Genome-wide association study of behavioral, physiological and gene expression traits in commercially available outbred CFW mice.

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Abstract

Although mice are the most widely used model organism, genetic studies have suffered from limited mapping resolution due to the extensive linkage disequilibrium (LD) that is characteristic of crosses among inbred strains. Carworth Farms White (CFW) mice, which are a commercially available outbred mouse population, exhibit rapid LD decay. We performed a genome-wide association study (GWAS) of behavioral, physiological and gene expression phenotypes using 1,200 CFW male mice. Because genotyping arrays for model organisms remain expensive and provide limited coverage, we used genotyping-by-sequencing (GBS) to obtain genotypes at 92,734 single nucleotide polymorphisms (SNPs). Because expression polymorphisms appear to underlie most human GWAS results, we also measured gene expression using RNA-Sequencing (RNA-Seq) in three brain regions (prefrontal cortex, hippocampus and striatum) that are known to influence the behavioral traits we studied. Our study identified numerous behavioral, physiological and expression quantitative trait loci (QTLs) and elucidated important population genetic parameters of CFW mice. Many QTLs identified small regions that allowed us to implicate specific genes including *Inhba* for testis weight, *Fst* and *Tp53inp2* for muscle mass, and *Pthlh, Abcf2, Slc4a2* and *Col1a1* for bone shape and mass. We integrated the behavioral QTL and expression QTL results to prioritize among genes within each locus of interest, which implicated *Azi2* in sensitivity to the locomotor effects of methamphetamine and *Zmynd11* in anxiety-like behavior. In summary, the combination of highly recombinant outbred mice, GBS and RNA-Seq allowed us to rapidly implicate specific genes for a variety of complex traits. Our results demonstrate a novel approach to GWAS that takes advantage of highly recombinant outbred populations.

Introduction

In the last decade, genome-wide association studies (GWAS) have demonstrated that common alleles influence susceptibility to virtually all common diseases [Manolio-2008, Manolio-2013, Schizophrenia Working Group 2014]. The success of GWAS in elucidating the genetic determinants of disease in humans is due in part to the large number of recombinations among unrelated individuals, which permits high-resolution mapping across the genome. One important conclusion from these studies is that most causal loci appear to be due to regulatory rather than coding polymorphisms [Albert-2015].

Mice offer a powerful tool for elucidating the genetic architecture of complex traits: environmental factors can be held constant or systematically varied; genome editing permits experimental testing of identified genotype-phenotype relationships; most mouse genes have a human homolog, allowing rapid translation to humans; and relevant tissues can be obtained under highly controlled laboratory conditions and used to identify gene expression QTLs (eQTLs). However, the mouse populations used in most prior studies lacked sufficient recombination to narrow the implicated loci to a tractable size and have thus generally failed to identify specific genes [Mott-2013, Parker-2011].

Carworth Farms White (CFW) mice are a commercially available outbred population [Lynch-1969]. While CFW mice were not developed for genetic research, they have several attractive properties. CFW mice were derived from a small number of founders and have been subsequently maintained as an outbred population for more than 100 generations, thus degrading linkage disequilibrium (LD) between nearby alleles [Rice-1980, Yalcin-2010, Chia-2005]. Although CFW mice still have longer range LD compared to most human populations, they have less LD than other commercially available laboratory mice [Yalcin et al 2010], and therefore should provide fine-scale mapping resolution. When compared to humans, the more extensive LD in CFW mice means that fewer

markers are needed to perform a GWAS and correspondingly lower levels of significance are required because fewer independent hypotheses are tested.

We used genotyping-by-sequencing (GBS) to obtain high-quality genotypes at over 90,000 single nucleotide polymorphisms (SNPs). This addressed another barrier to GWAS in mice, which is the high cost and limited coverage of extant SNP genotyping arrays. To assess the suitability of CFW mice for mapping complex traits, we quantified population genetic properties of the CFW mice such as inbreeding, LD patterns, and genetic similarity to other mouse populations. We used these data to map loci associated with a wide range of behavioral and physiological traits. To better elucidate the genetic basis of these traits, we used RNA-Sequencing (RNA-Seq) [Wang-2009, Pickrell-2010] to identify expression quantitative trait loci (eQTLs) in three brain regions that are strongly implicated in the behavioral traits under study. Our focus on eQTLs was inspired by the importance of regulatory variation suggested by human GWAS [Albert-2015, Gusev-2014]. By searching for eQTLs that co-mapped with behavioral QTLs, we improved our ability to identify the most likely causal genes.

Results

The workflow of this study is summarized in Figure 1: phenotyping, genotyping, RNA-Seq and GWAS. We phenotyped 1,200 male CFW mice for conditioned fear, anxiety-like behavior, methamphetamine sensitivity, prepulse inhibition, fasting glucose, body weight, tail length, testis weight, the weight of five hindlimb muscles, bone mineral density, bone morphology and gene expression in prefrontal cortex, hippocampus and striatum (see Online Methods, Supplemental Table 1 and Supplementary Material).

Genotyping

Existing mouse SNP genotyping technologies, such as the Mouse Universal Genotyping Array (MUGA), MegaMUGA [Gatti-2014], the more recent GigaMUGA [Morgan-2015] and the Mouse Diversity Array (MDA) [Yang-2009] were not designed to capture common genetic variation in the CFW population. Furthermore, we sought to reduce the cost of genotyping, which has been a barrier to GWAS in mice. Therefore, we adapted GBS, which was originally developed in maize [Elshire-2011], for use in mice. We used GBS to genotype 1,024 CFW mice, and identified 92,734 autosomal bi-allelic SNPs after filtering, 79,284 (86%) of which were present in dbSNP (v137; see Supplementary Materials Section 2.3.3 for details). The remaining 13,450 SNPs (14%) represent novel SNPs that had not been previously reported. The distribution of GBS SNPs on autosomal chromosomes is shown in Figure 2A. The non-uniform distribution of SNPs is likely due to a combination of factors, including differences in the numbers of polymorphic markers among all laboratory mice (Supplementary Figure 6) as well as regions that are identical-by-descent among CFW mice. The uneven distribution of polymorphic SNPs appears to be a characteristic of CFW mice since polymorphic SNPs identified by the MegaMUGA array showed a similar pattern (Figure 2A; $r^2 = 0.43$ on log-scale). To assess the quality of GBS genotypes, we estimated the genotyping error rate in two ways. First, we compared GBS SNPs against those that were also present on the MegaMUGA genotyping platform among 24 CFW mice genotyped using both platforms. This comparison yielded an overall discordance rate of 3% (see Supplementary Materials Section 3.3). We obtained a second estimate of the error rate by comparing genotypes in pairs of haplotypes that were identical-by-descent, which yielded an error rate of 1.6% (see Supplementary Materials Section 3.2). Based on these results, we concluded that GBS provided an efficient means of genotyping a larger number of polymorphic SNPs than were found using MegaMUGA. Significantly, we did not have to preselect particular SNPs because GBS integrates SNP discovery and SNP genotyping into a single process.

Genetic Architecture of the CFW Population

Comparing LD in different populations is useful for gauging mapping resolution [Pritchard-2001]. We found that LD (r^2) decays rapidly in CFW mice compared to other populations (Figure 2B), consistent with previous findings based on a much smaller number of SNPs [Yalcin-2010]. The extent of LD in CFW mice was less than that observed in other commonly used laboratory mouse populations (Figure 2B, also see [Laurie-2007]), supporting their suitability for high-resolution mapping. Importantly, the majority of the SNPs we identified in CFW mice segregate among *domesticus*-derived laboratory strains (Figure 2C). Unlike the Collaborative Cross (CC) and the Diversity Outbred (DO), few of the SNPs found in CFW are derived from the *castaneous* and *musculus* subspecies [Chesler-2014, Churchill-2012, Collaborative Cross Consortium-2012]. When compared to a panel of well-characterized inbred mouse strains, CFW are most genetically similar to FVB/NJ mice (Figure 2C).

Next, we considered the distribution of minor allele frequencies (MAF) of SNPs genotyped in the CFW mice (Supplementary Figure 10). The majority of SNPs (73%) had relatively high allele frequencies (MAF > 0.05). This profile is consistent with the reported history of CFW mice; namely, a severe bottleneck at the inception of the CFW population, followed by expansion to create an outbred population with a modest effective population size [Yalcin-2010]. The mean MAF of novel SNPs was lower than for previously reported SNPs, consistent with the hypothesis that some of these novel SNPs are recent mutations or are unique to the CFW population. The generally high MAF (MAF>0.05) is advantageous for GWAS because most association tests will be well-powered.

Although we requested only a single mouse from each litter, we were concerned that individuals in our study might have close familial relationships because they were sampled from a finite breeding population; however, we did not detect widespread population structure or cryptic relatedness in the CFW mice (Supplementary Figures 11-14, 16).

SNP Heritability

For each of the quantitative traits, we estimated the "SNP heritability" [Wray-2013], which is the proportion of variance in the trait explained by available SNP genotypes (Supplementary Table 1). SNP heritability estimates ranged from 9–60%, with a mean of 28%. The mean SNP heritability for physiological traits tended to be slightly higher (32%) than those for behavioral traits (27%). Therefore, genetic variation in CFW mice contributed to a substantial fraction of the phenotypic diversity for the range of traits we measured in this population.

GWAS

We mapped QTLs for 66 behavioral and physiological phenotypes (Supplementary Tables 1 and 2; Figure 3A). We used GEMMA to fit a linear mixed model (LMM) and quantify support for an association at each SNP. We also used a simpler linear model that did not correct for population structure (also implemented in GEMMA) and observed that it produced broadly similar results (see Supplementary Figure 17 for an example). However, we have presented the results from the LMM-based analysis because it may reduce subtle inflation of the test statistics due to close relationships or fine-scale population structure. We calculated a threshold via permutation, which is a standard approach for QTL mapping in mice that controls for the type I error rate [Cheng-2013, Churchill-1994; also see Supplemental Figure 25)]. Using this approach, we identified numerous QTLs for physiological and behavioral traits (Figure 3A, Supplementary Table 2) that exceeded 2×10^{-6} ($p < 0.1$, also see Supplementary Figures 4, 22).

Examples of GWAS results for specific traits

For testis weight we found a strong association with rs6279141 on chromosome 13 (Figure 3B, Supplementary Table 2; p-value: 4.51×10^{-18}) that accounted for 7.5% of variation in that trait. The implicated region contained few genes (Figure 3D), one of which was *Inhba*, a gene that has been previously shown to affect testis morphogenesis, testicular cell proliferation and testis weight in

mice [Mendis-2011, Mithraprabhu-2010, Tomaszewski-2007], and is therefore a promising candidate gene.

The strongest association for soleus muscle weight mapped to rs30535702 on chromosome 13 and explained 2.8% of trait variance (Supplementary Table 2; *p*-value: 8.33×10^{-8}). One of the genes in this interval, *Fst*, is known to influence muscle mass [Lee-2007, Lee-2010] and therefore is a strong candidate to explain this association.

We identified several examples of pleiotropy. For example, two independently measured muscle weights, tibialis anterior (TA) and extensor digitorum longus (EDL), were both associated with rs27338905 on chromosome 2 (Supplementary Table 2; *p*-value: 1.62×10^{-6}, 2.92×10^{-6}, respectively), in each case accounting for 2.3% of the variation. *Tp53inp2* is near the peak marker and is abundantly expressed in skeletal muscle [Lionikas-2012], where it functions as a negative regulator of muscle mass [Sala-2014]. Likewise, the weight of three muscles (gastrocnemius, EDL and soleus) mapped to the proximal end of chromosome 13; in each case, the minor allele was associated with increased muscle weight (Supplementary Table 2). Finally, on chromosome 12 we identified pleiotropic effects on tibia length and EDL weight (Supplementary Table 2).

Unexpectedly, we found that CFW mice appear to be predisposed toward abnormally high bone mineral density (BMD). This is a characteristic of CFW mice that does not appear to be shared with commonly used inbred laboratory strains (Supplementary Figure 5). This “abnormal BMD” phenotype was strongly associated with rs33583459 on chromosome 5 and rs29477109 on chromosome 11 (Supplementary Table 2; *p*-values: 1.57×10^{-9}, 1.12×10^{-14}, respectively). The locus on chromosome 5 contains a large number of genes, including *Abcf2* and *Slc4a2*. The human ortholog, *ABCF2,* has been associated with BMD in the largest GWAS of BMD completed to date [Estrada-2012], and is highly expressed in osteoblasts [Zheng-2015]. *Slc4a2* plays a critical role in osteoclasts [Coury-2013] and homozygous deletion of *Slc4a2* is associated with the osteopetrosis-

like phenotype “Marble Bone Disease” in Red Angus cattle [Meyers-2010]. Thus, both *Abcf2* and *Slc4a2* are viable candidates for this region. The association on chromosome 11 contains the gene *Col1a1*. In humans, Osteogenesis Imperfecta Type I can be caused by a null allele of *COL1A1* and results in gracile bones with decreased strength [Sillence-1979, Sykes-1986]. Furthermore, *COL1A1* is also associated with other bone size phenotypes [Long-2004], making *Col1a1* a likely causal gene for this locus.

Finally, we identified several associations for behavioral traits, including methamphetamine sensitivity on chromosome 6 (Supplementary Table 2; *p*-value at rs22397909: 9.03×10^{-7}) and chromosome 9 (Supplementary Table 2; *p*-value at rs46497021: 1.58×10^{-6}); these associations account for 2.6% and 2.1% of the phenotype variance, respectively (Figure 4, Supplementary Table 2). We also identified an association for anxiety-like behavior with rs238465220 on chromosome 13 (Supplementary Table 2; *p*-value: 7.31×10^{-8}) that explained 3% of the variance. For prepulse inhibition (12 db), we identified associations with rs264716939 on chromosome 7 (Figure 3C) and rs230308064 on chromosome 13 (Supplementary Table 2; *p*-values: 1.18×10^{-6} and 2.17×10^{-6}, respectively). In contrast to the testis weight QTL, there were many genes in the ~3Mb region on chromosome 7 that were associated with PPI (Figure 3E), making it difficult to identify the causal gene(s). Candidate genes for the associations with behavioral traits are discussed below.

Mapping eQTLs in striatum, hippocampus, and prefrontal cortex

In an effort to identify causal genes within the regions associated with behavioral traits, we mapped eQTLs for three brain regions that are critical for the behaviors that we studied. We performed RNA-Seq on messenger RNA (mRNA) from three brain regions: hippocampus (n=79), striatum (n=55) and prefrontal cortex (n=54). In a *cis*-eQTL scan that was limited to the region flanking the gene being interrogated, we identified a total of 6,045 associations for 4,174 genes (Figure 4A) at a permutation-derived significance threshold of $p < 0.05$ (this threshold reflects a per-gene, per-brain

region). For 534 of those genes we identified a *cis*-eQTL in all three tissues. For an additional 803 genes, we identified a *cis*-eQTL in two of the three tissues (Figure 4B). The RNA-Seq data were generated from different individuals; therefore, we did not perform a joint analysis of the three brain tissues [e.g. Flutre-2013]. As a result, we assume the observed overlap in Figure 4B is an underestimate. In addition, we searched for *cis*-eQTLs by examining allele-specific expression (ASE), which measures relative expression of the two possible RNA alleles derived from a heterozygous SNP [Serre-2008, Pickrell-2010]. After discarding SNPs that did not have at least 10 heterozygote individuals in a given tissue, we were left with 103,802 SNPs (hippocampus: 38,492, striatum: 32,313, prefrontal cortex: 32,997) in 17,888 genes (hippocampus: 6,439, striatum: 5,680, prefrontal cortex: 5,769) that could be interrogated for ASE. We identified 655 genes with ASE in at least one of the three tissues that exceeded a Bonferroni-corrected significance threshold of 1×10^{-6} (corresponding to an uncorrected significance threshold of about 0.05). Of these, 380 (58%) were found only using ASE, and 275 (42%) were also identified in the conventional *cis*-eQTL scan, suggesting that there was more overlap than would be expected by chance. Overlap was likely limited by several factors, including type I errors in the ASE and type II errors in both the ASE and conventional *cis*-eQTL mapping.

We also mapped eQTLs genome-wide for each gene in an effort to detect *trans*-eQTLs. We identified 2,278 *trans*-eQTLs that were significant ($p < 0.05$ permutation-based threshold) after testing 43,414 transcripts across the three brain regions. We expected almost that many tests to be positive under the null hypothesis. Consistent with this, a quantile-quantile (QQ) plot of these results suggested that only a small number of the detected results were true positives. As expected, most true positive results appear to be from the hippocampus, which had the largest sample size (n=79; Supplemental Figure 26).

Integration of brain eQTL and behavioral QTL results

Based on evidence from human GWAS, we anticipated that heritable gene expression polymorphisms (eQTLs) would be responsible for most of the observed behavioral associations. Therefore, we tried to identify eQTLs that co-mapped with behavioral QTLs, under the assumption that the eQTL might be the molecular cause of the behavioral QTL. For example, we observed an association between methamphetamine sensitivity and rs46497021 on chromosome 9 (p-value = 1.6×10^{-6}; Figure 4C). The implicated region was small (<1 Mb) and contained only 2 genes: *Cmc1* and *Azi2* (Figure 4D). We identified *cis*-eQTLs for both genes in the striatum, which is the tissue that is most relevant for methamphetamine sensitivity. However, rs46497021 was most strongly correlated with *Azi2* expression (p-value = 1.2×10^{-8}; Figure 4E). In addition, the pattern of SNPs associated with methamphetamine sensitivity and *Azi2* expression showed obvious overlap. Therefore, while both *Cmc1* and *Azi2* are credible positional candidates, the eQTL data suggest that *Azi2* is most likely to be the causative gene. Neither gene has been previously implicated in dopaminergic/striatal processes, suggesting that this observation may offer novel insights into the biology of this drug abuse-relevant trait.

Additionally, we identified an association between anxiety-like behavior and rs238465220 on chromosome 13 (p-value = 7.3×10^{-8}; Supplementary Table 2; Supplemental Figure 23). The implicated region spanned ~1.5 Mb and contained 4 genes: *Chrm3*, *Larp4b*, *Dip2c*, and *Zmynd11*. Among those 4 genes, rs238465220 was also associated with expression of *Zmynd11* in the hippocampus (Supplementary Figure 24), suggesting that this locus may influence anxiety-like behavior through its regulation of *Zmynd11* expression. *Zmydn11* has not been previously implicated in anxiety; however, copy number variants in *ZMYND11* were recently shown to be associated with autistic tendencies and aggressive behaviors in humans [Coe-2014]. These examples illustrate the utility of combining GWAS with eQTL data to identify the molecular mechanism by which a chromosomal region influences a complex trait.

Discussion

We performed a GWAS in a commercially available outbred mouse population, which identified numerous physiological, behavioral, and expression QTLs. In several cases the implicated loci were smaller than 1 Mb and contained just a handful of genes that included an obvious candidate. In addition, we used the eQTL results to further parse among the genes in the intervals that were implicated in the behavioral traits.

Utility of CFW mice as a mapping population

One of our major goals in selecting the CFW mice was to enhance our mapping resolution. CFW have been reported to have shorter-range LD than other commercially available populations [Yalcin-2010] which suggested they would provide excellent mapping resolution. Using the GBS genotypes, we estimated LD in our cohort of CFW mice and compared it to other commonly used mapping populations (Figure 2B). The mean rate of decay in LD as a function of physical distance in CFW was substantially greater than the 34th generation of the SM/J x LG/J advanced intercross line (AIL) that we have used in prior studies [Cheng-2010, Samocha-2011, Parker-2011, Parker- 2014] (that AIL is now at generation 62). Various outbred heterogeneous stocks, typically made up of 8 inbred strains, have also been used in prior mapping efforts [e.g. Talbot-1999; Demarest-2001; Valdar-2006]. We examined the outbred heterogeneous stock used by Valdar et al [2006], and found that it had longer range LD compared to CFW. The Hybrid Mouse Diversity Panel (HMDP) [Ghazalpour-2012, Orozco-2012], which is a collection of approximately 100 inbred strains of mice that can be used to analyze complex traits, also showed a longer range LD compared to CFW, as did a smaller cohort of 30 strains selected from the HMDP [Sittig-2016]. Another heterogeneous stock called the JAX Diversity Outbred (DO) [Svenson et al 2012, Churchill-2012, Chesler-2014, Gatti-2014], which includes three wild-derived strains, exhibited LD decay that was most similar to the CFW. Outbred populations like the AIL and HS (including the DO) are expected to show decreased

LD in the future due to the accumulation of additional recombinations. MF-1 is another commercially available outbred population that has been used to map QTLs [Yalcin-2004, Ghalzapour-2008], but we were unable to obtain the data needed to estimate LD decay in this population. Comparing LD patterns in different populations is a common method for estimating mapping resolution [Pritchard-2001], however additional factors including the allele frequency distribution [Eberle-2006], population structure [Mangin-2012], error rates and the number, effect size and frequency of causal variants all influence power and mapping resolution. Despite these limitations, our comparison of LD (Figure 2B) and our mapping results (Figures 3, 4, Supplementary Table 2, Supplementary Figures 22-24, 29) suggest that CFW mice are an attractive option for fine-mapping studies.

Another important parameter for GWAS studies is allele frequency, since power to identify associations increases with greater MAF. Laboratory mouse populations have higher MAF than humans or wild populations [Laurie-2007] presumably due to their population history. We found that 73% of SNPs genotyped in this study had allele frequencies greater than 5%, although our SNP filtering steps may have underestimated the number of rare SNPs. Populations produced by crossing inbred strains, such as F_2 crosses, recombinant inbred lines, AILs and heterogeneous stocks typically have even more desirable MAF distributions [e.g. see Cheng-2010]. Because the ascertainment of SNPs included in genotyping platforms directly influences the estimated MAF distribution in a population, we did not attempt to use publicly available data to compare MAF in commonly used mapping populations.

We found that CFW mice lacked genetic variability in certain regions; for example, Chromosome 16 had a low density of polymorphic markers as measured using both GBS and MegaMUGA (Figure 2A) and no significant QTLs (Figure 3A). This is an example of a well-known tendency for outbred

laboratory mouse populations to harbor regions that are identical by descent [e.g. Cheng-2010, Yang-2011].

Finally, several other advantages of CFW mice include their commercial availability, their low cost, and the ability to acquire non-siblings upon request. We also found that the CFW mice were easy to handle, and their uniform coat color simplified automated scoring of certain behavioral traits.

GBS

One barrier to more widespread adoption of GWAS in rodents has been the lack of universal and economical SNP genotyping platforms. One innovative aspect of this paper is the use of GBS to overcome this obstacle. GBS is a reduced-representation sequencing approach in which a small fraction of the genome (in our case, ~1%) is sequenced at moderate depth (in our case an average of ~15x) in order to obtain genotypes at a subset of markers. While GBS shares some characteristics with low-coverage whole-genome sequencing [CONVERGE consortium-2015, Le-2011, Li-2011], GBS yields high SNP density for a subset of the genome, thus obtaining fewer SNPs with greater confidence. Our GBS methods included a custom-designed library preparation protocol (which allowed us to further reduce per-sample costs), and used the standard software toolkits GATK [McKenna-2010] and IMPUTE2 [Howie-2009]. An advantage of GBS is that it does not require prior knowledge of SNPs in the population. We chose conservative criteria for SNP calling, which yielded 92,734 SNPs, of which 14% were newly discovered and possibly unique to CFW. These 92,734 SNPs provided extensive coverage of the genome (Figure 2A) and allowed for fine-mapping (Figures 3C, 3D, 4D, 4E and Supplemental Figures 23 and 24). The number of markers obtained using GBS can be titrated by varying the restriction enzymes used, the fragment sizes selected and the degree of sample multiplexing. GBS requires imputation to correct errors and to populate missing genotypes, which requires greater expertise compared to the analysis of SNP genotyping arrays. Compared to conventional array-based SNP genotyping, GBS had a higher error rate. The

higher genotype error rate is expected to modestly decrease power, but should not produce false positive QTLs since the errors will not be correlated with the traits. We are currently improving genotype imputation methods for populations in which the founder haplotypes are known, such as two-strain AILs [Parker-2011, Parker-2012a, Parker-2012b, Parker-2014] and eight-strain intercrosses similar to the DO [Gatti-2014]. Because the monetary advantage of GBS over array-based genotyping will continue to improve as sequencing prices decrease, we anticipate that GBS and other sequencing-based approaches will supplant array-based methods in the coming years.

Using eQTLs to identify causal genes

The majority of human GWAS findings implicate regulatory rather than coding differences [Albert-2015, Gusev-2014]. The identified regions frequently contain several genes that are in LD with one another. It is now widely appreciated that even when an association can be localized to a single gene, that gene may not be the cause of the association [Smemo-2014], meaning that proximity to the peak SNP is not sufficient to identify the causal gene. Therefore, a major goal of our study was to integrate behavioral QTL and eQTL data. eQTLs can provide the crucial link between a region implicated by GWAS and the biological processes that underlie that association. We exploited the easy access to tissue, which is a critical advantage of model organisms, to map eQTLs. Specifically, we used RNA-Seq to examine gene expression in three brain regions that are known to be important for the behavioral traits that we studied. Although *Azi2* was not an obvious candidate for the behavioral QTL for methamphetamine sensitivity, our data showing the co-mapping of an eQTL for *Azi2* expression in the striatum provide an additional layer of evidence. Similarly, *Zmynd11* has not been previously implicated in anxiety-like behavior, but the eQTL for *Zmynd11* expression in the hippocampus suggests that it is the most promising of the four genes that fall within the behavioral QTL. These examples demonstrate the power of integrating fine-mapping of behavioral QTLs and expression QTLs and extend on multiple prior mouse studies have used similar

approaches in conjunction with F_2 crosses [Schadt-2003], recombinant inbred lines [Chesler-2004, Chesler-2005, Bystrykh-2005], selected lines [Palmer-2005], heterogeneous stocks [Huang-2009], outbred MF-1 mice [Ghalzapour-2008] and the HMDP [Farber-2011, Calabrese-2012, Orozco-2012].

RNA-Seq offers a number of advantages relative to traditional array-based gene expression measurements [de Klert-2015, Mortazavi-2008, Mane-2009, Tang-2009, Trapnell-2009, Trapnell-2012, Walter-2009]. In particular, we were able to map *cis*- and *trans*-eQTLs using a traditional mapping approach, and simultaneously map *cis*-eQTLs by quantifying ASE. Since only a fraction of genes can be studied using ASE, we did not anticipate complete overlap between genes identified using these two approaches. Using ASE we identified 655 *cis*-eQTLs among the 6,000 genes considered (11%), of which 42% were also identified as *cis*-eQTLs using conventional mapping.

<u>Synthesis of our findings</u>

We found that the physiological traits typically had slightly higher heritabilities than the behavioral traits (Supplementary Table 1). We also found that the effect sizes of individual associations tended to be higher for physiological traits (Supplementary Table 2), consistent with findings from another recent study in rats [e.g. Baud-2013]. However, it was not always true that traits with the highest heritabilities also showed the largest effect sizes for individual associations. Because the effect size of individual QTL alleles is of paramount importance for assessing power at a given sample size, and because this parameter is never known in advance, it is not possible to provide general guidelines about the sample size that should be used for similar studies in the future. Based on our experience with this study, we believe that a sample size of 1,000 or more CFW mice should be used for most traits, though some of the traits we studied (e.g. testis weight, abnormal BMD), would have yielded significant results with just a few hundred mice, and our studies of gene expression identified *cis*-eQTLs with as few as 54 individuals. While our use of the CFW was intended to

increase mapping precision, there is a direct tradeoff between mapping precision and statistical power [Parker-2011], therefore sample sizes required for studies using CFW will necessarily be larger than for F_2, recombinant inbred, or other traditional mapping populations that offer less precision.

Our data do not directly address the reasons that the effect sizes we observed are so much larger than the effect sizes observed in most human genetic studies. We can speculate that the unique population history of laboratory mice (domestication, selection and repeated population bottlenecks) have increased the frequency of alleles that may have been rare in ancestral wild mouse populations. It is also true that, unlike many traits studied in human GWAS, the traits we are examining are not disease traits (and thus may not influence fitness), and therefore may not have been influenced by natural selection even among ancestral wild mouse populations from which laboratory populations were originally derived. Furthermore, laboratory mice are drawn from a much more uniform environment, potentially diminishing gene-by-environment interactions that may reduce the size of genetic effects in human GWAS. Finally, because LD in the CFW mice is more extensive as compared to humans, we are effectively testing fewer hypotheses and therefore have a lower (permutation-derived) threshold to obtain genome-wide significance.

Based on our prior experience with other fine-mapping populations [e.g. Cheng-2010, Cheng-2013, Parker-2014], we were concerned that relatedness among individuals might distort our association test statistics. As detailed in the Supplementary Materials, we found only modest evidence of cryptic relatedness among individuals. When we compared the results of GWAS using a simple linear model and a linear mixed model that included a genetic relationship matrix, the overall distributions of *p*-values were remarkably similar (Supplemental Figure 17). This suggests that the CFW breeding colony was large enough that non-littermates could have been safely be treated as unrelated subjects, however we nevertheless used a mixed model that accounted for relatedness.

We have shown that use of CFW mice in conjunction with GBS and RNA-Seq provides a powerful and efficient means for identifying genetic associations, and for nominating candidate genes within the associated regions. Compared to other outbred mouse populations, CFW mice show rapid decay of LD (Figure 2B), are less expensive, and primarily allow examination of *domesticus* derived alleles (Figure 2C). Compared to human GWAS, this approach provides dramatically reduced costs, the ability to examine phenotypes that include experimental manipulations that would be impractical or unethical in humans, the ability to obtain tissue samples for expression analysis, and the ability to exert exquisite control over environmental variables. Identified genes can be manipulated via genome engineering [Sander-2014]. Thus, our approach can be used to rapidly generate gene-specific, testable hypotheses for a wide array of complex traits. More broadly, our results demonstrate methods and principles that apply to a variety of other model systems.

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Figure Legends

Figure 1: Components of study. Each of the 4 panels illustrates a component of the study: (A) behavioral testing and measurement of physiological traits; (B) genotyping-by-sequencing (GBS); (C) measurement of gene expression in brain tissues using RNA-Seq; (D) QTL mapping for physiological and behavioral traits, and for gene expression.

Figure 2: Genetic characteristics of CFW mouse population. (A) Density of GBS SNPs on autosomal chromosomes; (B) mean LD (r^2) decay rates estimated using frequency-matched SNPs [Eberle-2006], with MAF > 20% in a 34th generation AIL derived from LG/J and SM/J strains [Cheng-2010, Parker-2014], heterogeneous stock (HS) mice bred for >50 generations [Valdar-2006], the Hybrid Mouse Diversity Panel (HMDP) [Bennett-2010], a panel of 30 inbred lab strains [Yang-2009, Sittig-2016], Diversity Outbred mice [Gatti-2014], and CFW mice; (C) *treemix* analysis summarizing genetic relationship between CFW mice and inbred strains in Wellcome Trust sequencing panel.

Figure 3: QTLs for physiological and behavioral traits. (A) Minimum *p*-values for association across all tested behavioral and physiological phenotypes (see Supplementary Table 1 and 2 for details). (B) Genome-wide scan for testis weight and (C) pre-pulse inhibition in response to +12 dB pre-pulse. (D) Association signal for testis weight near the QTL on chromosome on 13. (E) Association signal for pre-pulse inhibition near the QTL on chromosome 7. Dotted red lines indicate thresholds ($p<0.1$) estimated via permutation tests.

Figure 4: Overview of eQTL mapping. (A) Color of each pixel in the matrix depicts the lowest *p*-value among all eQTLs using a 10 Mb × 10 Mb window. (B) Overlap of genes with eQTLs in the three brain tissues detected using the traditional *cis*-eQTL mapping method (not ASE). The permutation based *p*-value threshold for each eQTL is 0.05. (C) Genome-wide scan for total locomotor activity on day 3 of the methamphetamine sensitivity tests. (D) Association signal for total locomotor activity in the QTL region on chromosome 9. (E) Association signal for expression of *Azi2* in the striatum, in the same region as panel D. Dotted red lines indicate thresholds ($p<0.1$) estimated via permutation tests.

Online Methods

Animal Models

We phenotyped 1,200 male Carworth Farms White (CFW) mice (*Mus musculus*) that were obtained from the Charles River Laboratories facility in Portage, Michigan, USA (**CRL**; strain code: CRL:CFW(SW); facility code: P08). Since our study was completed, the Portage colony has been relocated to Kingston, New York (new code K92). It has been reported that ancestors of the CFW mice were obtained from a large colony of Swiss mice in 1926, and maintained by Dr. Webster at the Rockefeller Institute. A single pair of highly inbred albino mice were later acquired by Carworth Farms and used to initiate an outbred mouse stock. Several mice from this colony were later acquired in 1974 by CRL and were subsequently maintained as an outbred population [Rice-1980, Chia-2005, Yalcin-2010].

Every two weeks, 48 male CFW mice were shipped from CRL in Portage, MI to our laboratory in Chicago, IL. We requested that CRL send only one mouse from each litter to avoid obtaining siblings, since close relatives reduce power to map QTLs, and complicate analysis. The average age of the mice upon arrival in our labs was 35 days (ranging from 34 to 46 days), and their average weight was 25.5 g (ranging from 13.4 g to 38.7 g). Mice were housed 4 per cage and given ~15 days to adapt to their new environment (Supplementary Figure 1). Standard lab chow and water were available *ad libitum*, except during the behavioral procedures and prior to testing for fasting glucose. Mice were maintained on a standard 12:12h light-dark cycle (lights on at 06:30). All phenotyping occurred during the light phase between 08:00 and 16:00 hours, over the period of August 2011 to December 2012. All procedures were approved by the University of Chicago Institutional Animal Care and Use Committee (IACUC) in accordance with National Institute of Health guidelines for the care and use of laboratory animals.

Phenotyping

The order of phenotyping was identical for each mouse, and is shown schematically in Supplementary Figure 1. One day after arrival, mice were fasted for four hours prior to measurement of blood glucose levels. Fourteen days later, we assessed their response to a novel environment and to administration of 1.5 mg/kg of methamphetamine in a 3-day paradigm [Parker-2012a]. Twelve days later, we tested mice for conditioned fear [Parker-2014]. Nine days after that, we tested mice for prepulse inhibition (PPI; [Samocha-2010]). Finally, after 15 days we weighed and sacrificed the mice. Immediately after sacrifice, we weighed testis, and collected one leg for measurement of muscle phenotypes, and collected the other leg for measurement of bone-phenotypes. We also measured tail length at this time. See Supplementary Material for further details.

RNA-Seq

After sacrifice, we collected brain tissue from a subset of mice as a source of mRNA from the hippocampus (n=79), striatum (n=55) and frontal cortex (n=54). We used RNA-Seq [Majewski-2011, Wang-2009] to quantify gene transcript abundance in these brain tissues. Library preparation was performed with the TruSeq RNA Sample Kit (Illumina). Samples were multiplexed 5-per lane and sequenced on an Illumina HiSeq 2000 sequencer, using single-end 100-bp reads. We processed the RNA-Seq short reads using the *Tuxedo* software suite [Trapnell-2012]: (1) first, we aligned the short reads to the reference genome assembly (NCBI release 38, mm10) with *bowtie2* [Langmead-2009]; (2) next, we used tophat2 [Trapnell-2012] to align the short reads to known splice junctions; (3) finally, we used *cufflinks* [Roberts-2011] to calculate, for each gene, a gene-level measure of expression based on the mapped reads. This measure is reported in reads per kilobase per million reads mapped (RPKM). This measure does not depend on length of coding sequences or sequencing depth of each sample (so mapping expression QTLs will not be biased by these factors). We focused on this gene-level measurement for subsequent investigation, including eQTL mapping and assessment of allele-specific expression (ASE). See Supplementary Methods for further details.

Genotyping-by-sequencing (GBS)

Genotyping-by-sequencing (GBS) is a reduced-representation genotyping method for obtaining genotyping information by sequencing only regions that are proximal to a restriction enzyme cut site [Elshire-2011]. Our protocol was adapted from the procedures described in Grabowski *et al* [Grabowski-2014]. GBS libraries were prepared by digesting genomic DNA with a restriction enzyme, *PstI*, and annealing oligonucleotide adapters to the resulting overhangs. Samples were multiplexed 12-per lane, and sequenced on an Illumina HiSeq 2000 sequencer using single-end 100-bp reads. We obtained an average of 4.8M reads per sample. By focusing the sequencing effort on the *Pstl* restriction sites, we obtained high coverage (~15x) at a subset of genomic loci, although those reads were very non-uniformly distributed. We aligned the 100-bp single-end reads to Mouse Reference Assembly 38 from the NCBI database (mm10) using *bwa* [Li-2009]. We used GATK [McKenna-2010, Van-der-Auwera-2013] to discover variants and to obtain genotype probabilities. For the Variant Quality Score Recalibration (VQSR) step, we calibrated variant discovery against (1) whole-genome sequencing (WGS) data that we ascertained from a small set of CFW mice, (2) SNPs and indels from the Wellcome Trust Sanger Mouse Genome project [Keane-2011], and SNPs available in dbSNP release 137. We used IMPUTE2 [Howie-2009] to improve low-confidence genotypes, or genotypes that were not called in individual mice. A small number of SNPs for which a large proportion of the genotypes were imputed with low certainty were discarded from the study. See Supplementary Material for further details.

Treemix analysis

We estimated phylogenetic relationships between the CFW mice and different lab strains sequenced as part of the Wellcome Trust mouse genome sequencing project using *treemix* [Pickrell-2012]. We used the genotypes for the lab strains sequenced by the Wellcome Trust to obtain the locations of SNPs that were identified in the CFW mice using our GBS pipeline. We excluded the *mus spretus* strain from the Wellcome Trust data, since this strain was included as an outgroup. Since

the lab strains are all inbred, we assumed that the allele frequency was 1 or 0. We represented each strain by only a single individual. We used a subset of 100 CFW mice to compute the allele frequencies from the genotype likelihoods of GBS SNPs in our sample. Treemix was used to fit a maximum-likelihood tree to all the lab strains and CFW samples.

QTL mapping for behavioral and physiological traits

We performed a GWAS for the behavioral and physiological phenotypes using all SNPs with MAF >2% and good imputation quality (defined as 95% of the samples having a maximum probability genotype greater than 0.5). Although our analyses did not suggest the presence of close relatives or population structure, we used the linear-mixed model implemented in the program GEMMA [Zhou-2013]. GEMMA is similar to a standard linear regression, in which the quantitative trait (Y) is modeled as a linear combination of the genotype (X) and the covariates (Z), except that it includes an additional "random" or "polygenic" effect capturing the covariance structure in the phenotype that is attributed to genome-wide genetic sharing:

$$y_i = \mu + z_{i1}\alpha_1 + \cdots + z_{im}\alpha_m + x_{ij}\beta_j + u_i + \varepsilon_i$$

The notation in this expression is defined as follows: y_i is the ith phenotype sample; z_{ik} is ith sample of covariate k, in which k ranges from 1 to the number of covariates included in the regression (m); α_k is the coefficient corresponding to covariate k; x_{ij} is the genotype of sample i at SNP j; β_j is the coefficient corresponding to SNP j; u_i is the polygenic effect for the ith sample; ε_i is the residual error; and μ is the intercept. The genotype, x_{ij}, is represented as the expected allele count, in which 0 represents a homozygous major allele, and 2 represents a homozygous minor allele, and β_j is the additive effect of the expected allele count on the phenotype. The residuals ε_i are assumed to be *i.i.d.* normal with zero mean and covariance σ^2, whereas the polygenic effect $u = (u_1, \ldots, u_n)^T$ is a random vector drawn from the multivariate normal distribution with mean zero and $n \times n$ covariance matrix $\sigma^2\lambda K$, where n is the number of samples.

We estimated the relatedness matrix, K, from the genotype data. We specified the covariance matrix using the realized relationship matrix $K = XX^T/p$, where p is the number of SNPs, and X is the $n \times p$ genotype matrix with entries x_{ij}. This formulation was derived from a polygenic model of the phenotype in which all SNPs helped explain variance in the phenotype, and the contributions of individual SNPs were *i.i.d.* normal [Hayes-2009, Listgarten-2012, Zhou-2012].

The inclusion of a genetic marker in both the fixed and random terms can deflate the test statistic for this marker, leading to a loss of power to detect a QTL; this problem has been termed "proximal contamination" [Listgarten-2012]. To avoid proximal contamination, we computed 19 different K matrices, each one excluding one of the 19 autosomes. To scan markers on a given chromosome, we used the version of K that did not include that chromosome. We have previously proposed this leave-one-chromosome-out (LOCO) approach as a simple solution for avoiding the problem of proximal contamination [Cheng et al 2013].

We used a permutation-based approach to calculate the genome-wide significance threshold for p-values calculated in GEMMA. We estimated the distribution of p-values under the null hypothesis by mapping QTLs in 1,000 randomly permuted data sets, then taking the threshold to be the 100(1 – α)th percentile of this distribution, with α = 0.1. Although this permutation test is technically only valid under the assumption that the samples are exchangeable [Abney-2015], we have previously suggested that 'naïve' permutations are generally sufficient [Cheng-2013]. Furthermore, given our observation that population structure is subtle, we expect that this simulation provides a good approximation to the null. See the Supplementary Materials for more details.

Heritability estimates

Instead of computing a point estimate for h^2, which is the usual approach (e.g. using the REML estimate [Yang-2010]), we evaluated the likelihood over a regular grid of values for h^2, which allowed us to directly quantify uncertainty in h^2 under the reasonable assumption of a uniform prior for the proportion of variance explained [Zhou-2012].

We estimated the SNP heritability, h^2, of our phenotypes [Wray-2013]. Because the GBS SNPs did not completely tag all casual variants (and because we excluded the sex chromosomes), our estimates of h^2 underestimated a trait's true narrow-sense heritability. To estimate h^2, we assumed that all genetic markers made some small contribution to variation in the trait, and that these contributions were normally distributed with the same variance [Speed-2011, Yang-2010, Zhou-2013]. Under this polygenic model, the covariance of the phenotype measurements was $Cov(Y_1, \ldots, Y_n) = \sigma^2 H$, where $H = (I + \sigma_a^2 K)$, I is the $n \times n$ identity matrix, K is the $n \times n$ realized relatedness matrix, σ_a^2 is the variance of the additive genetic effects, and σ^2 is the variance of the residuals. Under this formulation, σ_a^2 represents the relative contribution of the additive genetic variance, and we can use this parameter to provide an estimate for h^2:

$$h^2 = \sigma_a^2\, s_a / (\sigma_a^2 s_a + 1)$$

where s_a is the mean sample variances of all the available SNPs, or the mean of the diagonal entries of K assuming that the columns of X are centered so that each of the columns have a mean of zero. See Supplementary Methods for further details.

Expression QTL (eQTL) mapping

We used the RPKM measurements from RNA-Seq and the GBS genotype data to map expression QTLs (eQTLs). We performed an eQTL scan separately in each brain tissue (hippocampus, striatum, prefrontal cortex). First, we discarded genes with low levels of expression (RPKM < 1), and genes that showed no variability in expression. For the remaining genes, we quantile-normalized the expression data. To account for unknown confounders, we removed linear effects of the first few principal components (PCs) calculated from the $K \times N$ gene expression matrix (20 PCs for hippocampus, 10 PCs for striatum, 20 PCs for prefrontal cortex), similar to [Pickrell-2010]. After

removing linear effects of the PCs, we again quantile-normalized the expression data. We then used an LMM as implemented in GEMMA to scan for *cis*-eQTLs, as described above for the behavioral and physiological phenotypes. To define *cis*-eQTLs, we only considered SNPs within 1 Mb of the gene's transcribed region (preliminary analyses indicated that 1 MB captured most of the significant signals; Supplemental Figure 19). We used a permutation-based approach to calculate significance thresholds for *p*-values in the *cis*-eQTL mapping. We used 1,000 permutations of the expression values to compute a separate significance threshold for each gene, using only the SNPs that were included in the *cis*-eQTL scan. In addition to *cis*-eQTL scans, we also performed genome-wide *trans*-eQTLs scans for all the genes. The genome-wide scans were performed using the same LMM that was used for *cis*-eQTL analyses, except that all the SNPs outside a 2 Mb region around the gene were included in the *trans*-eQTL analysis. The significance threshold for *trans*-eQTLs was computed using permutations of 1,000 randomly selected genes in each tissue; this approach is permissible because all expression traits were quantile-normalized. See Supplementary Material for further details.

Allele-specific expression (ASE)

Finally, we performed an analysis of allele-specific expression (ASE) to identify genes that had ASE QTLs. This analysis was performed independently from the mapping of *cis*-eQTLs described above. We identified variants that had at least 10 samples with high-confidence heterozygote genotype calls. For genes that contained at least one such variant, we compared the relative expression of the two alleles across these heterozygote samples. To account for overdispersion, we used a beta-binomial model to fit the counts of the two alleles for each sample. We then used a likelihood-ratio test to test for significant deviation of the observed data from the expectation of equal counts from both alleles. See Supplementary Material for further details.