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

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Running Title: Genetic loci associated with heroin vulnerability

Abstract

The increased prevalence of opioid use disorder (OUD) makes it imperative to disentangle the biological mechanisms contributing to individual differences in OUD vulnerability. OUD shows strong heritability, however genetic variants contributing to vulnerability remain poorly defined. We performed a genome-wide association study using over 850 male and female heterogeneous stock (HS) rats to identify genes underlying behaviors associated with OUD such as nociception, as well as heroin-taking, extinction 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 used a novel non-linear network-based clustering approach to characterize rats based on OUD vulnerability to assess genetic variants associated with OUD susceptibility. Our findings confirm the heritability of several OUD-like behaviors, including OUD susceptibility. Additionally, several genetic variants associated with nociceptive threshold prior to heroin experience, heroin consumption, escalation of intake, and motivation to obtain heroin were identified. *Tom1*, a microglial component, was implicated for nociception. Several genes involved in dopaminergic signaling, neuroplasticity and substance use disorders, including *Brwd1*, *Pcp4, Phb1l2* and *Mmp15* were implicated for the heroin traits. Additionally, an OUD vulnerable phenotype was associated with genetic variants for consumption and break point, suggesting a specific genetic contribution for OUD-like traits contributing to vulnerability. Together, these findings identify novel genetic markers related to the susceptibility to OUD-relevant behaviors in HS rats.

56 **Introduction**

57 Opioid use disorder (OUD) is characterized by compulsive opioid use and relapse propensity, despite
58 efforts to maintain abstinence. OUD diagnosis has increased substantially in the past decade (1), affecting
59 approximately 16 million people per year worldwide (2). The rise in diagnosis is not demographically
60 constrained (1), but is prevalent in individuals across socio-economic class, age and sex (3), increasing the
61 urgency to better understand the biological mechanisms contributing to OUD vulnerability. In twin
62 studies, OUD shows high heritability (~50%) (4, 5), indicating that specific genetic variants contribute to
63 biological basis of OUD susceptibility.

64 Genome-wide association studies (GWAS) are a useful tool for identifying the genetic variants that
65 influence heritable traits contributing to OUD. GWAS of OUD, though limited, have already yielded
66 valuable insights into genetic contributions to diagnostic vulnerability (6-13). However, human GWAS of
67 OUD are limited to observational data and are thus confounded by numerous poorly defined
68 environmental factors. Understandably, due to a lack of empirical data from study participants, human
69 GWAS have largely focused on OUD diagnosis, which is comprised of poorly quantified amounts of drug-
70 taking, extinction and seeking behaviors. Using rodent models of OUD, we can quantify specific traits (e.g.,
71 consumption, escalation, reinstatement) associated with addiction-related behaviors to directly
72 interrogate the relationship between these metrics and genetic variants. By doing so, we can discover
73 novel genetic variants that may contribute to the different phases of OUD, which can then be compared
74 to data from human studies. Crucially, such genes may also provide targets for the development of novel
75 OUD treatments.

76 In the current study, we assessed several measures of heroin-taking, extinction and seeking behaviors in
77 outbred male and female heterogeneous stock (HS) rats at two different testing locations. Additionally,
78 anxiety- and stress-like behaviors, as well as nociceptive threshold, were assessed prior to and following
79 heroin experience. HS rats are a diverse outbred population that was created by interbreeding 8 inbred

strains at NIH in 1984 to capture genetic and behavioral heterogeneity reflective of the human population (14-16). Compared to inbred and commercially available outbred rat lines, HS rats exhibit comparatively greater diversity (16) and have been used in several studies assessing addiction-related behaviors (17-24), including those focusing on genetic analyses (25-27). In addition, HS rats are supported by complementary expression quantitative trait loci datasets (28), furthering the translational utility of our findings. In this study, we found genetic variants specific to different OUD behavioral traits, many of which had known involvement in neurobiological processes associated with substance use disorder (SUD). Additionally, we employed a novel non-linear network-based clustering approach to identify subpopulations of OUD vulnerable and resilient rats (23, 24) and thereby identify genetic variants associated with OUD susceptibility.

101 **Methods**

102 Behavioral testing occurred at two geographically distinct locations: Medical University of South Carolina
103 (MUSC, USA) and the University of Camerino (UCAM, Italy). Two testing locations rendered the advantage
104 of accounting for environmental differences on behavior, akin to the human experience, while
105 maintaining standardized experimental procedures and equipment used between the sites. All
106 experimental procedures were approved by the MUSC Institutional Animal Care and Use Committee and
107 the Italian Ministry of Health (UCAM). All testing procedures were compliant with the National Institute
108 of Health Guide for the Care and Use of Laboratory Animals and the Assessment and Accreditation of
109 Laboratory Animals Care, as well as the European Community Council Directive for Care and Use of
110 Laboratory Animals.

111 *Subjects*

112 Male and female HS rats (NMcwiWFsm #13673907, RRID:RGD13673907) bred at Wake Forest University
113 were shipped to MUSC and UCAM in batches of 40 (20 females and 20 males/site) at 5 weeks of age. Each
114 site had an equal representation of littermates thereby creating genetically comparable testing cohorts.
115 Whenever possible, each shipment contained only one male and one female per litter, to reduce genetic
116 relationships, which can reduce power in GWAS. Upon arrival, rats were left undisturbed for 3 weeks in a
117 climate-controlled vivarium room with a standard 12-hr light/dark cycle prior to testing. Rats were pair-
118 housed and received ad libitum access to food and water throughout the duration of testing. Behavioral
119 tests for stress, anxiety and nociception occurred during the light cycle, whereas all heroin-associated
120 testing occurred during the dark phase. A total of 1,160 rats underwent testing from February of 2019
121 through December of 2022, with the following animals excluded from all analyses: 147 for behavioral
122 control studies (e.g., did not undergo heroin self-administration procedures), 107 due to death (post-
123 surgical illness, n=84; surgery, n=23); 29 for behavioral data collection technical issues, and 3 for spleen

124 identification issues. Final behavioral and genetic analyses were conducted on 874 rats (MUSC, n=479;
125 UCAM, n=395).

126 *Drugs*

127 Heroin hydrochloride was supplied to both sites by the National Institute on Drug Abuse (Bethesda, MD)
128 and dissolved in 0.9% sterile saline.

129 *Behavioral testing*

130 The experimental timeline is shown in Figure 1a. Experimental testing occurred as previously described
131 (17, 23, 24). Extensive behavioral analyses for these animals, including sex and site differences, have
132 been conducted and reported in prior publications (17, 23, 24), and we refer the reader to these
133 manuscripts for further edification. The focus of this manuscript is on identifying the genetic variants that
134 contribute to individual differences in these behaviors. A brief description of behavioral testing
135 procedures is provided below with greater detail in the Supplemental Information.

136 *Stress, anxiety and nociceptive-like behavioral measures*

137 Stress and anxiety-related behaviors were assessed in the elevated-plus maze (EPM) and the open field
138 test (OFT). Nociception under baseline (administration of 1 mg/kg saline, sc) and test (0.75 mg/kg heroin,
139 sc) conditions were evaluated using the tail flick (TF) test. All tests were performed prior to and following
140 heroin self-administration (see Supplemental Information and Kuhn et al., 2022 and 2024).

141 *Heroin taking, extinction and seeking measures*

142 After EPM, OFT and TF tests, rats were outfitted with an indwelling jugular catheter prior to undergoing
143 heroin self-administration training procedures. During training, presses on the active lever resulted in
144 presentation of a tone and cue light stimulus, as well an infusion of heroin (20 µg/kg/100 µl infusion over
145 3-sec). Training occurred for 3 weeks with four 12-hr sessions/week. Next, motivation to take heroin was

146 assessed using a progressive ratio test whereupon the number of lever presses needed to earn an infusion
147 exponentially increased according to the following formula: $(5*e^{0.2n})-5$ (29). The maximum number of
148 lever presses exerted to receive an infusion was considered the break point. Heroin self-administration
149 training was then re-established for 3 days, followed by a within-session extinction-prime test lasting 6
150 hours. Rats were in extinction training conditions (i.e., no cue presentation or heroin delivery) for the
151 entire 6-h session. However, with two hours left in the session, animals received a heroin prime injection
152 (0.25 mg/kg, sc; heroin primed reinstatement test). Animals next underwent 6 daily 2-h extinction training
153 sessions prior to a 2-h test for cue-induced reinstatement (i.e., active lever presses resulted in cue
154 presentation but no heroin infusion). The EPM, OFT and TF tests were then repeated. See Supplemental
155 Information for further details.

156 *Tissue collection and genotyping*

157 Following all behavioral testing, animals were sacrificed and spleen samples were collected and shipped
158 to University of California San Diego for genotyping. Genotyping-by-sequencing was employed as
159 previously described to assess genotypes (30, 31). A total of 7,955,513 single nucleotide polymorphisms
160 (SNPs) were identified with an error rate <1%. However, prior to GWAS analysis, the SNPs were filtered
161 for missingness not above 10%, minor allele frequency (MAF) of no less than 0.5% and the Hardy-
162 Weinberg equilibrium (HWE) deviation p-value no less than $1e^{-10}$, resulting in a final set of 5,308,726 SNPs
163 for analysis.

164 *Statistical analyses*

165 <u>Behavioral traits assessed in GWAS:</u> Behaviors included in GWAS analysis included behavioral testing traits
166 (i.e., EPM, OFT and TF) both prior to (time point 1) and following (time point 2) heroin experience, as well
167 as the difference in individual testing measures between the time points (Supplemental Table 1). Traits
168 representative of heroin taking, extinction and seeking measures were also analyzed (Supplemental Table

169 1). Heroin-taking behaviors included: escalation of intake in the first hour of the session across training
170 (ug/kg; average consumption hour 1 days 1-3 subtracted from hour 1 days 10-12; Escalation of intake:
171 Hour 1), escalation of intake across the entire session (ug/kg; average consumption days 1-3 subtracted
172 from days 10-12; Escalation of intake: Hour 1-12), total consumption (ug/kg) across training sessions 1-
173 12, and break point achieved during the progressive ratio test. Extinction traits include active lever presses
174 made during the following: hours 1-2 (Extinction: Burst) and 3-4 (Extinction: Prime) of the 6-h within-
175 session extinction-heroin prime reinstatement test, the last day of extinction training prior to the test for
176 cued reinstatement (Extinction: Day 6) and the difference in lever presses between the first and last day
177 of extinction training (Extinction de-escalation). Active lever presses made during the tests for heroin
178 prime and cued reinstatement were assessed for seeking measures.

179 Behavioral clustering: In addition to specific traits, we wanted to determine if any genetic variants were
180 associated with OUD susceptibility. To do so, we employed a non-linear network-based clustering
181 approach (32) using an R package 'mlsbm' (33) and characterized rats into OUD vulnerable, intermediate
182 or resilient clusters. Behaviors included for analysis are indicated in Supplemental Table 1. Briefly,
183 behavioral data was standardized within sex and site and combined to create a rat-rat similarity network,
184 whereupon a Bayesian stochastic block model was employed to define clusters assignments (32).
185 Behavioral and neurobiological validation of this model are extensively detailed in prior publications (23,
186 24). Cluster assignment was used as phenotypes (Vulnerable vs intermediate/resilient; Intermediate vs
187 vulnerable/resilient; Resilient vs vulnerable/intermediate) for GWAS. Differences in cluster composition
188 between the top SNP allelic variants were assessed using a Chi-square test.

189 Phenotypic correlations: To address difference in data variance between sites (MUSC vs UCAM),
190 covariates (including sex) that explained more than 2% of trait variance (Supplemental Table 2 and 3) were
191 regressed out and data was quantile normalized within site. This resulted in trait variances being equal
192 between sites, thus weighing data from MUSC and UCAM equally. Next, data from both MUSC and UCAM

193 were merged and quantile normalized again to maintain normality. Behavioral co-variance between traits
194 was assessed using Spearman correlations with Bonferroni adjustment to correct for multiple
195 comparisons using GraphPad Prism version 10. SNP heritability for behavioral traits was determined using
196 GCTA-GREML analysis with significance determined by the p-value of rejecting the null hypothesis that
197 the heritability is zero ($p<0.05$).

198 Quantitative trait loci (QTL) and SNP identification: To perform the GWAS we used GCTA software
199 employing a genetic relatedness matrix and the Leave One Chromosome Out method to account for
200 proximal contamination (34, 35). Because all data was quantile normalized, only one significance
201 threshold, which was determined by permutation, was necessary (36). We report QTLs that surpassed the
202 permutation-derived threshold of $-\log_{10} p>5.58$ ($p<0.05$). Significant QTLs are only reported when there
203 was at least one additional SNP located within 0.5 Mb with a significance within 2 $-\log_{10}(p)$ units of the
204 first SNP (37). Independence of QTLs on the same chromosome was established by making the top SNP in
205 the most significant QTL a covariate and then repeating the GWAS analysis for the chromosome in
206 question. This process was repeated until no more significant QTLs were identified (37). Linkage
207 disequilibrium (LD; $r^2 \geq 0.6$) was used to define the boundaries of each QTL. SNPEff was used to identify
208 coding SNPs within each QTL interval (38). To identify associations from other GWAS that have been
209 performed in HS rats that map to the same loci, a phenome wide association study (PheWAS) was
210 conducted. To explore functional mechanisms whereby SNPs may affect OUD-like traits, we assessed the
211 co-location of the peak SNP in each QTL with known SNPs that can alter gene expression (eQTL), slicing
212 patterns (sQTL) or directly alter the amino acid sequence of genes within a 3 Mb window using
213 RatGTEx.org (28). The reference database for eQTLs was comprised of animals not in this study, therefore,
214 simple correlations between expression and behavior were not possible. Importantly, the absence of
215 coding variants, eQTLs or sQTLs could reflect the fact that RNA sequencing for the eQTL/sQTL analysis
216 used a polyA pulldown protocol, limiting our findings to RNAs that are polyadenylated.

217 Principal component analysis: To assess whether variance in behavioral traits within significant QTLs could
218 be explained by common factors, a principal component analysis was applied to the data. Number of
219 components was determined using a minimum threshold of Eigenvalue of 1 and Varimax method with a
220 minimum factor loading of 0.3 was used to rotate identified components. Analyses used SPSS version 27.

221 **Results**

222 *Behavioral correlations*

223 Behavioral traits showed weak to moderate correlations overall (Fig. 1b, Supplemental Table 4). However,
224 behavior during the progressive ratio test, cued reinstatement and total heroin consumption were
225 positively correlated ($r^2 \geq 0.48$), suggesting overlapping behavioral mechanisms guiding these behaviors.

226 *Heritability estimates*

227 SNP heritability (h^2) estimates the proportion of the phenotypic variance explained by the genetic variance
228 and is dependent on the genetic diversity of the population and environmental sources of variability.
229 Aspects of heroin taking (Consumption, h^2=0.17) and the inability to initially refrain from seeking
230 (Extinction: Burst, h^2=0.10) were heritable (Fig. 2a, Supplemental Table 5). While some measures of heroin
231 taking, extinction and seeking were not heritable, OUD susceptibility cluster assignment (23, 24) was
232 heritable (Vulnerable vs resilient/intermediate, h^2=0.17; Intermediate vs vulnerable/resilient, h^2=0.09;
233 Resilient vs vulnerable/intermediate, h^2=0.15), suggesting a heritable genetic contribution to OUD
234 susceptibility (Fig. 2a, Supplemental Table 5).

235 Several behavioral measures prior to and following heroin administration experience showed significant
236 heritability (Fig. 2a, Supplemental Table 5). Nociceptive threshold under basal conditions both prior to (TF
237 baseline 1, h^2=0.11) and following (TF baseline 2, h^2=0.15) heroin experience were both heritable. Heroin-
238 induced nociception after prolonged heroin exposure was also heritable (TF test 2, h^2=0.12), reflecting the

239 genetic contribution to the long-term effects of heroin exposure on nociceptive threshold. Aspects of
240 anxiety- and stress-like behaviors were also heritable, including high anxiety prior to heroin experience
241 (EPM closed 1, h^2=0.08), but lower levels following heroin self-administration (EPM open 2, h^2=0.07). Our
242 results support previous findings that locomotor behavior is heritable (25, 26) (OFT distance 1, h^2=0.14;
243 OFT time 1, h^2=0.10) and show that this heritability persists even after exposure to heroin administration
244 (OFT distance 2, h^2=0.23; OFT time 2, h^2=0.16).

245 *GWAS*

246 We identified genome-wide significant associations for several traits: escalation of intake across the entire
247 session on Chromosome 10, total heroin consumption on Chromosome 11, break point achieved during
248 the progressive ratio test on Chromosome 19, and baseline nociception prior to heroin experience (TF
249 baseline 1) on Chromosomes 2 and 19 (Fig. 2b). Shared behavioral variance between these traits was
250 assessed using PCA, with heroin taking traits loading onto one factor and baseline nociception onto its
251 own factor, reflecting the independence of these measures in our data (Supplemental Fig. 1). While both
252 nociception and break point were associated with loci on Chromosome 19, the associated loci are
253 separated by approximately 4 Mb and are not in strong LD with one another, indicating that they are due
254 to separate causal loci. See Supplemental Table 6 for MAF and HWE values for each significant loci and
255 the Supplementary Information for a detailed report of all GWAS findings. Both sites contributed
256 comparably to significant loci (Supplemental Table 7).

257 *Escalation of intake:* We identified a significant locus on Chromosome 10 for escalation of heroin intake
258 (Fig. 3). The peak SNP was located at 10:35,034,818 and spans from positions 34,961,844 to 35,103,415.
259 This locus did not contain any coding variants that are in strong LD with the top SNP. Though eQTLs and
260 sQTLs were identified, none are in strong LD (i.e., r^2) with the top SNP, suggesting they are unlikely to be
261 causally related to the escalation of heroin intake behavior. Identified eQTLs include metabotropic

262 glutamate receptor 6 (*Grm6*) in whole brain (r^2=0.63), collagen type XXIII alpha 1 chain (*Col23a1*) in the
263 orbitofrontal cortex (OFC; r^2=0.63) and zinc finger protein (*Zfp2*) in the prelimbic cortex (PrL; r^2=0.64). Two
264 sQTLs were also identified, jade family PHD finger 2 (*Jade2*) in the nucleus accumbens core (NAcc) and
265 basolateral amygdala (BLA; both r^2=0.63), and *Col23a1* in the PrL (r^2=0.63). The lack of any putatively
266 causal variants in this interval suggests that the causal variant remains unknown, possibly because there
267 is an eQTL or sQTL in a tissue that is not represented in the RatGTEx database, or an eQTL that was not
268 detected, for example, because it is significant at a different developmental time point or under different
269 environmental conditions.

270 *Total consumption:* Total heroin consumed across training (Supplemental Fig. 2) was associated with a
271 locus on Chromosome 11 (Fig. 4a) that spanned from positions 35,033,985 to 36,454,941 with the peak
272 SNP at 35,034,818. Several significant causal coding variants were identified that are in strong LD with this
273 locus, including bromodomain and WD repeat domain containing 1 (*Brwd1*: Leu2187Pro, r^2=0.99),
274 Purkinje cell protein 4 (*Pcp4*: Lys12Arg, r^2=0.99) and two different amino acid coding variants for SH3
275 domain binding glutamate-rich protein (*Sh3bgr:* Glu169Lys, r^2=0.99; Glu177Lys, r^2=0.99). There were also
276 eQTLs for *Sh3bgr* that was in high LD with this locus (r^2=0.99 for all) in whole brain, BLA, NAcc, PrL, lateral
277 habenula (LHb) and OFC. Two additional genes with eQTLs in high LD with the locus were identified: *Pcp4*
278 (PrL, r^2=0.88) and a long non-coding RNA (*ENSRNOG00000068065*; infralimbic cortex (IL), r^2=0.99). We
279 also identified an sQTL for *ENSRNOG00000068065* in several brain regions (whole brain, BLA, LHb, OFC,
280 NAcc, PrL, and PrL2; r^2=0.99-1.00). Lastly, there was an sQTL for the gene guided entry of tail-anchored
281 proteins factor 1 (*Get1*; r^2=0.99) in the IL. Heroin consumption is likely mediated, in part, by these coding
282 differences and or by heritable differences in expression of one or more of these genes. Because several
283 plausible variants are in LD with one another, mechanistic or other complementary approaches will be
284 needed to identify the causal gene. Additionally, PheWAS analysis showed that OUD vulnerability

285 (Vulnerable vs Intermediate/resilient cluster) was associated with the top SNP at a suggestive level (-
286 log(p)=4.5), with cluster composition differing between the allelic variants (x^2=18.12, p<0.0001; Fig. 4b,c).

287 *Break point:* The break point achieved during the progressive ratio test was associated with a locus on
288 Chromosome 19 (Fig, 5a) spanning from positions 8,647,094 to 8,801,084 with the peak SNP at 8,648,697.
289 Two potentially causal coding variants in prohibitin 1 like 2 (*Phb1l2*; Lys70Asn and Ile231Asn), which were
290 in perfect LD with the top SNP (r^2=1.0), were identified. An eQTL with perfect LD with the locus for the
291 gene matrix metallopeptidase 15 (*Mmp15*; r^2=1.0) was also identified in the whole brain. An additional
292 eQTL (solute carrier family 38 member 7, *Slc38a7*) and sQTL (casein kinase 1 alpha 2, *Csnk2a2*) were
293 identified in the BLA, however, the relationship between these genes and the locus are modest (r^2=0.65
294 for both) rendering them much less likely to be the causes of the association with break point behavior.
295 Similar to the Chromosome 11 association for total heroin consumption, PheWAS analysis showed a
296 suggestive association between the top SNP and the OUD vulnerable cluster (Vulnerable vs
297 intermediate/resilient clusters; -log(p)=5.16; Fig. 5b). OUD cluster composition differed between the
298 alleles at the peak SNP (x^2=18.91, p<0.0001; Fig. 5c). While there is a small bias toward the AA variant
299 conferring OUD resiliency, the minor variant, AC, strongly coincides with OUD vulnerability.

300 *Baseline nociception prior to heroin experience:* A locus on Chromosome 2 spanning positions 51,450,191
301 to 52,982,078 with the peak SNP at 51,588,876 was identified for basal nociception prior to heroin taking
302 procedures (Fig 6a). Several coding variants in modest LD with the peak SNP were identified in genes
303 within this locus, including coiled-coil domain containing 152 (*Ccdc152*: Ala242Ser and Thr14Asn, both
304 r^2=0.77), growth hormone receptor (*Ghr*: Ala546Val and Lys541Asn, both r^2=0.77), zinc finger protein 131
305 (*Zfp131*: Gly506Ser, r^2=0.82) and serine/threonine-protein kinase NIM1 (*Nim1k;* r^2=0.64). The gene
306 selenoprotein P (*Selenop*) had an sQTL in both BLA and whole brain (r^2=0.78-0.79), and an eQTL for both
307 whole brain and the NAcc (both r^2=0.66). These data provide support of the involvement of these genes
308 in the Chromosome 2 association with baseline nociception.

309 An additional significant association for this behavior was identified on Chromosome 19 (Fig. 6b). The peak
310 SNP was located at 19:12,470,318 with the QTL spanning from positions 11,508,674 to 12,474,455. A
311 potentially causal coding variant in modest LD with the top SNP in the gene LARGE xylosyl- and
312 glucuronyltransferase 1 *(Large1*: Ala92Val, r^2=0.75) was present. There were also several eQTLs for *Large1*
313 (whole brain, r^2=0.69; BLA, r^2=0.78; IL, r^2=0.68; NAcc, r^2=0.70; OFC, r^2=0.69; PrL, r^2=0.70; and PrL2, r^2=0.75).
314 In addition, there was an eQTL for ribosomal protein S18 (*Rps18l2*) within the LHb (r^2=0.98). An sQTL for
315 the gene myb1 trafficking protein (*Tom1*) was in strong LD with this locus (r^2=1.0) within the PrL. Finally,
316 there was a significant sQTL for metallothionein 3 (*Mt3*) in the BLA (r^2=0.75). These coding variants, eQTLs
317 and sQTLs could point to heritable differences and underlying biological mechanisms for basal nociception
318 prior to drug experience at the Chromosome 19 locus.

319 **Discussion**

320 The economic toll on society for OUD totals over one trillion dollars annually (39). Furthermore, over one
321 million individuals seek treatment for OUD annually (40) and more than 80,000 people die from opioid
322 overdoses every year in the US alone (41). Thus, there is an urgent need to understand the factors that
323 influence vulnerability to OUD. Using a rat line emulating within species genetic and phenotypic variability
324 (14) akin to humans, we assessed the genetic basis of several OUD-like behaviors and further extended
325 analysis to account for OUD susceptibility. While heroin was used, we expect these findings will be
326 relevant for other opioids of abuse whose primary site of action is mu opioid receptors. We found several
327 traits related to OUD are heritable, including overall OUD susceptibility. Genetic variants associated with
328 nociception prior to heroin experience as well as heroin consummatory behaviors were identified, several
329 of which are either biomarkers for other SUDs or are involved in neuroplasticity. Using this model, we are
330 able to identify genetic variants possibly associated with an OUD diagnosis, but also specific behavioral
331 traits that comprise vulnerability or resiliency thereby further contributing to our knowledge of the
332 genetic basis of OUD.

333 *Heritability*

334 OUD is heritable in humans and we found several OUD-like behaviors were also heritable in HS rats.
335 Human twin studies have yielded heritability estimates between 23-54% for OUD (4, 5) and human GWAS
336 of OUD have produced SNP heritability estimates, which are expected to be lower, ranging from 6-12.7%
337 (9-13). However, given the complex and multi-symptomatic nature of OUD (42), assessing heritability of
338 distinct behavioral traits associated with human OUD vulnerability is difficult. In a large sample of HS rats,
339 we identified several heritable heroin addiction-like traits that model different aspects of human OUD.
340 Both heroin consumption and initial extinction behavior were significantly heritable, with SNP heritability
341 estimates for these traits similar to SNP heritability obtained from human studies of OUD. However,
342 behaviors following prolonged heroin use and forced abstinence, such as cued reinstatement, did not
343 exhibit significant heritability, suggesting weaker genetics influences and/or limitations in our procedure's
344 ability to detect the cascade of neuroplastic adaptations that occur following prolonged heroin self-
345 administration (43, 44).

346 Novelty-induced locomotion, a heritable behavior (25, 26), has been associated with vulnerability in
347 opioid addiction-related behaviors in both rodent models (17, 45-49) and humans (50). We confirmed the
348 heritability of locomotor behavior both prior to and following heroin experience in HS rats, suggesting an
349 enduring genetic contribution to this behavior that remains relatively unaffected by factors (e.g.,
350 epigenetic changes) associated with prolonged heroin experience. Lastly, in agreement with prior work in
351 humans, we showed that nociceptive threshold to heat stimuli under baseline conditions is heritable (51).
352 Following opioid use, both humans (52, 53) and rats (54) demonstrate opioid-induced hyperalgesia in
353 response to a painful stimuli (17), a finding replicated in our study. However, the heritability of nociception
354 in humans is unknown. Given the inherent behavioral and genetic heterogeneity present in HS rats, and
355 the translational relevance of the findings reported here, HS rats offer an opportunity to disentangle the
356 genetic factors contributing to opioid-induced nociception.

357 *GWAS*

358 Genetic variants associated with OUD-like behaviors in rats can contribute novel insights that improve our
359 understanding of the neurobiological mechanisms underlying distinct behavioral traits associated with
360 OUD in humans. This study implicated several loci and genes within those loci that had coding variants,
361 eQTLs and/or sQTLs associated with affective or behavioral traits associated with SUD (Supplemental
362 Table 8).

363 Escalation of heroin intake across the entire session: While no causal coding variants, eQTLs or sQTLs were
364 in strong LD with the QTL on Chromosome 10 for escalation of heroin-taking behavior across the entire
365 session, several genes in this interval are of potential interest. For example, *Adamts2*, an extracellular
366 matrix proteinase (55) localized within the mesolimbic and mesocortical regions within the brain (56), is
367 a biomarker for heavy tobacco smoking and is downregulated following long-term morphine exposure
368 and withdrawal (57). Similarly, the gene heterogeneous nuclear ribonucleoprotein H1 (*Hnrnph1*) is located
369 in this interval. *Hnrnph1* was identified as the causal gene for a mouse QTL for methamphetamine
370 response (58-61) and subsequent work has shown it also modulates the response to fentanyl (62) and
371 alcohol (63). Further investigation is required to determine if there are overlooked coding variants or
372 eQTLs for *Adamts2* or *Hnrnph1*.

373 Heroin consumption: The region on Chromosome 11 that was associated with total consumption of heroin
374 across all training sessions contained a number of genes with coding and expression variants. Three genes
375 harbored coding variants that were in strong LD with the peak SNP: *Brwd1*, *Pcp4* and *Sh3bgr*. In humans,
376 *BRWD1* has been associated with potentially relevant traits including cigarette smoking (64-69),
377 externalizing behaviors (70), ADHD (67, 71) and hyperthymia in bipolar disorder (72). *PCP4* mediates
378 calcium signaling and through downstream mechanisms also affects dopamine and acetylcholine signaling
379 [76]. *PCP4* levels are attenuated in post-mortem tissue of individuals diagnosed with alcohol use disorder

380 [77] as well as in the NAc of rats following 2-weeks of nicotine withdrawal [78]. This gene has also been
381 linked to cigarette smoking (64, 73) and peripheral effects of alcohol drinking (74) in humans. We
382 identified an eQTL for *Pcp4* in the PrL, a critical component of drug-reward circuitry and plasticity (75, 76).
383 The relationship between *PCP4* and OUD is unknown, but our results suggest it may act to alter cortical
384 to NAc transmission as a consequence of sustained heroin use. Finally, *Sh3bgr* had two coding variants
385 and numerous eQTLs in whole brain, BLA, NAcc, OFC, LHb, and PrL, however the homologous human gene
386 has not been associated with any psychiatric traits in prior human GWAS. In addition to these genes, a
387 long non-coding RNA (ENSRNOG00000068065) and the gene *Get1* had sQTLs, but there is no supporting
388 literature for their role in OUD-relevant behaviors.

389 *Ets2*, which contained the peak SNP for this locus, did not identify any coding variants or an e/sQTL. *Ets2*
390 encodes a ubiquitous transcription factor that is found in many cells and has be linked to molecular
391 processes (77) and cell types (78) that modulate synaptic remodeling, including those associated with
392 OUD (79). Our results suggest *Ets2* and its allelic variants may impact OUD susceptibility, though further
393 investigation is necessary.

394 While the functional association between heroin consumption and the other identified e/sQTLs are not
395 known, *DSCAM* is correlated with the top SNP and encodes a neuronal cell adhesion molecule that
396 regulates cellular connectivity (80) and glutamatergic plasticity associated with learning (81, 82).
397 Glutamate-mediated neuroplasticity occurs following heroin experience and is considered a driving force
398 behind relapse vulnerability (44). *DSCAM* is necessary for tasks requiring memory formation (83, 84),
399 including those associated with rewarded behaviors (84), and was identified in human GWAS as a
400 biomarker for cigarette cessation success (85). Based on these data, *DSCAM* warrants further investigation
401 for OUD.

402 Break point: The QTL on Chromosome 19 for break point achieved during the progressive ratio test
403 contained two coding variants in strong LD with the top variant for the poorly characterized gene *Phb1l2*.
404 *Phb1l2* is localized to both neuronal and non-neural cell types in the central nervous system and helps to
405 maintain mitochondria respiration by attenuating reactive oxygen species which contribute to cellular
406 dysfunction (86). Chronic morphine administration disrupts mitochondrial respiration and restoring
407 proper functioning attenuates opioid withdrawal (87). Additionally, *Phb1*, an analogue of *Phb1l2*, has been
408 implicated in mediating glutamatergic and dopaminergic neural transmission and becomes dysregulated
409 in several disorders, including alcohol use disorder (86). It is thus possible that differences in the coding
410 sequence of *Phb1l2* influence break point through glutamate and dopamine dependent processes.
411 Additionally, an eQTL for *Mmp15* in whole brain that was in strong LD with the top SNP was identified,
412 suggesting *Mmp15* expression could modulate break point. MMP15 activates MMP2 (88) which
413 contributes to NAcc cell-specific regulation of extinction behavior following heroin self-administration
414 (89). It is possible that attenuated *Mmp15*-induced *Mmp2* expression contributes to higher break point
415 levels. Furthermore, PheWAS analysis identified this locus as being associated with OUD vulnerability with
416 the coding variant likely impacting OUD susceptibility.

417 Several of the heroin taking traits are both phenotypically and genetically correlated. Despite this, the
418 significant loci identified for these traits are not the same (Supplemental Table 9). We attribute this
419 observation to the fact that these traits are highly polygenic, meaning that many loci make small
420 contributions to the observed phenotype. Because our power is limited, we only expect to identify a small
421 subset of the true positive loci, and those subsets are expected to differ from one trait to another for
422 purely stochastic reasons.

423 Baseline nociceptive threshold prior to heroin experience: Genetic variants on two separate
424 chromosomes were found for nociceptive threshold under baseline conditions, prior to heroin experience.
425 On Chromosome 19, *Large1* was notable because it contained both putatively causal coding variants and

426 an eQTL. Large1 has been associated with various behavioral traits in humans, including educational
427 attainment (90-92), smoking (64), alcohol dependence (93), neuroticism (94) and depression (94, 95);
428 however, no prior evidence that we are aware of link it to nociception. Heritable differences in splicing
429 patterns (sQTLs) for both *Tom1* in the PrL and *Mt3* in the BLA are also candidates for nociception, though
430 neither has been previously directly linked to pain processing. *Mt3* is a brain zinc binding protein involved
431 in several metabolic processes and has been implicated in cellular dysfunction of diseases including
432 Alzheimer and Huntington disease (96). *Tom1* is an integral component of microglial function (97), which
433 is involved in neuropathic pain (98), but has yet to be implicated in acute painful stimuli. This gene has
434 also been associated with cigarette smoking in humans (73).

435 On chromosome 2, two genes that operate in tandem with one another were also associated with
436 nociception. There was modest LD between the top SNP for this QTL and splicing patterns in *Selenop* which
437 encodes for a selenoprotein. This protein is secreted by the liver and acts to deliver selenium, a trace
438 element necessary for proper brain function (99), via the bloodstream to regions including the brain.
439 Selenium has been implicated in mediating dopamine release and transmission within the substantia nigra
440 (100, 101) and the NAc (102). The presence of *Selenop* on NAc dopamine terminals is proposed to
441 attenuate dopamine release into the synaptic cleft via direct mediation of dopamine 2 autoreceptors,
442 protecting against neurotoxic effects of drugs of abuse such as methamphetamine (102). *Ccdc152*, which
443 also had coding variants that were in modest LD with this QTL, is encoded within the *Selenop* gene, and
444 acts to attenuate *Selenop* levels via post-translational modification (103). Though the periaqueductal gray
445 is the predominant brain nuclei processing pain signaling (104), striatal dopamine signaling has recently
446 been implicated in affecting pain processing and perception (105, 106) and warrants further investigation.

447 **Conclusion and Future Directions**

448 We used a rodent model to identify several heritable traits and genetic variants associated with OUD.
449 Unlike GWAS performed in humans, we were able to identify genetic variants associated with distinct
450 OUD behaviors that contribute toward diagnosis. Several of the identified genes are supported by prior
451 relationships to SUDs and other relevant behavioral and psychiatric traits, suggesting that they warrant
452 further investigation for OUD. Furthermore, using an established rodent model that characterizes rats
453 using a non-linear compilation of behaviors across the SUD phases to confer OUD susceptibility, we
454 identified genetic variants for heroin consumption and motivation that were associated specifically with
455 OUD vulnerability. These findings strengthen the translational utility of the behavioral model and can help
456 to assess genetic variants and biomarkers associated with OUD vulnerability versus resiliency. However,
457 complementing human OUD (5), phenotypic variance can only be partially explained by the genetic
458 variants discovered here. Other genetic variants as well as epigenetic (107) and uncontrolled
459 environmental factors also contribute to OUD behaviors in HS rats. Furthermore, both preclinical and
460 clinical GWAS analysis focusing on genetic variants associated with OUD resiliency, a neurobiological
461 active process (108, 109), should be considered in future analyses.

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467 **Conflict of Interest**

468 The authors declare no conflict of interest.

469

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750 **Figure 1** Experimental timeline and correlational analyses. **a)** Rats underwent testing for stress- and
751 anxiety-related behaviors (OFT: open field test; EPM: elevated-plus maze) and nociceptive threshold (TF:
752 tail flick) prior to jugular catheterization surgery, with tests repeated following heroin experience. Heroin
753 self-administration occurred for 3 weeks (12-h/session, 4 days a week) followed by a progressive ratio test
754 and an additional 3 days of self-administration training. Next, rats underwent a within-session extinction-
755 heroin prime reinstatement test (6-h). Extinction training sessions (2-h/session) then occurred followed
756 by a cue-induced reinstatement test (2-h). Spleen sample were collected for genetic analyses at the
757 conclusion of training. **b)** Correlation matrix for behavioral trait covariance. Behavioral testing that
758 occurred prior to heroin experience is designated with a 1, and testing following heroin training as 2. Traits
759 in bold are those with at least one significant QTL identified in GWAS analysis. (n=874)

760 **Figure 2** Heritability and genome-wide association results. **a)** SNP heritability (h^2) measure ± SEM for all
761 behavioral traits assessed. Significant h^2 measures ($p<0.05$) are indicated in blue, and non-significant traits
762 are in orange. **b)** Porcupine plot for selected behavioral traits. Top SNPs exceeding the genome-wide
763 significance threshold are highlighted and identified (red: -log(p)=5.36 and blue: -log(p)=5.58, which
764 correspond to permutation derived p values of 0.10 and 0.05, respectively. (n=874)

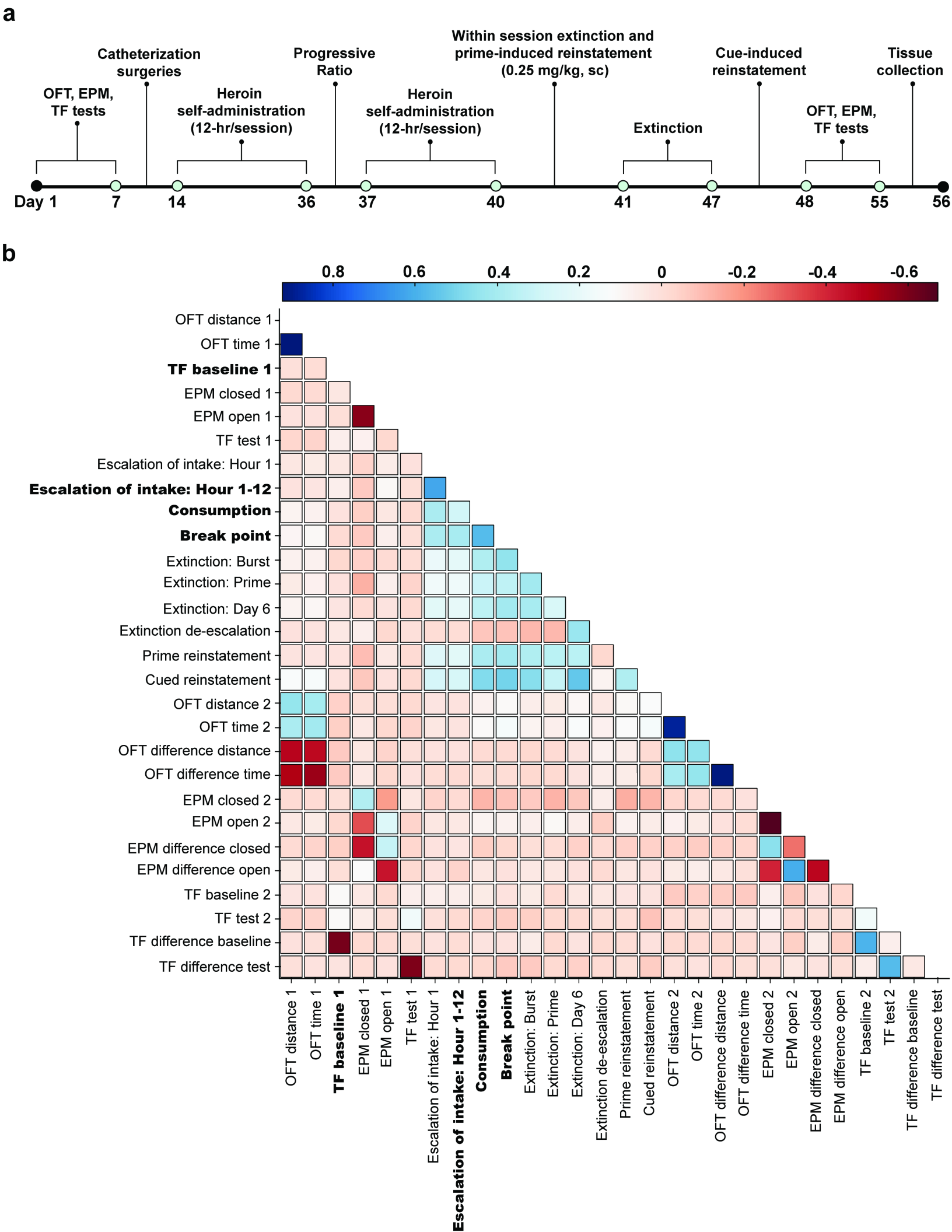
765 **Figure 3** GWAS results for escalation of heroin intake across the entire session. Regional association plot
766 for chromosome 10, with the top SNP in purple and chromosome location indicated on the plot. Other
767 SNPs are colored based on their linkage disequilibrium with the top SNP. Known genes of SNPs are
768 indicated below the plots. Significance threshold: red line, $p<0.10$; blue line, $p<0.05$. (n=874)

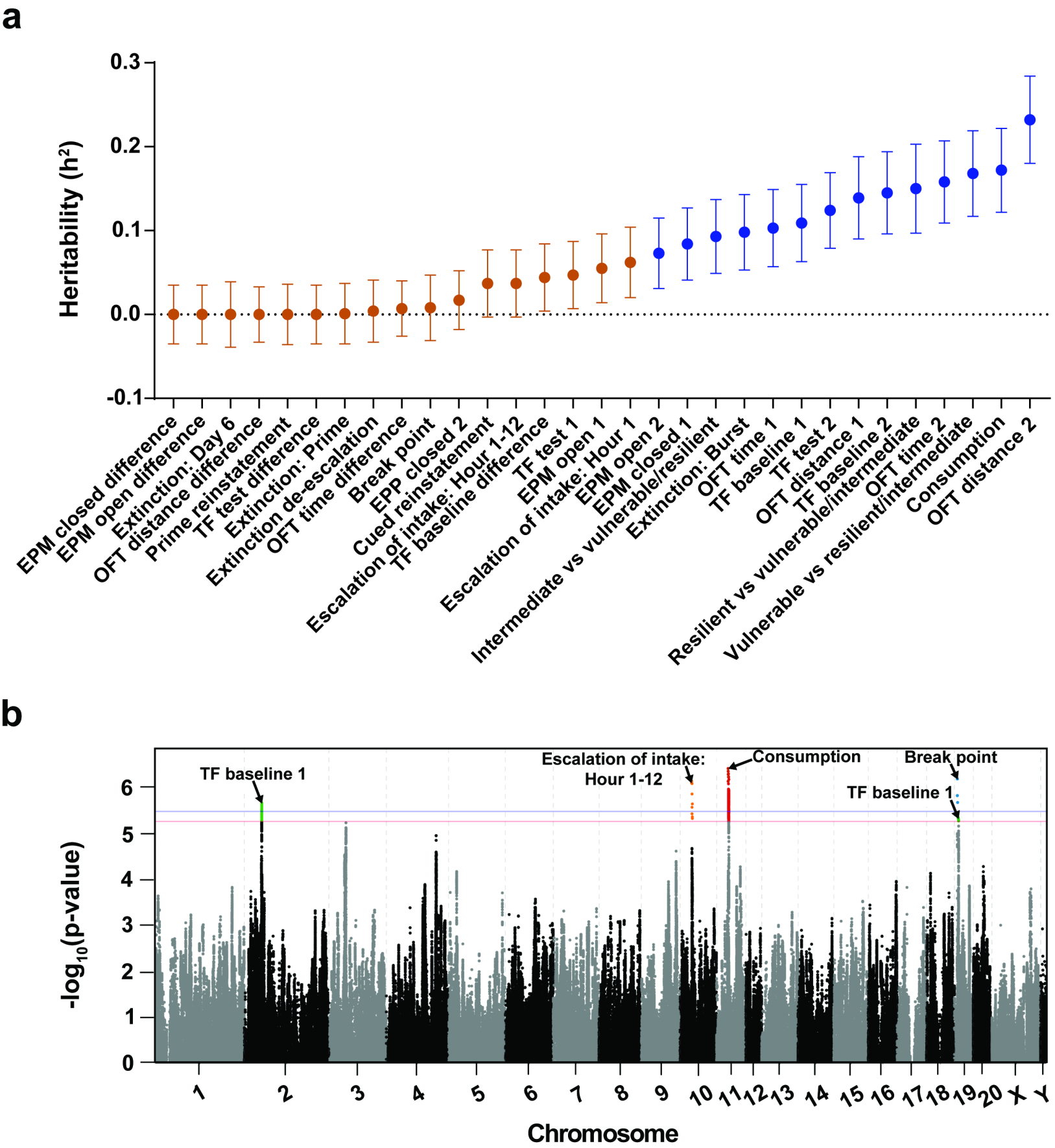
769 **Figure 4** GWAS results for total heroin consumed across self-administration training. **a)** Regional
770 association plot for chromosome 11, with the purple dot indicating the top SNP and location on
771 chromosome indicated on the plot. The color of additional SNPS refers to extent of linkage disequilibrium
772 with the top SNP. Known genes of SNPs are indicated below the plots. **b)** Effect plots for total heroin

773 consumption during training (left: normalized data; right: raw data) for OUD phenotypes (Vulnerable:
774 pink; Intermediate/resilient: yellow) according to peak SNP allelic variant. Half of a violin plot for each
775 phenotype is shown, with the solid line indicating the median value of the data and the dash lines
776 representing the upper and lower interquartile range. **c)** Top SNP allelic variant OUD phenotype cluster
777 composition differ ($x^2(1, 853)=18.12$, $p<0.0001$), with the minor allele associated with greater OUD
778 resiliency. Significance threshold: red line, $p<0.10$; blue line, $p<0.05$. (n=853; Vulnerable (pink), n= 361;
779 Intermediate/resilient (yellow), n= 492)

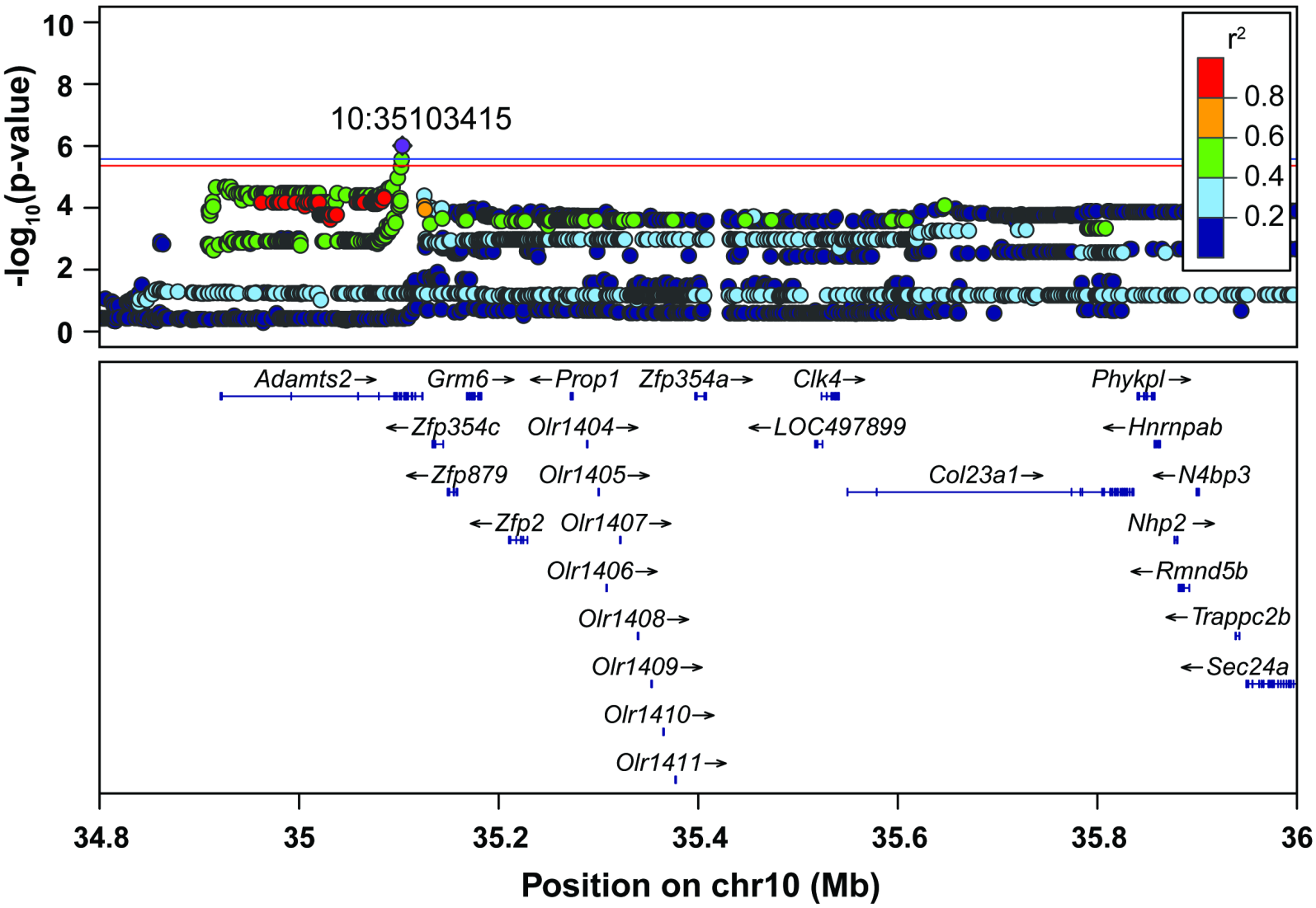
780 **Figure 5** GWAS results for break point achieved during the progressive ratio test. **a)** Regional association
781 plot for chromosome 19, with the top SNP in purple and the color of other significant SNPs referring to
782 level of linkage disequilibrium with the top SNPs. The location of the top SNP is indicated on the plot.
783 Known genes of SNPs are indicated below the plots. **b)** Effect plots showing break point achieved during
784 the progressive ratio test (left: normalized data, right: raw data) for OUD vulnerable (pink) and
785 intermediate/resilient (yellow) rats in relation to the top SNP allelic variant. Half a violin plot is shown for
786 each OUD phenotype, with the solid line indicating the median value of the data set, whereas the dashed
787 lines represent the upper and lower interquartile range. **c)** OUD phenotypic cluster composition for both
788 peak SNP allelic variants. Cluster composition between variants differed ($x^2(1, 864)=18.91$, $p<0.0001$), with
789 the minor allele heavily biased toward an OUD vulnerable phenotype. Significance threshold: red line,
790 $p<0.10$; blue line, $p<0.05$. (n=864; Vulnerable (pink), n= 345; Intermediate/resilient (yellow), n= 519)

791 **Figure 6** GWAS results for analgesic threshold during the TF baseline session prior to heroin experience.
792 Regional association plots for **a)** chromosome 2 and **b)** chromosome 19. The top SNP identified for each
793 chromosome is in purple with chromosome location indicated on the plot. The color of other SNPs
794 corresponds to extent of linkage disequilibrium with the top SNP. Known genes of SNPs are indicated
795 below the plots. Significance threshold: red line, $p<0.10$; blue line, $p<0.05$. (n= 874)


a
Catheterization surgeries
Progressive Ratio
Within session extinction and prime-induced reinstatement (0.25 mg/kg, sc)
Cue-induced reinstatement
Tissue collection
OFT, EPM, TF tests
Heroin self-administration (12-hr/session)
Heroin self-administration (12-hr/session)
Extinction
OFT, EPM, TF tests
Day 1
7
14
36
37
40
41
47
48
55
56
b
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
OFT distance 1
OFT time 1
TF baseline 1
EPM closed 1
EPM open 1
TF test 1
Escalation of intake: Hour 1
Escalation of intake: Hour 1-12
Consumption
Break point
Extinction: Burst
Extinction: Prime
Extinction: Day 6
Extinction de-escalation
Prime reinstatement
Cued reinstatement
OFT distance 2
OFT time 2
OFT difference distance
OFT difference time
EPM closed 2
EPM open 2
EPM difference closed
EPM difference open
TF baseline 2
TF test 2
TF difference baseline
TF difference test


a
Heritability (h^2)
0.3
0.2
0.1
0.0
-0.1
EPM closed difference
EPM open difference
Extinction: Day 6
OFT distance difference
Prime reinstatement
TF test difference
Extinction: Prime
Extinction de-escalation
OFT time difference
Break point
EPP closed 2
Cued reinstatement
Escalation of intake: Hour 1-12
TF baseline difference
TF test 1
EPM open 1
Escalation of intake: Hour 1
EPM open 2
EPM closed 1
Intermediate vs vulnerable/resilient
Extinction: Burst
OFT time 1
TF baseline 1
TF test 2
OFT distance 1
TF baseline 2
Resilient vs vulnerable/intermediate
OFT time 2
Vulnerable vs resilient/intermediate
Consumption
OFT distance 2
b
-log10(p-value)
TF baseline 1
Escalation of intake:
Hour 1-12
Consumption
Break point
TF baseline 1
6
5
4
3
2
1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
X
Y
Chromosome

a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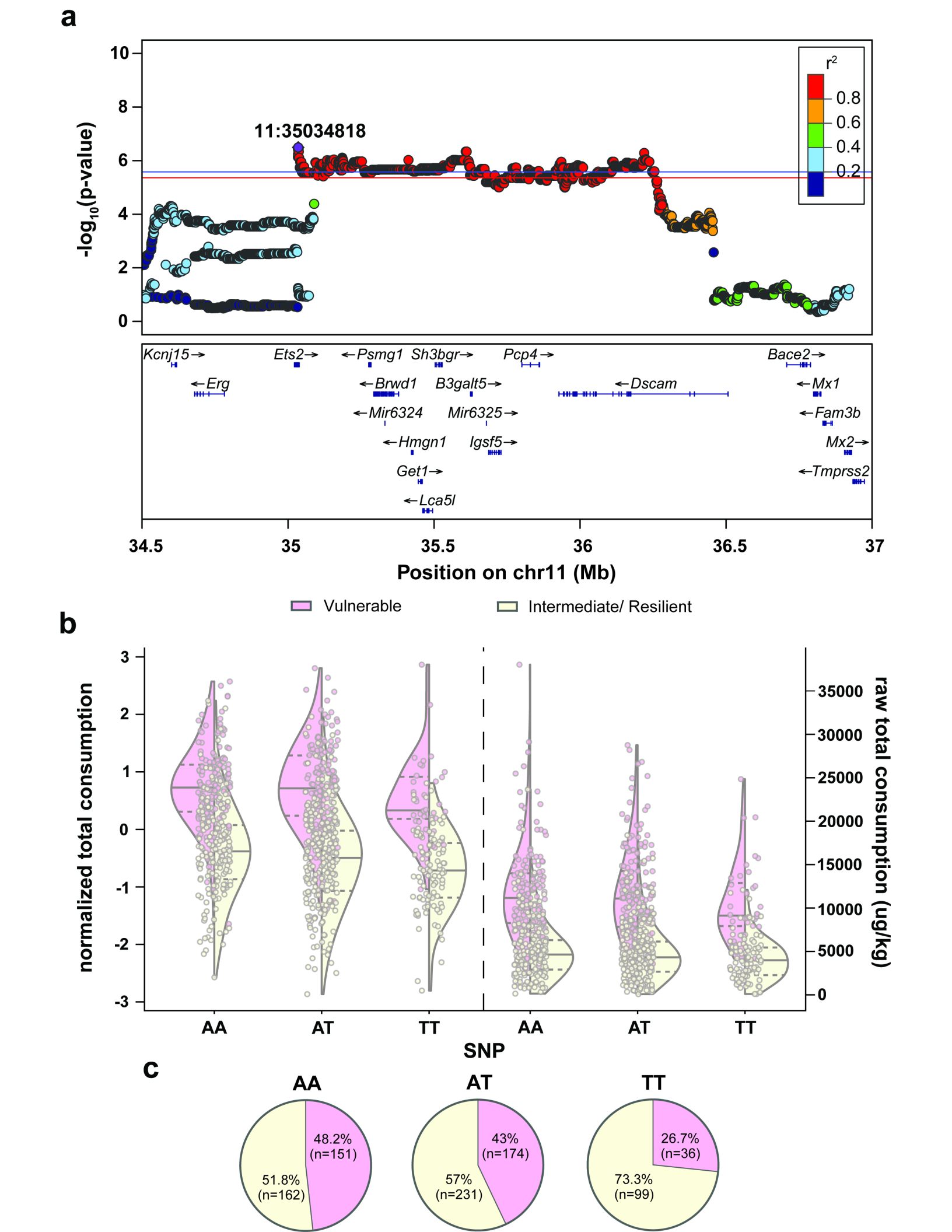
0.6
0.4
0.2
Adamts2
Grm6
Prop1
Zfp354a
Clk4
Phykpl
Zfp354c
Olr1404
LOC497899
Hnrnpab
Zfp879
Olr1405
Col23a1
N4bp3
Zfp2
Olr1407
Nhp2
Olr1406
Rmnd5b
Olr1408
Trappc2b
Olr1409
Sec24a
Olr1410
Olr1411
34.8
35
35.2
35.4
35.6
35.8
36
Position on chr10 (Mb)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r2
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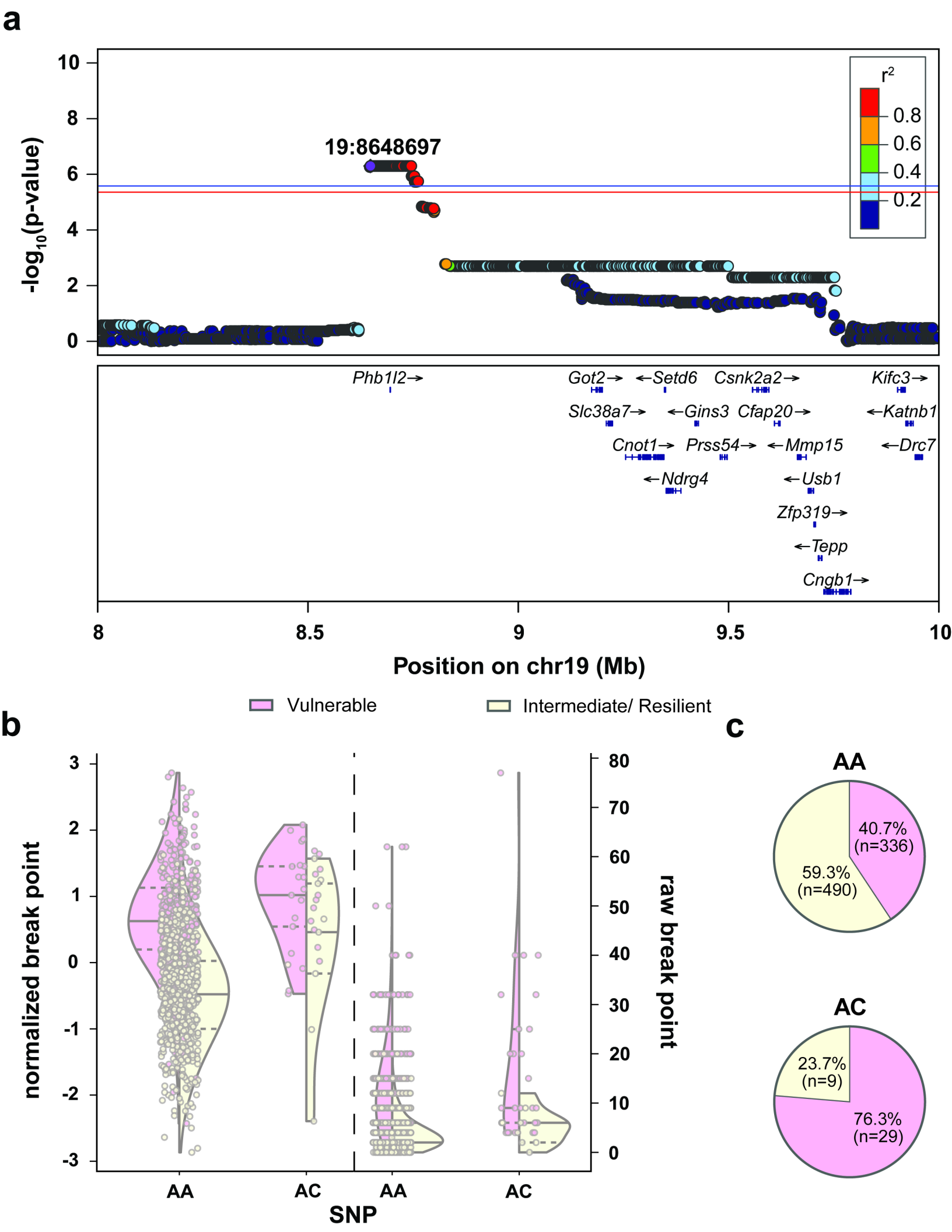
0.6
0.4
0.2
Kcnj15
Ets2
Psmg1
Sh3bgr
Pcp4
Bace2
Erg
Brwd1
B3galt5
Dscam
Mx1
Mir6324
Mir6325
Fam3b
Hmgn1
Igsf5
Mx2
Get1
Tmprss2
Lca5l
Position on chr11 (Mb)
Vulnerable
Intermediate/ Resilient
b
normalized total consumption
raw total consumption (ug/kg)
AA
AT
TT
SNP
c
48.2% (n=151)
51.8% (n=162)
43% (n=174)
57% (n=231)
26.7% (n=36)
73.3% (n=99)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r²
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0.6
0.4
0.2
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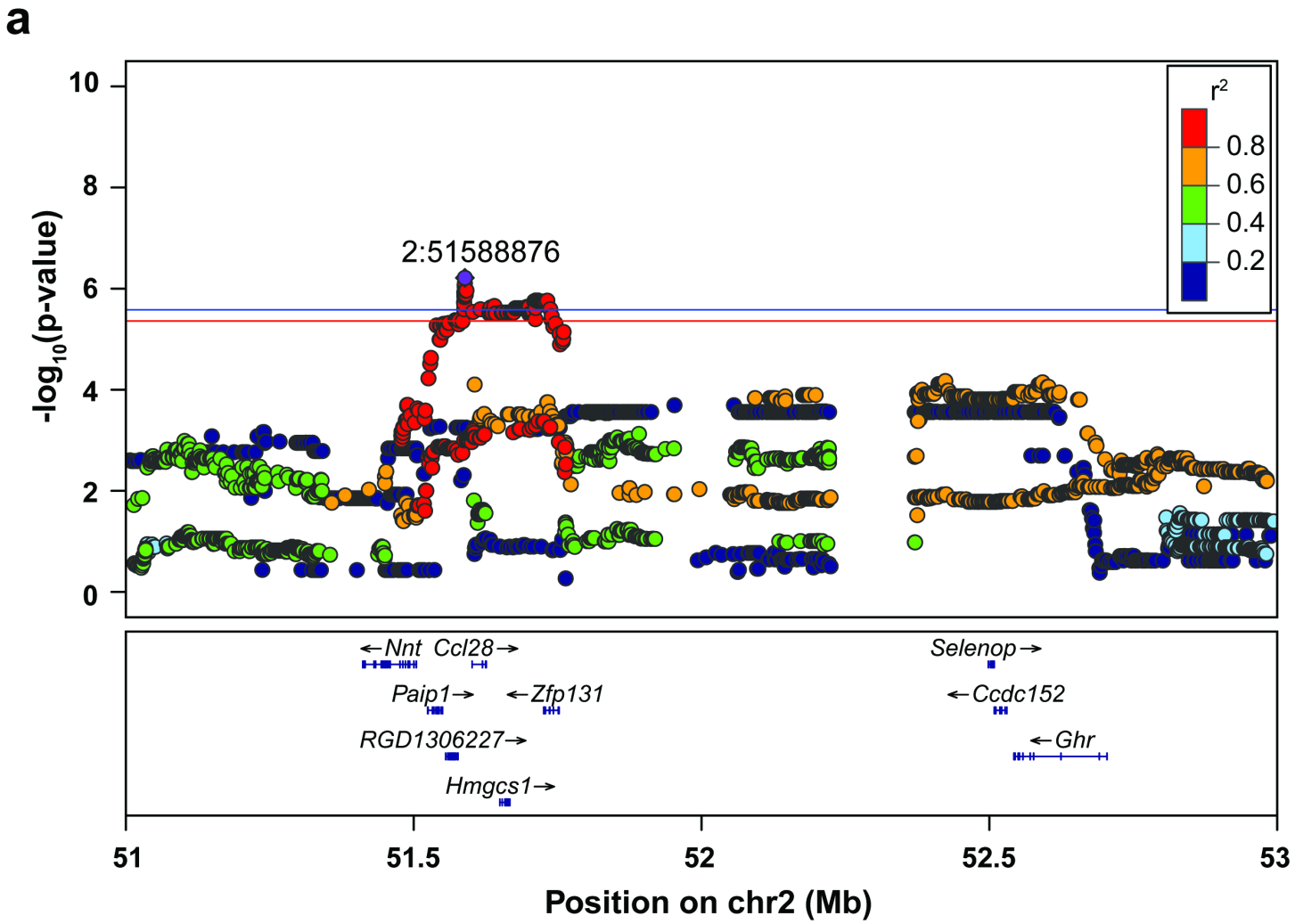
Got2→
←Setd6
Csnk2a2→
Kifc3→
Slc38a7→
←Gins3
Cfap20→
←Katnb1
Cnot1→
Prss54→
←Mmp15
←Drc7
←Ndrg4
←Usb1
Zfp319→
←Tepp
Cngb1→
Position on chr19 (Mb)
b
Vulnerable
Intermediate/ Resilient
normalized break point
raw break point
AA
AC
AA
AC
SNP
c
AA
40.7%
(n=336)
59.3%
(n=490)
AC
23.7%
(n=9)
76.3%
(n=29)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10
8
6
4
2
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Ccl28
Paip1
Zfp131
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Selenop
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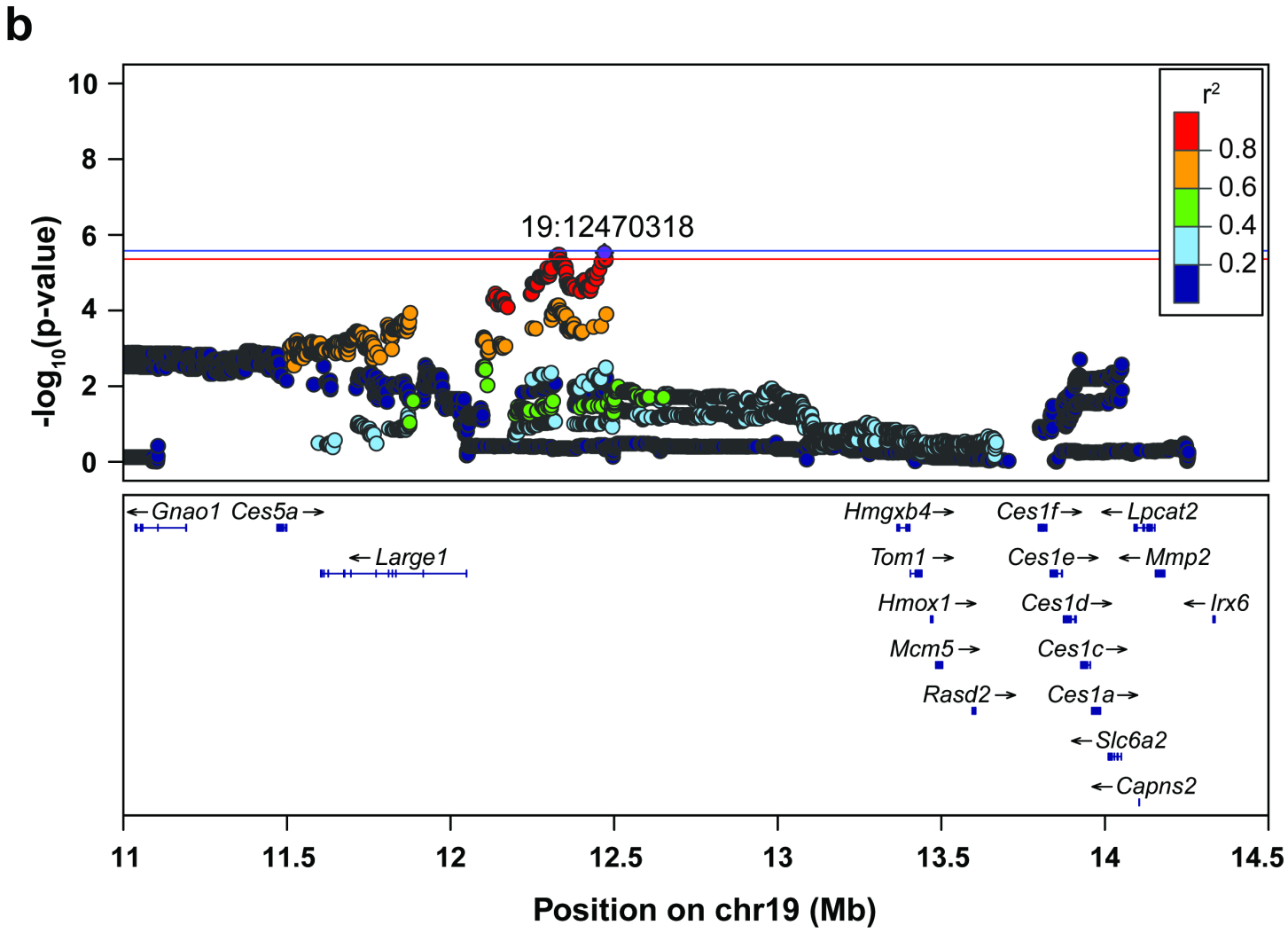
Ghr
51
51.5
52
52.5
53
Position on chr2 (Mb)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Gnao1
Ces5a
Large1
Hmgxb4
Ces1f
Lpcat2
Tom1
Ces1e
Mmp2
Hmox1
Ces1d
Irx6
Mcm5
Ces1c
Rasd2
Ces1a
Slc6a2
Capns2
11
11.5
12
12.5
13
13.5
14
14.5
Position on chr19 (Mb)

Supplementary Information

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 Electronics, 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 ratio 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 resulted 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.

GWAS analysis

A detailed report of all GWAS findings may be found in the included ZIP file.

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
*Breakpoint	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 (time point 1) and following (time point 2) heroin experience, and the difference between the two time points, along with several behaviors associated with heroin taking, refraining and seeking.

Traits used in the non-linear clustering approach to categorize rats into OUD phenotypes are designated with an asterisk.

Trait	Covariates
Prior to heroin experience	
OFT distance 1	Weight at time of surgery; Batch 6
OFT time 1	Batch 6
EPM closed 1	Weight at time of surgery; Age at time of experiment; Sex; Batch 2, 3, 4, 6, 12, 13, 15 and 17; Two specific testing chambers
EPM open 1	Weight at time of surgery; Sex; Batch 2, 3, 4, 12 and 17 ; Coat color: black hood; Three specific testing chambers
TF baseline 1	Batch 3 and 4; Coat color: black and brown hood
TF test 1	Weight at time of surgery; Sex; Three specific testing chambers
Heroin measures	
Escalation of intake: Hour 1	None
Escalation of intake: Hour 1-12	None
Consumption	Weight at time of surgery; Age at time of first self-administration session; Sex; Batch 14 and 15; Two specific testing chambers
Break point	Age at time of experiment; Behavior for Batch 14 and 15; One specific testing chamber
Extinction burst	Weight at time of surgery; Batch 4 and 17; 1 specific testing chamber
Extinction: Prime	Weight at time of surgery; Batch 2, 4, 14, 17; Two specific testing chambers
Extinction: Day 6	Batch 3
Extinction de-escalation	Batch 2
Prime reinstatement	Weight at time of surgery; Weight at time of experiment; Sex; batch 3; One specific testing chamber
Cued reinstatement	Weight at time of surgery for; Age at time of experiment; Behavior for Batch 2, 3, 15 and 17; Two specific testing chambers
Following heroin experience	
OFT distance 2	Age at time of experiment for Batch 2
OFT time 2	Age at time of experiment; Batch 2
OFT difference distance	Batch 6
OFT time difference	Batch 6
EPM closed 2	Weight at time of surgery; Sex; Batch 2, 6, 12, 16. One specific testing chamber
EPM open 2	Weight at time of surgery; Sex; Batch 2, 6, and 16; Coat color: albino and black hood; Two specific testing chambers
EPM difference closed	Batch 2, 3, 4, 5, 6, 7, 9, 10, 11, 13 and 16
EPM difference open	Batch 7 and 16. Coat color: albino
TF baseline 2	Weight at time of surgery; Batch 2, 3, 4, 8, 14
TF test 2	Weight at time of surgery; Sex; Batch 3, 4, 7; Three specific testing chambers
TF difference baseline	Batch 2
TF difference test	Weight at time of surgery

Supplemental Table 2 Covariates for behavioral traits from rats tested at MUSC. Covariates that explained >2% of variance were regressed out prior to further analyses. Batches of 40 rats (20 males and 20 females) were shipped every few months from early 2019 through the end of 2022. Testing chamber refers to behavioral testing chamber that all heroin taking, extinction and seeking occurred in. Heterogeneous rats have 5 distinct coat colors: albino, black, brown, black hood and brown hood.

Trait	Covariates
Prior to heroin experience	
OFT distance 1	Weight at time of surgery; Sex; Four specific testing chambers
OFT time 1	Weight at time of surgery; Sex; Batch 8 and 14; Two specific testing chambers
EPM closed 1	Batch 3, 9, 11, 14 and 16
EPM open 1	Weight at time of surgery; Age at time of experiment; Sex; Batch 9 and 11; One specific testing chamber
TF baseline 1	Weight at time of surgery; Batch 5, 7, 8 and 14; Coat color: brown, albino and brown hood
TF test 1	Weight at time of surgery; Sex; Batch 3, 4, 5, 6, 7, 13
Heroin measures	
Escalation of intake: Hour 1	Batch 4, 5, 12 and 13
Escalation of intake: Hour 1-12	Batch 4, 5, 6, 12 and 14
Consumption	Weight at time of surgery; Age at time of first self-administration session; Sex; One specific testing chambers
Break point	Weight at time of surgery; Sex; Batch 4 and 11
Extinction burst	Weight at time of surgery; Sex
Extinction: Prime	Weight at time of surgery; Sex; Batch 6
Extinction: Day 6	Weight at time of surgery; Sex; Batch 6, 12 and 14; One specific testing chamber
Extinction de-escalation	Batch 6
Prime reinstatement	Weight at time of surgery; Sex; batch 8, 10 and 12; One specific testing chamber
Cued reinstatement	Weight at time of surgery for; Sex; Batch 12, 13, 14; One specific testing chamber
Following heroin experience	
OFT distance 2	Weight at time of surgery; Age at time of experiment; Sex; Two specific testing chambers
OFT time 2	Weight at time of surgery; Sex; Batch 8 and 16
OFT difference distance	Age at time of experiment; Sex; Batch 8; Two specific testing chambers
OFT time difference	Age at time of experiment; Sex; Two specific testing chambers
EPM closed 2	Weight at time of surgery; Batch 4 and 8
EPM open 2	Weight at time of surgery; Sex; Batch 6; One specific testing chamber
EPM difference closed	Batch 3 and 11
EPM difference open	Two specific testing chambers
TF baseline 2	Weight at time of surgery; Sex; Batch 7 and 16; Coat color: albino; One specific testing chamber
TF test 2	Weight at time of surgery; Age at time of experiment; Batch 4, 5, 8, 10, 11, 12, 13, 14 and 16
TF difference baseline	None
TF difference test	Batch 3, 5, 7, 8, 9, 10, 11, 12 and 14

46 **Supplemental Table 3** Covariates for behavioral traits from rats tested at UCAM. All covariates that
47 explained >2% of variance for a trait were regressed out prior to continuing analyses. Rats were shipped
48 every few months from early 2019 through the end of 2022 in batches of 40 rats (20 males and 20
49 females). Testing chamber refers to behavioral testing chamber that all heroin taking, extinction and
50 seeking occurred in. Heterogeneous rats have 5 distinct coat colors: albino, black, brown, black hood and
51 brown hood.

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Behavioral trait correlations

Trait 1	Trait2	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^{-7}	874
	Extinction: Burst	0.19	9.72×10^{-9}	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^{-5}	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	7.64×10^{-5}	873
Extinction: Day 6	Cued reinstatement	0.54	1.21×10^{-67}	866
	Extinction de-escalation	0.44	7.92×10^{-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	8.33×10^{-5}	862
	OFT time 2	0.15	9.46×10^{-6}	860
	EPM closed 2	-0.11	0.0008	865
OFT distance 1	OFT distance 2	0.45	5.13×10^{-44}	864
	OFT distance difference	-0.49	4.07×10^{-52}	864
	OFT time 2	0.39	2.64×10^{-33}	864
	OFT time difference	-0.51	4.28×10^{-58}	864
OFT distance 2	OFT distance difference	0.46	1.53×10^{-46}	864
	OFT time difference	0.40	1.29×10^{-33}	864
	OFT time 1	0.40	7.59×10^{-35}	864
	OFT time 2	0.89	5.40×10^{-291}	868
OFT distance difference	OFT time difference	0.92	0.00	864
	OFT time 1	-0.47	3.15×10^{-49}	864
	OFT time 2	0.45	2.54×10^{-44}	864
OFT time 1	OFT time difference	-0.54	8.73×10^{-68}	864
	OFT time 2	0.41	4.28×10^{-36}	864
OFT time 2	OFT time difference	0.45	2.52×10^{-43}	864
EPM open 1	EPM open 2	0.25	8.36×10^{-14}	872
	EPM open difference	-0.44	7.78×10^{-43}	872
	EPM closed 1	-0.58	3.65×10^{-78}	872
	EPM closed 2	-0.18	1.33×10^{-7}	872
	EPM closed difference	0.32	1.80×10^{-22}	872
EPM open 2	EPM open difference	0.61	1.82×10^{-90}	872
	EPM closed 1	-0.32	1.32×10^{-22}	873
	EPM closed 2	-0.67	9.29×10^{-166}	873
	EPM closed difference	-0.26	7.65×10^{-15}	873
EPM open difference	EPM closed 1	0.12	0.0005	872
	EPM closed 2	-0.41	5.88×10^{-36}	872
	EPM closed difference	-0.48	8.16×10^{-51}	872
EPM closed 1	EPM closed 2	0.37	2.77×10^{-30}	873
	EPM closed difference	-0.45	0.0005	873
EPM closed 2	EPM closed difference	0.47	2.38×10^{-48}	873
TF baseline 1	TF baseline 2	0.11	0.0012	874
	TF baseline difference	-0.60	7.67×10^{-88}	874
	TF test 2	0.11	0.0013	874
TF baseline 2	TF baseline difference	0.59	7.30×10^{-84}	874
	TF test 2	0.16	1.39×10^{-6}	874
TF test 1	TF test 2	0.17	9.78×10^{-7}	874
	TF test difference	-0.59	3.40×10^{-84}	874
TF test 2	TF test difference	0.57	2.29×10^{-77}	874

Supplemental Table 4 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 nociceptive threshold exhibited greater within-test covariance. Direction and magnitude 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 adjustment for correction for multiple comparisons was implemented, resulting in a $p<0.0018$ in order to attain significance.

Trait	h^2SNP	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 5 SNP 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.

SNP	CHR	MISS	Genotype	HWE	MAF	A1	A2
19:8648697	19	0.00000	0/38/836	1.00000	0.02174	C	A
10:35103415	10	0.09725	44/288/457	0.92210	0.23830	A	G
2:51588876	2	0.05378	156/437/234	0.06793	0.45280	C	G
19:12470318	19	0.03661	103/371/368	0.54060	0.34260	T	C
11:35034818	11	0.01259	138/409/316	0.77620	0.39690	T	A

Supplemental Table 6 Minor allele frequency and Hardy-Weinberg equilibrium values for significant QTLs. For each SNP identified, the chromosome number (CHR), missingness (MISS), genotype (count of reference/reference, reference/alternate, and alternate/alternate alleles), p-value of Hardy-Weinberg equilibrium (HWE; assumes H=0 is in HWE), minor allele frequency (MAF), non-reference allele (A1) and reference allele (A2). All SNPs passed the filters for MISS, HWE and MAF.

Trait	Top SNP location	-Log$_{(10)}$ (p-value): MUSC	-Log$_{(10)}$ (p-value): UCAM	-Log$_{(10)}$ (p-value): Combined
Escalation of intake: Hour 1-12	10:35103415	3.324	3.238	6.007
Consumption	11:35034818	3.559	3.673	6.493
Break point	19:8648697	2.918	3.763	6.298
TF baseline 1	2:51588876	4.661	2.200	6.213
TF baseline 1	19:12470318	2.912	3.646	5.531

Supplemental Table 7 Comparison of GWAS results between sites. Table shows significant QTLs conserved between GWAS analysis when combining sites versus considering each side separately. Each trait, p-value (-$\log_{10}$) and chromosome location (chromosome: location) is recorded for each significant QTL. (MUSC, n=479; UCAM, n=395)

Total consumption					
Gene	**Effect**	**LD with top SNP**	**Relevant psychiatric function**	**Model**	**Citation**
Brwd1	Coding variant	0.99	Cigarette smoking	Human	63-68
			Externalizing behaviors	Human	69
			ADHD	Human	66, 70
			Hyperthymia in bipolar disorder	Human	71
PCP4	Coding variant; eQTL	0.99; 0.88	Alcohol drinking and use disorder	Human	73, 77
			Nicotine use withdrawal	Rodent	78
			Cigarette smoking	Human	63, 72
Sh3bgr	Coding variant; eQTL	0.99; 0.99	Unknown		
ENSRNOG 00000068065	eQTL; sQTL	0.99-1.00	Unknown		
Get1	sQTL	0.99	Unknown		
Break point					
Phb1l2	Coding variant	1.0	Alcohol use disorder (analogue)	Human	85
Mmp15	eQTL	1.0	MMP2 necessary for heroin extinction	Rodent	88
Baseline nociception prior to heroin experience (Chromosome 2)					
Ccdc152	Coding variant	0.77	Unknown		
Ghr	Coding variant	0.77	Unknown		
Zfp131	Coding variant	0.82	Unknown		
Selenop	eQTL; sQTL	0.66; 0.78-79	Methamphetamine toxicity	Rodent	91
Baseline nociception prior to heroin experience (Chromosome 19)					
Large1	Coding variant; eQTL	0.75; 0.69-0.75	Educational attainment	Human	89-91
			Cigarette smoking	Human	63
			Alcohol dependence	Human	92
			Neuroticism	Human	93
			Depression	Human	93-94
Rps18l2	eQTL	0.98	Unknown		
Tom1	sQTL	1.0	Cigarette smoking	Human	72
Mt3	sQTL	0.75	Alzheimer's and Huntington's disease	Human	85

112 **Supplemental Table 8** Summarization of significant QTLs for selected traits and relevance to psychiatric
113 disorders. The gene, effect (relationship to peak SNP), linkage disequilibrium (LD) to peak SNP, known
114 functional role in psychiatric illnesses, model organisms used and citation for the study are recorded.

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	SNP				
Trait	*2:51588876*	*10:35103415*	*11:35034818*	*19:12470318*	*19:8648697*
OFT distance 1	0.64	0.26	0.01	2.20	0.14
OFT time 1	1.15	0.07	0.10	2.29	0.22
EPM open 1	0.97	0.19	0.21	0.04	0.34
EPM closed 1	0.03	0.38	0.04	0.42	0.04
TF baseline 1	**6.21**	0.48	1.26	**5.53**	0.31
TF test 1	0.55	0.08	0.29	0.52	0.98
Escalation of intake: Hour 1	0.34	4.01	3.63	0.85	0.17
Escalation of intake: Hour 1-12	0.24	**6.01**	2.11	0.52	2.21
Consumption	<0.01	1.93	**6.49**	0.91	3.81
Break point	0.39	0.90	3.04	2.09	**6.30**
Extinction: Burst	0.08	0.72	2.06	0.35	2.09
Extinction: Prime	0.06	0.31	2.00	1.05	0.44
Extinction: Day 6	0.81	0.24	0.06	0.21	1.68
Extinction de-escalation	0.48	0.38	0.44	0.08	1.07
Prime reinstatement	0.74	0.25	2.36	1.04	2.11
Cued reinstatement	0.70	0.63	2.67	0.17	0.73
OFT distance 2	0.33	0.81	0.27	0.31	0.24
OFT distance difference	0.81	0.05	0.07	0.84	0.10
OFT time 2	0.35	0.25	0.04	0.34	0.37
OFT time difference	0.55	0.12	0.03	0.59	0.21
EPM open 2	0.33	0.19	0.64	0.04	0.18
EPM Open difference	0.32	0.63	0.13	0.30	0.11
EPM closed 2	0.01	0.96	1.35	0.04	0.28
EPM closed difference	0.89	0.44	0.85	0.46	0.01
TF baseline 2	0.01	0.33	0.26	0.58	0.46
TF baseline difference	3.81	0.04	0.64	1.89	1.07
TF test 2	0.14	0.31	0.22	2.20	0.19
TF test difference	0.63	0.23	0.02	1.07	0.99
Vulnerable vs resilient/intermediate	0.007	1.72	4.52	1.58	5.16
Intermediate vs vulnerable/resilient	1.05	0.54	0.29	0.78	1.43
Resilient vs vulnerable/intermediate	0.98	0.78	3.82	0.43	2.27

132 **Supplemental Table 9** Significant SNPs across all behavioral traits tested. The p-value ($-\log_{10}$) for the
133 significant SNPs identified in the GWAS analysis across all behavioral traits. Values in bold indicate
134 genome-wide significant p-values; underlines indicate p-values greater than -log(p) = 1.3 (-log(p) of 1.3 =
135 0.05) but below the threshold for genome-wide significance of -log(p)=5.58 (-log(p) = 5.58 = 0.00000263)

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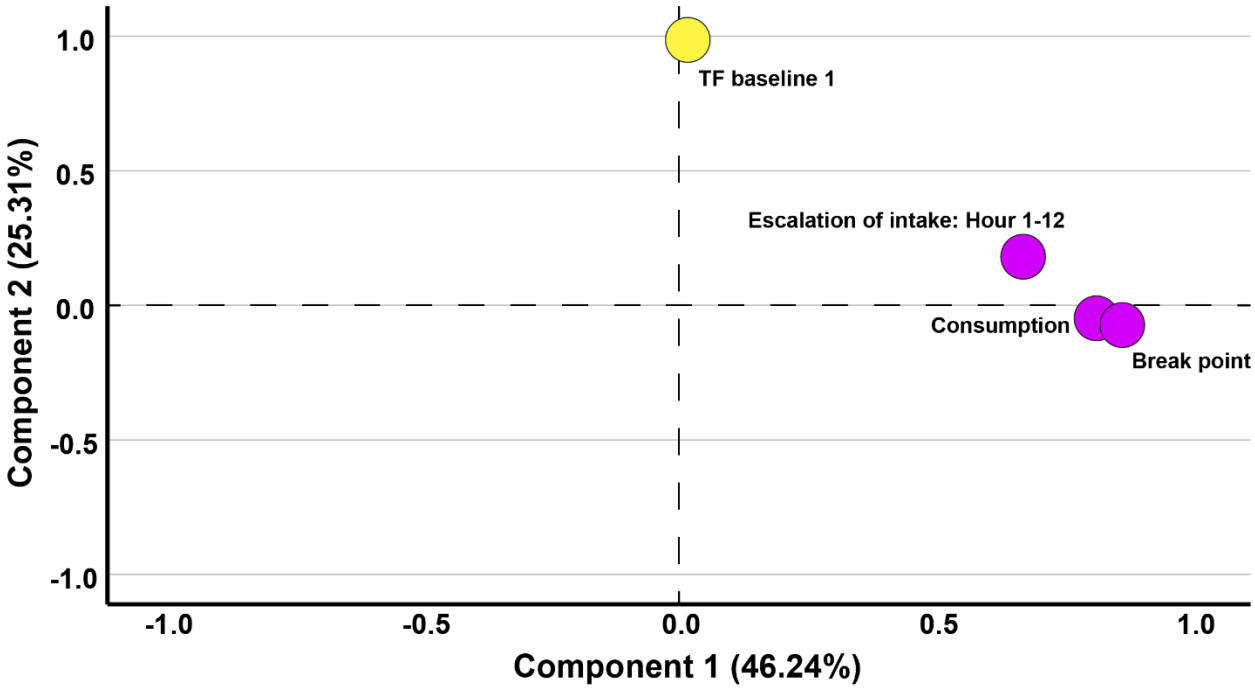

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153 **Supplemental Figure 1** Principal component analysis for behavioral traits with significant QTLs. Two
154 factors were identified and comprised 71.55% of total variance in the data. Heroin-taking behaviors
155 accounted for 46.24% of variance (component 1, purple) with traits exhibiting strong loading (Break point:
156 0.86; Consumption: 0.81; and Escalation of intake: Hour 1-12: 0.67), and was mutually exclusive from
157 baseline nociception prior to heroin experience (component 2, yellow) which accounted for 25.31% of
158 variance with strong loading (0.99). Axes represent factor loading values for each trait within respective
159 component. (n=874)

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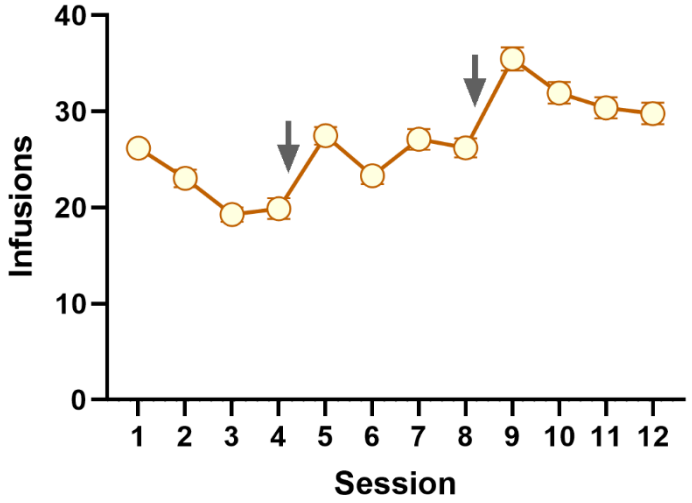

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172 **Supplemental Figure 2** Total number of heroin infusions earned across training sessions. Mean ± SEM for

173 total heroin infusions (20 µg/kg/100 µl infusion) earned during heroin self-administration across training

174 sessions. Animals underwent four training sessions a week with one day random day off between

175 Monday-Friday. Grey arrows indicate brief periods of forced abstinence between training weeks. Rats

176 increased heroin intake over the course of training (mixed effects ANOVA; $F(5.90,5399)=43.24$, $p<0.001$).

177 (n=874)