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Systems genetic analysis in GeneNetwork.org

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Abstract

Genome-wide association studies (GWAS) have emerged as a powerful tool to identify alleles and molecular pathways that influence susceptibility to psychiatric disorders and other diseases. Forward genetics using mouse mapping populations allows for a complementary approach which provides rigorous genetic and environmental control. In this protocol, we describe techniques and tools that reduce the technical burden traditionally associated with genetic mapping in mice and enhance their translational utility to human psychiatric disorders. We provide guidance on choosing the appropriate mapping population, discuss the importance of phenotype, and offer detailed instructions on using the Web-based resource GeneNetwork to aid neuroscientists in better understanding the mechanisms through which genes influence behavior. We believe that the continued development of mouse mapping populations, genetic tools, bioinformatics resources, and statistical methodologies should remain a parallel strategy by which to investigate the genetic and environmental underpinnings of psychiatric disorders and other diseases in humans.

Keywords

quantitative trait locus mapping; recombinant inbred strains; systems genetics; GeneNetwork

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INTERNET RESOURCES

http://genenetwork.org/webqtl/main.py

GeneNetwork is a free scientific web resource used to study relationships between differences in genes, environmental factors, phenotypes, and disease risk.

INTRODUCTION

In the last decade, genome-wide association studies (GWAS) have emerged as a powerful tool to identify alleles and molecular pathways that influence susceptibility to psychiatric disorders (Manolio et al. 2008; Manolio et al. 2013; SWGPGC 2014). To perform GWAS, researchers measure the correlation between phenotypes and DNA markers (often referred to as single nucleotide polymorphisms, or SNPs) across the genome. It is considered a forward genetic approach because it starts with naturally occurring trait variation and looks for naturally occurring genetic variation that is associated with it. If a SNP appears to be highly correlated with a trait of interest, this indicates that the SNP lies physically close to the causative genetic variant.

Despite recent successes, human GWAS are somewhat limited in their scope and utility due to numerous practical, financial, and ethical concerns (Kapp 2006; Gelernter 2015; Montalvo-Ortiz et al. 2016). One complementary approach to human GWAS relies on the use of mouse mapping populations to clarify the biological mechanisms of characteristics relevant to psychiatric disorders and provide deeper insight into the genetic and environmental interactions associated with complex traits. Mouse models allow for environmental factors including drug exposure to be held constant or systematically varied in order to explore interactions between genotype and environment. Since external factors partially regulate how psychiatric symptoms manifest, utilizing rodents exploits this important source of variability that GWAS are often unable to account for (Hovatta et al. 2008). In addition, almost all human genes known to be associated with disease have a mouse orthologue (Adams et al. 2015; MGSC 2002) and most disease genes identified in mice have also been shown to play a role in human diseases (Aitman et al. 2008). Another advantage of mice is that brain tissue can be obtained under idealized laboratory conditions and used to identify gene expression quantitative trait loci (eQTLs). This is especially important because most causal loci implicated in human GWAS appear to be due to regulatory rather than coding polymorphisms (Albert & Kruglyak 2015). Furthermore, replication of initial results can easily be performed in mice, but have become increasingly more challenging in human GWAS as successively larger sample sizes are needed to detect significant associations (Hunter & Crawford 2008). Finally, the genomes of mice can be easily and directly manipulated through a wide range of techniques, making them an incredibly powerful genetic tool (Thomas & Capecchi 1990; Sander & Joung 2014).

Genetic mapping studies in mice have proven to be successful in part because they permit analysis at the genomic, cellular, pharmacological, physiological, behavioral, and bioinformatics levels (Williams & Lu 2008). However, integrating the many different types of data that can be acquired from mouse models has proven to be a technically demanding approach for gene identification. Phenome, genome, and transcriptome data-sets contain massive amounts of information, and the analysis of the many different levels of data is often beyond the reach of an individual neuroscience laboratory. In this protocol, we describe techniques and tools that reduce the technical burden traditionally associated with genetic mapping in mice and enhance their translational utility to human psychiatric disorders. We provide guidance on choosing the appropriate mapping population, discuss the importance of phenotype, and offer detailed instructions on using the Web-based resource

GeneNetwork to aid neuroscientists in better understanding the mechanisms through which genes influence behavior. We believe that the continued development of mouse mapping populations, genetic tools, bioinformatics resources, and statistical methodologies should remain a parallel strategy by which to investigate the genetic and environmental underpinnings of psychiatric disorders in humans.

STRATEGIC PLANNING *(optional)*

Choosing a quantitative trait: the importance of phenotype

To perform systems genetic analysis or QTL mapping, one starts with an operationally defined quantitative trait, i.e. one that is heritable, varies continuously, and is influenced by multiple genetic and environmental factors. Thus, when choosing a trait to study, it is of the utmost importance that the trait does, in fact, vary across genotypes in the population of mice being studied. While this may be difficult to know ahead of time, one promising (but not essential) indicator that a quantitative trait will vary across genotypes is if the trait differs between progenitor strains in a mapping population. It is also important to choose testing conditions and manipulations that prevent a floor or ceiling effect from occurring across a major proportion of the population, as these might conceal any effects of phenotypic variation due to genotype. Another criterion for the trait under investigation is validity: typically, traits with high construct and face validity are going to be the most informative for the study of human disease. Similarly, one should be vigilant about maintaining phenotyping standards over the course of the experiment to avoid the confounding role of laboratory environment. Ideally, sampling of the population should occur in a manner well randomized with respect to genotype, such that if a population consisting of multiple strains is used, one should attempt to study as many strains as possible and to test replicate members of the strains in independent cohorts, rather than to test small groups of strains sequentially.

Choosing a population—There are many mouse populations to choose from, including recombinant inbred panels (e.g. BXD, AXB/BXA, LXS), common inbred panels (e.g. the Mouse Phenome Project strains), the Hybrid Mouse Diversity Panel (a combination of the former two panels), the new Collaborative Cross, Diversity Outbred or other hybrid populations. Availability, genetic and phenotypic variability, and cost may influence the choice of panel further. Populations consisting of inbred strains enable seamless data integration across multiple studies, and thus have long-term capabilities for extension of findings. In populations consisting of outbred mice, power and precision are limitless, but typically, all measures must be obtained from the same population.

BASIC PROTOCOL 1. BASIC PROTOCOL TITLE: Genetic mapping and systems genetics using GeneNetwork

Introductory paragraph

GeneNetwork (www.genenetwork.org) is a free online resource for systems genetics that stores and analyzes behavioral phenotypes, physiological phenotypes, and large gene expression data-sets with matched genomic data for numerous species, including mice. GeneNetwork can analyze a variety of mouse mapping populations, (including F_2

intercrosses, backcrosses, recombinant inbred (RI) panels, heterogeneous stock, and the mouse diversity panel) but is not currently compatible with some complex multi-generation crosses such as advanced intercross lines (AILs) or the Collaborative Cross (CC). It is intended for rapid exploratory analysis by neuroscientists and does not require specialized training in bioinformatics or statistical genetics. Data can be downloaded for more extensive or customized analysis as needed. GeneNetwork includes extensive multivariate statistical tools implemented in Python, C and the R statistical language and can be used to map quantitative trait loci (QTLs) and expression QTLs (eQTLs). It can also be used to examine genetic covariation among large numbers of phenotypes and to perform data mining in genomic regions containing candidates for quantitative trait genes (Chesler et al. 2003; 2005; Andreux et al. 2012; Wang, et al. 2003). Finally, GeneNetwork links to other informatics resources such as NCBI (www.ncbi.nlm.gov), the UCSC genome browser (www.genome.ucsc.edu), the Allen Brain Atlas (www.brain-map.org) and GeneWeaver (www.GeneWeaver.org) to drastically streamline the process of QTL and eQTL mapping, candidate gene identification, and co-expression analyses.

Materials

Here we will provide detailed instructions for using GeneNetwork along with some "worked" examples taken from the recent study of intravenous cocaine self-administration by Dickson et al. (2016) in BXD RI mice. A complete overview of GeneNetwork is beyond the scope of this protocol, but is extensively covered in elsewhere (see Mulligan et al. 2016; Williams & Mulligan 2012 for excellent reviews on GeneNetwork).

A computer with an internet connection and current web browser. See the GeneNetwork.org site for information on supported browser versions.

Method

Entering Data

1. Link to http://www.genenetwork.org. To submit multiple phenotypes at the same time, select the option for **Batch Submission** under the **Home** tab. This allows users to submit up to 100 traits for analysis by GeneNetwork. Here, select *BXD* as the **cross or RI set** to analyze from the first pull-down menu. The phenotype file should follow the format described in the Sample text (http://genenetwork.org/sample.txt). After uploading the appropriate file using the **Browse** button, enter a name for the file in the **Dataset** space. The data will be stored in the GeneNetwork server for 24 hours. Click **Next**.

2. Alternatively, one can analyze data for a single phenotype. Select the option to **Enter Trait Data** under the **Home** tab. Here, choose *BXD* from the pull-down menu. Data can be entered either from a file or by copying and pasting multiple trait values into the box provided. In both cases, the values can be on one line separated by spaces or tabs, or they can be on separate lines. Include one value for each progeny individual or recombinant inbred line. Represent missing values with a non-numeric character such as "x". If you have chosen a recombinant inbred set, your data will be displayed in a form where you can

confirm and/or edit them. GeneNetwork provides sample data so that you can ensure you have the correct format. As an example for using both methods, data for a phenotype related to intravenous self-administration (IVSA) of cocaine in 29 BXD RIs is provided below that can be saved and pasted in as:

x	x	x	x	0.093	x	x	x	x	−1.183
−1.575	x	0.763	0.402	−1.892	x	x	x	−0.981	x
x	x	x	x	x	0.23	x	1.113	x	1.235
1.048	−2.734	1.875	x	x	x	x	−0.687	−0.722	x
x	−0.264	x	x	x	x	x	0.928	x	x
x	x	x	1.76	x	x	0.255	x	−0.559	x
x	x	2.019	x	x	x	−1.512	−2.009	x	x
x	x	x	x	−0.694	x	1.071	x	x	x
x	x	x	x	x	x	x	0.902	x	x
x	x	1.278	−0.623	0.464	x	x	x	x	x

These data represent a principal component associated (see “Multitrait QTL Mapping for additional details) with the number of cocaine infusions on the ascending limb of the dose-response curve (IAL, Dickson et al. 2016) and can also be accessed in GeneNetwork by entering **Record ID** *18494* in the **Get Any** space on the Search page and clicking on the **Search** button. Alternatively, enter data by hand into the designated boxes provided by GeneNetwork. These latter options also allow for the inclusion of trait variance. It is a good idea to name the trait in the box provided. Then click **Next**, and manually enter the data for each RI strain, F_1, and founder strain.

3 After entering the data, click on the blue plus sign button called **Add**. This will add the dataset to the collection where it can be saved for future analyses by clicking on the button **Save Collection**.

Initial Analyses

4 To begin analyzing the data, click on the **Basic Statistics** tab on the **Data Editing** page. This provides the number of strains in the dataset, along with measures of central tendency and dispersion. It also contains a **Probability Plot** that can be used to detect the influence of major effect loci by displaying whether or not the data follows a normal distribution. If the distribution is normal, then the actual values and the predicted values based on a z score will form an approximate straight line. Deviations from normality may indicate probable QTLs. Probability plots can also be used to easily identify outliers, skewness, and kurtosis. Outliers on either tail of the distribution may require special statistical handling (see section on **Critical Parameters/ Troubleshooting**, below). Figure 1 shows that the IAL data are approximately normally distributed. The **Bar Graph** tabs show the IAL scores for all strains tested arranged by name or by rank.

5 Traits that are influenced by similar sets of genes would be expected to be correlated across the RI panel and perhaps functionally related in some way

(Rosen et al.2007). GeneNetwork can identify phenotypes that may be functionally related by searching for those whose average trait values are correlated.

a. Using GeneNetwork, click on the **Calculate Correlations** tab to assess genetic correlations of the trait of interest with all other records in the database, including BXD published phenotypes, BXD genotypes, and mRNA from various brain regions as well as other tissues. To begin, select *BXD Published Phenotypes* from the Database pull-down menu and click **Compute**. The default option returns the top 500 phenotypes associated with the trait of interest, but the **Return** pull-down bar allows researchers to choose how many results to display. Researchers can also choose between selecting Pearson or Spearman Rank correlations. Use Spearman Rank when the sample size is small (<20) or when there are influential outliers.

b. As an example, the top 10 phenotypes correlated with the IAL trait are shown in Table 1. The correlation analysis shows that the principal component corresponding to infusions on the ascending limb of the dose-response curve is correlated with cocaine infusions at two different doses of cocaine, as well as the number of active lever presses at a low dose of cocaine. The magnitude, not the direction of the correlation is constant, and a test of the correlation of the principal component with the original trait scores or examination of the factor loadings plot will reveal the polarity of the PC scores. Interestingly, IAL is negatively correlated with time spent in the light side of a light-dark box (**Record ID 11645**; Philip et al. 2010).

Single trait QTL Mapping

6 One particularly valuable function of GeneNetwork is QTL mapping (Wang et al. 2003). QTL mapping seeks to identify genes and/or sequence variants that modulate the trait of interest. It measures the association between SNPs and trait values across the genome. If a SNP appears to be highly correlated with the trait of interest, this indicates that the SNP is physically close to the causative mutation.

a. On the **Data Editing** page, the **Mapping Tools** tab can be used to run a variety of QTL mapping methods. **Interval** mapping is the standard for almost all non-human populations. It creates linkage maps for the entire genome and the consistency of linkage for a given trait can be evaluated using permutation tests. The permutation test is a method for establishing the significance of the likelihood ratio statistic (LRS) generated by the interval mapping procedure (Churchill & Doerge 1994). In this test, trait values are randomly permuted among the RI strains, destroying any relationship between the trait values and the genotypes of SNP loci. A regression model is fitted for the permuted

data at all genomic positions and the maximum LRS is recorded. This procedure is repeated thousands of times, and gives a distribution of likelihood ratio values that would be obtained by chance (if there were no QTL linked to any of the SNP loci). The logarithm of the odds ratio (LOD) score is also provided. Both the LRS and LOD scores give an estimate of the statistical strength of linkage (the LRS is equal to the LOD score multiplied by 4.61).

b. To perform interval mapping, select *All* from the **Chromosome** pull-down menu, choose *Megabase* from the **Mapping Scale** pull-down menu, and enter *5000* in the space for **Permutations**. Then click on the **Compute** button. This opens a new window that shows the QTL map for the entire genome (Figure 2a). Based on the permutation tests, GeneNetwork will display the exact values for suggestive and significant LOD/LRS scores. In this example, a suggestive LRS = 11.24, and a significant LRS = 18.57. Note that these numbers will change slightly each time you map the trait of interest. A positive additive coefficient is shown by the green line, and indicates that DBA/2J alleles increase trait values. In contrast, a negative additive coefficient is represented by a red line, and indicates that C57BL/6J alleles increase trait values. Thus, one can conclude there is a significant QTL on chromosome 11, and BXD RI mice with DBA/2J alleles at that locus have a decrease in the number of infusions at the lowest three cocaine doses

c. Zoom in on a particular chromosomal region by selecting the chromosome of interest (say, 11) from the **Chr**. pull-down menu, and clicking on the button **Remap** (Figure 2b) or by directly clicking on the chromosome number for the chromosome of interest in the plot. On the new page that opens, click on blue text **Download** near the top of the page to download the results in a tab-delimited text format. Open the data in an excel spreadsheet to find the exact marker and megabase location of the peak marker by clicking on the **Download** link. This also provides the genome-wide corrected *p*-value and additive effect at each marker. Note the megabase positions that define the 2-LOD support interval, which approximately reflects a 95% confidence interval (CI) around the peak locus. The 2-LOD support interval represents the most plausible location for the QTL and where the candidate gene(s) and SNP(s) are most likely to be located. It is calculated by subtracting the number 2 from the peak LOD score, and determining which SNPs are associated with that number on either side of the peak SNP. In this example, observe that the peak locus on Chromosome 11 occurs at 47.436 Mb, with LRS = 24.247 (LOD = 5.26). The 2-LOD interval is 46.03–50.39 Mb, and the nearest listed marker to the QTL peak is *rs13481014* located at 47.931 Mb.

Multi-trait QTL Mapping—GeneNetwork has a very useful feature that facilitates QTL mapping of multiple traits. This feature helps address the question of pleiotropy, inherent among many quantitative traits. The feature within GeneNetwork that facilitates such an analysis is based on Principal Component Analysis or PCA. PCA is a dimensionality reduction technique, which identifies the main sources of variation, called principal component, within a multi-trait dataset. These components are identified such that the first principal component is comprised of traits that explain the most amount of variation. Each subsequent principal component identifies groups of traits with lesser amounts of variation. In GeneNetwork, the user first selects the collection of traits that they would like to analyze (Figure 2c). Once selected, the user generates a matrix of correlations by choosing the **Matrix** tool. Along with correlations, this tool also derives new traits representing the principal components (Figure 2d). The user can add these principal components to their Trait Collection and proceed to perform QTL mapping, as in the case of a single trait QTL mapping. The R/QTL (Broman et al. 2003) and R/CAPE (Tyler et al. 2013) packages can be used for deeper analysis of epistasis and pleiotropy for multiple traits and multiple regulatory loci.

Prioritizing Candidate Genes

7 Following the identification of a significant QTL, focus shifts to identifying the particular gene(s) that cause the QTL. QTLminer (Alberts & Schughart 2010) on GeneNetwork can be used to integrate information about the candidate genes within the QTL region in order to prioritize among them. QTLminer automatically generates a list of genes within the interval and retrieves additional information for each gene using publicly available datasets. Not only does QTLminer provide basic annotation data such as gene name, description, and genomic position; it also supplies Gene Ontology (GO) terms and KEGG pathways in which the gene is implicated. In addition, it displays the number of non-synonymous SNPs (nsSNPs) within the gene. These nsSNPs are relevant because they result in amino acid changes in the corresponding protein, which may modify its structure and thus cause the phenotypic differences observed. Furthermore, you can select three GeneNetwork expression datasets. For each dataset, gene expression is included. This displays whether candidate genes are expressed in tissues of interest. Genes that are only expressed in the tissue of interest and not in others might even be better candidates.

a. Under the **Search** tab, select **QTLminer**. Next, use the **Chr** pull-down menu to select the chromosome under investigation (chromosome 11). Enter the megabase positions that define the 2-LOD support interval in the **View** spaces (46.03 and 50.39, respectively). Since we are utilizing the BXD RIs, leave C57BL/6J and DBA/2J as the two mouse strains for inclusion of the nsSNP count. Next, select three datasets for inclusion of expression and *cis*-activity. Given that we are examining a drug-related phenotype in this example, it is appropriate to choose the nucleus accumbens, the midbrain, and the pre-frontal cortex (**GeneNetwork study IDs: 44, 141, 36**; Wolen et al. 2012; Ye et al.

2014), as these regions have previously been implicated with cocaine IVSA (Bocklisch et al. 2013; Ito et al. 2004; Marinelli et al. 2003; Pettit et al. 1984; Roberts & Koob 1982; Vassoler et al. 2013; Weissenborn et al. 1997; Wise 2009). In all three datasets, choose the **Databases** associated with naïve or saline conditions on the pull-down menu, as we are interested in resting gene expression levels (Figure 3; midbrain = *VU BXD Midbrain Agilent SurePrint C3 Mouse GE (May 12) Quantile*, nucleus accumbens = *VCU BXD NAc Sal M430 2.0 (Oct07) RMA*, prefrontal cortex = *VCU BXD PFC Sal M430 2.0 (Dec06) RMA*). Click on the **Analyze QTL interval** button. Of the 68 genes in the QTL interval, 56 were expressed in at least one of the tissues of interest.

8 Information about *cis*-regulation can also help prioritize candidate genes. A gene is *cis*-regulated if its expression maps close to its own genomic position. Any *cis*-regulated gene within the QTL interval is an especially promising candidate gene, since *cis*-regulation indicates a difference in gene expression levels, which may regulate the trait. The results obtained from QTLminer show that of the 56 genes that were expressed in one of the tissues of interest, only two were also *cis*-regulated: *Cyfip2* and *Itk*. These two genes should be considered priority candidates. Notably, *Cyfip2* has been previously shown to play a critical role in behavioral sensitization to cocaine (Kumar et al. 2013). Therefore, the steps below will focus on examining the expression of the *Cyfip2* transcript in more detail, as it may be the driver of this QTL.

a. Click on the *Search Databases* option under the **Search** tab. Make sure that *Mouse* is selected as the **Species** and *BXD* as the **Group** on the pull-down menus. Under **Type**, select *Midbrain mRNA* and write *Cyfip2* into the **Get Any** space. Click on the blue **Search** button (Figure 4a).

b. In the new window (Figure 4b), click on the blue **Record ID** (**A_55_P1962771**). Perform QTL mapping for expression levels of *Cyfip2* the same way that was outlined for behavioral traits in step 6a. Under **Mapping Tools**, enter *5000* in the **Permutation** space, and then click **Compute**.

c. In the new window, there is a significant *cis*-eQTL (LRS = 62.7) for *Cyfip2* as well as a significant *trans*-eQTL (LRS = 18.8) for *Cyfip2* on chromosome 3 (Figure 4c). Notably, the gene *Fxr1* resides within this confidence interval (Chr 3 at 33.96 Mb) and is functionally related to *Cyfip2* (Schenck et al. 2001).

d. In order to examine the relationship between gene expression, genotype, and segregation pattern of parental alleles, check the **Haplotype Analyst** box and change **Chr** to *11* and **View** to *46.03* to *50.39*. Then click **Remap**. This shows the inheritance pattern for each BXD RI strain (Figure 5). Similar genotypes across markers define a

haplotype and are shown in blocks of green (inherited from the paternal strain) or red (inherited from the maternal strain), with undefined regions shown in grey. Therefore, BXD strains that have inherited the paternal DBA/2J allele (indicated in green) have high expression of *Cyfip2* and those strains that have inherited the maternal C57BL/6J allele (indicated in red) have lower expression.

9 Candidate genes can be further prioritized by determining if they exhibit expression levels that co-vary with the phenotype of interest in the brain region they are expressed in. On the **Trait Data and Analysis** page for **Record ID A_55_P1962771**, expand the **Calculate Correlations** tab. Select *BXD Published Phenotypes* from the pull-down menu, and click the **Compute** button. This opens a new window showing that expression of *Cyfip2* has a strong positive correlation ($r = .79$; $p = 0.00022$) with IAL (**Record ID 18494;** Table 2). Thus, without performing any additional experiments, steps 7, 8, and 9 establish that *Cyfip2* is expressed in a brain region that is implicated with cocaine IVSA, exhibits a *cis*- and a *trans*-eQTL in that brain region, and its expression levels co-vary with the cocaine IVSA phenotype.

Trans-eQTL associated with behavioral QTL

10 Genes exhibiting expression levels that map to the behavioral QTL in a brain region with known relevance to trait of interest may be functionally related to the gene(s) underlying those same behavioral QTL. Therefore, it is important to identify genes that exhibit genome-wide significant *trans*-eQTL ($p < 0.05$) with a peak located within the 2-LOD support interval of the IAL behavioral QTL.

a. From the **Search** tab, select **Search Databases**. Change **Type** to *Midbrain mRNA* and use the default **Data Set**. Enter the following text into the Combined search box: *MEAN = (9 16) LRS = (17 999 Chr11 46.03 50.39) transLRS = (17 999 5)* and then click the **Search** button (Figure 6a). This will retrieve all the genes and transcripts from this particular midbrain data set that have a mean expression between 9 and 16 *[(MEAN = 9 16)*] with a maximum LRS value between 17 and 999 [*transLRS = (17 999)*] located near the IAL behavioral QTL interval [*LRS = (17 999 Chr11 46.03 50.39)*]. This query returns six genes, *Fam53b, Mapk12, Lppr5, Chchd7, Serpini1*, and *Rps6ka6*, that exhibit genome-wide significant *trans*-eQTL located within the support interval of the IAL behavioral QTL on chromosome 11 (Figure 6b). Interestingly, a GWAS of cocaine dependence in humans identified *FAM53B* as the only candidate (Gelernter et al. 2014) to reach genome-wide significance.

b. To further explore the relationship between the highest priority candidate gene associated with the IAL QTL (*Cyfip2*) and *Fam53b*, we can determine if they are co-expressed genome-wide significantly in the midbrain. From the **Search** tab, select **Search Databases**. Select

Midbrain mRNA from the Type pull-down menu. Enter *Cyfip2* into the **Get Any** space and click on the **Search** button. Click on the blue **Record ID** (**A_55_P1962771**). Expand the **Calculate Correlations** tab, and select *VU BXD Midbrain Agilent Sureprint G3 Mouse GE (May 12) Quantile* from the pull-down menu to analyze midbrain RNA. Select the **Spearman Rank** button and click **Compute**. In the list of genes provided, scroll down and click on the correlation highlighted in blue (rho = 0.691) on the row for *Fam53b* to generate a correlation plot and see that *Fam53b* and *Cyfip2* are co-expressed genome-wide significantly in the midbrain (p = 7.11E-7; Figure 7). This suggests a functional relationship between *Cyfip2* and *Fam53b* in the context of cocaine use.

Genome-wide covariation of phenotypes and gene expression

11 Genes exhibiting expression levels that co-vary with a phenotype of interest in a relevant brain region may be part of a larger network involved in driving that trait. Therefore, it is informative to identify all genes across the genome exhibiting expression levels that co-vary genome-wide significantly with cocaine IVSA phenotypes associated with a behavioral QTL. To calculate the genome-wide error rate for each brain region, use the Bonferroni correction (.05/number of probe sets) and exclude probe sets that are not associated with any genes. In this example, Bonferroni-adjusted genome-wide significance levels for the nucleus accumbens, midbrain, and prefrontal cortex are 1.1×10^{-6}, 1.5×10^{-6}, and 1.1×10^{-6}, respectively.

a. Select **Search Databases** from the **Search** tab. Choose *BXD* from the pull-down menu for **Group**, *Phenotypes* from the pull-down menu for **Type**, and enter *IAL* in the **Get Any** space. Click on the **Search** button. In the new page that opens, click on the blue **Record ID** (**Record ID 18494**). Alternatively, enter or upload the data using the procedure described in step 2.

b. Expand the **Calculate Correlations** tab, and select *VU BXD Midbrain Agilent Sureprint G3 Mouse GE (May 12) Quantile* from the pull-down menu to analyze midbrain RNA. Click **Compute**. In the new page that opens, GeneNetwork provides a table showing all the genes and transcripts and their correlations with IAL. No transcripts expressed in the midbrain meet the criteria for genome-wide significance. Return to the **Trait Data and Analysis** page and select *VCU BXD PFC Sal M430 2.0 (Dec06) RMA* from the **Database** pull-down menu to analyze prefrontal cortex RNA. Click **Compute**. The expression of one gene in the prefrontal cortex, *Rpl31*, co-varied genome-wide significantly with IAL. Return to the **Trait Data and Analysis** page a final time and select *VCU BXD NAc Sal M430 2.0 (Oct07) RMA* from the **Database** pull-down menu to analyze nucleus accumbens RNA and then click **Compute**. In the new page that opens, the table shows that

Hist3h2a and *Skp1a* reached genome-wide statistical significance. This demonstrated that a total of three genes had expression values that significantly co-varied with the trait of interest.

SUPPORT PROTOCOL TITLE

Composite Interval Mapping

QTLs underlying complex traits may act independently, may be linked to another QTL, or may interact epistatically with other QTLs (Doerge 2002). Researchers are often interested in identifying interacting QTL, as they indicate genomic regions that might not otherwise be identified using an interval mapping approach. However, locating multiple, interacting QTL can be computationally demanding due to the large number of statistical tests that are required for this approach. Composite interval mapping (Zeng 1993; 1994) reduces the number of statistical tests by including additional marker loci as cofactors in order to remove any variation associated with other linked QTL. These markers are generally chosen because they are the peak marker located near a significant QTL. By controlling for a single background marker, GeneNetwork factors out a portion of the genetic variance produced by the major QTL and allows users to detect secondary QTLs.

Materials

Protocol steps—Step annotations—Here we will provide detailed instructions for composite interval mapping in GeneNetwork using an example taken from Crusio et al. (2016) who recently reanalyzed data from behavioral phenotyping of 64 BXD RI strains in the presence of morphine (Philip et al. 2010).

1. On the search page, select *Mouse* from the **Species** pull-down menu, *BXD* from the **Group** pull-down menu, *Phenotypes* from the **Type** pull-down menu, and *BXD Published Phenotypes* from the **Data Set** pull-down menu. In the Get Any space, enter the *11852*. This is the **Record ID** for distance traveled in the open field following 50 mg/kg morphine (Philip et al. 2010). Click on the **Search** button.

2. On the new page, click on the blue **Record ID 11852** to open up the **Trait Data and Analysis** page.

3. Marker regression computes and displays LRS values for individual markers. Determine which marker has the highest LRS value by expanding the **Mapping Tools** tab and clicking on the **Marker Regression** tab. Check the **Display all LRS** box, and click **Compute**. The genome-wide mapping results for morphine response are displayed. There is a highly significant QTL on chromosome 10, but no other QTLs reach significance (Figure 8). Scroll down the page to see the names and locations of markers ranked by LRS score. The SNP marker rs3721803 located at 3.342585 Mb on chromosome 10 is ranked highest, with an LRS of 31.591. The last column displays the additive effect value. This represents half the difference in the mean phenotype of all cases that are homozygous for one parental allele at this marker minus the mean of all cases

that are homozygous for the other parental allele at this marker. Here, a positive additive effect indicates that DBA/2J alleles increase trait values, while negative additive effects indicate that C57BL/6J alleles increase trait values. In this example, C57BL/6J alleles increase morphine sensitivity at the rs3721803 marker.

4. Return to the **Trait Data and Analysis** page and click on the **Composite** tab under **Mapping Tools**. In the **Control Locus** box, enter *rs3721803* into the space provided and click **Compute** (Figure 9a).

5. In the page that opens, the genome-wide mapping results now show a significant QTL on chromosome 7 (Figure 9b). Thus, composite interval mapping in the BXD RI panel resulted in the detection of an additional QTL for morphine-induced locomotor activity.

COMMENTARY

Background Information

Recombinant inbred panels are genetic reference populations that allow for well-powered and moderately precise QTL mapping. They are a key resource for the study of complex traits because genotyping is only required once. RI panels are homozygous at each locus throughout their entire genomes. Thus, each RI line consists of an inbred strain that can be infinitely replicated in large numbers. This makes them particularly useful for neuroscientific studies that require replication across individuals, treatment conditions, environments, and time. Importantly, the ability to resample individuals can boost heritability by reducing non-genetic variance (Belknap 1998).

The utility of RIs as a mapping population is dependent in part upon the availability of a sufficiently large panel that provides enough power to detect QTLs with small effect sizes (Gora-Maslak et al. 1991). Classical two-parent RI panels are created by crossing two inbred strains to produce the F_1 generation. This is followed by a minimum of 20 consecutive generations of brother × sister matings to obtain the different RI strains of mice. Currently, there are at least 10 different traditional RI panels, including BXD, AXB/BXA, AKXD, BXH, BRX58N, CXB, LXS, LGXSM, NXSM, and SWXJ.

The BXD RI panel is the most frequently used RI panel, and was initially derived through inbreeding the offspring of an intercross of C57BL/6J and DBA/2J mice (Taylor et al. 1977). Additional lines were created over the next 30 years (Taylor et al. 1999), including lines derived from an advanced intercross to increase mapping precision (Peirce et al. 2004). Today, the BXD RI panel consists of ~150 strains available from the Jackson Laboratory (Bar Harbor, ME) and the University of Tennessee Health Science Center (Memphis, TN). One significant advantage of BXD RIs is that both parental strains have been sequenced and differ at ~1.8 million SNPs across the genome. Combining this information with the BXD RI genotyping data makes it possible to determine the source of polymorphic alleles (either C57BL/6J or DBA/2J) throughout the genomes of BXD RI strains of mice (Hsu et al. 2007)

These RI strains have higher amounts of recombination as compared to backcrosses and F_2 intercrosses, but lower amounts of recombination as compared to heterogeneous stock mice, Diversity Outbred mice, commercially available outbred mice, most advanced intercross lines, and the Collaborative Cross RI panel. In addition, traditional two-parent RI panels segregate for only a fraction of all known polymorphisms. Thus, one can only study the alleles that segregate between the two founder strains, rather than the total number of alleles that segregate among laboratory or wild mice. For example, the BXD panel segregates ~5.2 million sequence variants, which represents only 44% of common variants among standard inbred strains (Roberts et al. 2007). Blindspots may be present in some regions of the genome that are identical-by-descent (Yang et al. 2007), and those regions will not contribute to trait variance (but see Li et al. 2010). The current BXD panel allows for mapping of QTL that are approximately 1–2 Mb in size and account for approximately 10% of the phenotypic variance (Peirce et al. 2004). Although this lower precision and diversity appear to be drawbacks, it should be noted that they also stabilize background variation, epistasis and GXE, possibly providing greater power to detect small effect loci in a cross of the RI panel. A caveat is that the earliest set of BXD RI lines was derived before the introduction of the genetic stability program and therefore, may harbor some loci that have since mutated in the founder stock. This can introduce sub-population effects (Philip et al. 2010).

Critical Parameters & Troubleshooting

Sex balance—Male and female mice should be used in roughly equal numbers and concurrently. This is important for several reasons. On a purely pragmatic level, NIH is mandating the inclusion of female subjects in biomedical research through program oversight, review, and through collaboration with publishers. Research may be less likely to be funded or even published if both sexes are not included. From a scientific perspective, results obtained by evaluating sex differences can improve the translational utility of mouse studies and provide mechanistic insight (for examples see Voskuhl & Palaszynski 2001; Wisdom et al. 2013; Fox et al. 2014). Currently, GeneNetwork does not allow sex to be treated as an interactive covariate. Instead, users can perform QTL mapping on each sex individually to identify sex-specific QTLs. Genetic analysis of the residuals from the regression of males against females will reveal sex specific effects.

Batch effects—To control for confounding batch effects, avoid using a single litter for each genotype. Instead, interleave phenotyping to test 10–15 different RI strains with 1–2 individuals per sex for the first phase of the experiment, and repeat cycles until the desired number of strains is obtained. If an interleaved design is not possible, at a minimum one should periodically re-phenotype a well-characterized strain to check whether litter or batch effects are contributing to phenotypic variation or drift.

Sample size—Genetic mapping studies rely on both the appropriate number of unique genotypes (i.e., strains) that are phenotyped as well as the number of replicates per genotype (Andreux et al. 2012; Belknap 1998). Increasing the number of total genotypes tested has a larger effect on power to detect QTLs than increasing the number of replicates (Belknap 1998). However, increasing the number of replicates within a strain can boost heritability by

reducing non-genetic variance (Belknap 1998). Therefore, determining the appropriate number of genotypes and replicates depends in part on the heritability of the trait under investigation. Because the effect size of individual QTLs is not generally known in advance, it is difficult to provide concrete guidelines about the sample size needed for genotypes and replicates. Two replicates per sex per strain is generally accepted, although this approach is unlikely to identify sex differences within any single strain. Therefore, some consider at 6 – 8 replicates per group to be optimal (Williams & Williams 2016).

Outliers—Outliers can have large, unwanted effects on correlations and QTL mapping results. GeneNetwork identifies all outliers in the **Trait Data and Analysis** window by highlighting them in yellow. Four options for dealing with outliers are: 1) include the outliers, 2) delete the outliers, 3) transform the data, or 4) Winsorize the outliers. Outliers will have the largest effects on results when the sample size is small or the outliers particularly extreme. It is recommended that if the obtained results depend on just one or two outliers, it is best to delete or Winsorize them.

Time Considerations

The computational steps described in this protocol are expected to take 1–2 hours to execute.

Acknowledgments

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KEY REFERENCE (optional)

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This is an excellent review that is intended to guide researchers through the process of designing mapping experiments and choosing appropriate mapping populations given the type of question under investigation.

Significance Statement

Despite recent successes in discovery of natural variation in biological mechanisms driving disease susceptibility, human genome-wide association studies (GWAS) are limited due to practical and financial concerns. Systems genetics in mice uses rigorous genetic and environmental control to offer a complementary approach for discovery of disease biology that has efficiency and versatility. Brain genomic analysis can be used to identify gene networks driving quantitative traits. Mouse genomes can be readily manipulated; consequently, enabling rapid, cost-effective validation. Mouse genetic reference populations are a powerful experimental system for neuroscientists wishing to study the molecular mechanisms underlying sophisticated, complex behavioral or neurobiological traits. Existing publicly available data resources make analyses in these populations facile, and all that is required is the quantification of traits of interest. Using recently published examples, this protocol demonstrates the process of systems genetic analysis in the mouse and its relevance to a human genetic variant.

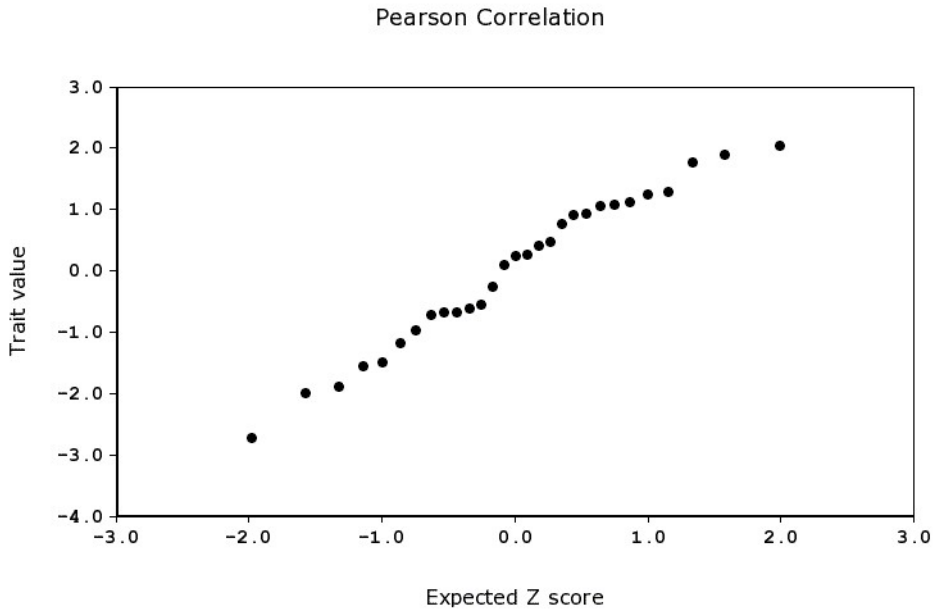


Figure 1.

Probability Plot. The normal probability plot evaluates whether data are normally distributed. In this example, the IAL trait values are plotted on the y-axis and the expected Z score value on the x-axis.

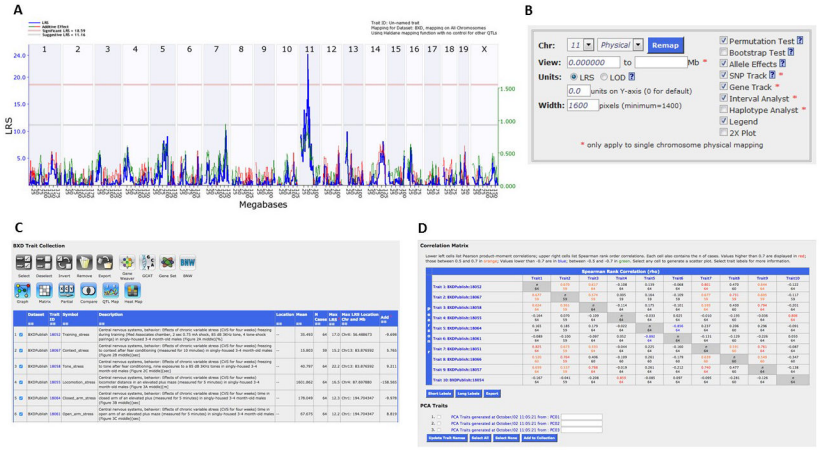


Figure 2.

Genome-wide scan of QTLs for IAL in BXD RI mice. (**A**) The QTL mapping results are shown for the entire mouse genome. The chromosomes are displayed across the top of the x-axis, and the megabase position is displayed along the bottom x-axis. The LRS value is plotted along the y-axis. Grey horizontal lines indicate suggestive QTL and the red horizontal line indicates a significant QTL. A positive additive coefficient (green line) indicates that DBA/2J alleles increase trait values; whereas a negative additive coefficient (red line) indicates that C57BL/6J alleles increase trait values. (**B**) This box allows the researcher to zoom in on a region of interest. (**C**) Selected traits to analyze in multi-trait QTL mapping. (**D**) Matrix correlations used to derive new traits representing principal components for QTL mapping.

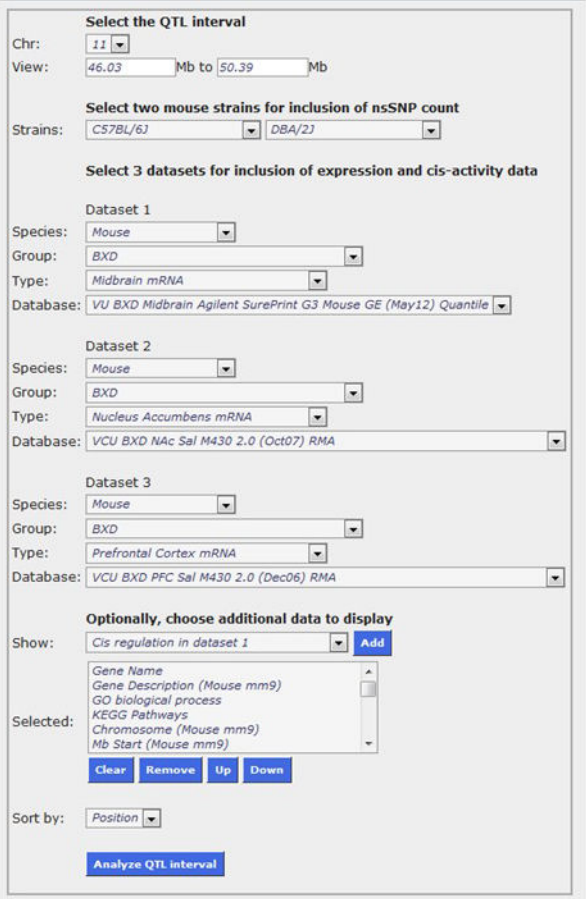


Figure 3.

Using QTLminer to prioritize candidate genes. QTLminer performs QTL region analysis. It provides the user with a list of candidate genes located within the QTL interval; as well as annotation data, number of non-synonymous SNPs, GO terms, and KEGG pathways. It also allows the user to select three GeneNetwork expression data sets to gene expression and *cis*-regulation.

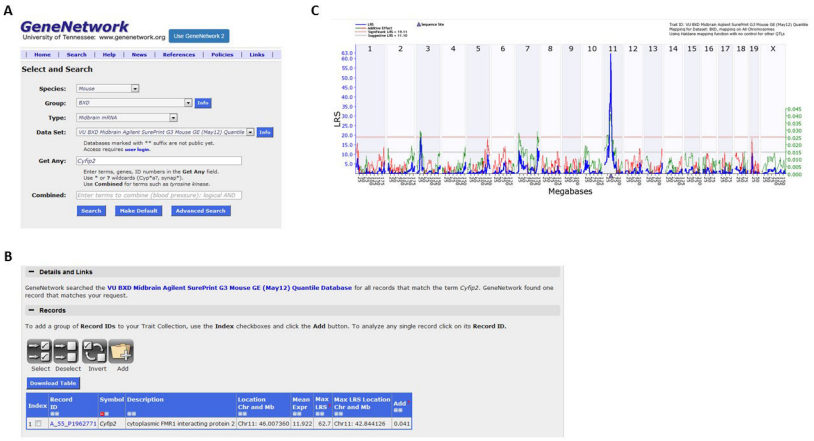


Figure 4.

Cis-regulation of candidate genes. (**A**) Searching for *Cyfip2* gene expression in BXD RI midbrain. (**B**) Record ID for *Cyfip2*. (C) eQTL mapping results for Cyfip2 expression in the midbrain.

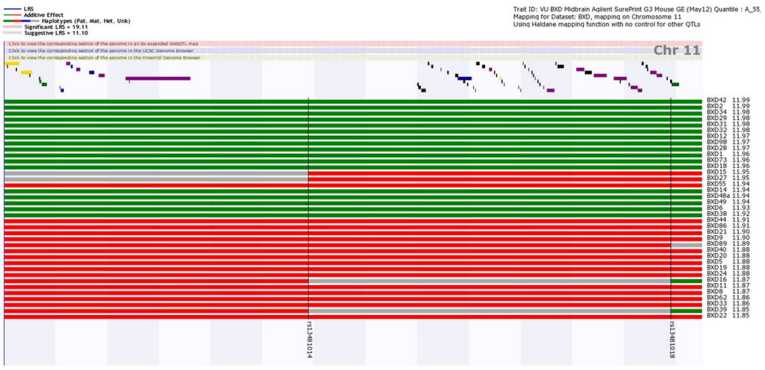


Figure 5.

Haplotype Analyst. Displayed is the inheritance pattern for each BXD RI strain. The location of the genes is displayed at the top of the plot, and a map of the chromosome for each strain lies below, with the strain IDs on the right y-axis. The trait values are sorted from highest to lowest and the numerical expression value for *Cyfip2* is listed to the right of the strain ID. The markers reveal whether that position in the genome is inherited from C57BL/6J or DBA/2J mice, and are indicated in black. Blocks of green are inherited from the paternal strain and blocks of red are inherited from the maternal strain.

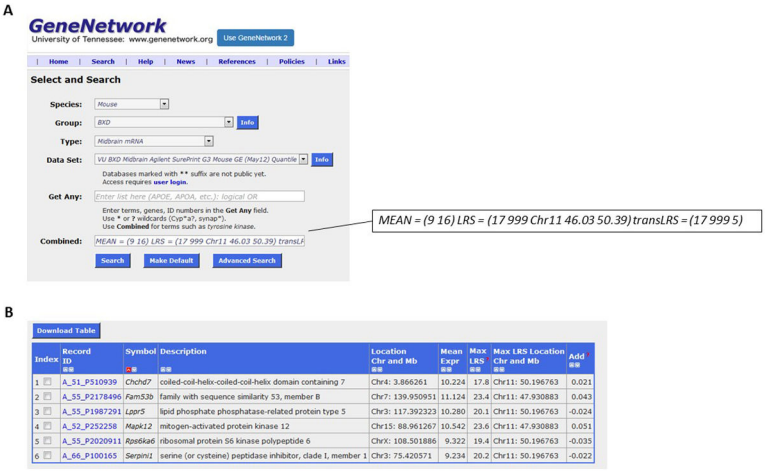


Figure 6.

Trans-eQTL associated with behavioral QTL. (**A**) Use the combined search box to retrieve all transcripts from the VU BXD midbrain data set that have a mean expression between 9 and 16 with a maximum LRS value between 17 and 999 located near the IAL behavioral QTL interval. (**B**) Six genes exhibited genome-wide significant *trans*-eQTL within the 2-LOD support interval of the IAL QTL.

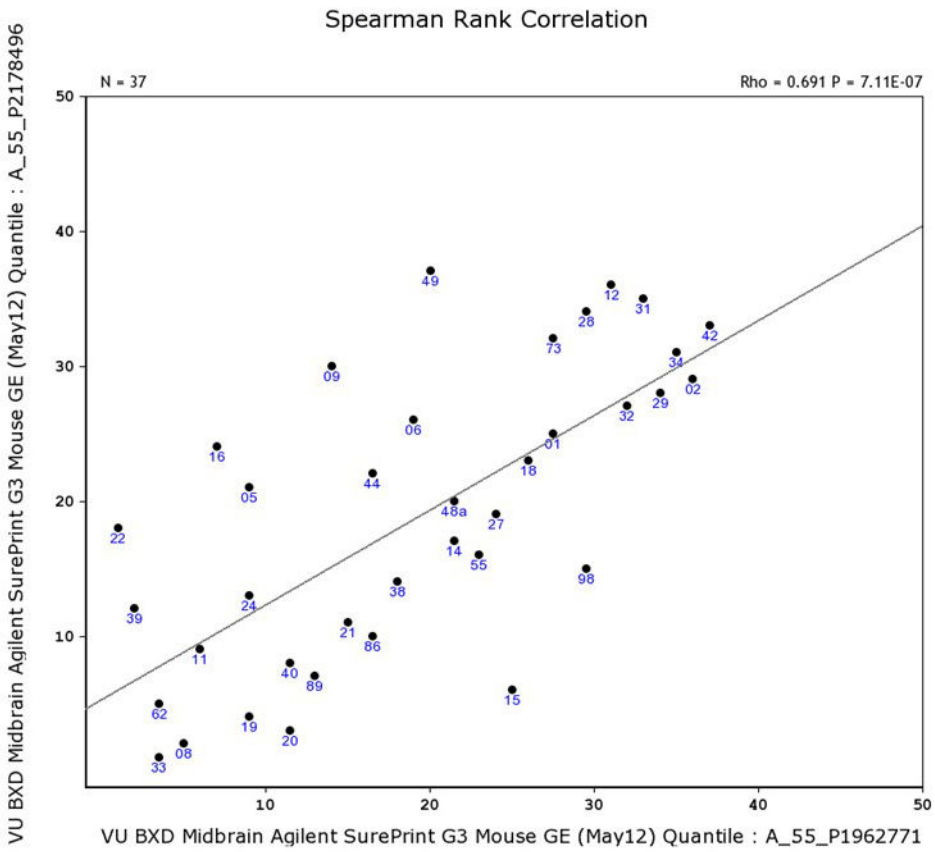


Figure 7.

Gene expression correlations. *Fam53b* (y-axis) and *Cyfip2* (x-axis) are co-expressed genome-side significantly in the midbrain.

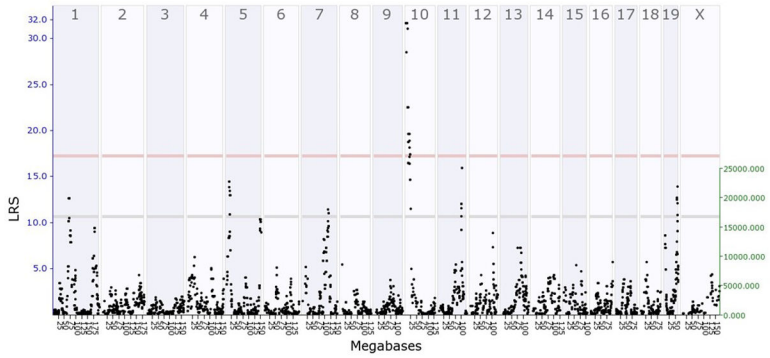


Figure 8.

Marker regression. Genome-wide mapping for morphine sensitivity using marker regression shows a significant QTL on chromosome 10. Each dot represents a genetic marker. The chromosomes are displayed across the top of the x-axis, and the megabase position is displayed along the bottom x-axis. The LRS value is plotted along the y-axis. Grey horizontal lines indicate suggestive QTL and the red horizontal line indicates a significant QTL.

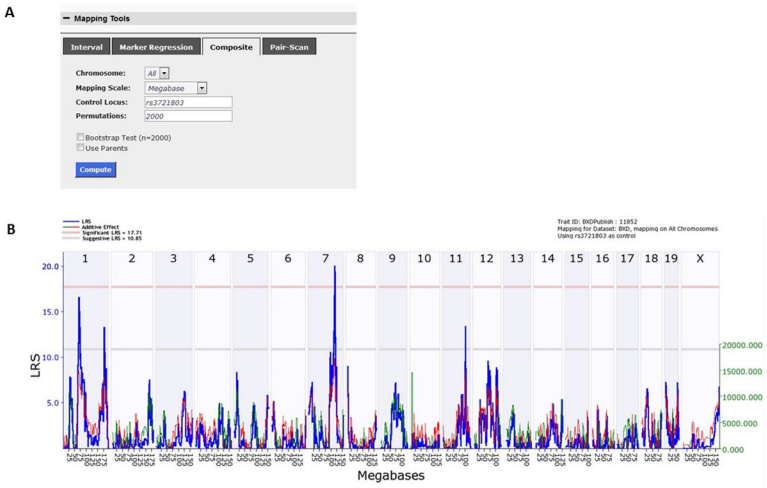
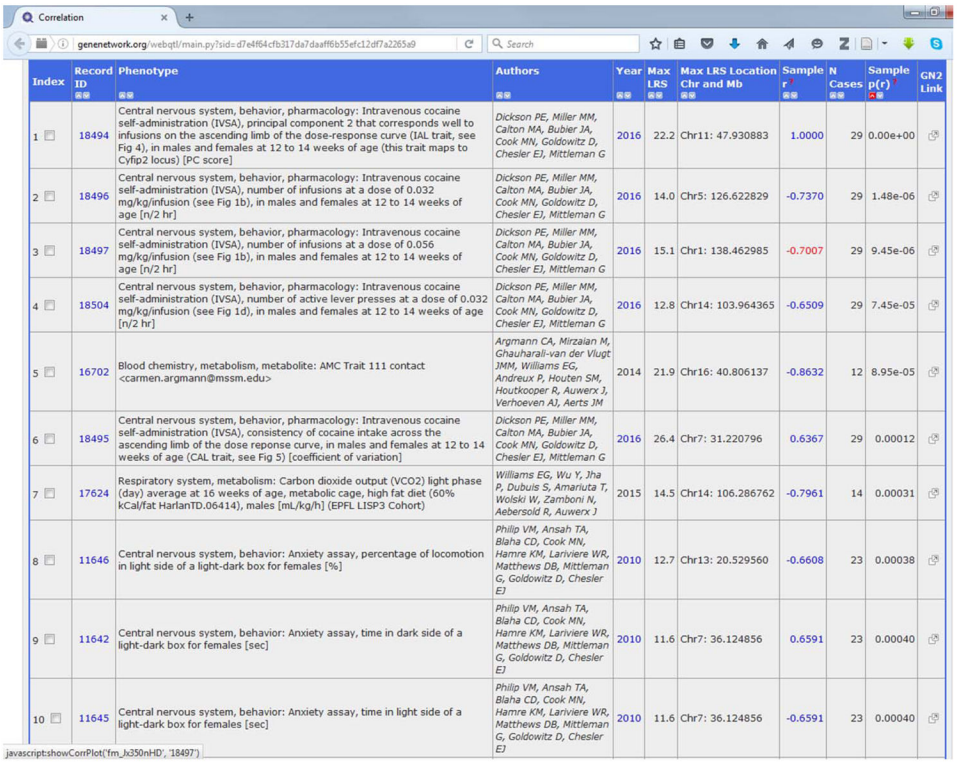


Figure 9.

Composite interval mapping. (**A**) This box allows researchers to include the peak marker (*rs3721803*) from the chromosome 11 QTL as a cofactor. (**B**) Genome-wide mapping using the composite interval approach identifies a novel QTL on chromosome 7 associated with morphine sensitivity.

Table 1

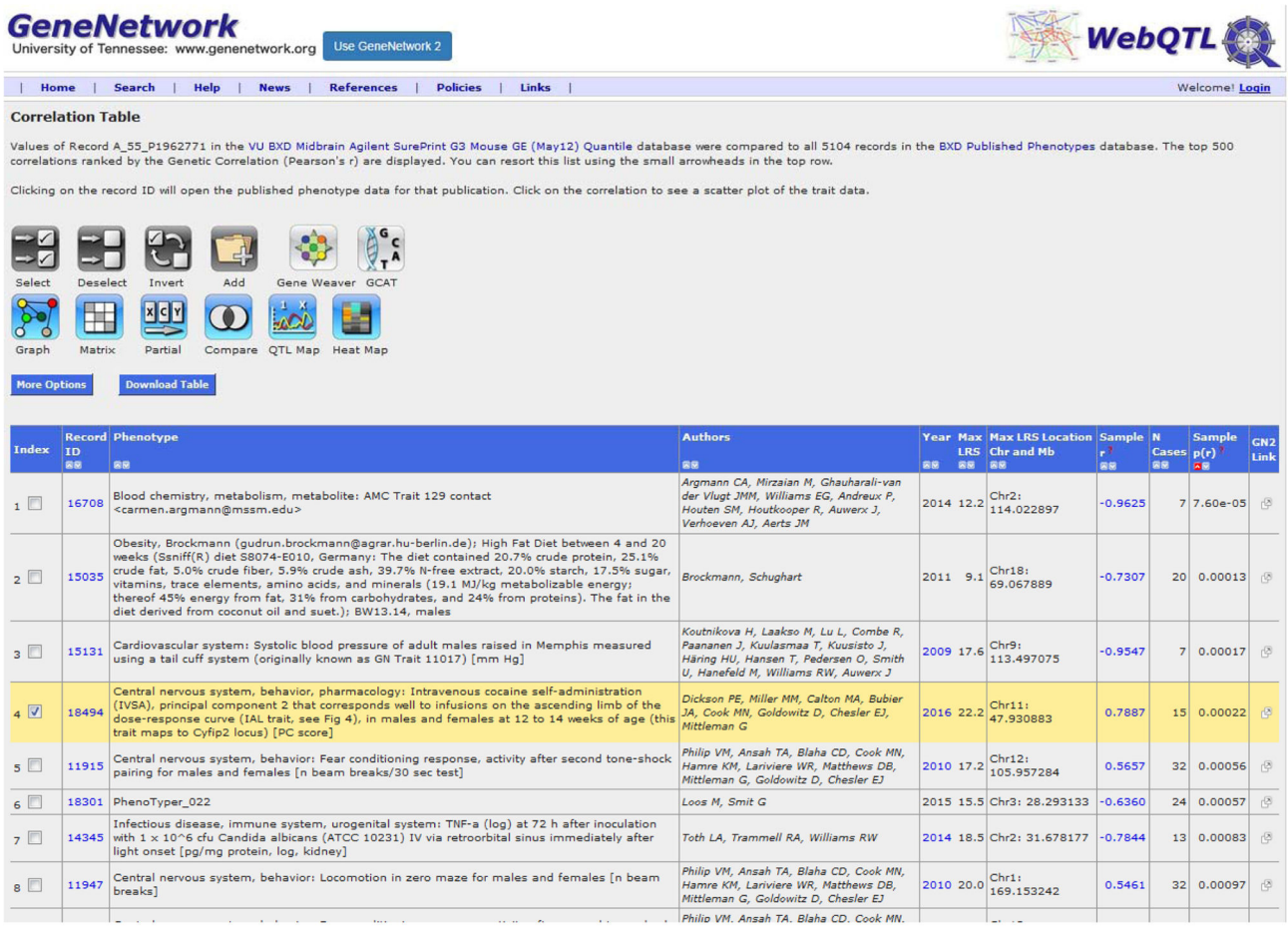
Trait Correlation Table. Values of IAL were compared to all 5104 traits in BXD Published Phenotypes database. The top 500 correlations ranked by the Genetic Correlation (Pearson's r) are displayed.



Index	Record ID	Phenotype	Authors	Year	Max LRS	Max LRS Location Chr and Mb	Sample r	N Cases	Sample p(r)	GN2 Link
1	18494	Central nervous system, behavior, pharmacology: Intravenous cocaine self-administration (IVSA), principal component 2 that corresponds well to infusions on the ascending limb of the dose-response curve (IAL trait, see Fig 4), in males and females at 12 to 14 weeks of age (this trait maps to Cyfip2 locus) [PC score]	Dickson PE, Miller MM, Calton MA, Bubier JA, Cook MN, Goldowitz D, Chesler EJ, Mittleman G	2016	22.2	Chr11: 47.930883	1.0000	29	0.00e+00	
2	18496	Central nervous system, behavior, pharmacology: Intravenous cocaine self-administration (IVSA), number of infusions at a dose of 0.032 mg/kg/infusion (see Fig 1b), in males and females at 12 to 14 weeks of age [n/2 hr]	Dickson PE, Miller MM, Calton MA, Bubier JA, Cook MN, Goldowitz D, Chesler EJ, Mittleman G	2016	14.0	Chr5: 126.622829	-0.7370	29	1.48e-06	
3	18497	Central nervous system, behavior, pharmacology: Intravenous cocaine self-administration (IVSA), number of infusions at a dose of 0.056 mg/kg/infusion (see Fig 1b), in males and females at 12 to 14 weeks of age [n/2 hr]	Dickson PE, Miller MM, Calton MA, Bubier JA, Cook MN, Goldowitz D, Chesler EJ, Mittleman G	2016	15.1	Chr1: 138.462985	-0.7007	29	9.45e-06	
4	18504	Central nervous system, behavior, pharmacology: Intravenous cocaine self-administration (IVSA), number of active lever presses at a dose of 0.032 mg/kg/infusion (see Fig 1d), in males and females at 12 to 14 weeks of age [n/2 hr]	Dickson PE, Miller MM, Calton MA, Bubier JA, Cook MN, Goldowitz D, Chesler EJ, Mittleman G	2016	12.8	Chr14: 103.964365	-0.6509	29	7.45e-05	
5	16702	Blood chemistry, metabolism, metabolite: AMC Trait 111 contact <carmen.argmann@mssm.edu>	Argmann CA, Mirzaian M, Ghauharali-van der Vlugt JMM, Williams EG, Andreux P, Houten SM, Houtkooper R, Auwerx J, Verhoeven AJ, Aerts JM	2014	21.9	Chr16: 40.806137	-0.8632	12	8.95e-05	
6	18495	Central nervous system, behavior, pharmacology: Intravenous cocaine self-administration (IVSA), consistency of cocaine intake across the ascending limb of the dose reponse curve, in males and females at 12 to 14 weeks of age (CAL trait, see Fig 5) [coefficient of variation]	Dickson PE, Miller MM, Calton MA, Bubier JA, Cook MN, Goldowitz D, Chesler EJ, Mittleman G	2016	26.4	Chr7: 31.220796	0.6367	29	0.00012	
7	17624	Respiratory system, metabolism: Carbon dioxide output (VCO2) light phase (day) average at 16 weeks of age, metabolic cage, high fat diet (60% kCal/fat HarlanTD.06414), males [mL/kg/h] (EPFL LISP3 Cohort)	Williams EG, Wu Y, Jha P, Dubuis S, Amariuta T, Wolski W, Zamboni N, Aebersold R, Auwerx J	2015	14.5	Chr14: 106.286762	-0.7961	14	0.00031	
8	11646	Central nervous system, behavior: Anxiety assay, percentage of locomotion in light side of a light-dark box for females [%]	Philip VM, Ansah TA, Blaha CD, Cook MN, Hamre KM, Lariviere WR, Matthews DB, Mittleman G, Goldowitz D, Chesler EJ	2010	12.7	Chr13: 20.529560	-0.6608	23	0.00038	
9	11642	Central nervous system, behavior: Anxiety assay, time in dark side of a light-dark box for females [sec]	Philip VM, Ansah TA, Blaha CD, Cook MN, Hamre KM, Lariviere WR, Matthews DB, Mittleman G, Goldowitz D, Chesler EJ	2010	11.6	Chr7: 36.124856	0.6591	23	0.00040	
10	11645	Central nervous system, behavior: Anxiety assay, time in light side of a light-dark box for females [sec]	Philip VM, Ansah TA, Blaha CD, Cook MN, Hamre KM, Lariviere WR, Matthews DB, Mittleman G, Goldowitz D, Chesler EJ	2010	11.6	Chr7: 36.124856	-0.6591	23	0.00040	

Table 2

Genetic covariation between midbrain expression of *Cyfip2* and IAL. Expression of Cyfip2 has a strong positive (r = .79; $p = 0.00022$) correlation with IAL.



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Correlation Table

Values of Record A_55_P1962771 in the VU BXD Midbrain Agilent SurePrint G3 Mouse GE (May12) Quantile database were compared to all 5104 records in the BXD Published Phenotypes database. The top 500 correlations ranked by the Genetic Correlation (Pearson's r) are displayed. You can resort this list using the small arrowheads in the top row.

Clicking on the record ID will open the published phenotype data for that publication. Click on the correlation to see a scatter plot of the trait data.

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More Options Download Table

Index	Record ID	Phenotype	Authors	Year	Max LRS	Max LRS Location Chr and Mb	Sample r	N Cases	Sample p(r)	GN2 Link
1	16708	Blood chemistry, metabolism, metabolite: AMC Trait 129 contact <carmen.argmann@mssm.edu>	Argmann CA, Mirzaian M, Ghauharali-van der Vlugt JMM, Williams EG, Andreux P, Houten SM, Houtkooper R, Auwerx J, Verhoeven AJ, Aerts JM	2014	12.2	Chr2: 114.022897	-0.9625	7	7.60e-05	
2	15035	Obesity, Brockmann (gudrun.brockmann@agrar.hu-berlin.de); High Fat Diet between 4 and 20 weeks (Ssniff(R) diet S8074-E010, Germany: The diet contained 20.7% crude protein, 25.1% crude fat, 5.0% crude fiber, 5.9% crude ash, 39.7% N-free extract, 20.0% starch, 17.5% sugar, vitamins, trace elements, amino acids, and minerals (19.1 MJ/kg metabolizable energy; thereof 45% energy from fat, 31% from carbohydrates, and 24% from proteins). The fat in the diet derived from coconut oil and suet.); BW13.14, males	Brockmann, Schughart	2011	9.1	Chr18: 69.067889	-0.7307	20	0.00013	
3	15131	Cardiovascular system: Systolic blood pressure of adult males raised in Memphis measured using a tail cuff system (originally known as GN Trait 11017) [mm Hg]	Koutnikova H, Laakso M, Lu L, Combe R, Paananen J, Kuulasmaa T, Kuusisto J, Häring HU, Hansen T, Pedersen O, Smith U, Hanefeld M, Williams RW, Auwerx J	2009	17.6	Chr9: 113.497075	-0.9547	7	0.00017	
4	18494	Central nervous system, behavior, pharmacology: Intravenous cocaine self-administration (IVSA), principal component 2 that corresponds well to infusions on the ascending limb of the dose-response curve (IAL trait, see Fig 4), in males and females at 12 to 14 weeks of age (this trait maps to Cyfip2 locus) [PC score]	Dickson PE, Miller MM, Calton MA, Bubier JA, Cook MN, Goldowitz D, Chesler EJ, Mittleman G	2016	22.2	Chr11: 47.930883	0.7887	15	0.00022	
5	11915	Central nervous system, behavior: Fear conditioning response, activity after second tone-shock pairing for males and females [n beam breaks/30 sec test]	Philip VM, Ansah TA, Blaha CD, Cook MN, Hamre KM, Lariviere WR, Matthews DB, Mittleman G, Goldowitz D, Chesler EJ	2010	17.2	Chr12: 105.957284	0.5657	32	0.00056	
6	18301	PhenoTyper_022	Loos M, Smit G	2015	15.5	Chr3: 28.293133	-0.6360	24	0.00057	
7	14345	Infectious disease, immune system, urogenital system: TNF-a (log) at 72 h after inoculation with 1 x 10^6 cfu Candida albicans (ATCC 10231) IV via retroorbital sinus immediately after light onset [pg/mg protein, log, kidney]	Toth LA, Trammell RA, Williams RW	2014	18.5	Chr2: 31.678177	-0.7844	13	0.00083	
8	11947	Central nervous system, behavior: Locomotion in zero maze for males and females [n beam breaks]	Philip VM, Ansah TA, Blaha CD, Cook MN, Hamre KM, Lariviere WR, Matthews DB, Mittleman G, Goldowitz D, Chesler EJ	2010	20.0	Chr1: 169.153242	0.5461	32	0.00097	
			Philip VM, Ansah TA, Blaha CD, Cook MN,							