**Genome-wide association study reveals multiple loci for analgesia and opioid consumption behaviors associated with heroin vulnerability in outbred rats**

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**Abstract**

The increased prevalence of opioid use disorder (OUD) has made it imperative to disentangle the biological mechanisms contributing to individual differences in susceptibility to OUD. OUD shows strong heritability, however genetic variants contributing toward vulnerability remain poorly defined. We performed a genome-wide association study (GWAS) using over 850 male and female heterogeneous stock rats to identify genes underlying behaviors associated with OUD such as analgesia, as well as heroin-taking, refraining and seeking behaviors. By using an animal model of OUD, we were able to identify genetic variants associated with distinct OUD behaviors while maintaining a uniform environment, an experimental design not easily achieved in humans. Furthermore, we applied an animal model capturing individual variation in OUD propensity to assess if GWAS results were associated with OUD vulnerable versus resilient behavioral phenotypes. Our findings confirm the heritability of several OUD-like behaviors, including overall phenotype. We identified several genetic variants associated with basal analgesia prior to heroin experience, heroin consumption, escalation of intake, and motivation to obtain heroin. *Ets2*, a regulator of microglia functional plasticity, and its eQTL *PCP4* were identified for heroin consumption, and were associated with an OUD vulnerable phenotype through phenotype wide association study analysis. Furthermore, the coding variant *Phd1l2* and the eQTL *MMP15* for break point are both known mediators of addiction-related behaviors, and correlated with OUD vulnerability. These findings identify novel genetic markers related to individual differences in the susceptibility to OUD-relevant behaviors.

## 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], globally affecting approximately 16 million people annually [2]. The 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 studies (GWAS) are a useful tool for identifying genetic variants that influence heritable traits. GWAS of OUD, though limited, have already yielded valuable insights into genetic contributions to diagnostic vulnerability [8-11]. However, human GWAS of OUD are limited to observational data that is confounded by numerous environmental factors. SUD diagnosis is composed of drug-taking, extinction and seeking (e.g., relapse) behaviors. Understandably, due to a lack of empirical data from study participants, human GWAS have largely focused on the terminal end point of a diagnosis for analysis purposes. Using rodent models of addiction, we can quantify specific traits associated with addiction-related behaviors to directly assess the relationship between these metrics and genetic variants. 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 [12]. This allows for the possible development of individualized OUD treatment options based on the most prevalent behavioral traits contributing toward a SUD diagnosis.

In the current study, we assessed several measures of heroin-taking, extinction 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 outbred population that was created at NIH in 1984 to capture genetic and behavioral heterogeneity reflective of the human population [13-15]. Compared to inbred rat lines, HS rats exhibit comparatively greater diversity [15], and have been used in several studies assessing addiction-related behaviors [16-20], including those focusing on genetic analyses [21-23], 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. Furthermore, using a rat model that captures SUD diagnostic and behavioral heterogeneity conferring OUD susceptibility, we identified genes associated with OUD vulnerability versus resiliency.

## Methods

Behavioral testing occurred at two geographically distinct locations: Medical University of South Carolina (MUSC, USA) and the University of Camerino (UCAM, Italy). All experimental procedures were approved by the Institutional Animal Care and Use Committee at MUSC, 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 #13673907, RRID:RGD_13673907) bred at Wake Forest University were shipped to MUSC and UCAM 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 1,160 rats underwent testing, with the following animals excluded from all analyses: 147 for behavioral control studies (e.g., did not undergo heroin self-administration procedures), 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 [16]. Extensive behavioral analyses for these animals, including sex and site differences, have been conducted and reported in prior publications [16, 24], and we refer the reader to these manuscripts for further edification. Underlying genetic variants that may be contributing toward these behaviors are the focus of this manuscript. Regardless, a brief description of behavioral testing procedures is provided below and the Supplemental Methods.

*Behavioral testing*

Stress and anxiety-related behaviors were assessed in the elevated-plus maze (EPM) and the open field test (OFT; see Supplemental Methods). Analgesic threshold under baseline (administration of 1 mg/kg saline, sc) and test (0.75 mg/kg heroin) conditions were evaluated using the tail flick (TF) test. All tests were performed prior to and following heroin experience.

*Heroin taking, refraining and seeking measures*

Rats were outfitted with an indwelling jugular catheter prior to undergoing heroin self-administration training procedures. During training, presses on the active lever resulted in presentation of a tone and cue light stimulus, as well an infusion of heroin (20 µg/kg/100 µl infusion over 3-sec). Training occurred for 3 weeks with four 12-hr sessions/week. Next, motivation to take heroin was assessed using a progressive ratio test whereupon the number of lever presses needed to earn an infusion exponentially increased according to the following formula: $(5*e^{0.2n})-5$ [25]. The maximum number of lever presses exerted to receive an infusion was considered the break point. Heroin self-administration training was then re-established for 3 days, followed by a within-session extinction-prime test lasting 6 hours. 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 (i.e., no cue presentation or heroin delivery) 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. See Supplemental Methods for further details.

*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 genotyping. Genotyping-by-sequencing was employed as previously described to assess genotypes [26, 27]. 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[28] 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 (i.e., EPM, OFT and TF) 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 (ST1). Traits representative of heroin taking, refraining and seeking measures were included in analysis. Heroin-taking behaviors (ST1) included: 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), 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), total consumption (ug/kg) across training sessions 1-12, and break point achieved during the progressive ratio test. Refraining, or withholding from seeking, traits include active lever presses made during the following: the first (Extinction: Burst) and middle (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.

Behavioral clustering: To assess if GWAS results were associated with OUD vulnerability versus resiliency, behavior across the different OUD phases (taking, refraining and seeking) were assessed using a non-linear network-based clustering approach [29] with rats were characterized as OUD vulnerable, intermediate or resilient. Behaviors include: escalation of intake: hour 1-12, consumption, break point, extinction burst, extinction day 6, prime reinstatement and cued reinstatement (ST1). The parameters as well as behavioral and neurobiological validation of this model are extensively detailed in prior publications [24, 29]. OUD phenotype cluster composition for top SNP coding variants were assessed with a Chi-square test.

Genetic and phenotypic correlations: To address both sex difference and nonparametric distributions, all data were quantile normalized separately for males and females and then 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 [16, 24], were accounted for without affecting possible gene x sex interactions. To assess the degree of genetic similarity between two traits, we used the Genome-wide Complex Trait Analysis (GCTA) software package. Behavioral co-variance between traits was assessed using Spearman correlations with Bonferroni test to correct for multiple comparisons. SNP heritability for behavioral traits was determined using GCTA-GREML analysis.

Quantitative trait loci (QTL) and SNP identification: To perform the GWAS we used GCTA software employing a genetic relatedness matrix and the Leave One Chromosome Out method to account for proximal contamination [30, 31]. Because all data was quantile normalized, only one significance threshold determined by permutation was necessary [32]. We report QTLs that surpassed the permutation-derived threshold of $-\log_{10} p>5.58$ for which there was also an additional SNP located within 0.5 Mb with a significance within $2 -\log_{10}(p)$ units of the first SNP [33]. Independence of QTLs on the same chromosome was established by making the top SNP in the most significant QTL a covariate and then repeating the GWAS analysis for the chromosome in question. This process was repeated until no more significant QTLs were identified [33]. Linkage disequilibrium (LD), the relatedness of different genetics variants, ($r^2 \geq 0.6$) was used to define the boundaries of each QTL. SNPEff was used to identify coding SNPs within each QTL interval [34]. To assess if significant SNPS were associated with behaviors across a wide range of studies undergoing GWAS analysis at UCSD, a phenotype wide association study (PheWAS) was conducted. To explore functional mechanisms whereby SNPs may affect gene expression patterns, expression QTL (eQTL) and splice QTL( sQTL) analyses were performed on cis genes within a 3 Mb window size ($r^2>0.6$ with top SNP and false discovery rate of $q=0.05$) using tensorQTL [35].

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. Number of

components was determined using a minimum threshold of Eigenvalue of 1 and Varimax method with a minimum factor loading of 0.3 was used to rotate identified components. Analyses occurred in IBM SPSS version 27.

**Results**

*Behavioral and genetic correlations*

Behavioral traits showed weak to moderate correlations overall (Fig 1b, ST2), with the exception of measures within the OFT, EPM and TF tests. However, behavior during the progressive ratio test, cued reinstatement and total heroin consumption were all positively correlated ($r^2 \geq 0.48$), suggesting overlapping behavioral mechanisms guiding these behaviors. Genetic correlations were consistently stronger than their behavioral counterparts, with several traits exhibiting collinearity (Fig 1b), indicating shared genetic variability of these traits.

*Heritability estimates*

SNP heritability ($h^2$) estimates the proportion of the phenotypic variance explained by the genetic variance and are influenced by many factors, including environmental noise, the population being studied, and sample size. Several behavioral measures prior to and following heroin experience showed significant heritability (Fig 2a, ST3). Analgesic threshold under basal condition both prior to (TF baseline 1, $h^2$=0.11) and following (TF baseline 2, $h^2$=0.15) heroin experience were heritable. Heroin-induced analgesia after prolonged heroin exposure was also heritable (TF test 2, $h^2$=0.12), further suggesting a possible genetic contribution to the long term effects of heroin exposure on analgesia. Aspects of anxiety- and stress-like behaviors were also heritable, including time spent in the enclosed arm of the EPM, suggestive of enhanced anxiety-like behavior, prior to heroin experience (EPM closed 1, $h^2$=0.08). Conversely, following heroin use a heritable decrease in anxiety-like behavior (EPM open 2, $h^2$=0.07) was present. We support previous findings that locomotor behavior is heritable [21, 22] (OFT distance 1, $h^2$=0.14; OFT time 1,

$h^2$=0.10; OFT distance 2, $h^2$=0.23; OFT time 2, $h^2$=0.16), and further suggest an enduring genetic contribution to this trait exists regardless of environmental circumstances, such as prolonged drug exposure.

Aspects of compulsive heroin taking (Consumption, $h^2$=0.17) and the inability to initially refrain from seeking (Extinction: Burst, $h^2$=0.098) were heritable. While other aspects of heroin taking, refraining and seeking were not, OUD vulnerability cluster assignment was heritable (Vulnerable vs resilient/intermediate, $h^2$=0.17; Intermediate vs vulnerable/resilient, $h^2$=0.09; Resilient vs vulnerable/intermediate, $h^2$=0.15), suggesting a heritable genetic contribution to OUD propensity (Fig 2a, ST3).

*GWAS*

The following behavioral traits contained significant SNPs: baseline analgesia prior to heroin experience (TF baseline 1), escalation of intake across the entire session, total heroin consumption and break point achieved during the progressive ratio test (Fig 2b). Shared behavioral variance between these traits was assessed using PCA, which confirmed heroin taking traits were mutually exclusive from baseline analgesia, implicating a lack of behavioral relationship between these measures (SF1). While two nearby loci on Chromosome 19 contained significant SNPs for TF baseline 1 and break point, these loci are separated by approximately 4 Mb and do not overlap with one another (see Fig 3b and 6a). In addition to the significant SNP on Chromosome 19, TF baseline 1 contained an additional locus on Chromosome 2 (Fig 3a).

*Baseline analgesia prior to heroin experience:* Twelve genes were inside the Chromosome 2 locus (Fig 3a) and several coding variants were identified including coiled-coil domain containing 152 (*Ccdc152*), growth hormone receptor (*Ghr*), zinc finger protein 1331 (*Zfp131*) and serine/threonine-protein kinase NIM1 (*Nim1k*). Though an eQTL, selenoprotein P (*Selenop*), was identified, the relationship between the lead SNP and the eQTL was only modest for both whole brain and the nucleus accumbens core (NAcc; $r^2$=0.66

for both), suggestive of no causal relationship. However, *Selenop* was also identified as an sQTL with strong LD to the top SNP for both whole brain ($r^2$=0.77) and the basolateral amygdala (BLA; $r^2$=0.79), and a weaker LD for the prelimbic cortex (PrL; $r^2$=0.67), suggestive of differences in the splice pattern of *Selenop* may mediate the effects of this locus on baseline analgesia prior to opioid experience. An additional significant SNP with a causal coding variant, LARGE xylosyl- and glucuronyltransferase 1 *(Large1*), was present on Chromosome 19 for baseline analgesia. *Large1* was also identified as an eQTL in several brain regions (whole brain, $r^2$=0.69; BLA, $r^2$=0.78; infralimbic cortex (IL), $r^2$=0.68; NAcc, $r^2$=0.70; orbitofrontal cortex (OFC), $r^2$=0.69; PrL (SNP 19:11540272), $r^2$=0.70; and PrL (SNP 19:11784646, $r^2$=0.75)), along with ribosomal protein S18 (*Rps18l2*) within the lateral habenula (LHb; $r^2$=0.98). An sQTL for the gene myb1 trafficking protein (*Tom1*) was in strong LD with this locus ($r^2$=1.0) within the PrL, along with a moderate relationship with metallothionein 3 (*Mt3*) in the BLA ($r^2$=0.75). These eQTLs and sQTLs could point to heritable differences in the expression of these genes as the underlying biological mechanisms for basal analgesia prior to drug experience.

*Escalation of intake:* Four genes were implicated on Chromosome 10 (Fig 4), however none had coding polymorphisms. Though eQTLs and sQTLs were identified, none are in strong in LD with the peak SNP, suggesting they are unlikely to be casually related to the escalation of heroin intake behavior. Identified eQTLs include metabotropic glutamate receptor 6 (*Grm6*) in whole brain ($r^2$=0.63), collagen type XXIII alpha 1 chain (*Col23a1*) in the OFC ($r^2$=0.63) and zinc finger protein (*Zfp2*) in the PrL ($r^2$=0.64). Two sQTLs were also identified, jade family PHD finger 2 (*Jade2*) in the NAcc and BLA and *Col23a1* in the PrL. As with the eQTLs, these sQTLs are not likely to affect heroin escalation behavior as low LD ($r^2$=0.63 ) was observed for both relationships.

*Total consumption:* Total heroin consumed across training was associated with a locus on chromosome 11 (Fig 5a) that contained 13 genes. Several significant causal coding variants were identified in strong LD with this locus, and included bromodomain and WD repeat domain containing 1 (*Brwd1*; $r^2$=0.99), Purkinje

cell protein 4 (*Pcp4*; $r^2$=0.99) and two different amino acid coding variants for SH3 domain binding glutamate-rich protein (*Sh3bgr:* Glu169Lys, $r^2$=0.99; Glu177Lys, $r^2$=0.99). *Sh3bgr* was also an eQTL in high LD ($r^2$=0.99 for all) with the peak SNP in the whole brain, and more specifically in the BLA, NAcc, PrL, LHb and OFC. Two additional eQTLs in high LD with the locus were identified as *Pcp4* (PrL, $r^2$=0.88) and N-acetylneuraminate synthase (*NaN*; IL, $r^2$=0.99). *NaN* was also an sQTL with high LD to the loci and found in several brain regions (whole brain, $r^2$=0.99; BLA, $r^2$=0.99; LHb, $r^2$=0.99; OFC, $r^2$=0.99; NAcc, $r^2$=0.99; PrL (SNP 11:35477590), $r^2$=0.99; PrL (SNP 11:35093717), $r^2$=1.00). Lastly, there was a very strong sQTL for guided entry of tail-anchored proteins factor 1 (*Get1*; $r^2$=0.99) in the IL for this locus. Heroin consumption is likely mediated, in part, by the heritable expression of a combination of these genes. Additionally, PheWAS analysis showed that OUD vulnerability (Vulnerable vs Intermediate/resilient cluster) was significantly associated with the top SNP for this locus, ETS proto-oncogene 2 (*Ets2*). *Ets2* is comprised of three different amino acid coding variants (Fig 5b), and OUD cluster composition differs between these variants ($x^2(1, 853)=18.12$, $p<0.0001$; Fig 5c). Specifically, it appears that having the AA and AT variant yields relatively equal risk of OUD propensity, but the TT variant results in greater OUD resiliency. These data suggest that differences in the coding sequence for *Ets2* likely affects overall OUD vulnerability versus resiliency.

*Break point:* A final significant association on Chromosome 19 was observed for progressive ratio break point (Fig 6a). This locus contained 4 genes and one causal coding variant, prohibitin 1 like 2 (*Phb1l2*), which was in perfect LD with the top SNP ($r^2$=1.0). An eQTL with perfect LD with the locus, matrix metallopeptidase 15 (*Mmp15*; $r^2$=1.0) was also identified in the whole brain. An additional eQTL (solute carrier family 38 member 7, *Slc38a7*) and sQTL (casein kinase 1 alpha 2, *Csnk2a2*) were identified in the BLA, however, the relationship between these genes and the locus are modest ($r^2$=0.65 for both) rendering them unlikely to affect heroin break point behavior. PheWAS analysis showed that a perfect relationship existed between the top SNP and the OUD vulnerable cluster (Vulnerable vs

Intermediate/resilient clusters; $r^2$=1.0), and cluster composition differed between the two amino acid SNP coding variants ($x^2$(1, 864)=18.91, p<0.0001; Fig 6b,c). While there is a small bias toward the AA variant conferring OUD resiliency, the minor variant, AC, strongly coincides with OUD vulnerability.

**Discussion**

The economic toll on society for OUD totals over one trillion dollars annually [36]. Furthermore, over 1 million individuals seek treatment for OUD every year [37], posing a necessity to better understand the biological traits contributing to OUD vulnerability. Using a rat line emulating within species genetic and phenotypic variability [13] akin to humans, we assessed genetic heritability and variants 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 heritable traits associated with OUD. Both heroin consumption and initial refraining behavior (extinction burst) exhibited heritability, indicating aspects of heroin consumption and compulsivity are heritable and warrant consideration during treatment. However, behaviors following prolonged heroin use and forced abstinence, such as cued reinstatement, did not exhibit heritability and are likely being mediated by the cascade of neuroplastic adaptations that occur following heroin [38, 39].

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 [40]. Following opioid use, both humans [41, 42] and rats [43] demonstrate opioid-induced hyperalgesia in response to a painful stimuli, a finding replicated in these data [16]. While heritability factors associated with hyperalgesia are unknown, epigenetic modulating of this behavior in a rodent model has been investigated [44]. 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 [16, 45-49] and humans [50], and is a heritable behavior [21, 22]. 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, several genes were identified as coding variants, or as being functionally affected by the lead SNP (i.e., eQTL and sQTLs).

<u>Baseline analgesic threshold prior to heroin experience:</u> Genetic variants on two separate chromosomes were found for analgesic threshold under baseline conditions prior to heroin experience. *Large1* was both a causal coding variant and an eQTL for the peak SNP, however, the involvement of this gene is analgesia is currently unknown. Alterations in splicing patterns of both *Tom1* in the PrL and *Mt3* in the BLA are likely to contribute to basal analgesia levels, though neither has been directly linked to pain processing. *Mt3* is a brain zinc binding protein involved in several metabolic processes, and has been implicated in cellular dysfunction of diseases including Alzheimer and Huntington disease [51]. *Tom1* is an integral component of microglia function [52] , which in turn mediates neuropathic pain [53], but has yet to be implicated in acute painful stimuli.

On chromosome 2, two genes that operate in tandem with one another were also associated with pre-heroin experience baseline analgesia. The top SNP for this chromosome affects splicing patterns in *Selenop* which encodes for a selenoprotein. This protein is secreted by the liver and acts to deliver selenium, a trace element necessary for proper brain function [54], via the bloodstream to regions including the brain. Selenium has been implicated in mediating dopamine release and transmission within the substantia nigra [55, 56] and the NAc [57]. The presence of *Selenop* on NAc 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 [57]. *Ccdc152*, a causal coding variant gene on chromosome 2, is encoded within the *Selenop* gene, and acts to attenuate *Selenop* levels via post-translational modification [58]. Though the periaqueductal gray is the predominant brain nuclei processing pain signaling [59], striatal dopamine signaling has recently been implicated in affecting pain processing and perception [60, 61]. The association between *Selenop* and *Ccdc152* 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.

<u>Escalation of heroin intake across the entire session:</u> While no causal coding variants, eQTLs and sQTLs were association with escalation of heroin-taking behavior across the entire session, several genes correlate with the top SNP and may be functionally relevant to this behavior with increased sample size. For example, *Adamts2*, an extracellular matrix proteinase [62] localized within the mesolimbic and mesocortical regions within the brain [63], is a biomarker for heavy tobacco smoking and is downregulated following long-term morphine exposure and withdrawal [64]. Though further investigation is required, *Adamts2* may also serve as a biomarker for OUD consumption vulnerability.

<u>Heroin consumption:</u> Several relevant genes were associated with total consumption of heroin across all training sessions. The peak SNP, *Ets2*, is a ubiquitous transcription factor that is found, among other cells,

in microglia [65, 66]. Microglia, the resident immune cells in the brain, mediate several forms of synaptic plasticity [67], including those associated with OUD [68] such as behavioral tolerance [69], conditioned place preferences [70], and self-administration behavior [71, 72]. This regulation is due, in part, to degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) [73] resulting in opioid-induced synaptic remodeling [39]. *Ets2* promotes MMP induced ECM degradation [65], suggesting microglia *Ets2* as a promising candidate gene underlying OUD. Furthermore, OUD phenotypic vulnerability was associated with *Ets2* expression, strengthening the implication of *Ets2* as a promising biomarker of OUD propensity.

In addition to *Ets2* independently affecting heroin consumption, it also functionally regulates the expression of *PCP4* in the PrL, a critical component of drug-reward circuitry and plasticity [74, 75]. *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 NAc 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 cortical to NAc transmission as a consequence of sustained heroin use. While the functional association between the other identified e/sQTLs are not known for OUD, the *DSCAM* gene is in close proximity and highly correlated with *Ets2*. *DSCAM*, a neuronal cell adhesion molecule, regulates cellular connectivity [79] and glutamatergic plasticity associated with learning [80, 81]. Glutamate-mediated neuroplasticity occurs following heroin experience, and is considered a driving force behind relapse vulnerability [39]. *DSCAM* is necessary for tasks requiring memory formation [82, 83], including those associated with rewarded behaviors [83], and is upregulated in the hippocampus following a learned memory task [81]. Furthermore, human GWAS identified *DSCAM* acts as a biomarker for cigarette cessation success [84], and based on these data warrants further investigation for OUD.

Break point: The peak SNP for break point achieved during the progressive ratio test is unknown, however, it is in perfect LD with a causal coding variant for, *Phb1l2*, suggesting that this gene is likely a main contributor to the functional consequences of this locus. *Phb1l2* is localized to both neuronal and non-neural cells types in the central nervous system and helps to maintain mitochondria respiration by attenuating reactive oxygen species which contribute to cellular dysfunction [85]. Chronic morphine administration disrupts mitochondrial respiration and restoring proper functioning attenuates opioid withdrawal [86]. Additionally, *Phb1*, an analogue of *Phb1l2*, has been implicated in mediating glutamatergic and dopaminergic neural transmission and becomes dysregulated in several disorders, including alcohol use disorder [85]. It is thus likely that opioid use results in dysregulation of *Phb1l2* functioning and through glutamate and dopamine dependent processes affects motivation to obtain the drug. Additionally, *MMP15* was identified as an eQTL in the entire brain and was in perfect LD with the top SNP, suggesting *MMP15* activity is functionally dependent on the top SNP. MMP15 activates MMP2 [87], likely contributing to NAcc cell-specific regulation of extinction, or refraining from seeking, behavior following heroin self-administration [88]. It is possible that OUD vulnerable rats lack maximum expression of this gene, resulting in attenuated MMP15-induced MMP2 expression and overall higher break point levels. In support, PheWAS analysis identified this locus as associated with OUD vulnerability, with the top SNP containing two allelic coding variants. The minor variant is significantly biased toward the OUD vulnerable phenotype and higher break points. Together, these data suggest that OUD phenotypic vulnerability is likely associated with the peak SNP directly affecting the functioning and expression of *Phb1l2* and *MMP15* activity, both of which may serve as strong biomarkers for OUD propensity and warrant further investigation both pre-clinically and clinically.

**Conclusion and Future Directions**

We used a rodent model to identify several heritable traits and genetic variants associated with OUD. Unlike GWAS performed in humans, we were able to identify genetic variants associated with distinct

OUD behaviors that contribute toward diagnosis. Several of the identified genes are either biomarkers or have known functional regulation of SUD, but warrant further investigation for OUD. Furthermore, using an established rodent model that characterizes rats using a non-linear compilation of behaviors across the SUD phases to confer OUD propensity, we identified genetic variants for heroin consumption and motivation that were associated specifically with OUD vulnerability. These findings strengthen the translational utility of the behavioral model, and can help to assess genetic variants and biomarkers associated with OUD vulnerability versus resiliency. However, complementing human OUD [7], phenotypic variance can only be partially explained by genetic variability, and other factors such as epigenetic mechanisms [89] likely contribute to OUD behaviors. Furthermore, both preclinical and clinical GWAS analysis focusing on genetic variants associated with OUD resiliency, a neurobiological active process [90, 91], should be considered in future analyses.

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**Conflict of Interest**

The authors declare no conflict of interest.

**References**


1. Wide-ranging online data for epidemiologic research (WONDER). Atlanta, GA: CDC, National Center for Health Statistics. 2020. http://wonder.cdc.gov/
2. Chang, H.Y., et al., *Healthcare costs and utilization associated with high-risk prescription opioid use: a retrospective cohort study.* BMC Med, 2018. **16**(1): p. 69.
3. Jones, C.M., et al., *Vital Signs: Demographic and Substance Use Trends Among Heroin Users - United States, 2002-2013.* MMWR Morb Mortal Wkly Rep, 2015. **64**(26): p. 719-25.
4. Association, A.P., *Diagnostic and statistical manual of mental disorders*. 5 ed. 2013.
5. Carroll, K.M., *The profound heterogeneity of substance use disorders: Implications for treatment development.* Curr Dir Psychol Sci, 2021. **30**(4): p. 358-364.
6. Berrettini, W., *A brief review of the genetics and pharmacogenetics of opioid use disorders.* Dialogues Clin Neurosci, 2017. **19**(3): p. 229-236.
7. Crist, R.C., B.C. Reiner, and W.H. Berrettini, *A review of opioid addiction genetics.* Curr Opin Psychol, 2019. **27**: p. 31-35.
8. Cheng, Z., et al., *Genome-wide Association Study Identifies a Regulatory Variant of RGMA Associated With Opioid Dependence in European Americans.* Biol Psychiatry, 2018. **84**(10): p. 762-770.
9. Gelernter, J., et al., *Genome-wide association study of opioid dependence: multiple associations mapped to calcium and potassium pathways.* Biol Psychiatry, 2014. **76**(1): p. 66-74.
10. Nelson, E.C., et al., *Evidence of CNIH3 involvement in opioid dependence.* Mol Psychiatry, 2016. **21**(5): p. 608-14.
11. Sanchez-Roige, S., et al., *Genome-wide association study of problematic opioid prescription use in 132,113 23andMe research participants of European ancestry.* Mol Psychiatry, 2021. **26**(11): p. 6209-6217.
12. Sanchez-Roige, S. and A.A. Palmer, *Emerging phenotyping strategies will advance our understanding of psychiatric genetics.* Nat Neurosci, 2020. **23**(4): p. 475-480.
13. Consortium, S., et al., *SNP and haplotype mapping for genetic analysis in the rat.* Nat Genet, 2008. **40**(5): p. 560-6.
14. Johannesson, M., et al., *A resource for the simultaneous high-resolution mapping of multiple quantitative trait loci in rats: the NIH heterogeneous stock.* Genome Res, 2009. **19**(1): p. 150-8.
15. Hansen, C. and K. Spuhler, *Development of the National Institutes of Health genetically heterogeneous rat stock.* Alcohol Clin Exp Res, 1984. **8**(5): p. 477-9.
16. Kuhn, B.N., et al., *Novelty-induced locomotor behavior predicts heroin addiction vulnerability in male, but not female, rats.* Psychopharmacology (Berl), 2022. **239**(11): p. 3605-3620.
17. Kallupi, M., et al., *Nociceptin attenuates the escalation of oxycodone self-administration by normalizing CeA-GABA transmission in highly addicted rats.* Proc Natl Acad Sci U S A, 2020. **117**(4): p. 2140-2148.
18. Deal, A., et al., *Early life stress induces hyperactivity but not increased anxiety-like behavior or ethanol drinking in outbred heterogeneous stock rats.* Alcohol, 2021. **91**: p. 41-51.
19. Wang, T., et al., *Social and anxiety-like behaviors contribute to nicotine self-administration in adolescent outbred rats.* Sci Rep, 2018. **8**(1): p. 18069.
20. King, C.P., et al., *Sensitivity to food and cocaine cues are independent traits in a large sample of heterogeneous stock rats.* Sci Rep, 2021. **11**(1): p. 2223.
21. Chitre, A.S., et al., *Genome-wide association study in a rat model of temperament identifies multiple loci for exploratory locomotion and anxiety-like traits.* Front Genet, 2022. **13**: p. 1003074.

22. Gunturkun, M.H., et al., *Genome-Wide Association Study on Three Behaviors Tested in an Open Field in Heterogeneous Stock Rats Identifies Multiple Loci Implicated in Psychiatric Disorders.* Front Psychiatry, 2022. **13**: p. 790566.
23. Zhou, J.L., et al., *Single-nucleus genomics in outbred rats with divergent cocaine addiction-like behaviors reveals changes in gene amygdala GABAergic inhibition.* Nat Neurosci, 2023.
24. Kuhn, B.N., et al., *A multi-symptomatic model of heroin use disorder in rats reveals distinct behavioral profiles and neuronal correlates of heroin vulnerability versus resiliency.* BioRxiv, 2024.
25. Richardson, N.R. and D.C. Roberts, *Progressive ratio schedules in drug self-administration studies in rats: a method to evaluate reinforcing efficacy.* J Neurosci Methods, 1996. **66**(1): p. 1-11.
26. Parker, C.C., et al., *Genome-wide association study of behavioral, physiological and gene expression traits in outbred CFW mice.* Nat Genet, 2016. **48**(8): p. 919-26.
27. Gileta, A.F., et al., *Adapting Genotyping-by-Sequencing and Variant Calling for Heterogeneous Stock Rats.* G3 (Bethesda), 2020. **10**(7): p. 2195-2205.
28. Chang, C.C., et al., *Second-generation PLINK: rising to the challenge of larger and richer datasets.* Gigascience, 2015. **4**: p. 7.
29. Allen, C., et al., *Network-Based Discovery of Opioid Use Vulnerability in Rats Using the Bayesian Stochastic Block Model.* Front Psychiatry, 2021. **12**: p. 745468.
30. Yang, J., et al., *GCTA: a tool for genome-wide complex trait analysis.* Am J Hum Genet, 2011. **88**(1): p. 76-82.
31. Cheng, R., et al., *Practical considerations regarding the use of genotype and pedigree data to model relatedness in the context of genome-wide association studies.* G3 (Bethesda), 2013. **3**(10): p. 1861-7.
32. Cheng, R. and A.A. Palmer, *A simulation study of permutation, bootstrap, and gene dropping for assessing statistical significance in the case of unequal relatedness.* Genetics, 2013. **193**(3): p. 1015-8.
33. Chitre, A.S., et al., *Genome-Wide Association Study in 3,173 Outbred Rats Identifies Multiple Loci for Body Weight, Adiposity, and Fasting Glucose.* Obesity (Silver Spring), 2020. **28**(10): p. 1964-1973.
34. Cingolani, P., et al., *A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3.* Fly (Austin), 2012. **6**(2): p. 80-92.
35. Taylor-Weiner, A., et al., *Scaling computational genomics to millions of individuals with GPUs.* Genome Biol, 2019. **20**(1): p. 228.
36. Florence, C., F. Luo, and K. Rice, *The economic burden of opioid use disorder and fatal opioid overdose in the United States, 2017.* Drug Alcohol Depend, 2021. **218**: p. 108350.
37. National Survey of Substance Abuse Treatment Services (N-SSATS): 2020. Data on Substance Abuse Treatment Facilities. 2021. https://www.samhsa.gov/data/sites/default/files/reports/rpt35313/2020_NSSATS_FINAL.pdf
38. Mazei-Robison, M.S. and E.J. Nestler, *Opiate-induced molecular and cellular plasticity of ventral tegmental area and locus coeruleus catecholamine neurons.* Cold Spring Harb Perspect Med, 2012. **2**(7): p. a012070.
39. Kruyer, A., V.C. Chioma, and P.W. Kalivas, *The Opioid-Addicted Tetrapartite Synapse.* Biol Psychiatry, 2020. **87**(1): p. 34-43.
40. Angst, M.S., et al., *Pain sensitivity and opioid analgesia: a pharmacogenomic twin study.* Pain, 2012. **153**(7): p. 1397-1409.
41. Ren, Z.Y., et al., *Abnormal pain response in pain-sensitive opiate addicts after prolonged abstinence predicts increased drug craving.* Psychopharmacology (Berl), 2009. **204**(3): p. 423-9.

42. Carcoba, L.M., et al., *Negative affect heightens opiate withdrawal-induced hyperalgesia in heroin dependent individuals.* J Addict Dis, 2011. **30**(3): p. 258-70.
43. Marchette, R.C.N., et al., *kappa-Opioid receptor antagonism reverses heroin withdrawal-induced hyperalgesia in male and female rats.* Neurobiol Stress, 2021. **14**: p. 100325.
44. Liang, D.Y., X. Li, and J.D. Clark, *Epigenetic regulation of opioid-induced hyperalgesia, dependence, and tolerance in mice.* J Pain, 2013. **14**(1): p. 36-47.
45. Ambrosio, E., S.R. Goldberg, and G.I. Elmer, *Behavior genetic investigation of the relationship between spontaneous locomotor activity and the acquisition of morphine self-administration behavior.* Behav Pharmacol, 1995. **6**(3): p. 229-237.
46. Lamarque, S., K. Taghzouti, and H. Simon, *Chronic treatment with Delta(9)-tetrahydrocannabinol enhances the locomotor response to amphetamine and heroin. Implications for vulnerability to drug addiction.* Neuropharmacology, 2001. **41**(1): p. 118-29.
47. Xigeng, Z., et al., *Social crowding sensitizes high-responding rats to psychomotor-stimulant effects of morphine.* Pharmacol Biochem Behav, 2004. **79**(2): p. 213-8.
48. Swain, Y., et al., *Locomotor activity does not predict individual differences in morphine self-administration in rats.* Pharmacol Biochem Behav, 2018. **166**: p. 48-56.
49. Chang, S.E., L.D. Krueger, and S.B. Flagel, *Investigating individual differences in opioid-taking and opioid-seeking behavior in male rats.* Psychopharmacology (Berl), 2022.
50. Wingo, T., et al., *Novelty Seeking and Drug Addiction in Humans and Animals: From Behavior to Molecules.* J Neuroimmune Pharmacol, 2016. **11**(3): p. 456-70.
51. Koh, J.Y. and S.J. Lee, *Metallothionein-3 as a multifunctional player in the control of cellular processes and diseases.* Mol Brain, 2020. **13**(1): p. 116.
52. Wang, H., et al., *SENP1 modulates chronic intermittent hypoxia-induced inflammation of microglia and neuronal injury by inhibiting TOM1 pathway.* Int Immunopharmacol, 2023. **119**: p. 110230.
53. Inoue, K. and M. Tsuda, *Microglia in neuropathic pain: cellular and molecular mechanisms and therapeutic potential.* Nat Rev Neurosci, 2018. **19**(3): p. 138-152.
54. Pillai, R., J.H. Uyehara-Lock, and F.P. Bellinger, *Selenium and selenoprotein function in brain disorders.* IUBMB Life, 2014. **66**(4): p. 229-39.
55. Bellinger, F.P., et al., *Changes in selenoprotein P in substantia nigra and putamen in Parkinson's disease.* J Parkinsons Dis, 2012. **2**(2): p. 115-26.
56. Romero-Ramos, M., et al., *Low selenium diet induces tyrosine hydroxylase enzyme in nigrostriatal system of the rat.* Brain Res Mol Brain Res, 2000. **84**(1-2): p. 7-16.
57. Torres, D.J., et al., *Selenoprotein P Modulates Methamphetamine Enhancement of Vesicular Dopamine Release in Mouse Nucleus Accumbens Via Dopamine D2 Receptors.* Front Neurosci, 2021. **15**: p. 631825.
58. Mita, Y., et al., *Identification of a novel endogenous long non-coding RNA that inhibits selenoprotein P translation.* Nucleic Acids Res, 2021. **49**(12): p. 6893-6907.
59. Averitt, D.L., et al., *Neuronal and glial factors contributing to sex differences in opioid modulation of pain.* Neuropsychopharmacology, 2019. **44**(1): p. 155-165.
60. Taylor, A.M.W., et al., *Mesolimbic dopamine signaling in acute and chronic pain: implications for motivation, analgesia, and addiction.* Pain, 2016. **157**(6): p. 1194-1198.
61. Harris, H.N. and Y.B. Peng, *Evidence and explanation for the involvement of the nucleus accumbens in pain processing.* Neural Regen Res, 2020. **15**(4): p. 597-605.
62. Shiomi, T., et al., *Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases.* Pathol Int, 2010. **60**(7): p. 477-96.
63. Ruso-Julve, F., et al., *Dopaminergic control of ADAMTS2 expression through cAMP/CREB and ERK: molecular effects of antipsychotics.* Transl Psychiatry, 2019. **9**(1): p. 306.

64. Thiagarajan, S.K., et al., *Receptor-Mediated AKT/PI3K Signalling and Behavioural Alterations in Zebrafish Larvae Reveal Association between Schizophrenia and Opioid Use Disorder.* Int J Mol Sci, 2022. **23**(9).
65. Trojanowska, M., *Ets factors and regulation of the extracellular matrix.* Oncogene, 2000. **19**(55): p. 6464-71.
66. Lehmann, M.L., et al., *Decoding microglia responses to psychosocial stress reveals blood-brain barrier breakdown that may drive stress susceptibility.* Sci Rep, 2018. **8**(1): p. 11240.
67. Cornell, J., et al., *Microglia regulation of synaptic plasticity and learning and memory.* Neural Regen Res, 2022. **17**(4): p. 705-716.
68. Green, J.M., M.H. Sundman, and Y.H. Chou, *Opioid-induced microglia reactivity modulates opioid reward, analgesia, and behavior.* Neurosci Biobehav Rev, 2022. **135**: p. 104544.
69. Eidson, L.N., et al., *Toll-like Receptor 4 Mediates Morphine-Induced Neuroinflammation and Tolerance via Soluble Tumor Necrosis Factor Signaling.* Neuropsychopharmacology, 2017. **42**(3): p. 661-670.
70. Rivera, P.D., et al., *Removal of microglial-specific MyD88 signaling alters dentate gyrus doublecortin and enhances opioid addiction-like behaviors.* Brain Behav Immun, 2019. **76**: p. 104-115.
71. Lacagnina, M.J., P.D. Rivera, and S.D. Bilbo, *Glial and Neuroimmune Mechanisms as Critical Modulators of Drug Use and Abuse.* Neuropsychopharmacology, 2017. **42**(1): p. 156-177.
72. Cannella, N., et al., *Long-access heroin self-administration induces region specific reduction of grey matter volume and microglia reactivity in the rat.* Brain Behav Immun, 2024.
73. Crapser, J.D., et al., *Microglia as hackers of the matrix: sculpting synapses and the extracellular space.* Cell Mol Immunol, 2021. **18**(11): p. 2472-2488.
74. Hearing, M., *Prefrontal-accumbens opioid plasticity: Implications for relapse and dependence.* Pharmacol Res, 2019. **139**: p. 158-165.
75. Kalivas, P.W. and N.D. Volkow, *The neural basis of addiction: a pathology of motivation and choice.* Am J Psychiatry, 2005. **162**(8): p. 1403-13.
76. Harashima, S., et al., *Purkinje cell protein 4 positively regulates neurite outgrowth and neurotransmitter release.* J Neurosci Res, 2011. **89**(10): p. 1519-30.
77. Iwamoto, K., et al., *Decreased expression of NEFH and PCP4/PEP19 in the prefrontal cortex of alcoholics.* Neurosci Res, 2004. **49**(4): p. 379-85.
78. Mychasiuk, R., et al., *Persistent gene expression changes in NAc, mPFC, and OFC associated with previous nicotine or amphetamine exposure.* Behav Brain Res, 2013. **256**: p. 655-61.
79. Ronn, L.C., V. Berezin, and E. Bock, *The neural cell adhesion molecule in synaptic plasticity and ageing.* Int J Dev Neurosci, 2000. **18**(2-3): p. 193-9.
80. Li, H.L., et al., *Dscam mediates remodeling of glutamate receptors in Aplysia during de novo and learning-related synapse formation.* Neuron, 2009. **61**(4): p. 527-40.
81. Stachowicz, K., et al., *Changes in working memory induced by lipopolysaccharide administration in mice are associated with metabotropic glutamate receptors 5 and contrast with changes induced by cyclooxygenase-2: Involvement of postsynaptic density protein 95 and down syndrome cell adhesion molecule.* Neuropeptides, 2023. **100**: p. 102347.
82. Chen, B.E., et al., *The molecular diversity of Dscam is functionally required for neuronal wiring specificity in Drosophila.* Cell, 2006. **125**(3): p. 607-20.
83. Keene, A.C., et al., *Drosophila dorsal paired medial neurons provide a general mechanism for memory consolidation.* Curr Biol, 2006. **16**(15): p. 1524-30.
84. Uhl, G.R., et al., *Molecular genetics of successful smoking cessation: convergent genome-wide association study results.* Arch Gen Psychiatry, 2008. **65**(6): p. 683-93.

85. Bernstein, H.G., et al., *The many "Neurofaces" of Prohibitins 1 and 2: Crucial for the healthy brain, dysregulated in numerous brain disorders.* J Chem Neuroanat, 2023. **132**: p. 102321.
86. Kowalczyk, P., et al., *Mitochondrial Oxidative Stress-A Causative Factor and Therapeutic Target in Many Diseases.* Int J Mol Sci, 2021. **22**(24).
87. Wright, J.W. and J.W. Harding, *Contributions of matrix metalloproteinases to neural plasticity, habituation, associative learning and drug addiction.* Neural Plast, 2009. **2009**: p. 579382.
88. Chioma, V.C., et al., *Heroin Seeking and Extinction From Seeking Activate Matrix Metalloproteinases at Synapses on Distinct Subpopulations of Accumbens Cells.* Biol Psychiatry, 2021. **89**(10): p. 947-958.
89. Werner, C.T., et al., *Epigenetic Mechanisms in Drug Relapse.* Biol Psychiatry, 2021. **89**(4): p. 331-338.
90. Ersche, K.D., et al., *Brain networks underlying vulnerability and resilience to drug addiction.* Proc Natl Acad Sci U S A, 2020. **117**(26): p. 15253-15261.
91. Ersche, K.D., et al., *Meta-analysis of structural brain abnormalities associated with stimulant drug dependence and neuroimaging of addiction vulnerability and resilience.* Curr Opin Neurobiol, 2013. **23**(4): p. 615-24.

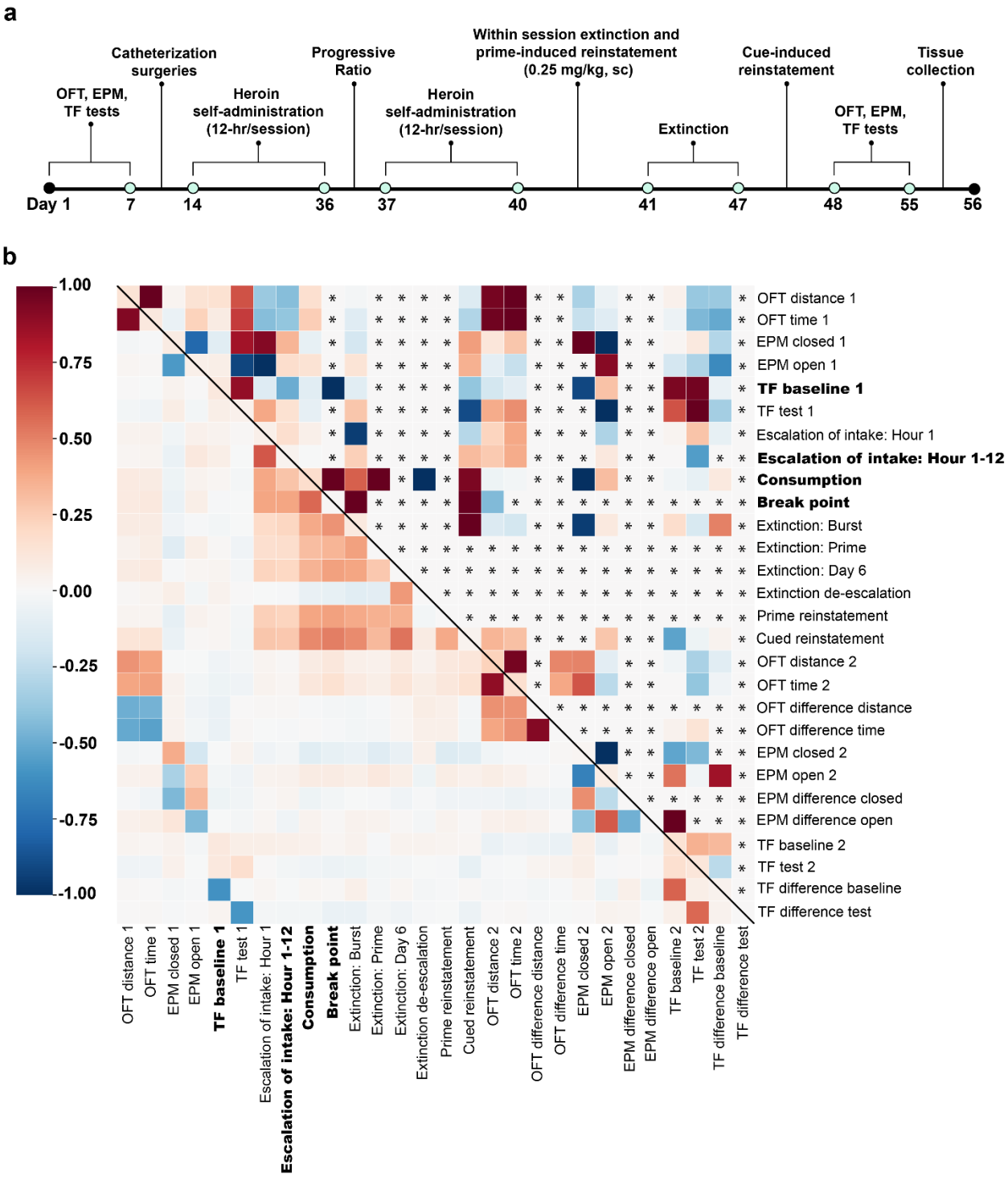



**Figure 1** Experimental timeline and correlational analyses. **a)** Rats underwent testing for stress- and anxiety-related behaviors (OFT: open field test; EPM: elevated-plus maze) and analgesic threshold (TF: tail flick) prior to jugular catheterization surgery, with tests repeated following heroine 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. The diagonal solid line intersects boxes showing the heritability of the trait. 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)

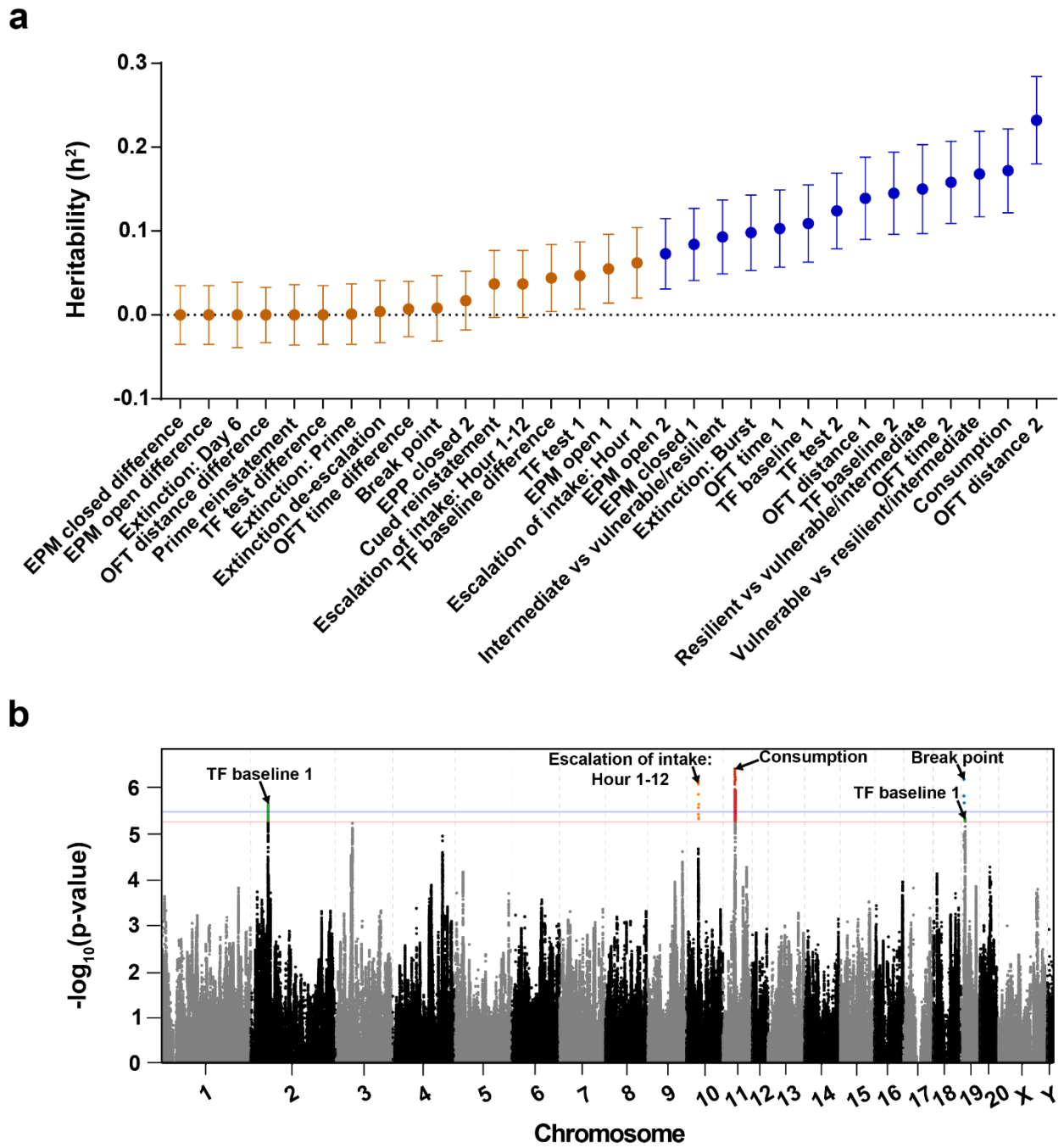
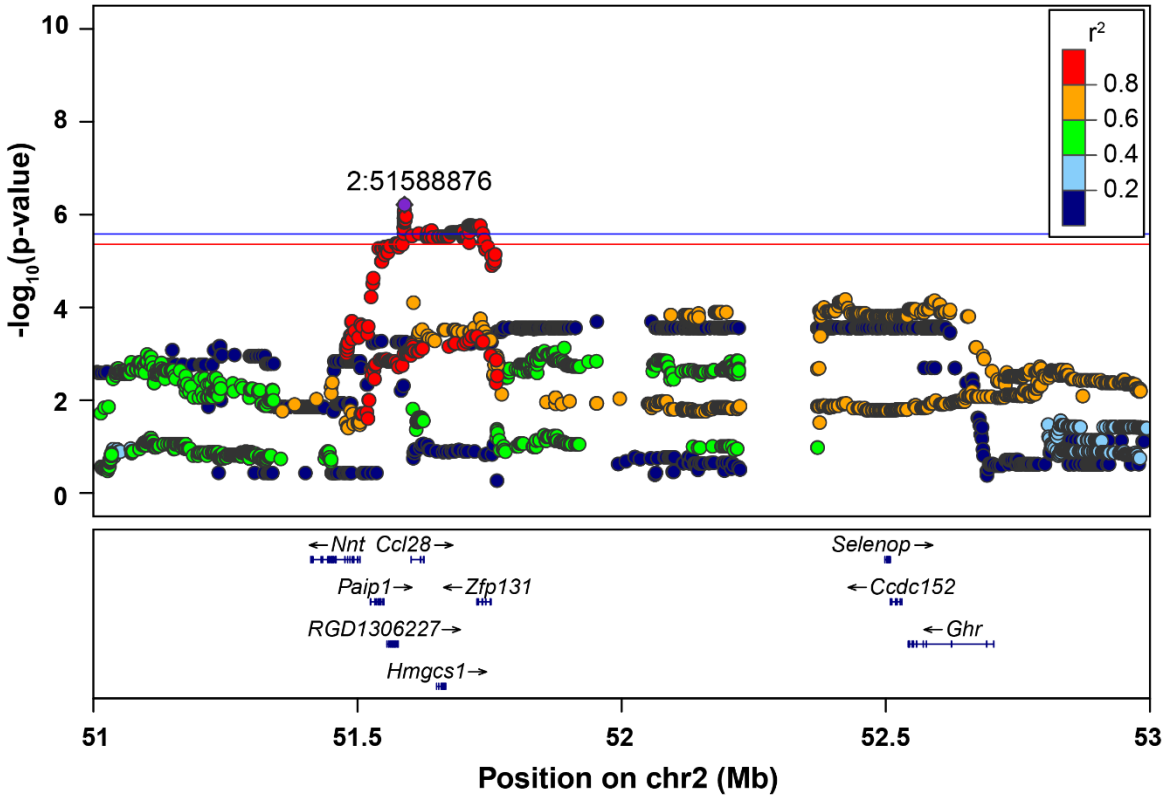



**Figure 2** Heritability and genome-wide association results. **a)** Heritability ($h^2$) measure ± SEM for all behavioral traits assessed. Significant $h^2$ measures ($p<0.05$) are indicated in blue, and non-significant traits are in orange. **b)** Porcupine plot for selected behavioral traits. Top 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. (n=874)

**a**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8
6
4
2
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Ccl28
Paip1
Zfp131
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Selenop
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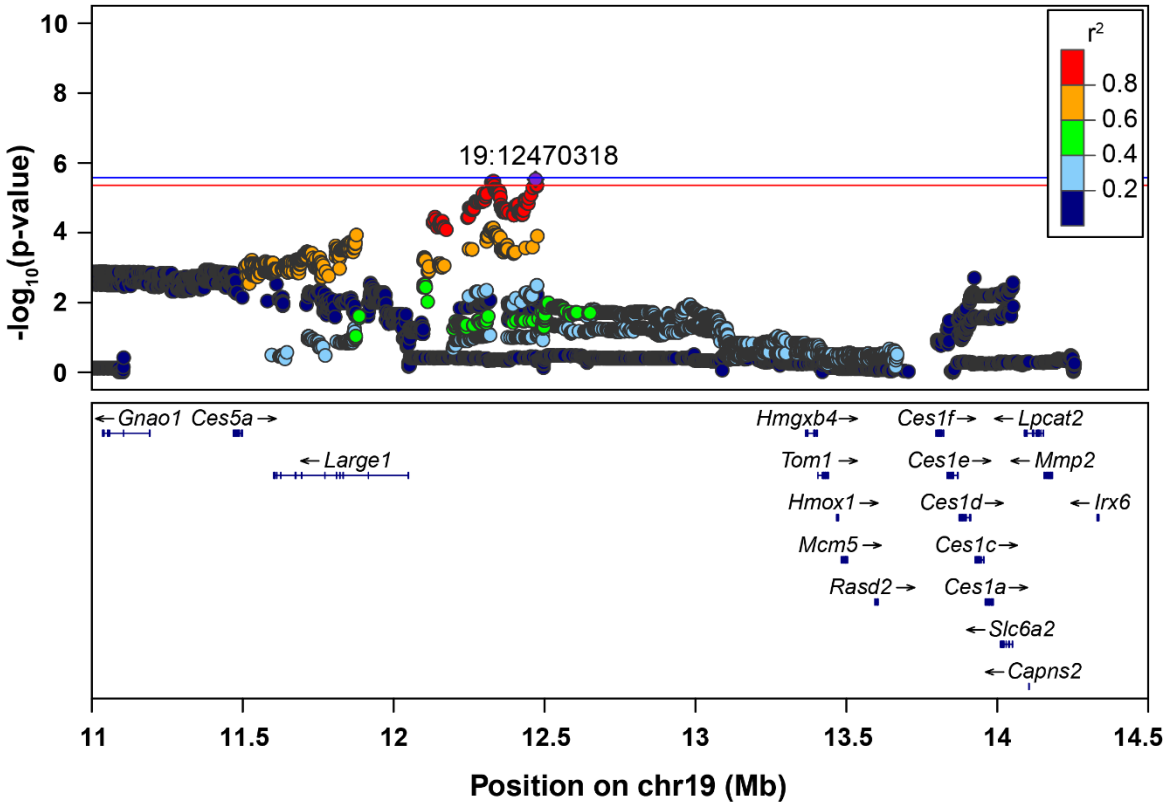
Ghr
51
51.5
52
52.5
53
Position on chr2 (Mb)

**b**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Large1
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Ces1f
Lpcat2
Tom1
Ces1e
Mmp2
Hmox1
Ces1d
Irx6
Mcm5
Ces1c
Rasd2
Ces1a
Slc6a2
Capns2
11
11.5
12
12.5
13
13.5
14
14.5
Position on chr19 (Mb)

**Figure 3** GWAS results for analgesic threshold during the TF baseline session prior to heroin experience. Regional association plots for **a)** chromosome 2 and **b)** 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. (n= 874)

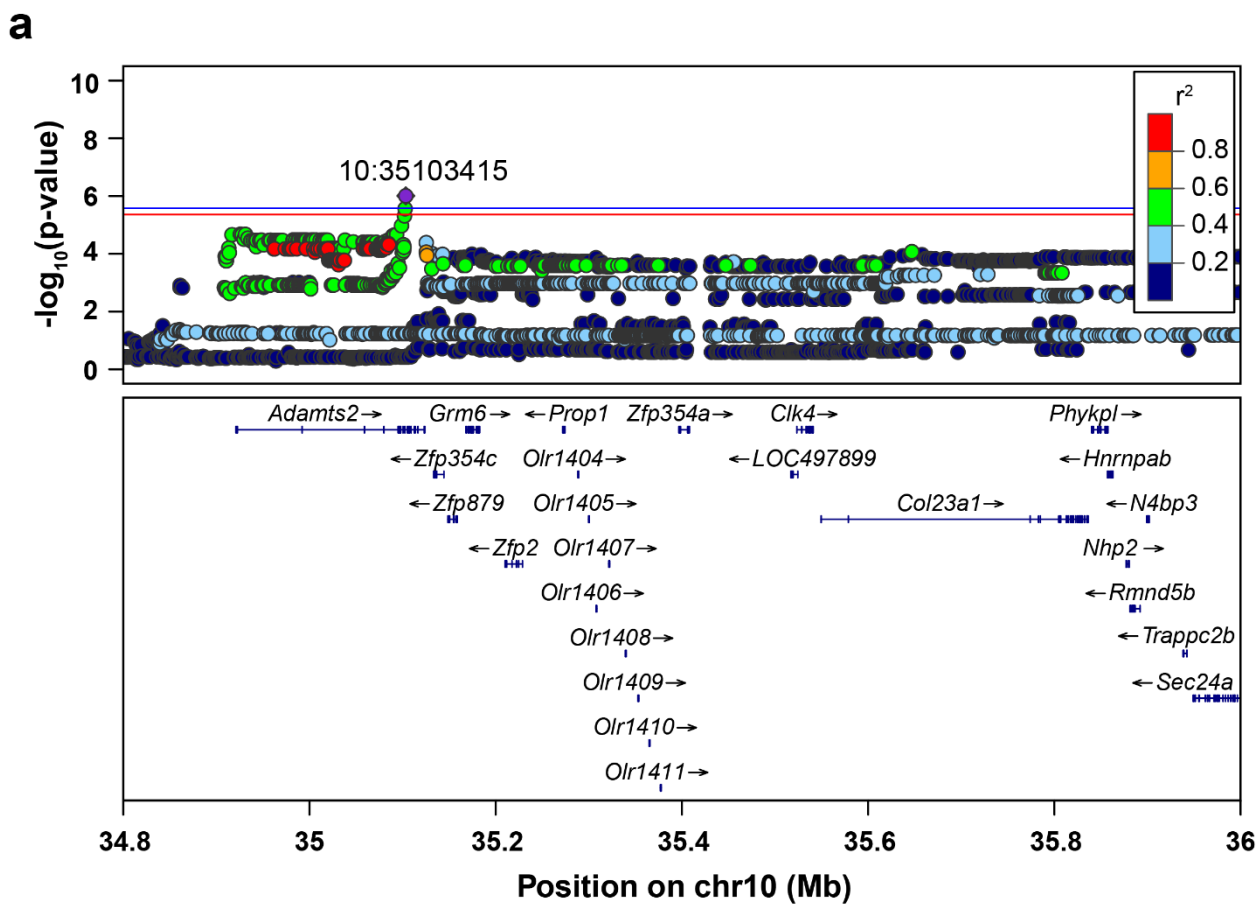
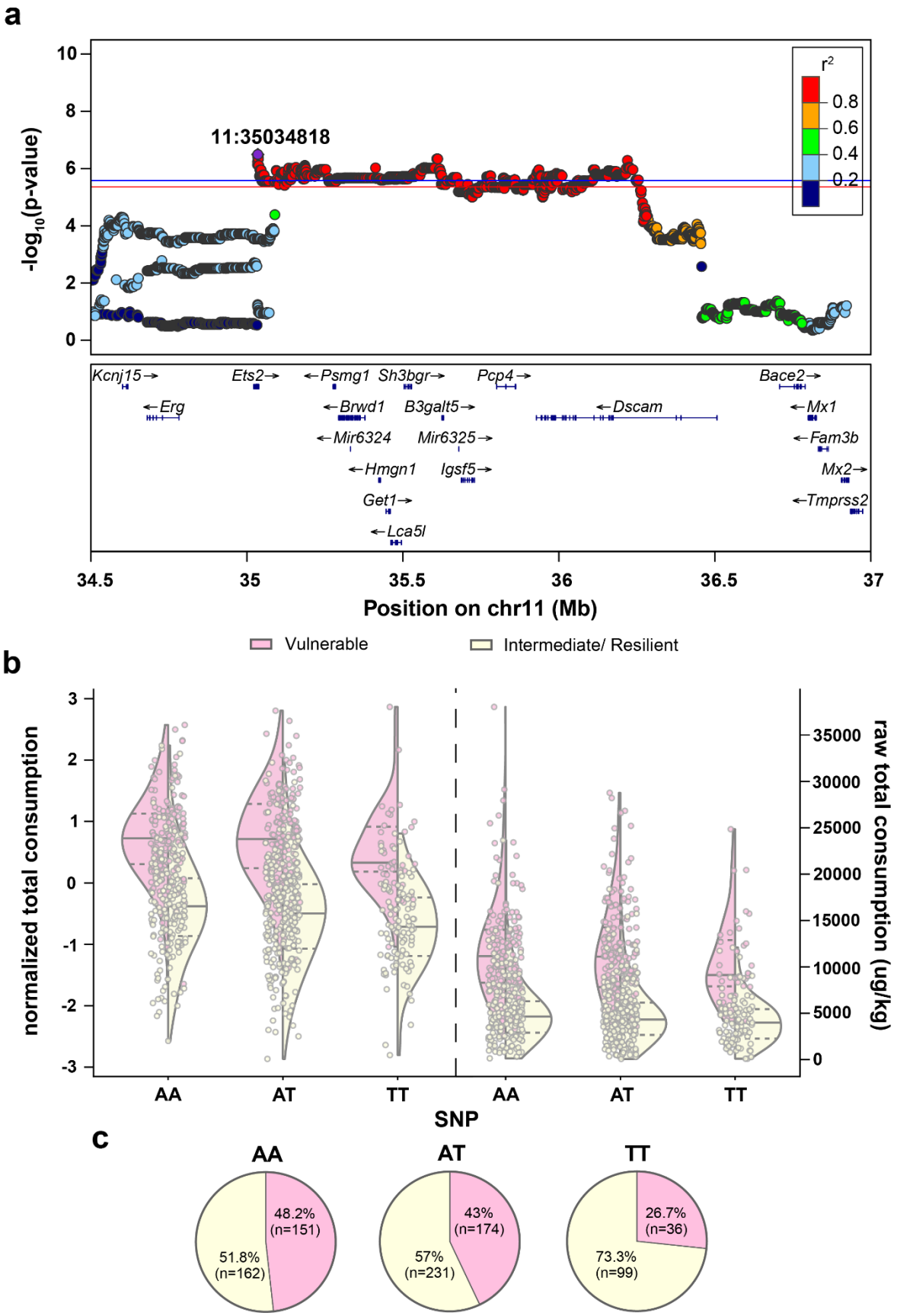



**Figure 4** GWAS results for escalation of heroin intake across the entire session. **a)** Regional association plot for chromosome 10, with the top SNP in purple and chromosome location indicated on the plot. Other SNPs are colored based on their linkage disequilibrium with the top SNP. Known genes of SNPs are indicated below the plots. (n=874)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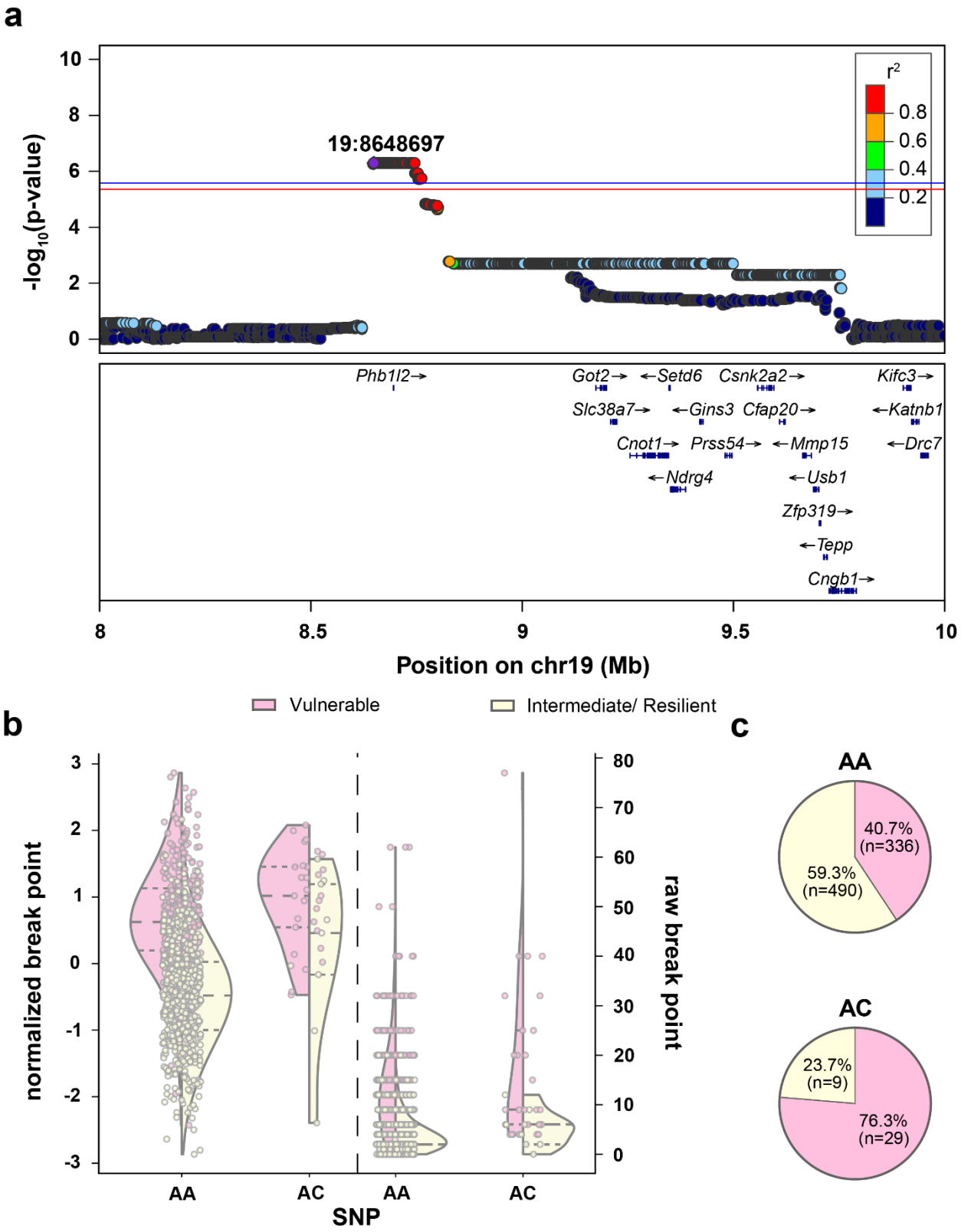
Pcp4
Bace2
Erg
Brwd1
B3galt5
Dscam
Mx1
Mir6324
Mir6325
Fam3b
Hmgn1
Igsf5
Mx2
Get1
Tmprss2
Lca5l
Position on chr11 (Mb)
b
Vulnerable
Intermediate/ Resilient
normalized total consumption
raw total consumption (ug/kg)
AA
AT
TT
SNP
c
48.2% (n=151)
51.8% (n=162)
43% (n=174)
57% (n=231)
26.7% (n=36)
73.3% (n=99)

**Figure 5** GWAS results for total heroin consumed across self-administration training. **a)** 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. **b)** Effect plots for total heroin consumption during training (left: normalized data; right: raw data) for OUD phenotypes (Vulnerable: pink; Intermediate/resilient: yellow) according to peak SNP allelic variant. Half of a violin plot for each phenotype is shown, with the solid line indicating the median value of the data and the dash lines representing the upper and lower interquartile range. **c)** Top SNP allelic variant OUD phenotype cluster composition different ($x^2(1, 853)=18.12$ , $p<0.0001$), with the minor allele associated with greater OUD resiliency. (n=853; Vulnerable, n= 361; Intermediate/resilient, n= 492)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Phb1l2
Got2
Setd6
Csnk2a2
Kifc3
Slc38a7
Gins3
Cfap20
Katnb1
Cnot1
Prss54
Mmp15
Drc7
Ndrg4
Usb1
Zfp319
Tepp
Cngb1
Position on chr19 (Mb)
b
Vulnerable
Intermediate/ Resilient
normalized break point
raw break point
SNP
AA
AC
AA
AC
c
AA
40.7% (n=336)
59.3% (n=490)
AC
23.7% (n=9)
76.3% (n=29)

**Figure 6** GWAS results for break point achieved during the progressive ratio test. **a)** 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. **b)** Effect plots showing break point achieved during the progressive ratio test (left: normalized data, right: raw data) for OUD vulnerable (pink) and OUD intermediate/resilient (yellow) rats in relation to the top SNP allelic variant. Half a violin plot is shown for each OUD phenotype, with the solid line indicating the median value of the data set, whereas the dashed lines represent the upper and lower interquartile range. **c)** OUD phenotypic cluster composition for both peak SNP allelic variants. Cluster composition between variants differed ($x^2(1, 864)=18.91$ , $p<0.0001$), with the minor allele heavily biased toward an OUD vulnerable phenotype. (n=864; Vulnerable, n= 345; Intermediate/resilient, n= 519)

| Trait | Description |
|---|---|
| *Prior to heroin experience* | |
| OFT distance 1 | Total distance travelled |
| OFT time 1 | Time spent moving |
| EPM closed 1 | Time spent in closed arms |
| EPM open 1 | Time spent in open arms |
| TF baseline 1 | Average latency of tail removal during baseline (i.e., saline) session |
| TF test 1 | Average latency of tail removal during test (i.e., heroin) session |
| *Heroin measures* | |
| Escalation of intake: Hour 1 | Average consumption (μg/kg) from hour 1 for sessions 1-3 subtracted from average hour 1 consumption for sessions 10-12 |
| Escalation of intake: Hour 1-12 | Average consumption (μg/kg) from entire session for sessions 1-3 subtracted from average total consumption for sessions 10-12 |
| Consumption | Cumulative heroin consumption (μg/kg) for sessions 1-12 |
| Break point | Break point (i.e., most active lever presses willing to expend to receive an infusion of heroin) during progressive ratio test |
| Extinction: Burst | Active lever presses during the first 2 hr of the within-session extinction-prime reinstatement test |
| Extinction: Prime | Active lever presses between hour 2-4 in the within-session extinction-prime reinstatement test |
| Extinction: Day 6 | Active lever presses made during extinction session 6 |
| Extinction de-escalation | Active lever presses during extinction session 1 subtracted from session 6 |
| Prime reinstatement | Active lever presses during prime reinstatement test |
| Cued reinstatement | Active lever presses during cued reinstatement test |
| *Following heroin experience* | |
| OFT distance 2 | Total distance travelled |
| OFT time 2 | Time spent moving |
| OFT difference distance | Total distance travelled during OFT 1 subtracted from OFT 2 |
| OFT time difference | Total time spent moving during OFT 1 subtracted from OFT 2 |
| EPM closed 2 | Time spent in closed arms |
| EPM open 2 | Time spent in open arms |
| EPM difference closed | Time spent in closed arm during EPM 1 subtracted from EPM 2 |
| EPM difference open | Time spent in open arm during EPM 1 subtracted from EPM 2 |
| TF baseline 2 | Average latency of tail removal during baseline (i.e., saline) session |
| TF test 2 | Average latency of tail removal during test (i.e., heroin) session |
| TF difference baseline | Average latency of tail removal during TF baseline 1 subtracted from TF baseline 2 |
| TF difference test | Average latency of tail removal during TF test 1 subtracted from TF test 2 |

**Supplemental Table 1** Description of behavioral traits assessed in GWAS. Traits included those measured both prior to and following heroin experience, as well as several behaviors associated with heroin taking, refraining and seeking.

**Behavioral trait correlations**

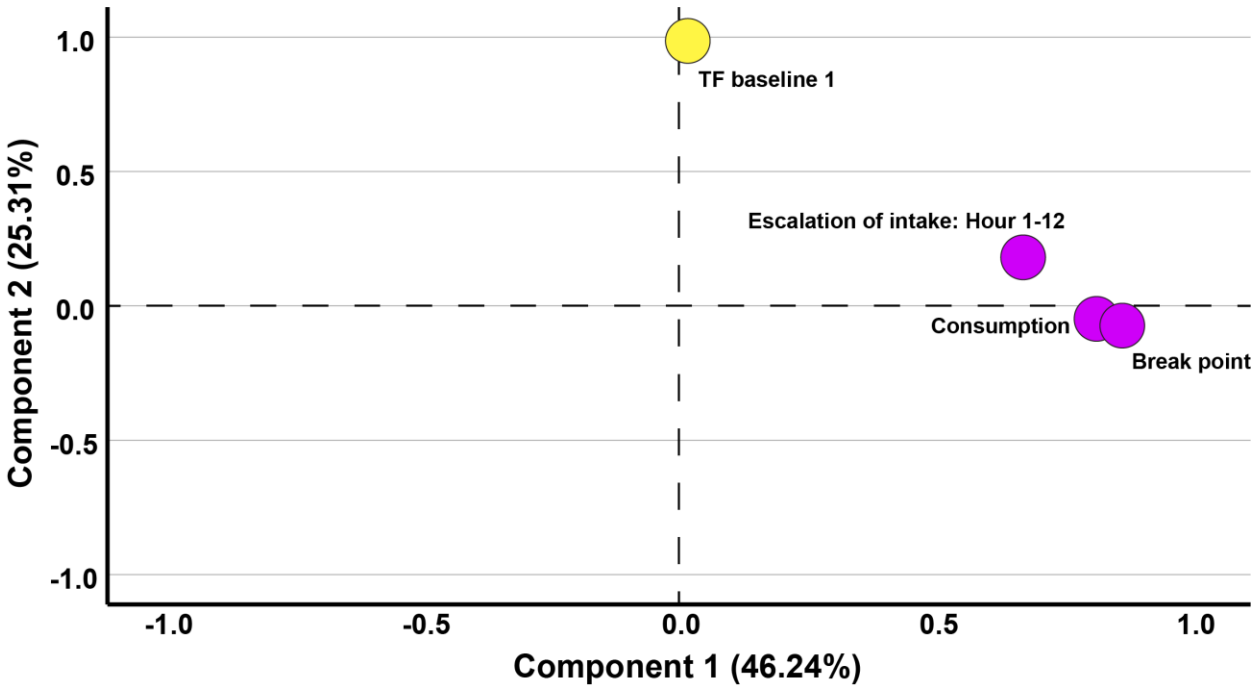
| Trait 1 | Trait2 | Spearman r | p-value | n |
|---|---|---|---|---|
| Escalation of intake: Hour 1 | Break point | 0.39 | $1.98 \times 10^{-33}$ | 874 |
| | Cued reinstatement | 0.28 | $1.26 \times 10^{-16}$ | 866 |
| | Escalation of intake: Hour 1-12 | 0.62 | $7.03 \times 10^{-94}$ | 874 |
| | Extinction: Prime | 0.17 | $4.93 \times 10^{-7}$ | 874 |
| | Extinction: Burst | 0.19 | $9.72 \times 10^{-9}$ | 874 |
| | Extinction: Day 6 | 0.22 | $4.02 \times 10^{-11}$ | 874 |
| | Consumption | 0.39 | $1.02 \times 10^{-32}$ | 874 |
| Escalation of intake: Hour 1-12 | Break point | 0.39 | $6.95 \times 10^{-34}$ | 874 |
| | Cued reinstatement | 0.27 | $2.87 \times 10^{-16}$ | 866 |
| | Extinction: Prime | 0.17 | $2.40 \times 10^{-32}$ | 874 |
| | Extinction: Burst | 0.21 | $2.36 \times 10^{-10}$ | 874 |
| | Extinction: Day 6 | 0.21 | $8.70 \times 10^{-10}$ | 874 |
| | Prime reinstatement | 0.22 | $2.46 \times 10^{-11}$ | 874 |
| | Consumption | 0.29 | $3.67 \times 10^{-18}$ | 874 |
| Consumption | Break point | 0.57 | $7.83 \times 10^{-78}$ | 874 |
| | Cued reinstatement | 0.48 | $1.12 \times 10^{-51}$ | 866 |
| | Extinction: Prime | 0.31 | $1.33 \times 10^{-20}$ | 874 |
| | Extinction: Burst | 0.38 | $1.05 \times 10^{-30}$ | 874 |
| | Extinction: Day 6 | 0.34 | $1.10 \times 10^{-25}$ | 874 |
| | Prime reinstatement | 0.39 | $1.20 \times 10^{-33}$ | 874 |
| | OFT time 2 | 0.11 | 0.0009 | 868 |
| | EPM closed 2 | -0.12 | 0.0006 | 873 |
| Break point | Cued reinstatement | 0.51 | $1.96 \times 10^{-58}$ | 866 |
| | Extinction: Prime | 0.34 | $6.60 \times 10^{-25}$ | 874 |
| | Extinction: Burst | 0.45 | $3.64 \times 10^{-45}$ | 874 |
| | Extinction: Day 6 | 0.41 | $1.54 \times 10^{-36}$ | 874 |
| | OFT time 2 | 0.15 | $1.01 \times 10^{-5}$ | 868 |
| | OFT distance 2 | 0.11 | 0.0001 | 868 |
| Extinction: Burst | Cued reinstatement | 0.47 | $5.72 \times 10^{-49}$ | 866 |
| | Extinction: Prime | 0.41 | $1.43 \times 10^{-36}$ | 874 |
| | Extinction: Day 6 | 0.40 | $1.57 \times 10^{-34}$ | 874 |
| | Extinction de-escalation | -0.11 | 0.001 | 874 |
| | Prime reinstatement | 0.39 | $6.52 \times 10^{-31}$ | 874 |
| Extinction: Prime | Cued reinstatement | 0.32 | $1.54 \times 10^{-22}$ | 866 |
| | Extinction: Day 6 | 0.27 | $9.39 \times 10^{-16}$ | 874 |
| | Extinction de-escalation | -0.11 | 0.001 | 874 |
| | Prime reinstatement | 0.36 | $1.71 \times 10^{-27}$ | 874 |
| | EPM closed 1 | -0.13 | 0.0002 | 873 |
| | EPM closed 2 | -0.12 | 0.0003 | 873 |
| Prime reinstatement | Cued reinstatement | 0.38 | $1.33 \times 10^{-30}$ | 866 |
| | OFT time 2 | 0.12 | 0.00006 | 868 |

| | | | | |
|---|---|---|---|---|
| | EPM closed 2 | -0.13 | $7.64 \times 10^{-5}$ | 873 |
| Extinction: Day 6 | Cued reinstatement | 0.54 | $1.21 \times 10^{-67}$ | 866 |
| | Extinction de-escalation | 0.44 | $7.92 \times 10^{-42}$ | 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 | $8.33 \times 10^{-5}$ | 862 |
| | OFT time 2 | 0.15 | $9.46 \times 10^{-6}$ | 860 |
| | EPM closed 2 | -0.11 | 0.0008 | 865 |
| OFT distance 1 | OFT distance 2 | 0.45 | $5.13 \times 10^{-44}$ | 864 |
| | OFT distance difference | -0.49 | $4.07 \times 10^{-52}$ | 864 |
| | OFT time 2 | 0.39 | $2.64 \times 10^{-33}$ | 864 |
| | OFT time difference | -0.51 | $4.28 \times 10^{-58}$ | 864 |
| OFT distance 2 | OFT distance difference | 0.46 | $1.53 \times 10^{-46}$ | 864 |
| | OFT time difference | 0.40 | $1.29 \times 10^{-33}$ | 864 |
| | OFT time 1 | 0.40 | $7.59 \times 10^{-35}$ | 864 |
| | OFT time 2 | 0.89 | $5.40 \times 10^{-291}$ | 868 |
| OFT distance difference | OFT time difference | 0.92 | 0.00 | 864 |
| | OFT time 1 | -0.47 | $3.15 \times 10^{-49}$ | 864 |
| | OFT time 2 | 0.45 | $2.54 \times 10^{-44}$ | 864 |
| OFT time 1 | OFT time difference | -0.54 | $8.73 \times 10^{-68}$ | 864 |
| | OFT time 2 | 0.41 | $4.28 \times 10^{-36}$ | 864 |
| OFT time 2 | OFT time difference | 0.45 | $2.52 \times 10^{-43}$ | 864 |
| EPM open 1 | EPM open 2 | 0.25 | $8.36 \times 10^{-14}$ | 872 |
| | EPM open difference | -0.44 | $7.78 \times 10^{-43}$ | 872 |
| | EPM closed 1 | -0.58 | $3.65 \times 10^{-78}$ | 872 |
| | EPM closed 2 | -0.18 | $1.33 \times 10^{-7}$ | 872 |
| | EPM closed difference | 0.32 | $1.80 \times 10^{-22}$ | 872 |
| EPM open 2 | EPM open difference | 0.61 | $1.82 \times 10^{-90}$ | 872 |
| | EPM closed 1 | -0.32 | $1.32 \times 10^{-22}$ | 873 |
| | EPM closed 2 | -0.67 | $9.29 \times 10^{-166}$ | 873 |
| | EPM closed difference | -0.26 | $7.65 \times 10^{-15}$ | 873 |
| EPM open difference | EPM closed 1 | 0.12 | 0.0005 | 872 |
| | EPM closed 2 | -0.41 | $5.88 \times 10^{-36}$ | 872 |
| | EPM closed difference | -0.48 | $8.16 \times 10^{-51}$ | 872 |
| EPM closed 1 | EPM closed 2 | 0.37 | $2.77 \times 10^{-30}$ | 873 |
| | EPM closed difference | -0.45 | 0.0005 | 873 |
| EPM closed 2 | EPM closed difference | 0.47 | $2.38 \times 10^{-48}$ | 873 |
| TF baseline 1 | TF baseline 2 | 0.11 | 0.0012 | 874 |
| | TF baseline difference | -0.60 | $7.67 \times 10^{-88}$ | 874 |
| | TF test 2 | 0.11 | 0.0013 | 874 |
| TF baseline 2 | TF baseline difference | 0.59 | $7.30 \times 10^{-84}$ | 874 |
| | TF test 2 | 0.16 | $1.39 \times 10^{-6}$ | 874 |
| TF test 1 | TF test 2 | 0.17 | $9.78 \times 10^{-7}$ | 874 |
| | TF test difference | -0.59 | $3.40 \times 10^{-84}$ | 874 |
| TF test 2 | TF test difference | 0.57 | $2.29 \times 10^{-77}$ | 874 |

**Supplemental Table 2** 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.

| Trait | $h^2$SNP | SE | p-value | n |
|---|---|---|---|---|
| OFT distance 1 | 0.139 | 0.048 | ***<0.001*** | 870 |
| OFT time 1 | 0.103 | 0.046 | ***0.004*** | 870 |
| EPM open 1 | 0.054 | 0.041 | 0.073 | 873 |
| EPM closed 1 | 0.083 | 0.043 | ***0.012*** | 873 |
| TF baseline 1 | 0.11 | 0.046 | ***0.003*** | 874 |
| TF test 1 | 0.047 | 0.039 | 0.1 | 874 |
| 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 |
| Extinction: Day 6 | 0 | 0.038 | 0.5 | 874 |
| Extinction de-escalation | 0.003 | 0.037 | 0.47 | 874 |
| Prime reinstatement | 0 | 0.036 | 0.5 | 874 |
| Cued reinstatement | 0.037 | 0.04 | 0.172 | 866 |
| OFT distance 2 | 0.232 | 0.052 | ***<0.001*** | 868 |
| OFT distance difference | 0 | 0.033 | 0.5 | 864 |
| OFT time 2 | 0.158 | 0.049 | ***<0.001*** | 868 |
| OFT time difference | 0.007 | 0.033 | 0.41 | 864 |
| EPM open 2 | 0.072 | 0.042 | ***0.024*** | 873 |
| EPM Open difference | 0 | 0.035 | 0.5 | 872 |
| EPM closed 2 | 0.016 | 0.035 | 0.31 | 873 |
| EPM closed difference | 0 | 0.034 | 0.5 | 873 |
| TF baseline 2 | 0.146 | 0.049 | ***<0.001*** | 874 |
| TF baseline difference | 0.044 | 0.04 | 0.12 | 974 |
| TF test 2 | 0.124 | 0.045 | ***<0.001*** | 874 |
| TF test difference | 0 | 0.035 | 0.5 | 874 |
| Vulnerable vs resilient/intermediate | 0.168 | 0.051 | ***<0.001*** | 862 |
| Intermediate vs vulnerable/resilient | 0.093 | 0.044 | ***0.007*** | 862 |
| Resilient vs vulnerable/intermediate | 0.15 | 0.053 | ***0.002*** | 862 |

**Supplemental Table 3** Heritability table for behavioral traits. Significant relationships are highlighted in bold. The heritability index ($h^2$SNP), standard error (SE), p-value and total number of animals in analysis (n) for each trait is recorded.




**Supplemental Figure 1** Principle component analysis for behavioral traits with significant SNPs. Two factors were identified and comprised 71.55% of total variance in the data. Heroin-taking behaviors accounted for 46.24% of variance (component 1, purple) with traits exhibiting strong loading (Break point: 0.86; Consumption: 0.81; and Escalation of intake: Hour 1-12: 0.67), and was mutually exclusive from baseline analgesia prior to heroin experience (component 2, yellow) which accounted for 25.31% of variance with strong loading (0.99). Axes represent factor loading values for each trait within respected component. (n=874)

**Supplemental Materials**

*Behavioral testing*

Elevated-plus maze (EPM): Sessions were conducted in an elevated apparatus (San Diego Instruments; 4 arms at 110.5 cm long and 10.2 cm wide) that consisted of two "closed" arms (30.5 cm 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; 40.6 cm L x 40.6 cm W x 30.5 cm 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 in 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 a 5-sec 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. The progressive ration test occurred next to assess heroin 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.). Extinction training session and a test for cued reinstatement followed. Throughout testing, presses on the inactive lever were recorded but had no consequence.