Genome wide association study of behavioral, physiological and gene expression traits in a multigenerational mouse intercross

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Author contributions

NMG maintained the AIL colony, phenotyped the mice, prepared RNA-sequencing libraries, and performed QTL/eQTL analysis under supervision of AAP and MA. AAP and MA also provided computational resources for the analyses in this paper. JS prepared RNA-sequencing data for eQTL mapping under supervision of SC. CLS assisted with colony maintenance, tissue collection, RNA extraction, and GBS library preparation. MGD performed experiments in mutant mice. AL, JSG and AIHC measured hind limb muscle and bone phenotypes. NMG co-wrote the manuscript with AAP, who designed the study.

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Abstract

Genome wide association analyses (**GWAS**) in model organisms have numerous advantages compared to human GWAS, including the ability to use populations with well-defined genetic diversity, the ability to collect tissue for gene expression analysis and the ability to perform experimental manipulations. We examined behavioral, physiological, and gene expression traits in 1,063 male and female mice from a 50-generation intercross between two inbred strains (LG/J and SM/J). We used genotyping by sequencing in conjunction with whole genome sequence data from the two founder strains to obtain genotypes at 4.3M SNPs. As expected, all alleles were common (mean MAF=0.35) and linkage disequilibrium degraded rapidly, providing excellent power and sub-megabase mapping precision. We identified 126 genome-wide significant loci for 50 traits and integrated this information with 7,081 *cis*-eQTLs and 1,476 *trans*-eQTLs identified in hippocampus, striatum and prefrontal cortex. We replicated several loci that were identified using an earlier generation of this intercross, including an association between locomotor activity and a locus containing a single gene, *Csmd1*. We also showed that *Csmd1* mutant mice recapitulated the locomotor phenotype. Our results demonstrate the utility of this population, identify numerous novel associations, and provide examples of replication in an independent cohort, which is customary in human genetics, and replication by experimental manipulation, which is a unique advantage of model organisms.

Introduction

Genome-wide association studies (**GWAS**) have revolutionized psychiatric genetics; however, they have also presented numerous challenges. Some of these challenges can be addressed by using model organisms. For example, human GWAS are confounded by environmental variables, such as childhood trauma, which can reduce power to detect genetic associations. In model organisms, environmental variables can be carefully controlled. Furthermore, it has become clear that phenotypic variation in humans is due to numerous common and rare variants of small effect. In model organisms, genetic diversity can be controlled such that all variants are common. In addition, allelic effect sizes in model organisms are dramatically larger than in humans[1,2]. Furthermore, because the majority of associated loci are in noncoding regions, expression quantitative trait loci (**eQTLs**) are useful for elucidating underlying molecular mechanisms[3,4]. However, it remains challenging to obtain large, high quality samples of human tissue, particularly from the brain. In contrast, tissue for gene expression studies can be collected from model organisms under optimal conditions. Finally, the genomes of model organisms can be edited to assess the functional consequences of specific mutations.

Model organism GWAS often employ multigenerational intercrosses because they promote recombination of ancestral haplotypes. We used an advanced intercross line (**AIL**) of mice, which is the simplest possible multigenerational intercross. AILs, originally proposed by Darvasi and Soller in 1995 (ref. 5), are produced by intercrossing two inbred strains beyond the F2 generation. Because the two inbred strains contribute equally to an AIL, all variants are common, and alleles that are identical by state are necessarily identical by descent (**IBD**), which greatly simplifies phasing and imputation. We performed a GWAS using the world's most advanced mouse AIL, which was created over 50 generations ago by crossing the LG/J (**LG**) and SM/J (**SM**) inbred strains[6]. We investigated over 100 traits using mice from generations 50-56 (**G50-56**), including locomotor activity, response to methamphetamine, prepulse inhibition (**PPI**), body weight, and various muscle and bone phenotypes. We also sequenced mRNA from three brain regions and used those data to map eQTLs and identify quantitative trait genes (**QTGs**) at each locus. Finally, we explored replication of previous associations identified in LG x SM G34[7–11] and used mutant mice to test one of our strongest candidate QTGs.

Results

We used genotyping by sequencing (**GBS**) to genotype 1,063 of the 1,123 mice that were phenotyped (60 were not successfully genotyped for technical reasons described in the **Supplementary Note**). After quality control, GBS yielded 38,238 autosomal SNPs. In the 24 AIL mice that were also genotyped on the Giga Mouse Universal Genotyping Array (**GigaMUGA**)[12], only 24,934 markers were polymorphic in LG and SM (**Supplementary Fig.1**). LG and SM have been re-sequenced[13], which allowed us to impute AIL genotypes at ~4.3 million single nucleotide polymorphisms (**SNPs**; **Fig. 1a**). Consistent with the expectation for an AIL, the average minor allele frequency (**MAF**) was high (**Fig. 1b**). Linkage disequilibrium (**LD**) decay, which is critical to mapping resolution, has improved since LG x SM G34 (**Fig. 1c**)[7].

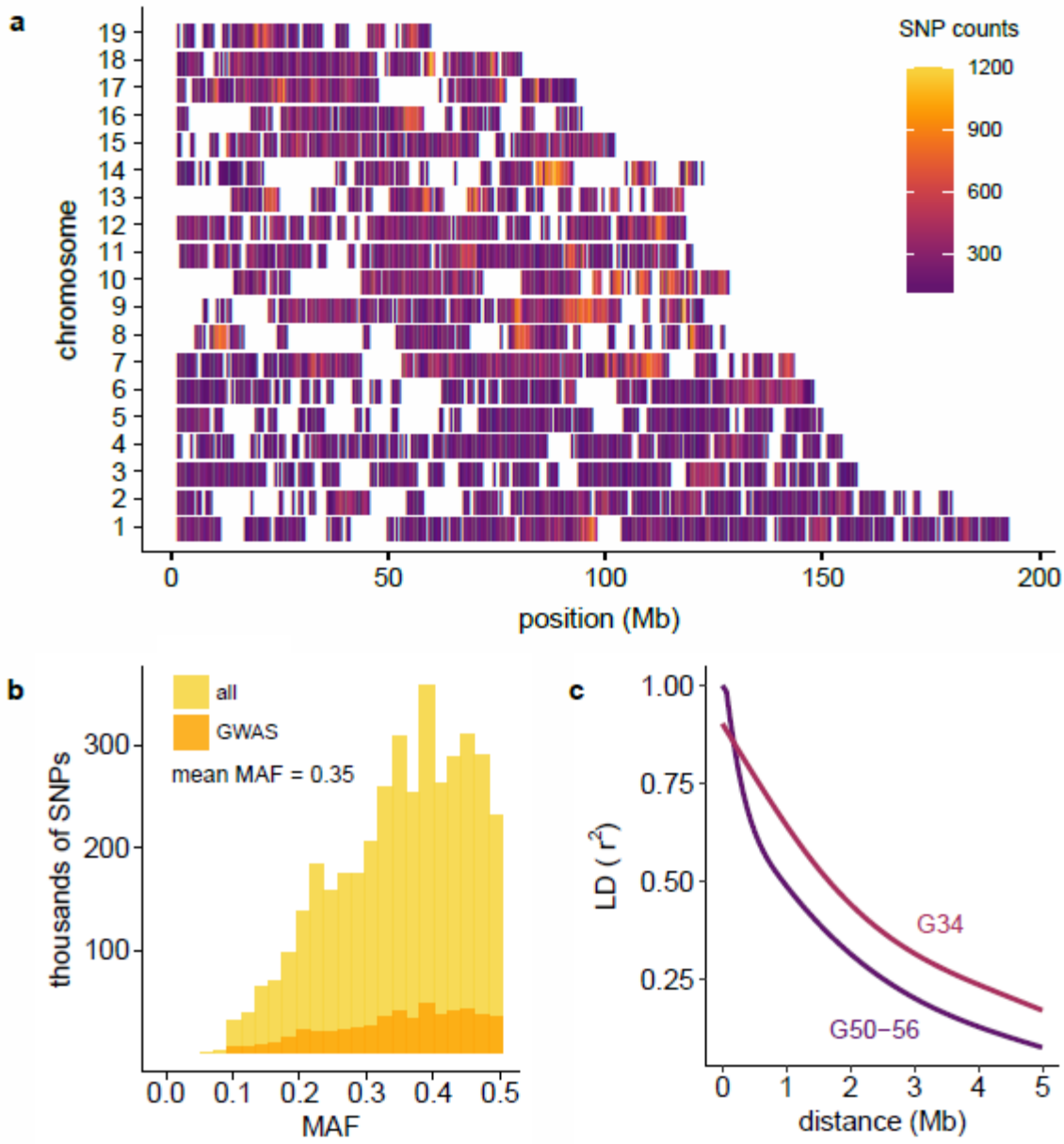

Figure 1. SNPs, minor allele frequencies (MAFs) and linkage disequilibrium (LD) decay in the LG x SM AIL. Imputation provided ~4.3 million SNPs. Filtering for LD ($r^2 \geq 0.95$), MAF < 0.1, and HWE ($p \leq 7.62 \times 10^{-6}$) resulted in 523,028 SNPs for GWAS. **(a)** SNP distribution and density of GWAS SNPs are plotted in 500 kb windows for each chromosome. As shown in **Supplementary Fig. 1**, regions with low SNP density correspond to regions predicted to be nearly IBD in LG and SM (Nikolskiy *et al*. 2015). **(b)** MAF distributions are shown for ~4.3 million imputed SNPs (gold; unfiltered) and for the 523,028 SNPs used for GWAS (orange; filtered). Mean MAF is the same in both SNP sets. **(c)** Comparison of LD decay in G50-56 (dark purple) and G34 (light purple) of the LG x SM AIL. Each curve was plotted using the 95th percentile of r^2 values for SNPs spaced up to 5 Mb apart.

LOCO-LMM effectively reduces the type II error rate

Linear mixed models (**LMMs**) are commonly used to perform GWAS in AILs and other populations that include close relatives[14]. SNP data are used to obtain a genetic relationship matrix (**GRM**); however, this can lead to an inflation of the type II error rate due to proximal contamination[15,16]. We previously proposed using a leave-one-chromosome-out LMM (**LOCO-LMM**) to address this issue[15]. To demonstrate the appropriateness of a LOCO-LMM, we performed a GWAS for albinism, which is a recessive Mendelian trait, using three approaches: a simple linear model, an LMM and a LOCO-LMM (**Fig. 2**). GWAS using a LOCO-LMM for albinism yielded an association on chromosome 7 (**Fig. 2a**); accurately identifying the albino locus (*Tyr*). As expected, p-values from a genome-wide scan using a linear model, which does not account for relatedness, appeared highly inflated (**Fig. 2b**). This inflation was greatly reduced by fitting a standard LMM, which included SNPs from chromosome 7 in both the fixed and random effects (**Fig. 2c**). The LOCO-LMM, which does not include SNPs from the chromosome being tested in the GRM, showed an intermediate level of inflation (**Fig. 2d**). Was the inflation observed in **Fig. 2b-d** due to true signal, or uncontrolled population structure? To

address this question, we repeated these analyses after excluding SNPs on chromosome 7 from the fixed effect (**Fig. 2e-g**). Even in the absence of the causal locus, the simple linear model showed substantial inflation, which can only be explained by population structure (**Fig. 2e**). The standard LMM appeared overly conservative, which we attributed to proximal contamination (**Fig. 2f**). The LOCO-LMM showed no inflation, consistent with the absence of *Tyr* and linked SNPs in the fixed effect (**Fig. 2g**). These results demonstrate the appropriateness of a LOCO-LMM.

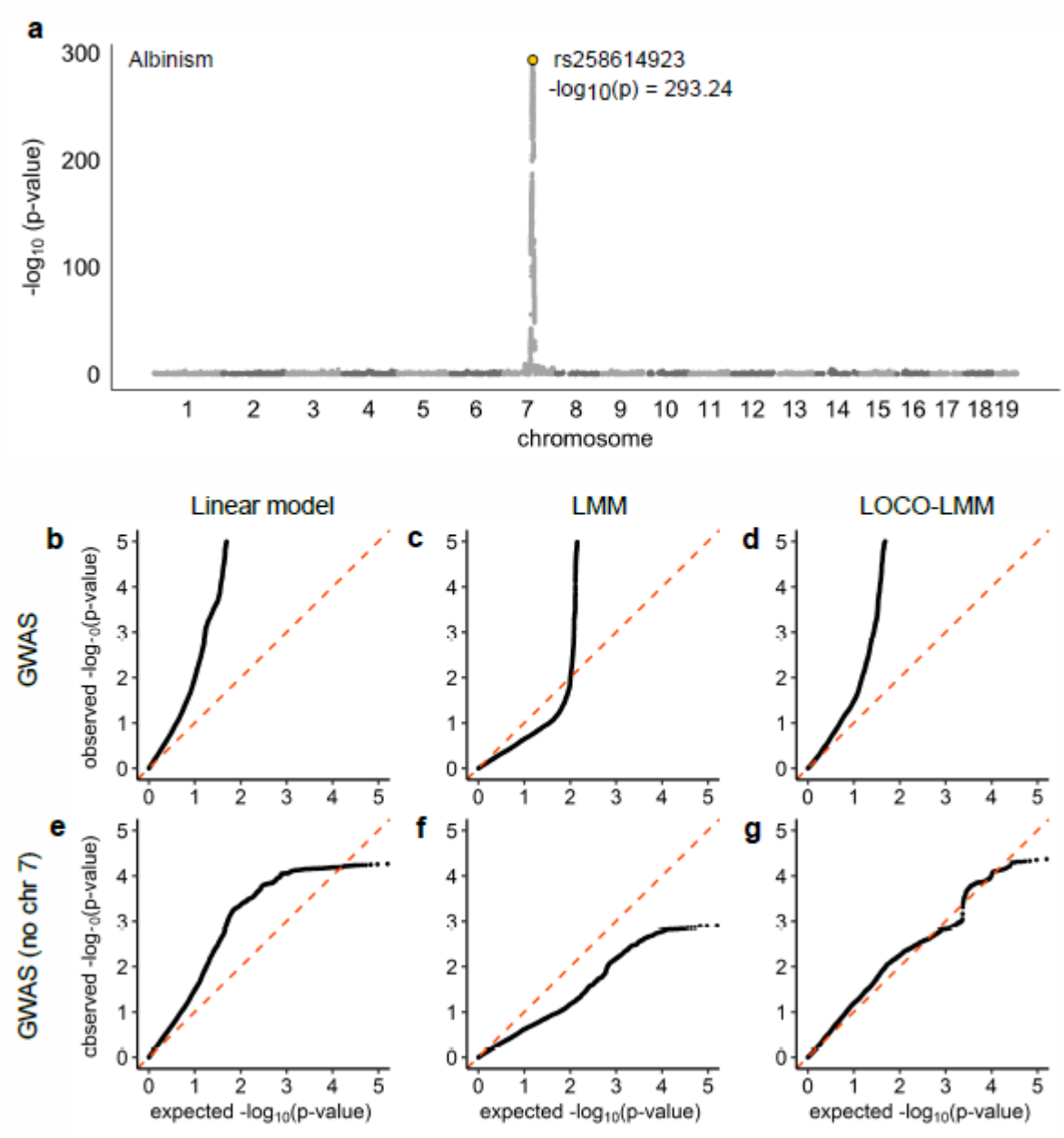

Figure 2. GWAS for albinism verifies that the LOCO-LMM effectively controls type I and type II error. We conducted a GWAS for albinism, a Mendelian trait caused by the *Tyr* locus on mouse chromosome 7, using three models: a linear model, an LMM, and a LOCO-LMM. We also repeated each scan after excluding SNPs on chromosome 7. A Manhattan plot of results from the LOCO-LMM is shown in (**a**). Quantile-quantile plots of expected vs. observed p-values are shown for (**b**) a simple linear model that does not account for relatedness; (**c**) a standard LMM that includes all GWAS SNPs in the genetic relatedness matrix (**GRM**; i.e. the random effect); and (**d**) a LOCO-LMM whose GRM excludes SNPs located on the chromosome being tested. Plots (**e-g**) show results after excluding chromosome 7 from the GWAS.

Genetic architecture of complex traits in the LG x SM AIL

We used an LD-pruned set of 523,028 autosomal SNPs genotyped in 1,063 mice from LG x SM G50-56 to perform GWAS for 120 behavioral and physiological traits using a LOCO-LMM (**Fig. 3a**). We used permutation to define a significance threshold of $p=8.06 \times 10^{-6}$ ($\alpha=0.05$). There were 52 loci associated with 33 behavioral traits and 74 loci associated with 17 physiological traits (**Fig. 3a**, **Supplementary Table 1; Supplementary Fig. 2**).

To estimate the heritability attributable to SNPs ('**SNP heritability**'), we calculated the proportion of trait variance explained by the additive effects of 523,028 SNPs. In general, heritability estimates were larger for physiological traits than for behavioral traits (**Fig. 3b, Supplementary Table 2**), which is consistent with findings in other rodent GWAS[17–19]. Mean heritability was 0.355 (se=0.045) for physiological traits and 0.168 (se=0.038) for behavioral traits (conditioned place preference, locomotor sensitization, and habituation to startle were not found to have a genetic component and were excluded from the mean). In general, traits with higher heritabilities yielded more associations (**Supplementary Fig. 2**). However, there was no significant relationship between heritability and effect size at individual loci (**Supplementary Fig. 3**), suggesting that high heritability does not reliably predict the presence of large-effect alleles.

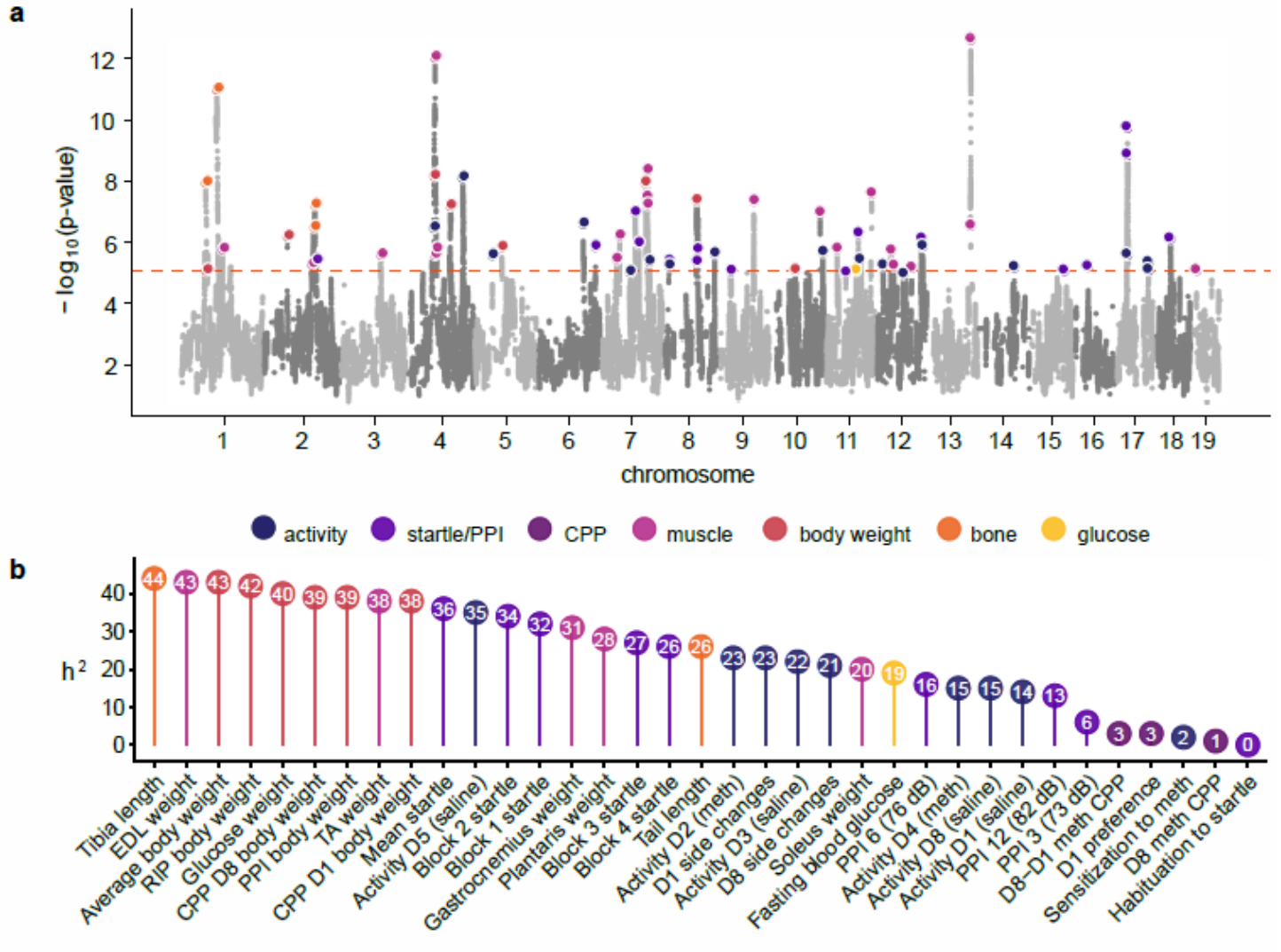

Figure 3. Manhattan plot and heritability for 120 traits measured in the LG x SM AIL. We identified 126 loci for behavioral and physiological traits using 1,063 mice from G50-56 of the LG x SM AIL. A Manhattan plot of GWAS results is shown in **(a)**. Associations for related traits are grouped by color. For clarity, related traits that mapped to the same locus (**Supplementary Table S1**) are highlighted only once. The dashed line indicates a permutation-derived significance threshold of $-\log_{10}(p)=5.09$ ($p=8.06\times10^{-6}$; $\alpha=0.05$). **(b)** For a representative subset of traits, SNP heritability estimates (percent trait variance explained by 523,028 GWAS SNPs) for a subset of traits are shown. Precise estimates of heritability with standard error are provided for all traits in **Supplementary Table 2**.

eQTLs

For a subset of phenotyped and genotyped mice, we used RNA-sequencing (**RNA-seq**) to measure gene expression in the hippocampus (**HIP**), prefrontal cortex (**PFC**) and striatum (**STR**) ($\alpha=0.05$; **Fig. 4, Supplementary Fig. 4**). We identified 2,902 *cis*-eQTLs in HIP, 2,125 *cis*-eQTLs in PFC and 2,054 *cis*-eQTLs in STR; 1,087 *cis*-eQTLs were

significant in all three tissues (FDR<0.05; **Supplementary Table 3**). We also identified 562 HIP *trans*-eQTLs, 408 PFC *trans*-eQTLs and 506 STR *trans*-eQTLs (p<0.05; **Supplementary Fig. 5; Supplementary Table 4**).

Previous studies in model organisms have identified *trans*-eQTLs that regulate the expression of many genes[4,20,21]; we refer to these as '**master eQTLs**'. We identified several master eQTLs, including one on chromosome 12 (70.19-73.72 Mb) that was associated with the expression of 85 genes distributed throughout the genome (**Fig. 4; Supplementary Table 4**). This locus was present in HIP, but not in PFC or STR.

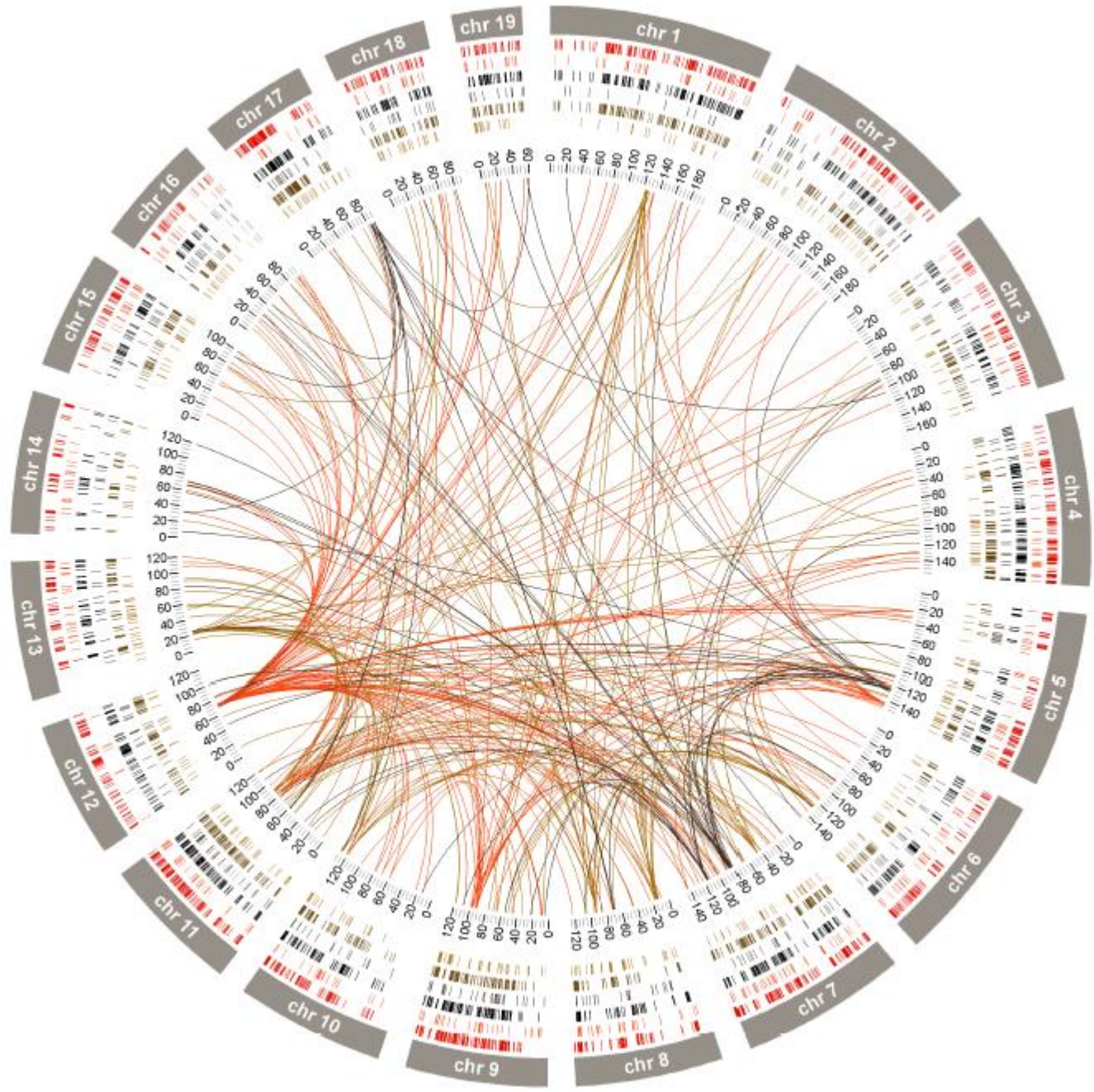

Figure 4. eQTLs in hippocampus (HIP), prefrontal cortex (PFC) and striatum (STR). We identified over 7,000 *cis*-eQTLs (FDR < 0.05) and over 1,400 *trans*-eQTLs (α=0.05) in HIP (n=208; outer red for *cis*-eQTLs, inner red for *trans*-eQTLs), PFC (n=185; outer black for *cis*-eQTLs, inner black for *trans*-eQTL) and STR (n=169; outer brown for *cis*-eQTLs, inner brown for *trans*-eQTLs). We also identified master eQTLs, which we defined as loci that regulate the expression of ten or more target eGenes in a given tissue (central lines link master eQTLs to eGenes).

Integration of eQTLs and behavioral GWAS results

Based on results from human GWAS[3,4], we hypothesized that most loci associated with behavior were due to gene expression differences. For example, four loci associated with locomotor behavior mapped to the same region on chromosome 17 (**Supplementary Table 1; Supplementary Fig. 2**). The narrowest of these (D1 side changes, 15-20 min; $p=3.60x10^{-6}$) identified a locus that contains a single gene, *Crim1* (cysteine rich transmembrane BMP regulator 1), which had a significant *cis*-eQTL in HIP. It would be tempting to conclude that *Crim1* is the best candidate to explain the associations with locomotor behavior; however, two nearby genes, *Qpct* (glutaminyl-peptide cyclotransferase) and *Vit* (vitrin), though physically located outside of the locus, also had *cis*-eQTLs within the locomotor-associated region (**Supplementary Table 3**). We therefore consider all three genes valid candidates to explain the association with locomotor behavior.

One of the most significant loci we identified was an association with the startle response, also on chromosome 17 ($p=5.28x10^{-10}$; **Fig. 3**; **Supplementary Fig. 2**). This result replicated a previous association with startle from a prior study using G34 mice[8]. We performed a phenome-wide association analysis (**PheWAS**) which showed that this region pleiotropically affected multiple other traits, including locomotor activity following saline and methamphetamine administration (**Supplementary Fig. 6**). This region was also implicated in conditioned fear and anxiety in our prior studies of G34 mice[9], demonstrating that it has extensive pleiotropic effects on behavior. Because the association with startle identifies a relatively large haplotype that includes over 25 eGenes, our data cannot clarify whether the pleiotropic effects are due to one or several genes in this interval.

We also identified a 0.49-Mb locus on chromosome 8 that was associated with locomotor activity (**Fig. 5a; Supplementary Table 1**); this region was nominally associated with PPI and multiple other activity traits (**Fig. 5b**). The region identified in the present study (**Fig. 5a-c**) replicates a finding from our previous study using G34 mice[7] (**Fig. 5d**). In both cases, the SM allele conferred increased activity (**Fig. 5c,d**) and the implicated locus contained only one gene: *Csmd1* (CUB and sushi multiple domains 1; **Fig. 5b; Supplementary Table 2**); furthermore, the only *cis*-eQTL that mapped to this region was for *Csmd1* (**Supplementary Fig. 7**). We obtained mice in which the first exon of *Csmd1* was deleted to test the hypothesis that *Csmd1* is the QTG for this locus. *Csmd1* mutant mice exhibited increased activity compared to heterozygous and wild-type mice (**Fig. 5e**), similar to the SM allele. This result is consistent with the hypothesis that *Csmd1* is the causal gene.

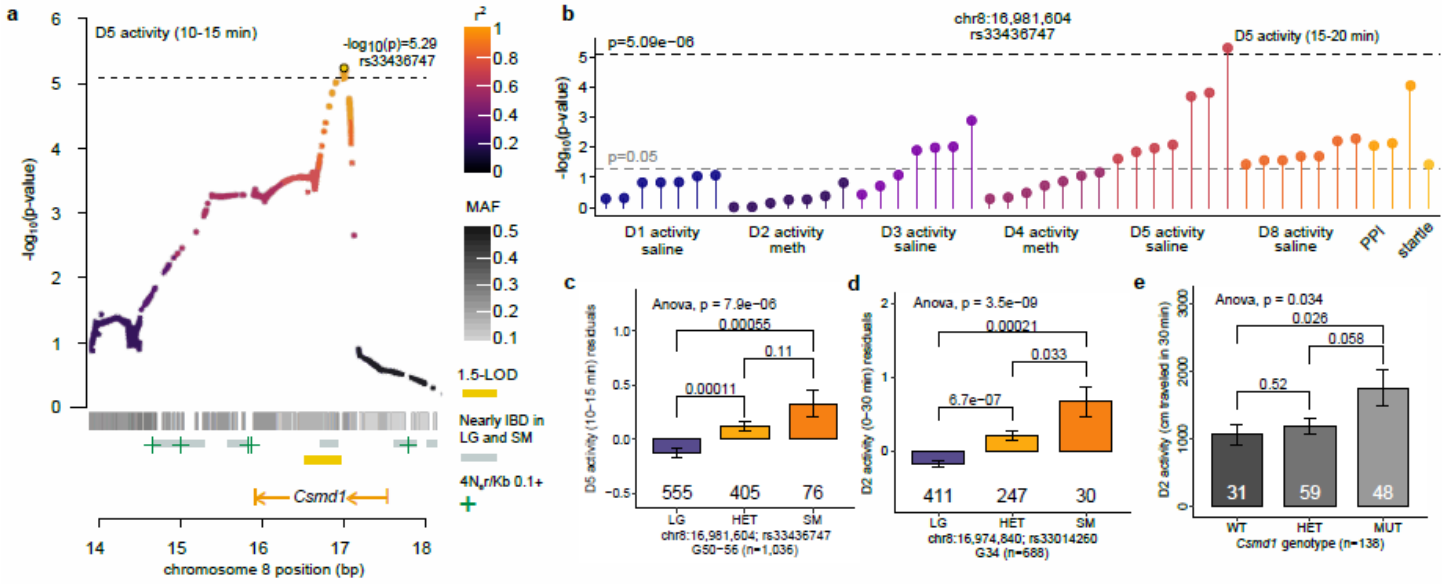

Figure 5. Replication of an association between *Csmd1* and locomotor activity. (a)) Regional plot drawn from all 4.3M SNPs showing the association between rs33436747 and D5 activity levels. The location of Csmd1, 1.5-LOD interval (gold bar), areas of elevated recombination **from Brunschwig et al. (ref. 22)** (green plus symbols), regions predicted by **Nikolskiy et al. (ref. 13)** to be nearly IBD between LG and SM (grey bars), and SNP MAFs (grey heatmap) are indicated. Points are colored by LD (r^2) with rs33436747. The dashed line indicates a significance threshold of $-\log_{10}(p)=5.09$ ($\alpha=0.05$). (**b**) PheWAS plot of associations between rs33436747 and other behavioral traits measured in G50-56 mice. **(b)** Bar plot of quantile-normalized residuals of locomotor activity at the Csmd1 locus are plotted for G50-56 mice. **(c)** Bar plot of quantile-normalized residuals of locomotor activity at the Csmd1 locus for G34 mice from **Cheng et al. (ref. 7)**. rs33436747 was not genotyped in G34; therefore, we plotted activity by genotype at the nearest SNP (rs33014260; 6,764 bp upstream of rs33436747). **(e)** Bar plot of locomotor activity data (distance

traveled in 0-30 min) for Csmd1 mutant mice**.** In panels **c-e** the number of mice in each genotype class is shown below the corresponding bar. ANOVA and two-sided t-test (95% confidence level) p-values are shown for each comparison.

We identified seven overlapping loci for locomotor activity on chromosome 4 (**Supplementary Table 1; Supplementary Fig. 2**). The strongest locus (D5 activity, 0-30 min; $p=6.75x10^{-9}$) spanned 2.31 Mb and completely encompassed the narrowest locus, which spanned 0.74 Mb (D5 activity, 25-30 min; $p=4.66x10^{-8}$); therefore, we focused on the smaller region. *Oprd1* (opioid receptor delta-1) was a *cis*-eGene in all three brain regions; the SM allele conferred an increase in locomotor activity and was associated with decreased expression of *Oprd1. Oprd1* knockout mice have been reported to display increased activity relative to wild-type mice[23], suggesting that differential expression of *Oprd1* could explain the locomotor effect at this locus. However, the presence of other genes and eGenes within the region make it difficult to determine whether *Oprd1* is the only QTG.

We identified an association with D1 locomotor behavior on chromosome 6 at rs108610974, which is located in an intron of *Itpr1* (inositol 1,4,5-trisphosphate receptor type 1; **Supplementary Fig. 8**). This locus contained three *cis*-eGenes and seven *trans*-eQTLs (**Supplementary Fig. 8**). One of the *trans*-eGenes targeted by the locus (*Capn5;* calpain 5) was most strongly associated with rs108610974 and may be the QTG (**Supplementary Table 4**). These results illustrate how the knowledge of both *cis*- and *trans*-eQTLs informed our search for QTGs.

Pleiotropic effects on physiological traits

Because LG and SM were created by selective breeding for large (LG) and small (SM) body size, this AIL is expected to segregate numerous body size alleles[11,24]. We measured body weight at ten timepoints throughout development and identified 46 associations. There was extensive pleiotropy among body weight, muscle mass, and bone length (**Supplementary Table 1; Supplementary Fig. 9-12**). Accounting for pleiotropic genetic architecture, eight major loci arose that influenced body weight at multiple timepoints (**Fig. 3a; Supplementary Table 1**).

For example, eight body weight timepoints mapped to a region on chromosome 2, where the LG allele was associated with smaller body mass (**Supplementary Table 1; Supplementary Fig. 2,9**). The narrowest region spanned 0.08 Mb and did not contain any genes. However, the 0.08-Mb interval contained a *cis*-eQTL SNP for *Nr4a2* (nuclear receptor subfamily 4, group A, member 2) in PFC. Mice lacking *Nr4a2* in midbrain dopamine neurons exhibit a 40% reduction in body weight[25]. Consistent with this, the LG allele was associated with decreased expression of *Nr4a2*.

All body weight timepoints were associated with a locus on chromosome 7 (**Supplementary Table 1; Supplementary Fig. 2**). We also identified associations for tibialis anterior (**TA**), gastrocnemius, plantaris weight that partially overlapped this region (**Supplementary Fig. 10**). Although the most significant SNP associated with muscle weight was ~5 Mb downstream of the top body weight SNP, the LG allele was associated with greater weight at both loci (**Supplementary Table 1**). For eight out of ten body weight timepoints, the most significant association fell within *Tpp1* (tripeptidyl peptidase 1), which was a *cis*-eGene in all tissues and a *trans*-eGene targeted by the master HIP eQTL on chromosome 12 (**Fig. 4**). To our knowledge, *Tpp1* has not been shown to affect body size in mice or humans; however, four other *cis*-eGenes in the region have been associated with human body mass index (*Rpl27a, Stk33, Trim66,* and *Tub*)[26,27]. Dysfunction of *Tub* (tubby bipartite transcription factor) causes late-onset obesity in mice, perhaps due to *Tub*'s role in insulin signaling[28]. In addition, several *trans*-eGenes map to this interval, including *Gnb1* (G protein subunit beta 1), which forms a complex with *Tub*[29]. Another *trans*-eGene associated with this interval, *Crebbp* (*CREB* binding protein), has been associated with juvenile obesity in human GWAS[30].

Multiple strong associations identified for muscle mass

We examined five hind limb muscle traits, identifying 22 loci (**Supplementary Table 1; Supplementary Fig. 2**). No loci were identified for soleus weight. The strongest association we identified in this study was for extensor digitorum longus (**EDL**) weight ($p=2.03x10^{-13}$; **Supplementary Table 1; Supplementary Fig. 11**). An association with gastrocnemius weight provided additional support for the region ($p=2.56x10^{-7}$; **Supplementary Fig. 11**); in both cases, the SM allele was associated with increased muscle mass. Each locus spans less than 0.5 Mb and is flanked by regions of low polymorphism between LG and SM (**Supplementary Fig. 11, Supplementary Table 1**). Two *cis*-eGenes within the region, *Trappc13* (trafficking protein particle complex 13) and *Nln* (neurolysin), are differentially expressed in LG and SM soleus[31] and TA[32], with LG exhibiting increased expression of both genes. While there is no known relationship between *Trappc13* and muscle, *Nln* has been shown to play a role in mouse skeletal muscle[33].

The LG allele was associated with greater EDL, plantaris, and TA weight at another locus on chromosome 4 (**Supplementary Table 1**; **Supplementary Fig. 12**). The loci for EDL and plantaris spanned ~0.5 Mb, defining a region

that contained six genes (**Supplementary Table 1**). The top SNPs for EDL (rs239008301; $p=7.88x10^{-13}$) and plantaris (rs246489756; $p=2.25x10^{-6}$) were located in an intron of *Astn2* (astrotactin 2), which is differentially expressed in LG and SM soleus[31]. SM, which exhibits lower expression of *Astn2* in soleus relative to LG[31], has a 16 bp insertion in an enhancer region 6.6 kb upstream of *Astn2* (ENSMUSR00000192783)[13]. Two other genes in this region have been associated with muscle or bone phenotypes traits in the mouse: *Tlr4* (toll-like receptor 4), which harbors one synonymous coding mutation on the SM background (rs13489095) and *Trim32* (tripartite motif-containing 32), which contains no coding polymorphisms between the LG and SM strains.

Discussion

Crosses among well-characterized inbred strains are a mainstay of model organism genetics. In the present study, we used 1,063 male and female mice from LG x SM G50-56 to identify 126 loci for a variety of traits selected for their relevance to human psychiatric and metabolic diseases[24,34,35] (**Fig. 3; Supplementary Table 1; Supplementary Fig. 2**). Whereas our previous work established AILs as an effective tool for fine-mapping loci identified in F2 crosses[7–11,14,31], this study demonstrates that AILs are also a powerful fine mapping population in their own right. We show that several QTGs we identified are corroborated by extant human and mouse genetic data. We also replicated a number of our earlier findings.

Classical crosses like F_2 and recombinant inbred strains provide poor mapping resolution because the ancestral chromosomes persist as extremely long haplotypes[2]. To address this limitation, we and others have used AILs[14]. Because both inbred strains contribute equally to an AIL, there are numerous common variants (**Fig. 1a,b**), and each successive generation further degrades LD between adjacent SNPs (**Fig. 1c**). In addition to AILs, a number of other outbred populations have been used in rodent GWAS, including CFW mice[17,18], Diversity Outbred (**DO**) mice[36,37], and N/NIH heterogeneous stock rats (**HS**)[19,38]. CFW mice are obtained from a commercial vendor, which avoids the expenses of maintaining a colony. In addition, non-siblings can be obtained, which reduces the complicating effects that can occur when close relatives are used in GWAS. However, the CFW founder strains are unavailable, and many alleles exist at low frequencies among CFWs, limiting power and introducing genetic noise[17,18]. Commercially available DO mice are more expensive than CFWs, but like AILs, the founder strains have been fully sequenced, which allows imputation of SNPs and founder haplotypes. However, three of the eight inbred strains used to produce the DO are so-called wild-derived strains; making DO mice very difficult to handle, which complicates many behavioral procedures[37]. Furthermore, the causal alleles in the DO are often from one of the wild derived strains, because 8 strains contributed equally to the DO, this means that the causal allele frequencies are often in the range of 0.125. Finally, N/NIH HS rats, which are conceptually very similar to DO mice, have also been used as a fine mapping population[19,38]. Among these options, AILs stand out for their simplicity, balanced allele frequency and ease of handling.

A major goal of this study was to identify the genes that are responsible for the loci implicated in behavioral and physiological traits. The mapping resolution of the LG x SM AIL was critically important for this goal. However, no matter how precise the resolution, proximity of a gene to the associated SNP is never sufficient to establish causality[4]. Therefore, we used RNA-seq to quantify mRNA abundance in three brain regions that are strongly implicated in the behavioral traits: HIP, PFC and STR. We used these data to identify 7,081 *cis*-eQTLs and 1,372 *trans*-eQTLs (**Fig. 4, Supplementary Fig. 4-5; Supplementary Tables 3-4**). In a few cases, loci contained only a single eQTL; however, in most cases multiple *cis*-eQTLs and *trans*-eQTLs mapped to the implicated loci. Thus, we frequently incorporated functional information, including data about tissue specific expression, coding SNP, mutant mice, and human genetic studies to parse among the implicated genes.

We have previously shown that GBS is a cost-effective strategy for genotyping CFW mice[39]. The advantages of GBS were even greater for this AIL because imputation allowed us to easily obtain 4.3M SNPs while using only half the sequencing depth (**Fig. 1a**). Even before imputation, GBS yielded nearly 50% more informative SNPs compared to the best available SNP genotyping chip[12] at about half the cost (**Supplementary Fig. 1**).

One of the goals of this study was to perform GWAS for conditioned place preference (**CPP**), which is a well-validated measure of the reinforcing effects of drugs[40]. Unfortunately, the heritability of CPP in this study was not significantly different from zero (**Fig. 3b**). This result was partially consistent with our prior study in which we used a higher dose of methamphetamine (2 vs. the 1 mg/kg used in the present study)[41]. The low heritability of CPP in this AIL likely reflects a lack of relevant genetic variation in this specific population since both panels of inbred strains and genetically engineered mutant alleles show differences in CPP[40,42,43], demonstrating the existence of heritable variance in other populations. It is possible that even lower doses of methamphetamine, which might fall on the ascending portion of the dose-response function, would have resulted in higher heritability. Similarly, responses to other drugs or different CPP methodology may have exhibited greater heritability.

We used PheWAS to identify pleiotropic effects of several loci identified in this study. In many cases, pleiotropy involved highly correlated traits such as body weight on different days or behavior at different time points within a single day (**Supplementary Fig. 9-13**; **Supplementary Table 1**). We also observed more surprising examples of pleiotropy, including pleiotropy between locomotor activity and gastrocnemius mass on chromosome 4 (**Supplementary Fig. 14**), and pleiotropy between locomotor activity and the startle response on chromosome 12 (**Supplementary Fig. 15**). We also observed extensive pleiotropy on chromosome 17; this locus influenced saline- and methamphetamine-induced locomotor activity and startle response (**Supplementary Fig. 6**). Moreover, this same region had been previously implicated in anxiety-like behavior[9], contextual and conditioned fear[9], and startle response[8] in prior studies of LG x SM G34, suggesting that the locus has a broad impact on many behavioral traits. These results support the idea that pleiotropy is a pervasive feature in this AIL and provide further evidence of the replicability of the loci identified by this and prior GWAS.

Discoveries from human GWAS are often considered preliminary until they are replicated in an independent cohort. In model organisms, replication using an independent cohort is rarely employed because it is possible to directly manipulate the implicated gene. We replicated one behavioral locus identified in this study using the criteria of both human and model organism genetics. We had previously identified an association with locomotor activity on chromosome 8 using G34 of this AIL[7], which was replicated in the present study (**Fig. 5**). In both cases, the SM allele was associated with lower activity (**Fig. 5c-d**). We also identified a locus for PPI (76 dB) in this region (**Fig. 5a**; **Supplementary Table 1, Supplementary Fig. 2**). The loci identified in both G34 and in G50-56 were small and contained just one gene: *Csmd1* (**Fig. 5b**). In the present study we also identified a *cis*-eQTL for *Csmd1* in HIP (**Supplementary Figure 7**). Finally, we obtained *Csmd1* mutant mice[44] and found that they also showed altered locomotor activity (**Fig. 5e**). Thus, we have demonstrated replication both by performing an independent GWAS and by performing an experimental manipulation that recapitulates the phenotype.

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Genetic background

The LG and SM inbred strains were independently selected for high and low body weight at 60 days[45]. The LG x SM AIL was derived from an F_1 intercross of SM females and LG males initiated by Dr. James Cheverud at Washington University in St. Louis[6]. Subsequent AIL generations were maintained using at least 65 breeder pairs selected by pseudo-random mating[46]. In 2006, we established an independent AIL colony using 140 G33 mice obtained from Dr. Cheverud (Jmc: LG,SM-G33). Since 2009, we have selected breeders using an R script (**Supplementary Note**) that leverages pairwise kinship coefficients estimated from the AIL pedigree to select the most unrelated pairs while also attempting to minimize mean kinship among individuals in the incipient generation (the full pedigree is included in **Supplementary File 1**). We maintained ~100 breeder pairs in G49-55 to produce the mice for this study. In each generation, we used one male and one female from each nuclear family for phenotyping, and reserved up to three of their siblings for breeding the next generation.

Phenotypes

We subjected 1,123 AIL mice (562 female, 561 male; Aap: LG,SM-G50-56) to a four-week battery of tests over the course of two years. This sample size was based on an analysis suggesting that 1,000 mice would provide 80% power to detect associations explaining 3% of the phenotypic variance (**Supplementary Fig. 16**). We measured CPP for 1 mg/kg methamphetamine, locomotor behavior, PPI, startle, body weight, muscle mass, bone length and other related traits (**Supplementary Table 2**). We tested mice during the light phase of a 12:12h light-dark cycle in 22 batches comprised of 24-71 individuals (median=53.5). Median age was 54 days (mean=55.09, range=35-101) at the start of testing and 83 days (mean=84.4, range=64-129) at death. Standard lab chow and water were available *ad libitum*, except during testing. Testing was performed during the light phase, starting one hour after lights on and ending one hour before lights off. No environmental enrichment was provided. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Chicago (**Supplementary File 2**). Traits are summarized briefly below; detailed descriptions are provided in the **Supplementary Note.**

CPP and locomotor behavior: CPP is an associative learning paradigm that has been used to measure the motivational properties of drugs in humans[47] and rodents[40]. We defined CPP as the number of seconds spent in a drug-associated environment relative to a neutral environment over the course of 30 minutes. The full procedure takes eight days, which we refer to as **D1-D8**. We measured baseline preference after administration of vehicle (0.9% saline, i.p.) on D1. On D2 and D4, mice were administered methamphetamine (1 mg/kg, i.p.) and restricted to one visually and tactically distinct environment; on D3 and D5 mice were administered vehicle and restricted to the other, contrasting environment. On D8, mice were allowed to choose between the two environments after administration of vehicle; we measured CPP at this time. Other variables measured during the CPP test include the distance traveled (cm) on all testing days, the number of side changes on D1 and D8, and locomotor sensitization to methamphetamine (the increase in activity on D4 relative to D2). We measured CPP and locomotor traits across six 5-minute intervals and summed them to generate a total phenotype for each day.

PPI and startle: PPI is the reduction of the acoustic startle response when a loud noise is immediately preceded by a low decibel (**dB**) prepulse[48]. PPI and startle are measured across multiple trials that occur over four consecutive blocks of time[8]. The primary startle trait is the mean startle amplitude across all pulse-alone trials in blocks 1-4. Habituation to startle is the difference between the mean startle response at the start of the test (block 1) and the end of the test (block 4). PPI, which we measured at three prepulse intensities (3, 6, and 12 dB above 70 dB background noise), is the mean startle response during pulse-alone trials in blocks 2-3 normalized by the mean startle response during prepulse trials in blocks 2-3.

Physiological traits: We measured body weight (g) on each testing day and at the time of death. One week after PPI, we measured blood glucose levels (mg/dL) after a four-hour fast. One week after glucose testing, we killed the mice, and measured tail length (cm from base to tip of the tail). We stored spleens in a 1.5 mL solution of 0.9% saline at -80°C until DNA extraction. We removed the left hind limb of each mouse just below the pelvis; hind limbs were stored at -80°C. Frozen hind limbs were phenotyped by Dr. Arimantas Lionikas at the University of Aberdeen. Phenotyped muscles include two dorsiflexors, TA and EDL, and three plantar flexors: gastrocnemius, plantaris and soleus. We isolated individual muscles under a dissection microscope and weighed them to 0.1 mg precision on a Pioneer balance

(Ohaus, Parsippany, NJ, USA). After removing soft tissue from the length of tibia, we measured its length to 0.01 mm precision with a Z22855 digital caliper (OWIM GmbH & Co., Neckarsulm, GER).

Brain tissue: We collected HIP, PFC and STR for RNA-seq from the brain of one mouse per cage. This allowed us to dissect each brain within five minutes of removing a cage from the colony room (rapid tissue collection was intended to limit stress-induced changes in gene expression). We preselected brain donors to prevent biased sampling of docile (easily caught) mice and to avoid sampling full siblings, which would reduce our power to detect eQTLs. Intact brains were extracted and submerged in chilled RNALater (Ambion, Carlsbad, CA, USA) for one minute before dissection. Individual tissues were stored separately in chilled 0.5-mL tubes of RNALater. All brain tissue was dissected by the same experimenter and subsequently stored at -80°C until extraction.

GBS variant calling and imputation

GBS is a reduced-representation genotyping method[49,50] that we have adapted for use in mice and rats[17,39]. We extracted DNA from spleen using a standard salting-out protocol and prepared GBS libraries by digesting DNA with the restriction enzyme *PstI*, as described previously[17]. We sequenced 24 uniquely barcoded samples per lane of an Illumina HiSeq 2500 using single-end, 100 bp reads. We aligned 1,110 GBS libraries to the mm10 reference genome before using GATK[51] to realign reads around known indels in LG and SM[13] (see **Supplementary Note** and **Supplementary File 3** for details and example commands). We obtained an average of 3.2M reads per sample. We discarded 32 samples with <1M reads aligned to the main chromosome contigs (1-19, X, Y) or with a primary alignment rate <77% (i.e. three s.d. below the mean of 97.4%; **Supplementary Fig. 17**).

We used ANGSD[52] to obtain genotype likelihoods for the remaining 1,078 mice and used Beagle[53] for variant calling, which we performed in two stages. We used first-pass variant calls as input for IBDLD[54,55], which we used to estimate kinship coefficients for the mice in our sample. Because our sample contained opposite-sex siblings, we were able to identify and resolve sample mix-ups by comparing genetic kinship estimates to kinship estimated from the LG x SM pedigree (described in the **Supplementary Note**). In addition, we re-genotyped 24 mice on the GigaMUGA[12] to evaluate GBS variant calls (**Supplementary Table 5** lists concordance rates at various stages of our pipeline; see **Supplementary Note** for details).

After identifying and correcting sample mix-ups, we discarded 15 samples whose identities could not be resolved (**Supplementary Note**). Next, we used Beagle[53,56], in conjunction with LG and SM haplotypes obtained from whole-genome sequencing data[13] to impute 4.3M additional SNPs into the final sample of 1,063 mice. We removed SNPs with low MAFs (<0.1), SNPs with Hardy-Weinberg Equilibrium (**HWE**) violations ($p \leq 7.62 \times 10^{-6}$, determined from gene dropping simulations as described in the **Supplementary Note**), and SNPs with low imputation quality (dosage r^2, $\mathbf{DR^2}$<0.9). We then pruned variants in high LD (r^2>0.95) to obtain the 523,028 SNPs that we used for GWAS.

LD decay

We used PLINK[57] to calculate r^2 for all pairs of autosomal GWAS SNPs typed in G50-56 (parameters are listed in **Supplementary File 3**). We repeated the procedure for 3,054 SNPs that were genotyped in G34 mice[7]. Next, we randomly sampled r^2 values calculated for ~40,000 SNP pairs from each population and used the data to visualize the rate of LD decay (**Fig. 1c**).

LOCO-LMM

We used a modified LMM implemented in GEMMA[58] to perform GWAS. An LMM accounts for relatedness by modeling the covariance between phenotypes and genotypes as a random, polygenic effect[14], which we also refer to as a genetic relationship matrix (**GRM**). Power to detect associations is reduced when the locus being tested is also included in the GRM because the effect of the locus is represented in both the fixed and random terms[15]. To address this issue, we calculated 19 separate GRMs, each one excluding a different chromosome. When testing SNPs on a given chromosome, we used the GRM that did not include markers from that chromosome as the polygenic effect in the model. Fixed covariates for each trait are listed in **Supplementary Table 2.**

We used a permutation-based approach implemented in **MultiTrans**[59] and **SLIDE**[60] to obtain a genome-wide significance threshold that accounts for LD between nearby markers (see **Supplementary Note** for details). We obtained a significance threshold of $p=8.06 \times 10^{-6}$ ($\alpha=0.05$) from 2.5M samplings. Because the phenotypic data were

quantile-normalized, we applied the same threshold to all traits. We converted p-values to LOD scores and used a 1.5-LOD support interval to approximate a critical region around each associated region, which enabled us to systematically identify overlap with eQTLs.

We estimated the proportion of phenotypic variance explained by the set of 523,028 LD-pruned SNPs using the restricted maximum likelihood algorithm in GEMMA[58]. We ran a second genome-wide scan for each trait, this time dropping the fixed effect of dosage and including the complete GRM estimated from SNPs on all 19 autosomes. We repeated the procedure using dosage at the most significant SNP as a covariate for each trait and interpreted the difference between the two estimates as the effect size of that locus.

RNA-sequencing and quality control

We extracted mRNA from HIP, PFC and STR as described in Parker *et al.* (ref. 17) and prepared cDNA libraries from 741 samples with RNA integrity scores ≥ 8.0 (265 HIP; 240 PFC; 236 STR)[61] as measured on a Bioanalyzer (Agilent, Wilmington, DE, USA). We used Quant-iT kits to quantify RNA (Ribogreen) and cDNA (Picogreen; Fisher Scientific, Pittsburgh, PA, USA). Barcoded sequencing libraries were prepared with the TruSeq RNA Kit (Illumina, San Diego, USA), pooled in sets of 24, and sequenced on two lanes of an Illumina HiSeq 2500 using 100 bp, single-end reads.

Because mapping quality tends to be higher for reads that closely match the reference genome[62], read mapping in an AIL may be biased toward the reference strain (C57BL/6J)[63]. We addressed this concern by aligning RNA-seq reads to custom genomes created from LG and SM using whole-genome sequence data[13]. We used default parameters in HISAT[64] for alignment and GenomicAlignments[65] for assembly, assigning each read to a gene as defined by Ensembl (*Mus_musculus.*GRCm38.85)[66]. We required that each read overlap one unique disjoint region of the gene. If a read contained a region overlapping multiple genes, genes were split into disjoint intervals, and any shared regions between them were hidden. If the read overlapped one of the remaining intervals, it was assigned to the gene that the interval originated from; otherwise, it was discarded. Next, we reassigned the mapping position and CIGAR strings for each read to match mm10 genome coordinates and combined the LG and SM alignment files for each sample by choosing the best mapping. Only uniquely mapped reads were included in the final alignment files. We then used DESeq[67] to obtain normalized read counts for each gene in HIP, PFC and STR. We excluded genes detected in <95% of samples within each tissue.

We also excluded 30 samples with <5M mapped reads or with an alignment rate <91.48% (i.e. less than 1 s.d. below the mean number of reads or the mean alignment rate across all samples and tissues; **Supplementary Fig. 18**). We merged expression data from HIP, PFC and STR and plotted the first two principal components (**PCs**) of the data to identify potential tissue swaps. Most samples clustered into distinct groups based on tissue. We reassigned 12 mismatched samples to new tissues and removed 35 apparently contaminated samples that did not cluster with the rest of the data (**Supplementary Fig. 19**). We also used agreement among GBS genotypes and genotypes called from RNA-seq data in the same individuals to identify and resolve mixed-up samples, as detailed in the **Supplementary Note.** We discarded 108 sample mix-ups that we were not able to resolve, 29 samples with low-quality GBS data, and 12 outliers (details are provided in the **Supplementary Note**). A total of 208 HIP, 185 PFC, and 169 STR samples were retained for further analyses.

Prior to eQTL mapping, we quantile-normalized gene expression data and used principal components analysis to remove the effects of unknown confounding variables[68]. For each tissue, we calculated the first 100 PCs of the gene x sample gene expression matrix. We quantile-normalized PCs and used GEMMA[58] to test for association with SNPs using sex and batch as covariates. We evaluated significance with the same permutation-based threshold used for GWAS. We retained PCs that showed evidence of association with a SNP in order to avoid removing *trans*-eQTL effects. We then used linear regression to remove the effects of the remaining PCs (71 in HIP, 81 in STR and 93 in PFC) and quantile-normalized the residuals.

eQTL mapping

We mapped *cis*- and *trans*-eQTLs using a LOCO-LMM[15] implemented in GEMMA[58], conservatively including sex and batch as covariates even though PC regression might have accounted for them (see **Supplementary Note** for details).

We considered intergenic SNPs and SNPs 1 Mb upstream or downstream of the gene as potential *cis*-eQTLs and excluded 2,143 genes that had no SNPs within their *cis*-regions. We used eigenMT[69] to obtain a gene-based p-value adjusted for the number of independent SNPs in each *cis* region. We declared *cis*-eQTLs significant at an FDR<0.05. We refer to genes with significant *cis*-eQTLs as ***cis*-eGenes**.

SNPs on chromosomes that did not contain the gene being tested were considered potential *trans*-eQTLs. We determined significance thresholds for *trans*-eQTLs by permuting data 1,000 times. Since expression data were quantile-normalized, we permuted one randomly chosen gene per tissue. The significance threshold for *trans*-eQTLs was 8.68×10^{-6} in STR, 9.01×10^{-6} in in PFC ($\alpha=0.05$). We used all SNPs for permutation; therefore, we expect these thresholds to be conservative. We refer to genes with *trans*-eQTLs as ***trans*-eGenes**. Finally, we defined *trans*-eQTL hotspots or **'master eQTLs'** as 5 Mb regions that contain ten or more *trans*-eQTLs. To identify master eQTLs, we divided chromosomes into 5 Mb bins and assigned each *trans*-eGene to the bin containing its most significant eQTL SNP.

Csmd1 mutant mice

Csmd1 mutants were created by Lexicon Genetics by inserting a Neomycin cassette into the first exon of *Csmd1* using embryonic stem cells derived from 129S5 mice[70] as described by Distler *et al.* (ref. [44]). The mice we used were the result of a C57BL/6 x 129S5 intercross designated B6;129S5-*Csmd1*^{tm1Lex}/Mmucd (the exact C57BL/6 substrain is unknown). We bred heterozygous males and females and tested littermate offspring to account for their mixed genetic background. *Csmd1* spans 1.6 Mb and has 70 exons. Its four major transcripts, termed *Csmd1-1* to *Csmd1-4*, are expressed in the central nervous system[44]. Distler *et al.* (ref. [44]) demonstrated that *Csmd1* homozygous mutant mice express <30% of wild-type *Csmd1* levels in the brain, and heterozygous mice show a 54% reduction in *Csmd1* expression. Residual expression of *Csmd1* in homozygous mutant mice is derived from *Csmd1-4*, the only transcript that does not include the first exon. We analyzed locomotor behavior on two days following a saline injection in 31 wild-type, 59 heterozygous, and 48 mutant mice.

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Introduction, Results and Discussion

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