptors are found in human heroin addicts[62], and are predictive of sensitivity to drugs of abuse[63]. It is possible, though requires further investigation, that Adamts2 may also serve as a biomarker for OUD consumption vulnerability. The gene for metabotropic glutamate receptor 6 (mGluR6; gene Grm6) was present within the escalation of heroin intake SNP as well. While other members of the mGluR family mediate motivated and addiction-like behaviors[64], the role of mGluR6 in these behaviors is less apparent. There is a high mGluR6 receptor density in the retina, however receptor expression is also present across several region of the brain, and interacts with periaqueductal grey-dependent pain processing[65]. mGluR6's are also present in microglia[66]. Through a TLR4-dependent signaling pathway, microglia regulate opioid addiction-like behaviors[67]. The absence of TLR4 either by inhibition or genetic knock-out prevents both morphine and oxycodone conditioned place preference in rats[68]. Furthermore, chronic antagonisms of TLR4 attenuates both remifentanil[68] and heroin self-administration[69]. There is currently no known relationship between microglia mGluR6 and TLR4 signaling in microglia, however, it is plausible these signaling cascades interact affecting opioid escalation behavior.

Heroin consumption: Several relevant genes were associated with total consumption of heroin across all training sessions. DSCAM, a neuronal cell adhesion molecule, regulates cellular connectivity[70] and glutamatergic plasticity associated with learning[71, 72]. Glutamate-mediated neuroplasticity occurs following heroin experience, and is considered a driving force behind relapse vulnerability[36]. DSCAM is necessary for tasks requiring memory formation[73, 74], including those associated with rewarded

behaviors[74], and is upregulated in the hippocampus following a learned memory task[72]. Furthermore, GWAS analysis in human showed DSCAM acts as a biomarker for cigarette cessation success[75], and should be explored for heroin consumption as well. Based on these findings, it is likely DSCAM contributes, at least in part, to making the learned associations necessary during heroin self-administration training, and that this process is mediated in a glutamate neuroplasticity-dependent manner, though further investigation is necessary. Another gene of interest detected for heroin consumption was PCP4. PCP4 mediates calcium signaling and through downstream mechanisms also affects dopamine and acetylcholine signaling[76]. PCP4 levels are attenuated in post-mortem tissue of individuals diagnosed with alcohol use disorder[77], as well as in the nucleus accumbens of rats following 2-weeks of nicotine withdrawal[78]. The relationship between PCP4 and OUD is unknown, but is likely to be a potential regulator of altered nucleus accumbens transmission as a consequence of sustained heroin use.

Break point: Genes associated with the chromosome 19 SNP for break point achieved during the progressive ratio task are largely unknown. However, Slc38a7, a gene present within neuronal cell bodies and axon terminals[79], was identified. Gene expression for Slc38a7 is biased toward GABA cells, with a high density within the ventral tegmental area and basolateral amygdala[79], both regions associated with SUD[80]. It is hypothesized that Slc38a7 contributes toward glutamate reuptake[79], a neuroplastic mechanism associated with different states of heroin dependence[36]. Interestingly, this genetic variant is not present in astrocytes[79], the main contributor of nucleus accumbens glutamate reuptake following heroin experience[36, 81]. The density and functional role of this genetic variant in mediating heroin addiction-related behaviors, specifically in the ventral striatum, is of great interest, specifically as it relates to a complex motivational task such as break point.

Conclusion and Future Directions

In this study, we identified, to the first of our knowledge using a rodent model, several heritable traits and genetic variants associated with OUD. Unlike GWAS studies performed in humans, we were able to identify several genetic variants associated with distinct OUD behaviors that contribute toward diagnosis. Several of the identified genes are known biomarkers of SUD, but have not been assessed for OUD. Other genes are involved in neurobiological mechanisms associated with SUD, and warrant further investigation for OUD vulnerability. However, complementing human OUD[7], phenotypic variance can only be partially explained by genetic variability, and other factors such as epigenetic mechanisms[82] likely contribute to OUD behaviors. Furthermore, GWAS analysis focusing on genetic variants associated with OUD resiliency, a neurobiological active process[83, 84], should be considered in future analyses.

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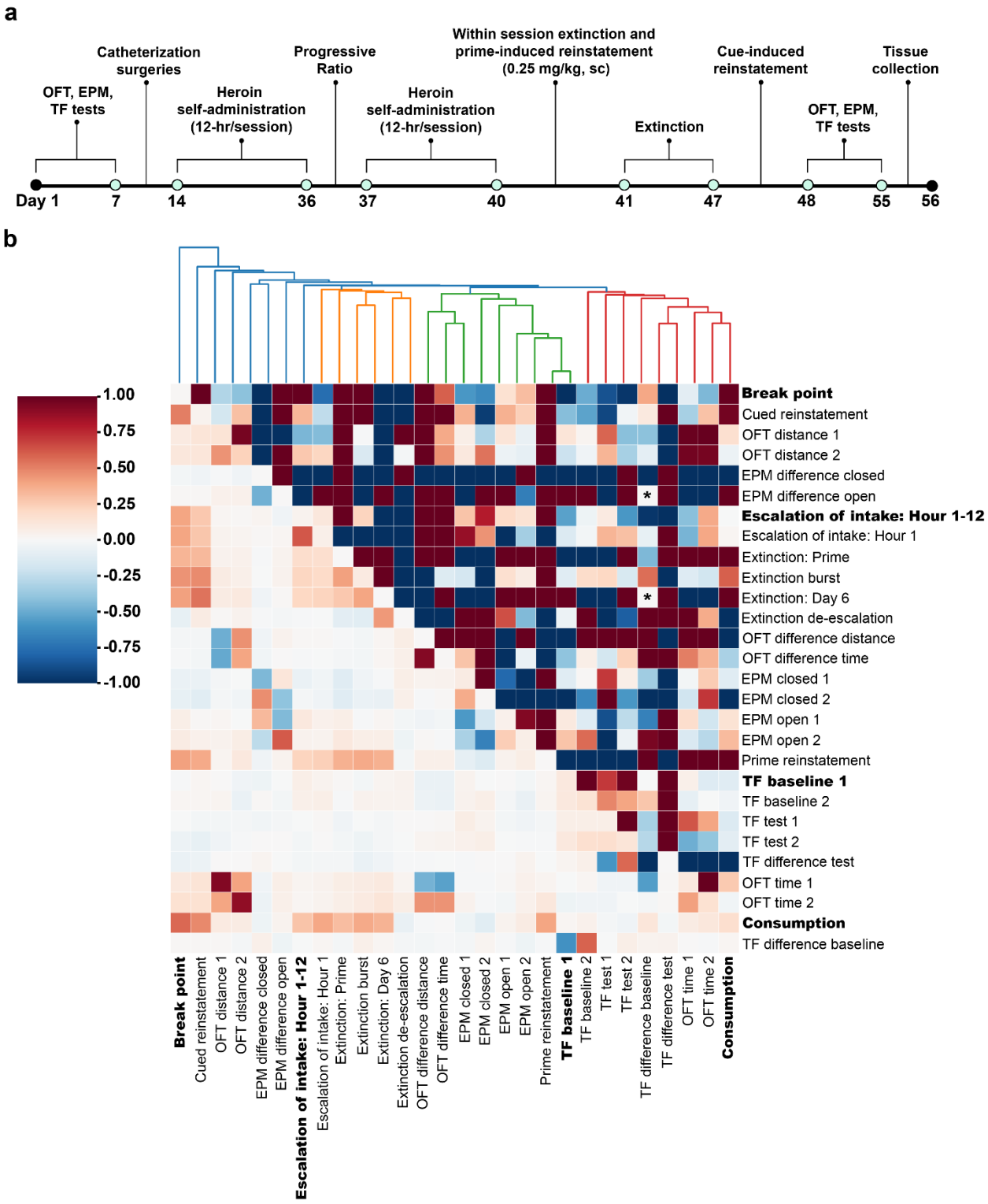


Figure 1 Experimental timeline and correlational analyses. **a)** Rats underwent testing for stress- and anxiety-related behaviors (OFT: open field test; EPM: elevated-plus maze) and analgesic threshold (TF: tail flick) prior to jugular catheterization surgery, with tests repeated following heroine experience. Heroin

self-administration occurred for 3 weeks (12-h/session, 4 days a week) followed by a progressive ratio test and an additional 3 days of self-administration training. Next, rats underwent a within-session extinction-heroin prime reinstatement test (6-h). Extinction training sessions (2-h/session) then occurred followed by a cue-induced reinstatement test (2-h). Spleen sample were collected for genetic analyses at the conclusion of training. **b)** Correlation matrix for phenotypic (lower left triangle) and genetic (upper right triangle) covariance. Four distinct genetic co-variance clusters within the matrix were identified and represented by the dendrogram. Behavioral testing that occurred prior to heroin experience is designated with a 1, and testing following heroin training as 2. Traits in bold are those with at least one significant QTL identified in GWAS analysis. *test failed due to collinearity between traits (n=874)

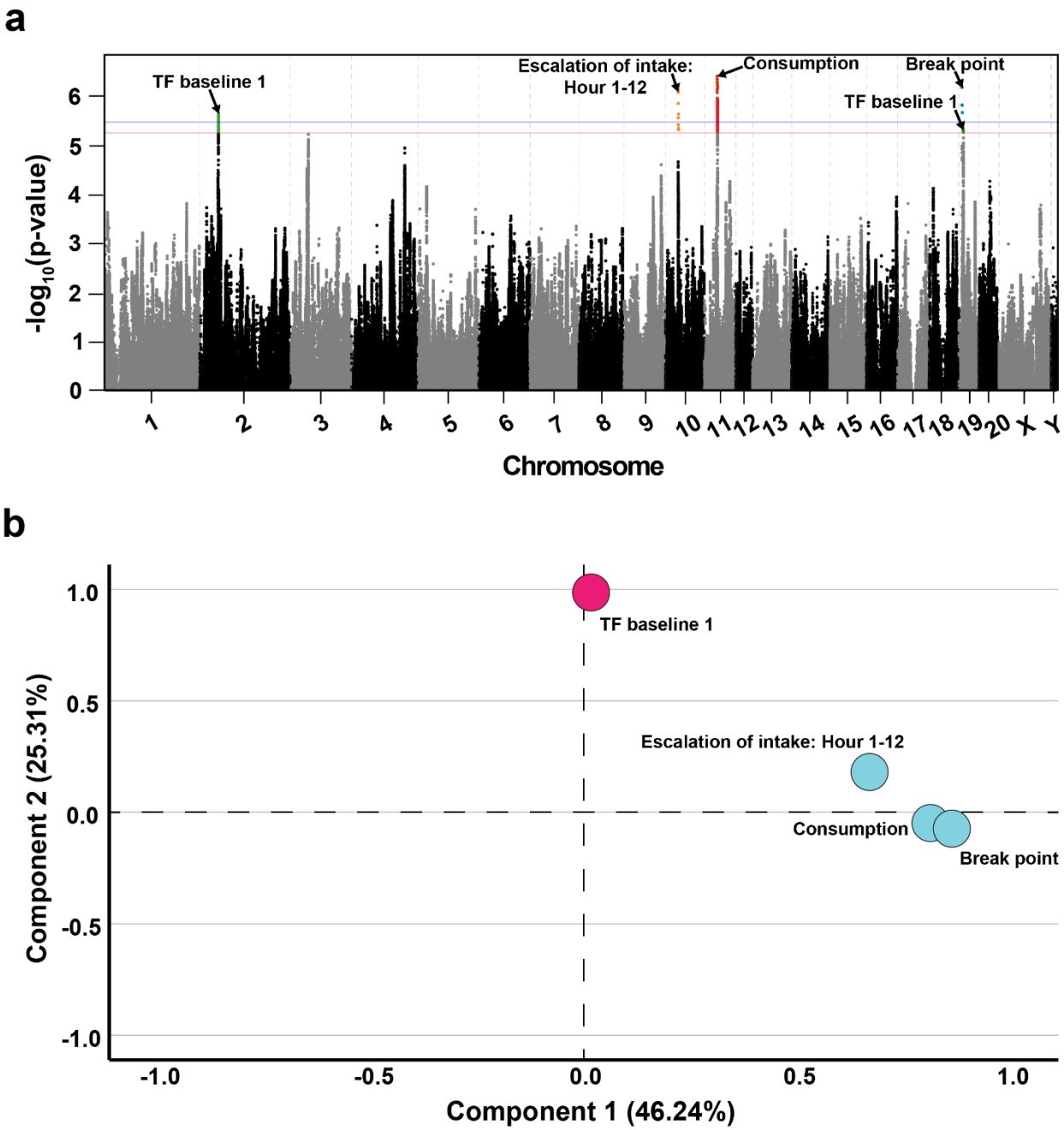
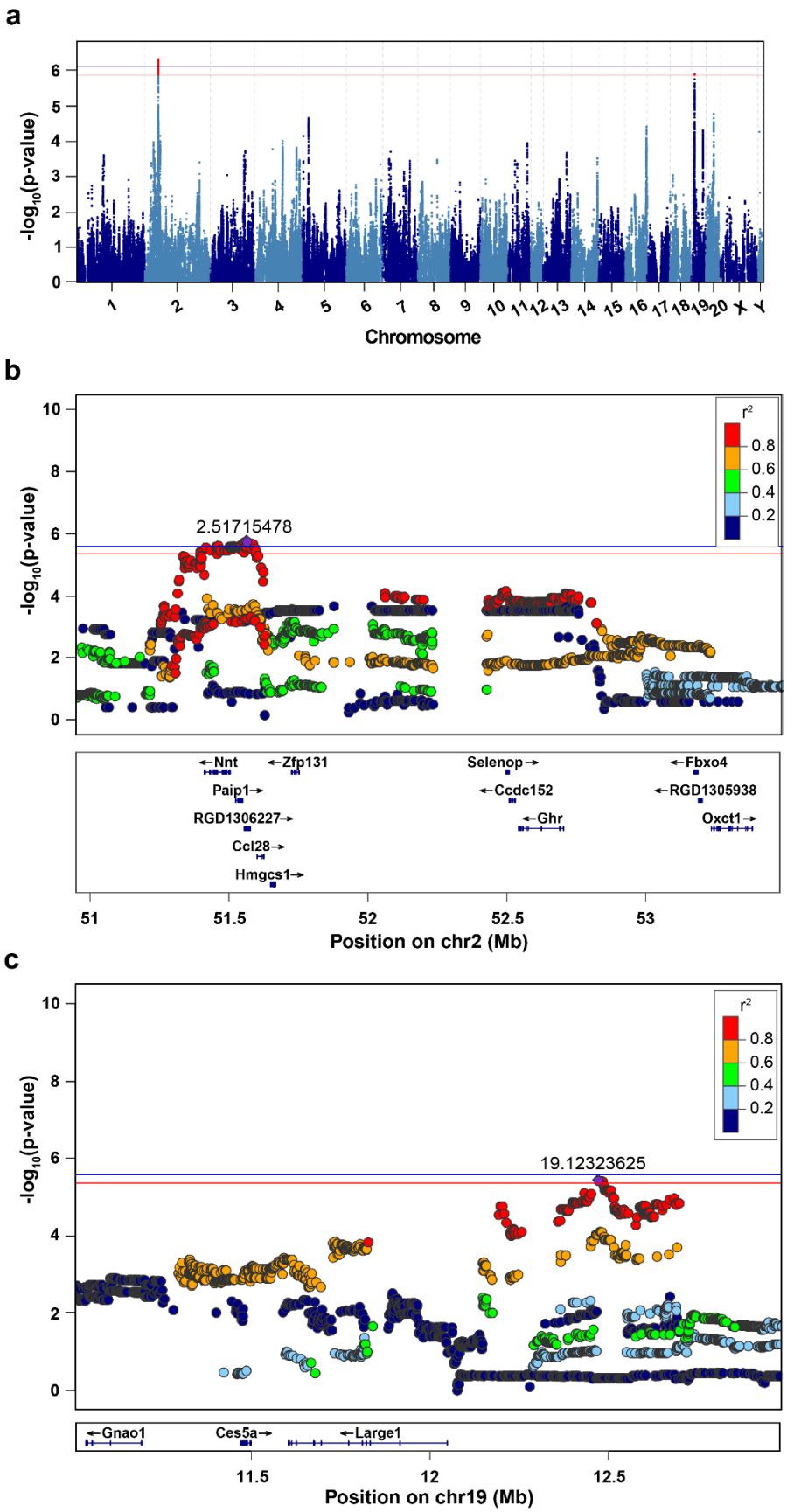


Figure 2 Genome-wide association results for behaviors and phenotypic covariance. **a)** Porcupine plot for selected behavioral traits. Top SNPs exceeding the genome-wide significance threshold (red: 5.36%, 10% false positive rate; blue: 5.58%, 5% false positive rate) are highlighted and identified. **b)** Principle component analysis for behavioral traits with significant SNPs. Two factors were identified and comprised 71.55% of total variance in the data. Heroin consummatory behaviors (Break point, Consumption and

Escalation of intake: Hour 1-12) are in component 1 (blue), whereas analgesic threshold is independent from the other traits in component 2 (pink). Axes represent factor loading values for each trait within respected component. (n=874)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Chromosome
b
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r2
0.8
0.6
0.4
0.2
Nnt
Zfp131
Selenop
Fbxo4
Paip1
Ccdc152
RGD1305938
RGD1306227
Ghr
Oxct1
Ccl28
Hmgcs1
Position on chr2 (Mb)
c
19.12323625
Gnao1
Ces5a
Large1
Position on chr19 (Mb)

Figure 3 GWAS results for analgesic threshold during the first TF baseline session. **a)** Manhattan plot for both SNPS exceeding the threshold of 5.36% (10% false positive rate), with one further exceeding a more stringent threshold of 5.58% (5% false positive rate; chromosome 2). Regional association plots for **(b)** chromosome 2 and **(c)** chromosome 19. The top SNP identified for each chromosome is in purple with chromosome location indicated on the plot. The color of other SNPs corresponds to extent of linkage disequilibrium with the top SNP. Known genes of SNPs are indicated below the plots.

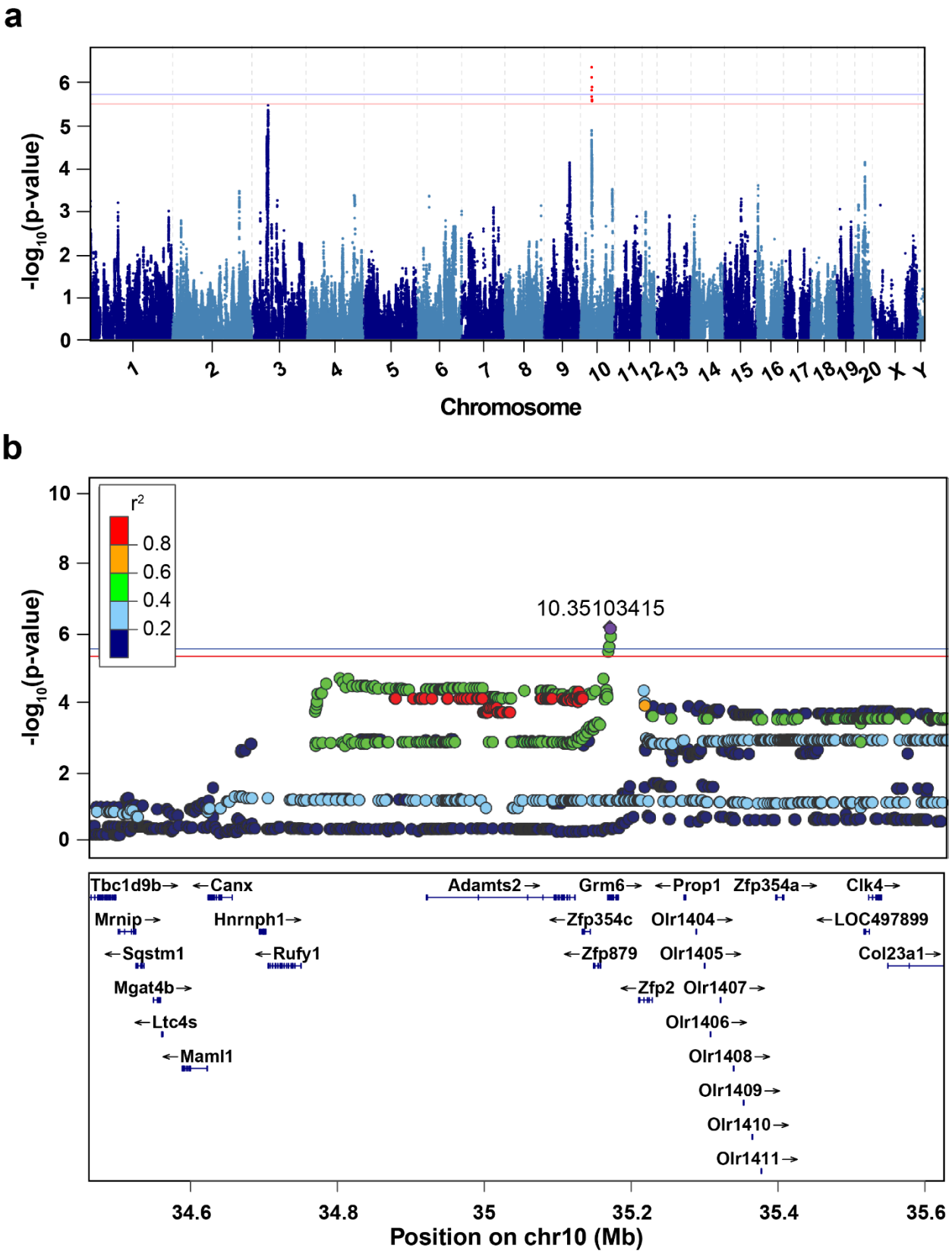
a
-log10(p-value)
Chromosome
b
r2
0.8
0.6
0.4
0.2
10.35103415
Tbc1d9b
Canx
Adamts2
Grm6
Prop1
Zfp354a
Clk4
Mrnip
Hnrnph1
Zfp354c
Olr1404
LOC497899
Sqstm1
Rufy1
Zfp879
Olr1405
Col23a1
Mgat4b
Zfp2
Olr1407
Ltc4s
Olr1406
Maml1
Olr1408
Olr1409
Olr1410
Olr1411
Position on chr10 (Mb)

Figure 4 GWAS results for escalation of heroin intake across the entire session. **a)** Manhattan plot for the SNP (chromosome 10) exceeding the threshold of 5.58% (5% false positive rate). **(b)** Regional association plot for chromosome 10, with the top SNP in purple and chromosome location indicated on the plot. Other SNPs are colored based on their linkage disequilibrium with the top SNP. Known genes of SNPs are indicated below the plots.

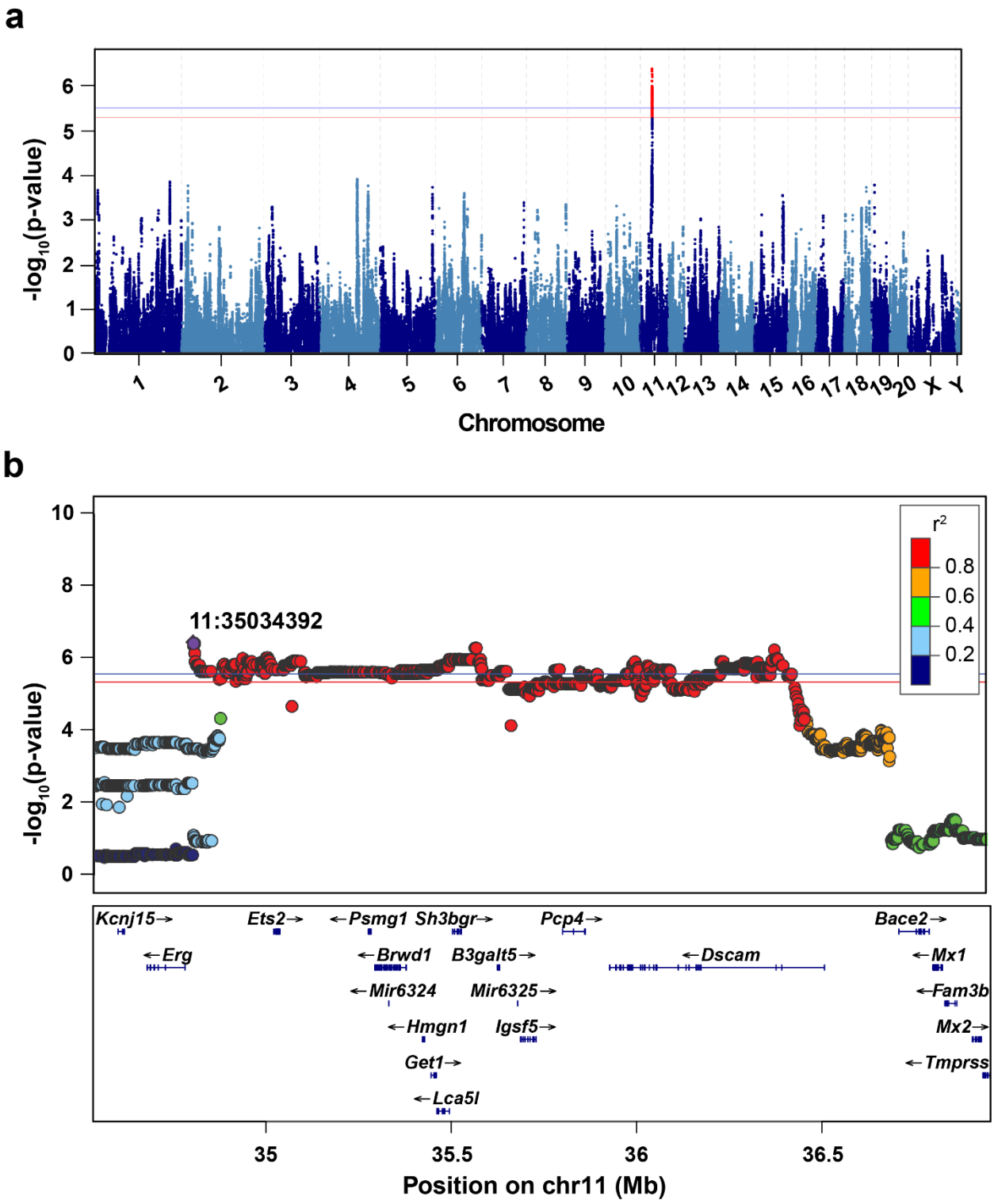

Figure 5 GWAS results for total heroin consumed across self-administration training. **a)** Manhattan plot for the SNP on chromosome 11 that exceeded the threshold of 5.58% (5% false positive rate). **(b)** Regional

association plot for chromosome 11, with the purple dot indicating the top SNP and location on chromosome indicated on the plot. The color of additional SNPS refers to extent of linkage disequilibrium with the top SNP. Known genes of SNPs are indicated below the plots.

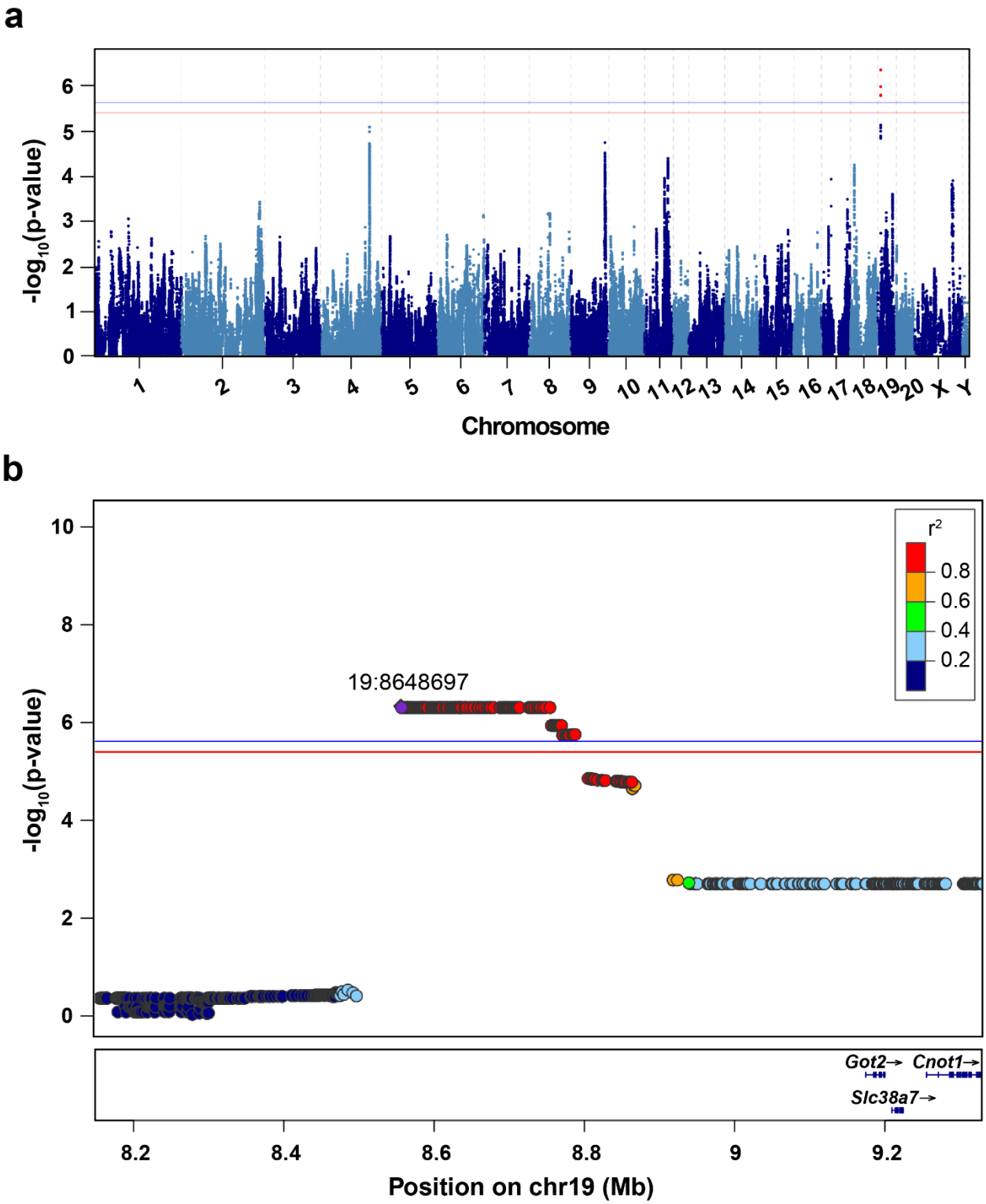

Figure 6 GWAS results for break point achieved during the progressive ratio test. **a)** Manhattan plot for the significant SNP that exceeded the threshold of 5.58% (5% false positive rate) on chromosome 19. **(b)**

Regional association plot for chromosome 19, with the top SNP in purple and the color of other significant SNPs referring to level of linkage disequilibrium with the top SNPs. The location of the top SNP is indicated on the plot. Known genes of SNPs are indicated below the plots.

Trait	h^2SNP	SE	p-value	n
Escalation of intake: Hour 1	0.062	0.042	0.059	874
Escalation of intake: Hour 1-12	0.037	0.04	0.168	874
Consumption	0.172	0.052	**<0.001**	874
Break point	0.009	0.039	0.42	874
Extinction: Burst	0.098	0.045	**0.007**	874
Extinction: Prime	0.002	0.036	0.48	874
Prime reinstatement	0	0.036	0.5	874
Extinction: Day 6	0	0.038	0.5	874
Extinction de-escalation	0.003	0.037	0.47	874
Cued reinstatement	0.037	0.04	0.172	866
OFT distance 1	0.139	0.048	**<0.001**	870
OFT distance 2	0.232	0.052	**<0.001**	868
OFT distance difference	0	0.033	0.5	864
OFT time 1	0.103	0.046	**0.004**	870
OFT time 2	0.158	0.049	**<0.001**	868
OFT time difference	0.007	0.033	0.41	864
EPM open 1	0.054	0.041	0.073	873
EPM open 2	0.072	0.042	**0.024**	873
EPM Open difference	0	0.035	0.5	872
EPM closed 1	0.083	0.043	**0.012**	873
EPM closed 2	0.016	0.035	0.31	873
EPM closed difference	0	0.034	0.5	873
TF baseline 1	0.11	0.046	**0.003**	874
TF baseline 2	0.146	0.049	**<0.001**	874
TF baseline difference	0.044	0.04	0.12	974
TF test 1	0.047	0.039	0.1	874
TF test 2	0.124	0.045	**<0.001**	874
TF test difference	0	0.035	0.5	874

Table 1 Heritability table for behavioral traits. Significant relationships are highlighted in bold. The heritability index (h^2SNP), standard error (SE), p-value and total number of animals in analysis (n) for each trait is recorded.

Behavioral trait correlations

Trait 1	Trait 2	Spearman r	p-value	n
Escalation of intake: Hour 1	Break point	0.39	1.98×10^{-33}	874
	Cued reinstatement	0.28	1.26×10^{-16}	866
	Escalation of intake: Hour 1-12	0.62	7.03×10^{-94}	874
	Extinction: Prime	0.17	4.93×10^{-07}	874
	Extinction: Burst	0.19	9.72×10^{-09}	874
	Extinction: Day 6	0.22	4.02×10^{-11}	874
	Consumption	0.39	1.02×10^{-32}	874
Escalation of intake: Hour 1-12	Break point	0.39	6.95×10^{-34}	874
	Cued reinstatement	0.27	2.87×10^{-16}	866
	Extinction: Prime	0.17	2.40×10^{-32}	874
	Extinction: Burst	0.21	2.36×10^{-10}	874
	Extinction: Day 6	0.21	8.70×10^{-10}	874
	Prime reinstatement	0.22	2.46×10^{-11}	874
	Consumption	0.29	3.67×10^{-18}	874
Consumption	Break point	0.57	7.83×10^{-78}	874
	Cued reinstatement	0.48	1.12×10^{-51}	866
	Extinction: Prime	0.31	1.33×10^{-20}	874
	Extinction: Burst	0.38	1.05×10^{-30}	874
	Extinction: Day 6	0.34	1.10×10^{-25}	874
	Prime reinstatement	0.39	1.20×10^{-33}	874
	OFT time 2	0.11	0.0009	868
	EPM closed 2	-0.12	0.0006	873
Break point	Cued reinstatement	0.51	1.96×10^{-58}	866
	Extinction: Prime	0.34	6.60×10^{-25}	874
	Extinction: Burst	0.45	3.64×10^{-45}	874
	Extinction: Day 6	0.41	1.54×10^{-36}	874
	OFT time 2	0.15	1.01×10^{-05}	868
	OFT distance 2	0.11	0.0001	868
Extinction: Burst	Cued reinstatement	0.47	5.72×10^{-49}	866
	Extinction: Prime	0.41	1.43×10^{-36}	874
	Extinction: Day 6	0.40	1.57×10^{-34}	874
	Extinction de-escalation	-0.11	0.001	874
	Prime reinstatement	0.39	6.52×10^{-31}	874
Extinction: Prime	Cued reinstatement	0.32	1.54×10^{-22}	866
	Extinction: Day 6	0.27	9.39×10^{-16}	874
	Extinction de-escalation	-0.11	0.001	874
	Prime reinstatement	0.36	1.71×10^{-27}	874
	EPM closed 1	-0.13	0.0002	873
	EPM closed 2	-0.12	0.0003	873
Prime reinstatement	Cued reinstatement	0.38	1.33×10^{-30}	866
	OFT time 2	0.12	0.00006	868

	EPM closed 2	-0.13	$7.64x10^{-05}$	873
Extinction: Day 6	Cued reinstatement	0.54	$1.21x10^{-67}$	866
	Extinction de-escalation	0.44	$7.92x10^{-42}$	874
	OFT time 2	0.13	0.0001	868
Cued reinstatement	OFT distance 1	0.13	0.0001	862
	OFT distance 2	0.12	0.0003	860
	OFT time 1	0.13	$8.33x10^{-05}$	862
	OFT time 2	0.15	$9.46x10^{-06}$	860
	EPM closed 2	-0.11	0.0008	865
OFT distance 1	OFT distance 2	0.45	$5.13x10^{-44}$	864
	OFT distance difference	-0.49	$4.07x10^{-52}$	864
	OFT time 2	0.39	$2.64x10^{-33}$	864
	OFT time difference	-0.51	$4.28x10^{-58}$	864
OFT distance 2	OFT distance difference	0.46	$1.53x10^{-46}$	864
	OFT time difference	0.40	$1.29x10^{-33}$	864
	OFT time 1	0.40	$7.59x10^{-35}$	864
	OFT time 2	0.89	$5.40x10^{-291}$	868
OFT distance difference	OFT time difference	0.92	0.00	864
	OFT time 1	-0.47	$3.15x10^{-49}$	864
	OFT time 2	0.45	$2.54x10^{-44}$	864
OFT time 1	OFT time difference	-0.54	$8.73x10^{-68}$	864
	OFT time 2	0.41	$4.28x10^{-36}$	864
OFT time 2	OFT time difference	0.45	$2.52x10^{-43}$	864
EPM open 1	EPM open 2	0.25	$8.36x10^{-14}$	872
	EPM open difference	-0.44	$7.78x10^{-43}$	872
	EPM closed 1	-0.58	$3.65x10^{-78}$	872
	EPM closed 2	-0.18	$1.33x10^{-07}$	872
	EPM closed difference	0.32	$1.80x10^{-22}$	872
EPM open 2	EPM open difference	0.61	$1.82x10^{-90}$	872
	EPM closed 1	-0.32	$1.32x10^{-22}$	873
	EPM closed 2	-0.67	$9.29x10^{-166}$	873
	EPM closed difference	-0.26	$7.65x10^{-15}$	873
EPM open difference	EPM closed 1	0.12	0.0005	872
	EPM closed 2	-0.41	$5.88x10^{-36}$	872
	EPM closed difference	-0.48	$8.16x10^{-51}$	872
EPM closed 1	EPM closed 2	0.37	$2.77x10^{-30}$	873
	EPM closed difference	-0.45	0.0005	873
EPM closed 2	EPM closed difference	0.47	$2.38x10^{-48}$	873
TF baseline 1	TF baseline 2	0.11	0.0012	874
	TF baseline difference	-0.60	$7.67x10^{-88}$	874
	TF test 2	0.11	0.0013	874
TF baseline 2	TF baseline difference	0.59	$7.30x10^{-84}$	874
	TF test 2	0.16	$1.39x10^{-06}$	874
TF test 1	TF test 2	0.17	$9.78x10^{-07}$	874
	TF test difference	-0.59	$3.40x10^{-84}$	874
TF test 2	TF test difference	0.57	$2.29x10^{-77}$	874

Supplemental Table 1 Data statistics for significant correlated behavioral traits. Heroin taking, refraining and seeking behaviors showed the tendency to co-vary with one another, whereas behaviors assessing stress- and anxiety-like behaviors as well as analgesic threshold exhibited greater within-test covariance. Direction of correlation (Spearman r) and total number of animals included in analysis (n) are included in the table. A significance threshold of $p<0.05$ with Bonferroni test for correction for multiple comparisons was implemented, resulting in a $p<0.0018$ in order to attain significance.