Dissection of a QTL Hotspot on Mouse Distal Chromosome 1 that Modulates Neurobehavioral Phenotypes and Gene Expression

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Abstract

A remarkably diverse set of traits maps to a region on mouse distal chromosome 1 (Chr 1) that corresponds to human Chr 1q21–q23. This region is highly enriched in quantitative trait loci (QTLs) that control neural and behavioral phenotypes, including motor behavior, escape latency, emotionality, seizure susceptibility (Sz3), and responses to ethanol, caffeine, pentobarbital, and haloperidol. This region also controls the expression of a remarkably large number of genes, including genes that are associated with some of the classical traits that map to distal Chr 1 (e.g., seizure susceptibility). Here, we ask whether this QTL-rich region on Chr 1 (Qrr1) consists of a single master locus or a mixture of linked, but functionally unrelated, QTLs. To answer this question and to evaluate candidate genes, we generated and analyzed several gene expression, haplotype, and sequence datasets. We exploited six complementary mouse crosses, and combed through 18 expression datasets to determine class membership of genes modulated by Qrr1. Qrr1 can be broadly divided into a proximal part (Qrr1p) and a distal part (Qrr1d), each associated with the expression of distinct subsets of genes. Qrr1d controls RNA metabolism and protein synthesis, including the expression of ~20 aminoacyl-tRNA synthetases. Qrr1d contains a tRNA cluster, and this is a functionally pertinent candidate for the tRNA synthetases. Rgs7 and Fmn2 are other strong candidates in Qrr1d. FMN2 protein has pronounced expression in neurons, including in the dendrites, and deletion of Fmn2 had a strong effect on the expression of few genes modulated by Qrr1d. Our analysis revealed a highly complex gene expression regulatory interval in Qrr1, composed of multiple loci modulating the expression of functionally cognate sets of genes.

Introduction

The distal part of mouse Chr 1 harbors a large number of QTLs that generate differences in behavior. Open field activity [1], fear conditioning [2], rearing behavior [3], and several other measures of emotionality [4,5] have been repeatedly mapped to distal Chr 1. This region is also notable because it appears to influence responses to a wide range of drugs including ethanol [6], caffeine [7], pentobarbital [8], and haloperidol [9]. In addition to the behavioral traits, a number of metabolic, physiological and immunological phenotypes have been mapped to this region (table 1) [10–36]. This QTL rich region on mouse distal Chr 1 exhibits reasonably compelling functional and genetic concordance with the orthologous region on human Chr 1q21–q23. Prime examples of genes in this region that have been associated with similar traits in mouse and human are Rgs2 (anxiety in both species), Apoa2 (atherosclerosis), and Kenj10 (seizure susceptibility) [37–42].

Studies of gene expression in the central nervous system (CNS) of mice have revealed major strain differences in the expression level of numerous genes located on distal Chr 1, e.g., Coda, Atp1a2, and Kenj9 [26,43–45]. These differentially expressed genes are strong candidates for the behavioral and neuropharmacological traits that map to this region. We have recently shown that sequence variants near each of these candidate genes are often responsible for the prominent differences in expression [26,46,47]. In other words, sequence differences near genes such as Kenj9 cause expression to differ, and variation in transcript level maps back to the location of the source gene itself. Transcripts of this type are associated with cis-QTLs.

These expression genetic studies have also uncovered another unusual characteristic of mouse distal Chr 1. In addition to the extensive cis-effects, a large number of transcripts of genes located on other chromosomes map into this same short interval on distal Chr 1 [47,48]. These types of QTLs are often referred to as trans-QTLs. The clustering of trans-QTLs to distal Chr 1 has been replicated in multiple crosses and CNS microarray datasets [47]. We refer to this region of Chr 1, extending from Fgs3 (172.5 Mb) to Rgs7 (177.5 Mb) as the QTL-rich region on Chr 1, or Qrr1. It is possible that these modular effects on expression are the first steps in a cascade of events that are ultimately responsible for many of the prominent differences in behavior and neuropharmacology. For example, Qrr1 modulates the expression of several genes that have been implicated in seizure (e.g., Scn1b, Pmp22, Cauna1g), and this may be a basis for the strong influence Qrr1 has on seizure susceptibility [41].

In this study, we exploited 18 diverse array datasets derived from different mouse crosses to systematically dissect the expression QTLs in Qrr1. The strong trans effects are consistently
detected in CNS tissues of C57BL/6J [B6×DBA/2] (D2) and B6×C3H/HeJ (C3H) crosses, but are largely absent in ILS/1Bgc (ILS)×ISS/1Bgc (ISS) and C57BL/6By (B6)y×BALB/cBy (BALB), and in all non-neural tissues we have examined. We applied high-resolution mapping and haplotype analysis of Qrr1 using a large panel of BXD recombinant inbred (RI) strains that included highly recombinant advanced intercross RI lines. Our analyses revealed multiple distinct loci in Qrr1 that regulate gene expression specifically in the CNS. The distal part of Qrr1 (Qrr1d) has a strong effect on the expression of numerous genes involved in RNA metabolism and protein synthesis, including more than half of all aminoacyl-tRNA synthetases. Fmr2 and Rgs7, and a cluster of tRNAs are the strongest candidates in Qrr1d.

Results

Enrichment in Classical QTLs

The Chr 1 interval, from 172–178 Mb, harbors 32 relatively precisely mapped QTLs for classical traits such as alcohol dependency, escape latency, and emotionality (Mouse Genome Informatics at www.informatics.jax.org, Table 1). To compare the enrichment of QTLs in Qrr1 with that in other regions, we counted classical QTLs in 100 non-overlapping intervals covering almost the entire autosomal genome (table S1). These intervals were selected to contain the same number of genes as Qrr1. Numbers of QTLs ranged from 0 to 23, and averaged at 9.16±2.53 SD (SD). Compared to these regions, Qrr1 had the highest QTL number, over 4 SD above the mean, and over three times higher than average.

Enrichment in Expression QTLs in Neural Tissues

In this section, we summarize the number of expression phenotypes that map to Qrr1 in different tissues and mouse crosses. The results are based on the analysis of 18 array datasets that provide estimates of global mRNA abundance in neural and non-neural tissues from six different crosses. These crosses are—(i) BXD RI and advanced intercross RI strains derived from B6 and D2, (ii) CXB RI strains derived from B6y×BALB, (iii) LXS RI strains derived from ILS and ISS, (iv) B6×C3H F2 intercrosses, and (v & vi) two separate B6×D2 F2 intercrosses. These datasets were generated by collaborative efforts over the last few years [46,47,49–52] and some were generated more recently (e.g., the Illumina datasets for BXD striatum and LXS hippocampus, and BXD Hippocampus UMIUTAlly Exon Array dataset). All datasets can be accessed from GeneNetwork (www.genenetwork.org).

We mapped loci that modulate transcript levels and selected only those transcripts that have peak QTLs in Qrr1 with a minimum LOD score of 3. This corresponds to a generally lenient threshold with genome-wide p-value of 0.1 to 0.05, but corresponds to a highly significant pointwise p-value. Because we are mainly interested in testing a short segment on Chr 1, a pointwise (region-wise) threshold is more appropriate to select those transcripts that are likely to be modulated by Qrr1. Qrr1 covers approximately 0.2% of the genome and extends from Fgr3 (more precisely, SNP rs8242852 at 172,887364 Mb using Mouse Genome Assembly NCBI m36, UCSC Genome Browser mm8) through to Rgs7 (SNP rs1436041 at 177,273526 Mb). We defined this region on the basis of the large number of transcripts that have maximal LOD scores associated with markers between these SNPs.

Hundreds of transcripts map to Qrr1 with LOD scores ≥3 in neural tissue datasets of BXD RI strains, B6D2F2 intercrosses, and B6C3H2F2 intercrosses (table 2). The QTL counts in Qrr1 are far higher than the average of 15 to 35 expression QTLs in a typical 6 Mb interval. The fraction of QTLs in Qrr1 is as high as 14% of all trans-QTLs, and 5% of all cis-QTLs in the whole genome (table 2). The enrichment in trans-QTLs in Qrr1 is even more pronounced when the QTL selection stringency is increased to a LOD threshold of 4 (genome-wide p-value of approximately 0.01). For example, 27% of all highly significant trans-QTLs in the BXD cerebellum dataset are in Qrr1 (table 2). The BXD hippocampus dataset that was assayed on the Affymetrix Exon ST array is an exception—there are over a million probe sets in this array, and the percent enrichment of QTLs in Qrr1 appears to be relatively low. Nevertheless, about 1000 transcripts map to Qrr1 in this exon dataset.

In contrast to the CNS datasets, relatively few transcripts map to Qrr1 in non-neural tissues of the BXD strains and B6C3H2F2 intercrosses. While the number of cis-QTLs is still relatively high (1–3%), Qrr1 has limited or no trans-effect in these datasets (table 2). Qrr1 does not have a strong trans-effect in the LXS and CXB hippocampus datasets (table 2). This indicates that the sequence variants underlying the trans-QTLs do not segregate to nearly the same extent in the LXS and CXB RI panels as they do in B6×D2 and B6×C3H crosses. This contrast across genes can be exploited to parse Qrr1 into sub-regions and identify stronger candidate genes.

Replication of trans-QTLs in Multiple Datasets

The trans-QTLs in Qrr1 are highly replicable. A large fraction of the transcripts, in some cases represented by multiple probes or probe sets, map to Qrr1 in multiple CNS datasets. For example, there are 747 unique trans-QTLs with LOD scores greater than 4 (genomic-wide p-value ≤ 0.01) in the BXD hippocampus dataset (assayed on Affymetrix M430v2 arrays). Out of these highly significant trans-QTLs, 155 are in Qrr1 and the remaining 592 are distributed across the rest of the genome (figure 1). We compared the trans-QTLs in the hippocampus dataset with a similar collection of trans-QTLs (LOD≥4) in the cerebellum dataset (assayed on Affymetrix M430v2 arrays). Only 101 trans-QTLs in the hippocampus are replicated in the cerebellum (for trans-QTLs that were declared as common, the average distance between peak QTL markers in the two datasets is 1.6 Mb). But it is remarkable that of the subset of common trans-QTLs, 64 are in Qrr1 (figure 1). The replication rate of trans-QTLs in Qrr1 is therefore about 6-fold higher relative to the rest of the genome. When we compared the BXD hippocampus dataset with the B6C3H2F2 brain dataset (assayed on Agilent arrays), we found 54 trans-QTLs common to
both datasets (for the common trans-QTLs, the average distance between peak markers in the two datasets is 2.7 Mb). Strikingly, out of the 54 trans-QTLs common to both crosses, 52 are in Qrr1 (figure 1).

Among the transcripts with the most consistent trans-QTLs are glyceryl-tRNA synthetase (Gars), cysteinyl-tRNA synthetase (Cars), asparaginyl-tRNA synthetase (Nars), isoleucyl tRNA synthetase (Iars), asparagine synthetase (Asns), and activating transcription factor 4 (Atf4). These transcripts map to Qrr1 in almost all datasets in which the strong trans-effect is detected. Gars, Cars, and Nars are aminoacyl-tRNA synthetases (ARS) that charge tRNAs with amino acids during translation. Asns and Atf4 are also involved in amino acid metabolism—Asns is required for asparagine synthesis and is under the regulation of Atf4, which in turn is sensitive to cellular amino acid levels [52]. Other transcripts that consistently map as trans-QTLs to Qrr1 include brain expressed X-linked 2 (Bex2), splicing factor Sfus3, ribonucleoproteins Sfus1, and Sfus2, ring-finger protein 6 (Rnf6), and RAS oncogene family member Ralb.

Candidates in Qrr1

Qrr1 contains 164 known genes. The proximal part of Qrr1 is gene-rich and has several genes with high expression in the CNS (e.g., Prtn3, Kcnj9, Kain1, Atp1a2). The middle to distal part of Qrr1 is relatively gene sparse and consists mostly of clusters of olfactory receptors and members of the interferon activated Rgs7 gene family. Though comparatively gene sparse, the middle to distal part of Qrr1 contains a small number of genes that have high expression in the CNS—Idg4b, Dfy, Fmna2, and Rgs7.

A subset of 35 genes were initially selected as high priority candidates based on the number of known and inferred sequence differences between the B6 allele (B) and D2 allele (D) and based on expression levels in multiple CNS datasets (table 3). Eleven of these candidates contain missense SNPs segregating in B6×D2

Table 1. Classical QTLs on Chr 1 from 172–178 Mb; listed by approximate position from proximal to distal end (adapted from Mouse Genome Informatics).

| MGI ID | Symbol | Name | Type | Cross | Reference |
|--------|--------|------|------|-------|-----------|
| 2389120 | Bmd5 | Bone mineral density 5 | bone | C3H/HeJ×C57BL/6J | [10] |
| 1349434 | Bmd1 | Bone mineral density 1 | bone | C57BL/6J×CAST/Ei | [11] |
| 3624655 | Scga1 | Spontaneous crescentic glomerulonephritis QTL 1 | kidney | C57BL/6J×SCG/Kj | [12] |
| 2680094 | Ropd1 | Rotator performance 1 | behavior | 129S6/SvEvTac×C57BL/6J | [13] |
| 1894174 | Tir3c | Trypansomiasis infection response 3c | immune | BALB/c×129S6/SvEvTac×C57BL/6J | [14] |
| 2387316 | Elnt | Escape latencies during navigation task | behavior | C57BL/6J×DBA/2J | [15] |
| 1350920 | Emo1 | Emotionality 1 | behavior | BALB/c×C57BL/6J | [5] |
| 3050452 | Alcdp1 | Alcohol dependency 1 | behavior | C57BL/6J×DBA/2J | [6] |
| 1309452 | Alce1 | Alcohol withdrawal 1 | behavior | C57BL/6J×DBA/2J | [6] |
| 2150827 | Caiq1 | Caffeine metabolism QTL 1 | metabolism | C3H/HeJ×APN | [7] |
| 1098770 | Szs1 | Seizure susceptibility 1 | CNS | C57BL/6J×DBA/2 | [17] |
| 2661242 | Cdbmt1 | CD8 T memory cell subset 1 | immune | BALB/c×C3H/HeJ×C57BL/6J×DBA/2 | [18] |
| 3613641 | Chiq1 | Circulating hormone level QTL 1 | endocrine | BALB/c×C3H/HeJ×C57BL/6J×DBA/2J | [19] |
| 1345638 | Pbw1 | Pentobarbital withdrawal QTL 1 | behavior | C57BL/6J×DBA/2J | [8] |
| 2661145 | Ssta2 | Susceptibility to Salmonella typhimurium antigens 2 | immune | Hill×UliI | [20] |
| 3522039 | Tglyd | Triglycerides metabolism C57BL/6J | susceptibility | LIII | [21] |
| 1346066 | Gvhd1 | Graft-versus-host disease 1 | immune | B10.D2-H2d×C57BL/10J | [22] |
| 2155287 | Radp2 | Radiation pulmonary fibrosis 2 | immune | C3H/Kam×C57BL/6J | [23] |
| 2151854 | Pbw2 | Pentobarbital withdrawal modifier | behavior | C57BL/6J×DBA/2J | [24] |
| 1890350 | Ath9 | Atherosclerosis 9 | metabolism | C57BL/6J×FVB/Ncr | [25] |
| 2682357 | Bslm4 | Basal locomotor activity 4 | behavior | BALB/c×C57BL/6J; C57BL/6J×DBA/2J; C57BL/6J×LP/J | [26] |
| 1891174 | Cbn1 | Cerebellum weight 1 | CNS | C57BL/6J×DBA/2J | [27] |
| 2137602 | Cq2 | Cholesterol QTL 2 | metabolism | C57BL/6J×KH-Ay | [28] |
| 2680927 | Eila1 | Ethanol induced locomotor activity | behavior | C3H/HeJ×C57BL/6J | [29] |
| 2660561 | Fglu2 | Fasting glucose 2 | metabolism | C57BL/6J×KK-Ay | [30] |
| 2137474 | Hpic2 | Haloperidol induced catalepsy 2 | behavior | C57BL/6J×DBA/2J | [9] |
| 1890554 | Melm2 | Melanoma modifier 2 | tumor | BALB/c×C57BL/6J | [31] |
| 2684308 | Mnotch | Modifier of Notch | 129X1/SvJ | C57BL/6J×DBA/2J | [32] |
| 2149094 | Ste9 | Systemic lupus erythematosus susceptibility 9 | immune | BXSB/J×C57BL/10J | [33] |
| 3579342 | Sphr1 | Spermatozoa heat stress resistance 1 | other | C57BL/6GSGl×MRL/MpJ×Sc/J | [34] |
| 2148891 | Yaad4 | Y-linked autoimmune acceleration | immune | BXSB/J×C57BL/10J | [35] |
| 3613551 | Bglu3 | Blood glucose level 3 | metabolism | C3H/HeJ×C57BL/6J | [36] |

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We also scanned *Qrr1* for variation in copy number [54,55]. Graubert et al. [55] reported segmental duplication in *Qrr1* with a copy number gain in D2 compared to B6 near the intellectin 1 (*Itlna*) gene at 173.352 Mb. We failed to detect any expression signatures of a copy number variation around *Itlna* in any of the GeneNetwork datasets. However, we did identify an apparent 150 kb deletion across the *Ifi200* gene cluster (175.584–175.733 Mb).

| Cross            | N* | Dataset & Normalization | Tissue          | Array            | LOD ≥ 3 % trans | % cis | LOD ≥ 4 % trans | % cis |
|------------------|----|-------------------------|-----------------|------------------|-----------------|------|-----------------|------|
| B6D2F2           | 58 | OHSU/VA (Sep05) PDNN    | Striatum        | Affymetrix M430v2| 197 56 8 5      |      | 18 5            |      |
| B6D2F2           | 56 | OHSU/VA mRNA (Aug05) PDNN| Whole brain     | Affymetrix M430  | 79 30 1 2       | 5 2  |
| BXD              | 45 | SUT (Mar05) PDNN        | Cerebellum      | Affymetrix M430  | 439 44 9 2      | 27 2 |
| BXD              | 69 | Hippocampus Consortium (Dec05) PDNN| Hippocampus | Affymetrix M430v2| 345 54 7 1      | 22 1 |
| BXD              | 39 | INIA (Jan06) PDNN       | Forebrain       | Affymetrix M430  | 279 39 5 1      | 13 1 |
| BXD              | 64 | Hamilton Eye Institute (Sep06) RMA| Eye            | Affymetrix M430v2| 156 43 2 1      | 2 1  |
| BXD              | 54 | HQF (Nov07) Rankinv     | Striatum        | Illumina M6.1    | 97 31 1 2       |      | 2 1             |      |
| BXD              | 29 | HBP/Rosen(Apr05) PDNN   | Striatum        | Affymetrix M430v2| 94 25 2 1       | 6 1  |
| BXD              | 63 | UMLUTaffy RMA (Mar08)   | Hippocampus     | Affymetrix Exon 1.0 ST| 700 302 0.4 | 1 0.5 |
| BXD              | 40 | UNC (Jan06) BothSexes LOWESS| Liver          | Agilent G4121A   | 9 20 0.3 1      | 0.7 1|
| BXD              | 53 | Kidney Consortium (Aug06) PDNN| Kidney        | Affymetrix M430v2| 8 33 0.2 1      | 0 1  |
| BXD              | 30 | GNF (Mar03) MASS        | Hematopoietic Cells| Affymetrix UTI4av2| 0 6 0 3        |      | 3 0             |      |
| LXS              | 75 | NIAAA INIA (May07) Rankinv| Hippocampus    | Illumina M6.1    | 10 28 0.4 1     |      | 1 1             |      |
| B6C3F2           | 238| UCLA BHBBF2 (2005) mlratio| Brain          | Agilent         | 516 51 14 3     | 23 2 |
| B6C3F2           | 306| UCLA BHBBF2 (2005) mlratio| Muscle         | Agilent         | 15 33 0.3 2     | 0.3 2|
| B6C3F2           | 298| UCLA BHBBF2 (2005) mlratio| Liver          | Agilent         | 63 46 0.7 3     | 0.6 3|
| B6C3F2           | 282| UCLA BHBBF2 (2005) mlratio| Adipose        | Agilent         | 56 34 0.5 3     | 0.4 3|
| CXB              | 13 | Hippocampus Consortium (Dec05) PDNN| Hippocampus | Affymetrix M430v2| 7 12 0.08 2     | 0.1 2|

*Number of RI strains or F2 mice.

*Number of cis- and trans-QTLs in *Qrr1* at minimum LOD of 3; complete list of these transcripts can be retrieved from www.genenetwork.org using search key “LRS = (15 500 Chr1 172 178)”.

*Percent of trans-QTLs in *Qrr1*= [number of trans-QTLs in *Qrr1*/total number of trans-QTLs in the whole genome]×100.

*Percent of cis-QTLs in *Qrr1*= [number of cis-QTLs in *Qrr1*/total number of cis-QTLs in the whole genome]×100.

Figure 1. Highly replicable trans-QTLs in *Qrr1*. The charts illustrate the total number of trans-QTLs (LOD ≥ 4) in *Qrr1* (shaded) and in other regions of the genome (non-shaded) in three datasets—BXD cerebellum, BXD hippocampus, and B6C3HF2 brain. The smaller charts represent the trans-QTLs in BXD hippocampus that are also detected in BXD cerebellum, and B6C3HF2 brain datasets. Out of the 101 trans-QTLs common to both BXD hippocampus and cerebellum, 64 are in *Qrr1* and the remaining 37 are located in other regions of the genome. The BXD hippocampus and B6C3HF2 brain datasets have 54 common trans-QTLs, and almost all (52 out of 54) are in *Qrr1*. doi:10.1371/journal.pgen.1000260.g001

Table 2. Expression QTLs in *Qrr1* in different crosses and tissues.
and 1452349_x_at detect Ifi204 and Mnda transcripts in B6 but not in D2. The expression difference is robust enough to generate cis-QTLs with very high LOD scores (>40). This gene cluster has low expression in the CNS (Affymetrix declares this probe sets to be “not present”), but high expression in tissues such as hematopoietic stem cells and kidney, in which the trans-effect of Ifi204 is not detected. The Ifi204 gene cluster was therefore excluded as a high priority candidate.

**cis-QTLs in Qrr1**

Transcripts of 26 of the 35 selected candidate genes map as cis-QTLs (LOD≥3) in the BXD CNS datasets (table 3). These putatively cis-regulated genes are among the strongest candidates in the QTL interval. The D allele in Qrr1 has the positive effect on the expression of Sdhc, Ndufs2, Adamts4, Dedd, Pfdn2, Ltap, Pfa15, Atpl1a2, Kcnj9, Kcnj10, Igsf4b, and Grem2. Increase in expression caused by the D allele ranges from about 10% for Adamts4 to over 2-fold for Atpl1a2. In contrast, the B allele has the positive effect on the expression of Pp441, For1g, B4galt3, Ppxx, Uf1, Nvl1, Uf1, Copa, Pex19, Wdr42a, Igsf8, Dfy, Finn2, and Rgs2. Increase in expression caused by the B allele ranges from about 7% for Usf1 to 40% for Pex19.

Individual probes were screened to assess if the strong cis-effects are due to hybridization artifacts caused by SNPs in probe targets. Thirteen candidate genes with cis-QTLs were then selected for further analysis and validation of cis-regulation by measuring allele specific expression (ASE) difference [56]. This method exploits transcribed SNPs, and uses single base extension to assess expression difference in F1 hybrids. By means of ASE, we validated the cis-regulation of 10 candidate genes—Ndufs2, Nvl1, Pfdn2, Usf1, Copa, Atpl1a2, Kcnj9, Kcnj10, Dfy, and Finn2 (table 4). Adamts4 and Igsf4b failed to show significant allelic expression difference. In the case of Usf1, the polarity of the allele effect failed to agree with the ASE result (D positive at p-value = 0.02).

High-Resolution cis-QTL Mapping

The BXD CNS datasets were generated from a combined panel of conventional RI strains and advanced RI strains that were derived by inbreeding advanced intercross progeny. The advanced RI strains have approximately twice as many recombinations compared to standard RI strains and the merged panel offers over a 3-fold increase in mapping resolution [57]. This expanded RI set combined with the relatively high intrinsic recombination rate within Qrr1 [58] provides comparatively high mapping resolution. Mapping precision can be empirically determined by analyzing cis-QTLs in multiple large datasets, particularly the BXD Hippocampus Consortium, UMITA/Hippocampus, and Hamilton Eye datasets. These three datasets were selected because they have expression measurements from six BXD strains with recombinations in Qrr1. These strains—BXD8, BXD29, BXD62, BXD64, BXD68, and BXD84—collectively provide six sets of informative markers and divide Qrr1 into six non-recombinant segments, labeled as segments 1–6 (haplotype structures shown in figure 2).

As cis-acting regulatory elements are usually located within a few kilobases of a gene’s coding sequence [59], we used the cis-QTLs as an internal metric of mapping precision by measuring the offset distance between a cis-QTL (position of peak QTL marker) and the parent gene (figure 3). For cis-QTLs with LOD scores between 3–4 (genome-wide p-value of 0.1–0.01) the mean gene-to-QTL peak distance is 900 kb. The offset decreases to a mean of 640 kb for cis-QTLs with LOD scores greater than 4 (p-value<0.001). Very strong cis-QTLs with LOD scores greater than 11 (p-value<10^-6) have a mean gene-to-QTL peak distance of only 450 kb. In all, 60% of cis-QTLs we examined have peak linkage on markers located precisely in the same non-recombinant segment as the parent gene, and 30% have peak linkage on markers in a segment adjacent to the parent gene (dataset S1). These cis-QTLs provide an empirical metric of mapping precision within Qrr1.

**Table 3. Candidate genes in Qrr1.**

| Gene   | Mb   | nsSNP* | Expb | BXDb | B6C3HFr2b | CXBb | LXSb |
|--------|------|--------|------|------|-----------|------|------|
| Egr3   | 172.981 | 2   | 8.2  | cis  | cis       |      |      |
| Sdhc   | 173.059 | 2   | 12.3 | cis  | cis       |      |      |
| Pp441  | 173.103 | 8.7  | cis  | cis  | cis       |      |      |
| Tomm40l| 173.148 | 9.67 | cis  | cis  | cis       |      |      |
| Apoa2  | 173.155 | 7.2  | cis  | cis  | cis       |      |      |
| Fcrr1g | 173.160 | 8.5  | cis  | cis  | cis       |      |      |
| Ndufs2 | 173.165 | 2   | 13.6 | cis  | cis       |      |      |
| Adamts4| 173.181 | 8.1  | cis  | cis  | cis       | cis  | cis  |
| B4galt3| 173.201 | 9.5  | cis  | cis  | cis       |      |      |
| Ppox   | 173.207 | 7.8  | cis  | cis  | cis       |      |      |
| Usp21  | 173.212 | 9.0  | cis  | cis  | cis       |      |      |
| Uf1    | 173.219 | 10.8 | cis  | cis  | cis       |      |      |
| Dedd   | 173.260 | 9.7  | cis  |      |           |      |      |
| Nl1    | 173.272 | 1    | 9.8  | cis  | cis       | cis  | cis  |
| Pfdn2  | 173.276 | 12.8 | cis  | cis  | cis       | cis  | cis  |
| Arhygp30| 173.319 | 4    | 7.6  |      |           |      |      |
| Ufs1   | 173.342 | 7.5  | cis  | cis  | cis       |      |      |
| Retbp2 | 173.434 | 2    | 9.7  | cis  | cis       |      |      |
| Vangl2 | 173.935 | 7.6  | cis  | cis  | cis       |      |      |
| Ncstn  | 173.996 | 8.5  | cis  |      |           |      |      |
| Copa   | 174.013 | 1    | 12.7 | cis  | cis       | cis  | cis  |
| Pex19  | 174.057 | 1    | 9.9  | cis  | cis       | cis  |      |
| Wdr42a | 174.078 | 10.3 | cis  | cis  | cis       |      |      |
| Pfa15  | 174.127 | 14.1 | cis  |      |           |      |      |
| Atpl1a2| 174.202 | 15.4 | cis  | cis  | cis       | cis  | cis  |
| Igsf8  | 174.243 | 12.1 | cis  |      |           |      |      |
| Kcnj9  | 174.251 | 9.1  | cis  | cis  | cis       | cis  |      |
| Kcnj10 | 174.271 | 1    | 11.2 | cis  | cis       | cis  |      |
| Tagln2 | 174.430 | 8.8  |      |      |           |      |      |
| Dusp23 | 174.561 | 7.4  | cis  |      |           |      |      |
| Dfy    | 175.262 | 10.3 | cis  | cis  | cis       |      |      |
| Igsf4b | 175.264 | 10.6 | cis  |      |           |      |      |
| Finn2  | 176.419 | 3    | 10.4 | cis  | cis       | cis  |      |
| Grem2  | 176.764 | 8.2  |      |      |           |      |      |
| Rgs7   | 176.989 | 11.5 | cis  |      |           |      |      |

*Number of missense mutations between B and D alleles.

**Parsing trans-QTLs by High-Resolution Mapping and Gene Functions**

Mapping precision of cis-QTLs is comparatively higher in the BXD hippocampus dataset (average offset of only 410 kb), and we used this set to examine the trans-QTLs (LOD≥3) at higher resolution. The trans-QTLs in Qrr1 were parsed into subgroups...
based on the location of peak LOD score markers (figure 4). This method of resolving trans QTLs effectively grouped subsets of transcripts into functionally related cohorts. For instance, all the QTLs for the aminoacyl-tRNA synthetases (ARS) have peak LOD scores only within the distal three segments of Qrr1 (figure 5). This consistency in QTL peaks for transcripts of the same gene family is itself a good indicator of mapping precision. In addition to the ARS, numerous other genes involved in amino acid metabolism and translation map to the distal part of Qrr1 (e.g., Atf4, Asns, Eif4g2, and Pum2).

We divided the trans QTLs into two broad subgroups—those with peak QTLs on markers in the proximal part of Qrr1 (Qrr1p; 172–174.5 Mb or segments 1, 2, 3 in figure 2), and those with peak QTLs on markers in the distal part of Qrr1 (Qrr1d; 174.5–177.5 Mb or segments 4, 5, and 6 in figure 2). While Qrr1p is relatively gene-rich, only 35% of the trans QTLs (129 out of 365 probe sets) have peak LOD scores in this region. The majority of trans QTLs—about 65% (236 out of 365 probe sets)—have peak QTLs in the relatively gene-sparse Qrr1d.

The two subsets of transcripts—those with trans QTLs in Qrr1p and those with trans QTLs in Qrr1d—were analyzed for overrepresented gene functions using the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/). This revealed distinct gene ontology (GO) categories enriched in the two subsets (dataset S2). Enriched GOs among the transcripts modulated by Qrr1p include GTPase-mediate signal transduction (modified Fisher’s exact test).

### Table 4. Validation of cis QTLs by measuring allele specific expression difference.

| Gene   | ProbeSet ID     | SNP ID  | Cis-LOD | Add. effect (QTL) | High allele (ASE) | P-value   |
|--------|-----------------|---------|---------|-------------------|-------------------|-----------|
| Ndufs2 | 1451096_at      | rs8245216 | 12      | 0.172             | D                 | 2.4×10⁻⁵  |
| Adams4 | 1455965_at      | rs31537832 | 25      | -0.376            | B                 | 0.2       |
| Ufc1   | 1416327_at      | rs13470410 | 21      | -0.262            | D                 | 0.02      |
| Nit1   | 1417468_at      | rs31552469 | 15      | -0.154            | B                 | 0.01      |
| Phdn2  | 1421950_at      | rs31549998 | 5       | 0.174             | D                 | 4.1×10⁻⁷  |
| U6f1   | 1426164_a_at    | rs31542370 | 5       | -0.166            | B                 | 0.004     |
| Copa   | 1415706_at      | rs13461812 | 9       | -0.148            | B                 | 3.9×10⁻⁵  |
| Atp1a2 | 1455136_at      | rs31570902 | 49      | 1.186             | B                 | 0.02      |
| Kcnj9  | 1450712_at      | rs31569118 | 19      | 0.511             | D                 | 0.01      |
| Kcnj10 | 1419601_at      | rs30789204 | 28      | 0.349             | D                 | 0.003     |
| Dfy    | 1432273_a_at    | rs31616337 | 24      | -0.337            | B                 | 0.006     |
| Igs4b  | 1418921_at      | rs31613626 | 7       | 0.171             | B                 | 0.3       |
| Fmn2   | 1450063_at      | rs33800912 | 17      | -0.286            | B                 | 5.5×10⁻⁶  |

*Additive effect is computed as [(mean expression in DD homozygote) – (mean expression in BB homozygote)]/2 on a log2 scale. Positive value means D high expression, and negative value means B high expression.

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Figure 2. Haplotype maps of Qrr1 recombinant BXD strains. BXD8, BXD29, BXD62, BXD64, BXD68, and BXD84 have recombinations in Qrr1. B haplotype is assigned blue (–), D haplotype is assigned pink (+), and recombination regions are shown in grey. The Qrr1 interval (in Mb scale) is shown above and approximate positions of recombination are highlighted (red). The recombinant strains collectively divide Qrr1 into six segments (labeled 1–6), and provide six sets of informative markers. Markers are shown below and approximate positions of candidate genes (yellow bars) and tRNA clusters (orange triangles) are indicated.

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Figure 3. QTL mapping precision in Qrr1. Mapping precision was empirically determined by measuring the distance between a cis-QTL peak and location of parent gene. Cis-QTLs in BXD Hippocampus Consortium, UMUTAFFY Hippocampus, and Hamilton Eye datasets were used for this purpose. Mean gene-to-QTL peak distance (y-axis) was plotted as a function of LOD score (LOD score range on x-axis). Number of probes sets in each LOD range is shown. Mapping precision increases with increase in LOD score. The mean offset for cis-QTLs with LOD scores 3–4 (genome-wide adjusted p-value of 0.1–0.01) is 900 kb, and the offset decreases to 650 kb at 4–5 LOD scores (p-value of 0.01–0.001). Cis-QTLs with LOD scores greater than 11 (p-value< 10^-5) have mean offset of only 450 kb.

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p = 0.001), and structural constituents of ribosomes (p = 0.003). Transcripts modulated by Qrr1d are highly enriched in genes involved in RNA metabolism (p = 4 × 10^-5), tRNA aminoacylation (p = 1 × 10^-3) and translation (p = 2 × 10^-5), RNA transport (p = 0.003), cell cycle (p = 0.004), and ubiquitin mediated protein catabolism (p = 0.006). Other GO categories show enrichment in both Qrr1p and Qrr1d. For example, genes involved in RNA metabolism and ubiquitin-mediated protein catabolism are also overrepresented among the transcripts modulated by Qrr1p (p = 0.002 for RNA metabolism and p = 0.005 for ubiquitin-protein ligases). This may either be due to limitations in QTL resolution, or due to multiple loci in Qrr1p and Qrr1d controlling these subsets of transcripts.

An Aminoacyl-tRNA Synthetase trans-QTL in Distal Qrr1

A remarkable number of transcripts of the ARS gene family map to Qrr1. A total of 16 ARS transcripts have trans-QTLs at a minimum LOD score of 3 in one or multiple BXD, B6D2F2, and B6C3H CNS datasets (table 5). In almost all cases, QTLs peak on markers on the distal part of Qrr1. Except for Hars, the B allele in Qrr1 consistently increases expression by 10% to 30%. In the case of Hars, the D allele has the positive additive effect and increases expression by about 10%.

We examined all probes or probe sets that target ARS and ARS-like genes in the B6×D2 CNS datasets. The Affymetrix platform measures the expression of 34 ARS and ARS-like genes; 24 of these map to Qrr1 at LOD scores ranging from a low of 2 to a high of 12. Even in the case of the suggestive trans-QTLs (i.e., LOD values between 2 and 3), the B allele in Qrr1 has the positive effect on expression. The ARS family is also highly represented among trans-QTLs in the B6C3H/F2 brain dataset. Thirty-seven probes in this dataset target the tRNA synthetases, eleven of these have trans-QTLs in Qrr1d (LOD scores ranging from 2 to 20), and almost all have a B positive additive effect (exceptions are Hars and Qrr1p). The co-localization of trans-QTLs to Qrr1d, the general consensus in parental allele effect, and their common biological function indicate that there is a single QTL in the distal part of Qrr1 modulating the expression of the ARS. It is crucial to note that this genetic modulation is only detected in CNS tissues.

In the LXS hippocampus dataset, Qrr1 has only a limited trans-effect on gene expression. Despite the weak effect, expression of Dars2 (probe ID ILM580427) maps to the distal part of Qrr1 at a LOD of 3. Although this is only a weak detection of the ARS QTL in the LXS dataset, it nonetheless demonstrates the strong regulatory effect of Qrr1 on the expression of this gene family. In the case of the CXB hippocampus dataset, not a single trans-QTL for the ARS is detected in Qrr1.

trans-QTLs for Transcripts Localized in Neuronal Processes

In addition to the high overrepresentation of transcripts involved in translation and RNA metabolism, several transcripts known to be transported to neuronal processes or involved in RNA transport also map to Qrr1d, including Camk2a, Bdh2, Cdk4, Eif4g2, Eif4g3, Hapab, Pppl1c, Pubp1, Eif3f, Rnbp1, Rhoq3, Siam2, and Pum2 [60–63]. An interesting example is provided by the brain derived neurotrophic factor (Bdnf). Two alternative forms of Bdnf mRNA are known—one isoform has a long 3’ UTR and is specifically transported into the dendrites; the other isoform has a short 3’ UTR and remains primarily in the somatic cytosol [64].

The Affymetrix M430 arrays contain two different probe sets that target these Bdnf isoforms. Probe set 1422169_a_at targets the distal 3’ UTR and is essentially specific for the dendritic isoform, and probe set 1422168_a_at targets a coding sequence common to both isoforms. Although both probe sets detect high expression signal in the hippocampus, only the dendritic isoform maps as a trans-QTL to Qrr1d. This enrichment in transcripts that are transported to neuronal processes raises the possibility that this CNS specific trans-effect may be related to local protein synthesis.

tRNAs in Qrr1

Prompted by the many ARS transcripts that consistently map to Qrr1d, we searched the genomic tRNA database [65] for tRNAs in this region. Interestingly, distal Chr 1 is one of many tRNA hotspots in the mouse genome and several predicted tRNAs are clustered in the non-coding regions of Qrr1 (figure 2). The majority of these tRNA sequences are in the proximal end of Qrr1, over 2 Mb away from Qrr1d. We scanned the intergenic non-coding regions in Qrr1d for tRNAs using the tRNAScan-SE software [65] and uncovered tRNAs for arginine and serine, and three pseudo-tRNA sequences between genes Igsf4b and Aum2 (175,204–175,257 Mb) in Qrr1d (dataset S3). Transfer RNAs are involved in regulating transcription of the ARS in response to cellular amino acid levels [66] and are functionally highly relevant candidates in Qrr1d. Polymorphism in the tRNA clusters (e.g., possible copy number variants, differences in tRNA species) may have significant impact on the expression of the ARS.

Sequence Analysis of Crosses

Trans-regulation of large number of transcripts by Qrr1 is a strong feature of crosses between B6 and D2—both the BXD RI
set and B6D2F2 intercrosses—and in the B6 and C3H intercrosses. The feature is much weaker in the large LXS RI set and in the small CXB panel. The effect specificity demonstrates that a major source of the Qrr1 signal is generated by variations between B and D, and B and C3H alleles [H], but not by variations between the ILS and ISS alleles (L and S, respectively), and B and BALB alleles (C). This contrast can be exploited to identify subregions that underlie the trans-QTLs [67].

SNPs were counted for all four pairs of parental haplotypes—B vs D, B vs H, B vs C, and L vs S—and SNP profiles for the four crosses were compared (figure 6). Qrr1 is a highly polymorphic interval in the B6×D2 crosses. The flanking regions, however, have few SNPs (170–172.25 Mb proximally, and 177.5–179.5 Mb distally) and are almost identical-by-descent between B6 and D2. The B6×BALB crosses, despite being negative for the trans-effect, have moderate to high SNP counts in Qrr1 and share a SNP profile somewhat similar to B6×D2 crosses. The B6×C3H crosses also have moderate to high SNP counts in Qrr1, with a relatively higher SNP count in Qrr1d compared to Qrr1p. In contrast, in the LXS, Qrr1p is more SNP-rich than Qrr1d. Most notably, the segments that harbor the tRNAs and candidates Fmn2, Grem2, and Rgs7 are almost identical by descent between ILS and ISS. This SNP

Figure 4. Segregation of trans-QTLs in Qrr1. Expression of Atp5j2, Cplx2, and Nars are modulated by trans-QTLs in Qrr1 (blue plot). D allele has the positive additive effect (green plot; allele effect scale shown on the right) on the expression of Atp5j2 and Cplx2; peak LOD scores are on markers near candidate genes Ndufs2 and Kcnj10. B allele has the positive additive effect (red plot) on the expression of Nars; peak LOD score is on markers near candidate gene Fmn2. The horizontal lines indicate the genome-wide significant thresholds (p-value = 0.05). Yellow seismograph tracks the SNP density between B and D alleles. Affymetrix probe set ID for each transcript in the BXD hippocampus dataset is shown.
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comparison indicates that the strongest \textit{trans}-effect is from \textit{Qrr1d}. A possible reason why the \textit{trans}-effect is not detected in the CXB RI strains, despite being SNP rich in \textit{Qrr1}, is that the crucial SNPs underlying the \textit{trans}-QTLs may not be segregating in this cross or that undetected copy number variants make important contributions to the \textit{Qrr1} effects. A final explanation may be that the small CXB dataset (13 strains) is simply underpowered.

High-Ranking Candidates Based on Cross Specificity of \textit{cis}-QTLs

We used the specificity of \textit{cis}-QTLs in the multiple crosses to identify higher priority candidates in \textit{Qrr1}. The assumption is that candidate genes whose transcripts have \textit{cis}-QTLs (LOD score above 3) in the B6×D2 and B6×C3H crosses but not in the LXS and CXB RI strains are stronger candidates for \textit{trans}-QTLs that are detected in the former two crosses but not in the latter two crosses. In contrast, \textit{cis}-QTLs with the inverse cross specificity are less likely to underlie these \textit{trans}-QTLs. Based on this criterion, there are four high-ranking candidates in \textit{Qrr1p}—Purkinje cell protein 4-like 1 (\textit{Pcp4l1}), prefoldin (\textit{Pfdn2}), WD repeat domain 42 a (\textit{Wdr42a}), and \textit{Kcnj10} (table 3). There are only two high-ranking candidates in \textit{Qrr1d}—formin 2 (\textit{Fmn2}), an actin binding protein involved in cytoskeletal organization, and regulator of G-protein signaling 7 (\textit{Rgs7}) (table 3).

Table 5. Transcripts of aminoacyl tRNA synthetases that have \textit{trans}-QTLs in \textit{Qrr1} (LOD$\geq$3) in one or multiple CNS datasets.

| Gene | Name                  | Probe ID$^*$ | Chr$^b$ | Dataset$^c$               | LOD$^d$ | B/D$^e$ |
|------|-----------------------|--------------|---------|---------------------------|---------|---------|
| \textit{Nars} | asparaginyl-tRS         | 1452866_at_A | Chr 18  | BXD cerebellum           | 12.0    | B       |
| \textit{Gars} | glycyl-tRS            | 1423784_at   | Chr 6   | BXD hippocampus          | 10.6    | B       |
| \textit{Aras} | arginyl-tRS           | 1416312_at_A | Chr 11  | BXD forebrain            | 8.9     | B       |
| \textit{Cars} | cysteinyl-tRS         | 10024406001  | Chr 7   | B6C3HF2 brain            | 8.9     | B       |
| \textit{Yars} | tyrosyl-tRS           | 1002439842   | Chr 4   | B6C3HF2 brain            | 8.0     | B       |
| \textit{Iars} | isoleucine-tRS        | 1426257_s_at | Chr 13  | BXD cerebellum           | 7.8     | B       |
| \textit{Sars} | seryl-tRS             | 1426257_a_at | Chr 3   | BXD cerebellum           | 6.9     | B       |
| \textit{Mars} | methionine-tRS        | 1455951_at   | Chr 10  | BXD hippocampus          | 6.5     | B       |
| \textit{Har}  | histidyl-tRS          | 1438510_a_at | Chr 18  | BXD hippocampus          | 5.2     | D       |
| \textit{Iars2} | isoleucine-tRS        | 1426735_at   | Chr 1   | BXD hippocampus          | 4.3     | B       |
| \textit{Tars} | threonyl-tRS          | 10024395655  | Chr 15  | B6C3HF2 brain            | 4.0     | B       |
| \textit{Aars} | alanyl-tRS            | 1451083_s_at | Chr 8   | BXD eye                  | 3.9     | B       |
| \textit{Lars} | leucyl-tRS            | 1448403_at_A | Chr 18  | BXD cerebellum           | 3.7     | B       |
| \textit{Ears2} | glutamyl-tRS          | ILM5290446   | Chr 7   | BXD ILM striatum         | 3.7     | B       |
| \textit{Aarsd1} | alanyl-tRS domain 1   | 1424006_at   | Chr 11  | B6DF2 brain              | 3.5     | B       |
| \textit{Dars}  | aspartyl-tRS          | 1423800_at_A | Chr 1   | BXD cerebellum           | 3.2     | B       |

$^*$Probe/Probe set ID.
$^b$Physical location of gene; \textit{Iars2} is located on Chr 1 at 186.9 Mb, and \textit{Dars} on Chr 1 at 130 Mb.
$^c$Dataset in which transcript has highest \textit{trans}-QTL in \textit{Qrr1}.
$^d$Highest LOD score in \textit{Qrr1}.
$^e$Allele that increases expression.

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Both Fmn2 and Rgs7 are almost exclusively expressed in the CNS and are high priority candidates for the CNS specific trans-QTLs. A point of distinction between the two candidates is that while expression of Rgs7 maps as a cis-QTL only in the B6 x D2 and B6 x C3H crosses, expression of Fmn2 maps as a cis-QTL in B6 x D2 and B6 x C3H crosses, and in the CXB RI strains in which the trans-effect is not detected (table 3). Based on the pattern of specificity of cis-QTLs in multiple crosses, Rgs7 is a more appealing candidate. However, Fmn2 has known missense SNPs that segregate in the B6 x D2 (Glu610Asp, Pro1077Leu, Asp1431Glu) and B6 x C3H crosses (Val372Ala). There are no known missense mutations in Fmn2 in the CXB and LXS RI strains, and no known missense mutation in Rgs7 in any of the four crosses.

Partial Correlation Analysis

Linkage disequilibrium (LD) is a major confounding factor that limits fine-scale discrimination among physically linked candidates in a QTL. To further evaluate the two high-priority candidates in

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**Figure 6. SNP comparison between crosses.** SNPs in Qrr1 were counted for (A) C57BL/6J (B6) x DBA/2J (D2), (B) B6 x BALB/cBy (BALB), (C) B6 x C3H/HeJ (C3H), and (D) ILS x ISS. The SNP distribution profiles were generated by plotting the number of SNPs in 250 kb bins. Vertical red lines mark the approximate positions of recombination (corresponds to figure 2). Region covered by Qrr1p (horizontal line), candidate genes in Qrr1d (yellow bars), and position of tRNA clusters (triangles) are shown above the graphs. The B6 x D2, B6 x BALB, and B6 x C3H crosses have moderate to high SNP counts throughout Qrr1. In the ILS x ISS cross, Qrr1p is relatively SNP-rich but Qrr1d is SNP-sparse.

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Effect of Fmn2 Deletion on Gene Expression

Fmn2 is almost exclusively expressed in the nervous system [70] and is a strong candidate for a trans-effect specific to neural tissues. However, its precise function in the brain has not been established. Fmn2-null mice do not have notable CNS abnormalities [71], but to evaluate a possible role of Fmn2 in expression of genes that map to Qrr1d, we generated array data from brains of Fmn2-null (Fmn2+/−) and coisogenic (Fmn2+/+) 129/SvEv controls. At a stringent statistical threshold (Bonferroni corrected α=0.05), only eight genes have significant expression differences between Fmn2+/+ and Fmn2+/− genotypes (table 6). Five out of the eight genes, including Pou6f1, Usp53, and Slc11a2, have trans-QTLs in Qrr1d. Deletion of Fmn2 had the most drastic effect on the expression of the transcription factor gene Pou6f1, a gene implicated in CNS development and regulation of brain-specific gene expression [72,73]. Expression of Pou6f1 maps as a trans-QTL (at LOD score of 3) to Qrr1d in the hippocampus dataset, and its expression was down-regulated more than 44-fold in the Fmn2−/− line. While the expression analysis of Fmn2-null mice does not definitively link all the trans-QTLs to Fmn2, variation in this gene is likely to underlie some of the trans-QTLs in Qrr1d. The possible compensatory mechanism in the Fmn2−/− and the different genetic background of the mice (129/SvEv) are factors that may have contributed to the weak detection of trans-effects in the knockout line.

Sub-Cellular Localization of FMN2 Protein in Hippocampal Neurons

We examined the intracellular distribution of FMN2 protein in neurons using immunocytochemical techniques. All hippocampal pyramidal neurons on a culture dish exhibited distinct and fine granular immunoreactivity for FMN2. The cell body itself had the strongest signal (figure 7A). This fine punctate labeling extended into proximal dendrites and could be followed into distal dendrites. In some instances very thin processes, possibly the axons, were also labeled.

Linking Expression and Classical QTLs: Szs1

The strong trans-effect that Qrr1 has on gene expression is a likely basis for the classical QTLs that map to this region. For example, the major seizure susceptibility QTL (Szs1) has been precisely narrowed to Qrr1p [74]. We found that 10 genes already known to be associated with seizure or epilepsy have trans-QTLs with peak LOD scores near Szs1 and in Qrr1p. These include Sels1, Cacna1g, Ppo, and Dopkl (Table S2) [75–84]. In every case, the D allele has the positive additive effect on the expression of these seizure related transcripts, increasing expression 5% to 20%. The two potassium channel genes, Kcnj9 and Kcnj10, are the primary candidates [74]. Both are strongly cis-regulated. The tight linkage between these genes (within 100 kb) limits further genetic dissection, but in situ expression data from the Allen Brain Atlas (ABA, www.brain-map.org) provides us with a powerful complementary approach to evaluate these candidates [85]. Kcnj9 (figure 8A) is expressed most heavily in neurons within the dentate gyrus, whereas Kcnj10 (figure 8B) is expressed diffusely in glial cells in all parts of the CNS. The seizure-related transcripts with trans-QTLs near Szs1 are most highly expressed in neurons, and all have comparatively high expression in the hippocampus. Furthermore, expression patterns of six of the seizure transcripts that map to Qrr1p show spatial correlations with Kcnj9, Dopkl and Cacna1g (figure 8C) and have expression pattern that match Kcnj9 with strong labeling in the dentate gyrus and CA1, and weaker labeling in CA2 and CA3. In contrast, Sels2 (figure 8D), Adora1, Ppo, and Kcnj1 complement the expression of Kcnj9 with comparatively strong expression in CA2 and CA3, and weak expression in CA1 and dentate gyrus.

| Gene     | ProbeID* | Chrb | Fmn2+/− | Fmn2−/− | Foldc | p*       | LODc | Dataset† |
|----------|----------|------|---------|---------|-------|---------|-------|----------|
| Pou6f1   | ILM6200168 | 15   | 11.96   | 6.48    | 45    | 3 × 10−6 | 3.0   | BXD Hippocampus |
| Zfp420   | ILM2570632 | 7    | 10.12   | 7.70    | 5     | 0.002   |       |           |
| Tnnt1    | ILM2850148 | 18   | 10.72   | 6.70    | 16    | 0.002   | 3.0   | B6D2F2 striatum |
| Usp53    | ILM103190068 | 3    | 7.17    | 9.32    | 4     | 0.009   | 3.3   | BXD Hippocampus |
| LOC331139| ILM103170273 | 4    | 14.45   | 10.59   | 15    | 0.01    |       |           |
| Slc11a2  | ILM104050242 | 15   | 9.92    | 9.17    | 2     | 0.02    | 3.9   | BXD Hippocampus |
| Pgdb5    | ILM103940435 | 8    | 13.40   | 12.12   | 2     | 0.02    | 3.3   | BXD HBP Striatum |
| 6330569M22Rik | ILM104570300 | 3    | 6.42    | 10.63   | 18    | 0.03    |       |           |

* Illumina probe ID.  
† Physical location of gene.  
‡ Average expression signal in Fmn2-null and wild-type lines.  
§ Fold difference in expression between Fmn2-null and wild-type lines  
¶ Bonferroni adjusted p-values; corrected for 46,620 tests.  
∥ Highest LOD in Qrr1 and dataset in which transcript has highest LOD in Qrr1.  

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transcripts. The distal portion of this gene, perhaps due to the promoter polymorphism in the proximal cause of variation in the expression of functionally coherent sets of transcripts. The cell body had the strongest signal. The fine granular staining extended into apical and distal dendrites (arrows). Thin axon-like processes were also labeled (arrow head). (B) The fine granular staining is not detected in controls of sister cultures processed in parallel without the first antibody. doi:10.1371/journal.pgen.1000260.g007

**Figure 8.** Expression patterns of seizure related genes with **Kcnj9** and **Kcnj10** vs. **Kcnj10 and Seizure Susceptibility**

The two inwardly rectifying potassium channel genes—**Kcnj9** and **Kcnj10**—are strong candidates for the seizure susceptibility QTL in **Qrr1p** that has been unambiguously narrowed to the short interval from **Atp1a2** to **Kcnj10** [74]. In BXD CNS datasets, **Qrr1** also modulates the expression of a set of genes implicated in the etiology of seizure and epilepsy, including **Ptnp1**, **Scn1b**, **Kcnma1**, and **Socs2**, and **Cacna1g**. Polymorphisms in the **Kcnj9/Kcnj10** interval that influence expression of these genes are excellent candidates for the **Szs1** locus.

**Discussion**

**Qrr1** is a complex regulatory region that modulates expression of many genes and classical phenotypes. By exploiting a variety of microarray datasets and by applying a combination of high-resolution mapping, sequence analysis, and multiple cross analysis, we have dissected **Qrr1** into segments that are primarily responsible for variation in the expression of functionally coherent sets of transcripts. The distal portion of **Qrr1** (**Qrr1d**) has a strong trans-regulatory effect on RNA metabolism, translation, tRNA aminoacylation, and transcripts that are transported into neuronal dendrites. **Fmn2**, **Rgs7** and a cluster of tRNAs are strong candidates in **Qrr1d**. We analyzed gene expression changes in the CNS of **Fmn2-null** mice and detected a profound effect on the expression of a small number of transcripts that map to **Qrr1d**, particularly on the expression of the transcription factor **Pou6f1**. We have shown that the **Fmn2** protein is highly expressed in the cell body and processes of neurons, and is a high priority candidate in **Qrr1d**.

**Multiple Loci in a Major QTL Interval**

Fine mapping of complex traits have often yielded multiple constituent loci within a QTL interval [87,88]. Our mapping analyses of expression traits also show that multiple gene variants, rather than one master regulatory gene, cause the aggregation of expression QTLs in **Qrr1**. Subgroups of genes with tight coexpression can be dissected from the dense cluster of QTLs. Most notable is the strong trans-regulatory effect of **Qrr1d** on genes involved in amino acid metabolism and translation, including a host of ARS transcripts. However, there are limits to our ability to dissect **Qrr1**, and genes associated with protein degradation and RNA metabolism map throughout the region. In part this may be due to inadequate mapping resolution, but it may also reflect...
clusters of functionally related loci and genes [89]. At this stage we are also unable to discern whether there is a single or multiple QTLs within Qrl1d. While it is likely that a single QTL modulates the expression of the ARS, there may be additional gene variants in Qrl1d that modulate other transcripts involved in translation and RNA metabolism. With increased resolving power it may be possible to further subdivide transcripts that map to Qrl1f and Qrl1d into smaller functional modules. There may be multiple loci in Qrl1 that modulate different stages of protein metabolism in the CNS. Maintenance of cellular protein homeostasis requires finely tuned cross talk between transcription and RNA processing, the translation machinery, and protein degradation [90–92], gene functions highly overrepresented among the transcripts that map to Qrl1. While these are generic cellular processes, there are unique demands on protein metabolism in the nervous system. Neurons are highly polarized cells and specialized mechanisms are in place to manage local protein synthesis and degradation in dendrites and axons [93]. The nervous system is also particularly sensitive to imbalances in protein homeostasis [94,95], a possible reason why the trans-effects of Qrl1 are detected only in neural tissues.

Candidates in Qrl1d and Possible Links with Local Protein Synthesis

Transfer RNAs are direct biological partners of the ARS, and the cluster of tRNAs in the highly polymorphic intergenic region of Qrl1d (figure 6) is an enticing candidate. In addition to their role in shuttling amino acids, tRNAs also act as sensors of cellular amino acid levels and regulate transcription of genes involved in amino acid metabolism and the ARS [66]. There is tissue specificity in the expression of different tRNA isoforms [96], and we speculate that the tRNA cluster in Qrl1d is specifically functional in neural tissues. Rgs7, a member of the RGS (regulator of G-protein signaling) family, is another high-ranking candidate in Qrl1d. RGS proteins are important regulators of G-protein mediated signal transduction. Rgs7 is predominantly expressed in the brain and has been implicated in regulation of neuronal excitability and synaptic transmission [97,98]. Although RGS proteins are usually localized in the plasma membrane, RGS7 has been found to shuttle between the membrane and the nucleus [99]. This implies a role for RGS7 in gene expression regulation in response to external stimuli. Our final high-ranking candidate in Qrl1d is Fmn2. It codes for an actin binding protein exclusively expressed in the CNS and oocytes, and is involved in the establishment of cell polarity [70,71]. In Drosophila, the formin homolog, cappuccino, has a role in RNA transport and in localizing the staufen protein to oocyte poles [100–102]. It is possible that FMN2 has parallel functions in mammalian neurons. Interestingly, Staufen 2 (Stau2), a gene involved in RNA transport to dendrites [62], maps to Qrl1d in BXD CNS datasets. Furthermore, deletion of formin homologs in yeast results in inhibition of protein translation [103], compelling evidence for an interaction between the protein translation system and formins. Evidence for a role for Fmn2 in dendrites also comes from our immunocytochemical analysis that clearly demonstrates the expression of FNM2 protein in dendrites. Taken together, Fmn2 is a functionally relevant candidate gene in Qrl1d and may be related to RNA transport and protein synthesis in the CNS.

Methods

Microarray Datasets

The microarray datasets used in this study (table 2) were generated by collaborative efforts [46,47,49–52]. All datasets can be accessed from www.genenetwork.org. They provide estimates of global mRNA abundance in neural and non-neural tissues in the BXD, LXS, and CXB RI strains, B6D2F2 intercrosses, and B6C3HF2 intercrosses. Detailed description of each set, tissue acquisition, RNA extraction and array hybridization methods, and data processing and normalization methods are provided in the “Info” page linked to each dataset. In brief, the datasets are:

1) BXD CNS transcriptomes: The BXD CNS datasets measure gene expression in the forebrain and midbrain (INIA Forebrain), striatum (HBPCRosen Striatum and HQF Striatum), hippocampus (Hippocampus Consortium and Umuta Hippocampus), cerebellum (SJUT Cerebellum mRNA), and eye (Hamilton Eye) of BXD RI strains (table 2). The INIA Brain and HBB Rosen Striatum datasets have been described in Peirce et al. [47]. The Hippocampus Consortium dataset measures gene expression in the adult hippocampus of 69 BXD RI strains, the parental B6 and D2 strains, and F1 hybrids. The SJUT Cerebellum dataset measures gene expression in the adult cerebellum of 45 BXD RI strains, parental strains, and F1 hybrids. The Eye dataset measures gene expression in the eyes of 64 BXD RI strains, parental strains, and F1 hybrids. The HQF BXD Striatum is one of the newest datasets and was generated on Illumina Sentrix Mouse-6.1 arrays. It is similar to the HBB/Rosen Striatum and measures gene expression in the striatum of 54 BXD RI strains, parental strains, and F1 hybrids.

2) BXD non-neural transcriptomes: The non-neural BXD array sets measure gene expression in the liver (UNC Liver) of 40 BXD strains, kidney (Kidney Consortium) of 53 BXD strains, and hematopoietic stem cells (GNF Hematopoietic Cells) of 30 BXD strains [49,50].

3) LXS hippocampus transcriptome: The LXS Hippocampus dataset measures gene expression in the adult hippocampus of 75 LXS RI strains and the parental ILS and ISS strains.

4) B6D2F2 CNS transcriptomes: The B6D2F2 datasets measure gene expression in the whole brain (OHsu/VA Brain), and striatum (OHsu/VA Striatum) of B6×D2 F2 intercrosses [47,52]. The whole brain dataset comprises of samples from 56 F2 animals, and the striatum dataset comprises of samples from 58 F2 animals.

5) B6C3HF2 transcriptomes: These datasets were generated from large numbers of B6×C3H F2 intercross progeny and assayed using Agilent arrays [51]. These datasets have been described in Yang et al [51].

Mouse Strains and Genotype Data

The conventional BXD RI strains were derived from the B6 and D2 inbred mice [104,105]. The newer sets of advanced RI strains were derived by inbreeding intercrosses of the RI strains [57]. The parental B6 and D2 strains differ significantly in sequence and have approximately 2 million informative SNP. A subset of 14,000 SNPs and microsatellite markers have been used to genotype the BXD strains [106,107]. We used 3,795 informative markers for QTL mapping. Thirty such informative markers are in Qrl1 and we queried these markers to identify strains with recombinations in Qrl1; genes with strong cis-QTLs (Silh, Apila2, Dfy, and Fmn2) were used as additional markers. Smaller sub-sets of markers were used to genotype the two F2 panels (total of 306 markers for the whole brain, and 75 markers for the striatum F2 datasets).
The LXS RI strains were derived from the ILS and ISS inbred strains. They have been genotyped using 13,377 SNPs, and some microsatellite markers [108]. 2,659 informative SNPs and microsatellite markers were used for QTL mapping. The CBX panel consists of 15 RI strains derived from C57BL/ 6By and BALB/cBy inbred strains. A total of 1304 informative markers were used for QTL mapping. The B6 x C3H/HeJ F2 intercrosses have been genotyped using 13,377 SNPs and microsatellite markers, and 8,311 informative markers were used for QTL mapping.

Animals and Tissue Acquisition
Majority of the BXD and LXS tissues (cerebellum, eye, forebrain, hippocampus, kidney, liver, and striatum for the HQF Illumina dataset) were dissected at the University of Tennessee Health Science Center (UTHSC). Mice were housed at the UTHSC in pathogen-free colonies, at an average of three mice per cage. All animal procedures were approved by the Animal Care and Use Committee. Mice were killed by cervical dislocation, and tissues were rapidly dissected and placed in RNAlater (Ambion, www.ambion.com) and kept overnight at 4 °C, and subsequently stored at −80 degree C. Tissue were then processed at UTHSC or shipped to other locations for processing.

RNA Isolation and Sample Preparation
For the tissues that were processed at UTHSC (all BXD and LXS CNS tissues except HBP Affymetrix striatum), RNA was isolated using RNA STAT-60 (Tel-Test Inc., www.tel-test.com) as per manufacturer’s instructions. Samples were then purified using standard sodium acetate methods prior to microarray hybridization. The eye samples required additional purification steps to remove eye pigment; this was done using the RNeasy MinElute Cleanup Kit (Qiagen, www.qiagen.com). RNA purity and concentration was evaluated with a spectrophotometer using 260/280 nm absorbance ratio, and RNA quality was checked using Agilent Bioanalyzer 2100 prior to hybridization. Array hybridizations were then done according to standard protocols.

Microarray Probe Set Annotation
We have re-annotated a majority of Affymetrix probe sets to ensure more accurate description of probe targets. Each probe set represents a concatenations of eleven 25-mer probes, and these have been aligned to the NCBI built 36 version of the mouse genome (mm8 in UCSC Genome Browser) by BLAT analysis. We have also re-annotated the Illumina probes and incorporated these annotations into GeneNetwork. Each probe in the Illumina Mouse−6 and Mouse−6.1 arrays is 50 nucleotides in length, and these have been aligned to NCBI built 36.

QTL Mapping
We used the strain average expression signal detected by a probe or probe set. QTL mapping was done for all transcripts using QTL Reapper [47]. The mapping algorithm combines simple regression mapping, linear interpolation, and standard Haley-Knott interval mapping [109]. QTL Reapper performs up to a million permutations of an expression trait to calculate the genome-wide empirical p-value and the LOD score associated with a marker. We selected only those transcripts that have highest LOD scores, i.e., genome-wide adjusted best p-values, on markers located on Chr 1 from 172 to 178 Mb. This selected transcripts that are primarily modulated by Qtl1 but excluded transcripts that have QTls in Qtl1 but have higher LOD scores on markers located on other chromosomal regions. cis- and trans-QTLs were distinguished based on criteria described by Peirce et al. [47]. To identify trans-QTLs common to multiple datasets, we selected probes/probe sets that target the same genes and have peak LOD scores within 10 Mb in the different datasets.

Screening Local QTLs
We screened all Affymetrix probe sets with cis-QTLs in Qtl1 for SNPs in target sequences. This step was taken to identify false cis-QTLs caused by differences in hybridization. As probe design is based on the B6 sequence, such spurious cis-QTLs show high expression for the B allele, and low expression for the D allele. Our screening identified only two probe sets in which SNPs result in spurios local QTLs—1429382_at (Tomm40), and 1452308_a_at (Atp1a2). The majority of cis-QTLs in Qtl1 are likely to be due to actual differences in mRNA abundance. We did not detect a bias in favor of the B allele on cis-regulated expression and the ratio of transcripts with B- and D- positive additive effects is close to 1:1.

Analysis of Allele-Specific Expression Difference
To measure expression difference between the B and D alleles, we explored transcribed SNPs to capture allelic expression difference in F1 hybrids [56] using a combination of RT-PCR and a single base extension technology (SNAPshot, Applied Biosystems, www.appliedbiosystems.com). For each transcript we analyzed, Primer 3 [110] was used to design a pair of PCR primers that target sequences on the same exon and flanking an informative SNP. We prepared four pools of RNA from the hippocampus, and four pools of genomic DNA from the spleen of F1 hybrids (male and female B6×D2 and D2×B6 F1 hybrids). To avoid contamination by genomic DNA, the four RNA pools were treated with Turbo DNase (Ambion, www.ambion.com), and then first strand cDNA was synthesized (GE Healthcare, www.gehealthcare.com). The genomic DNA samples were used as controls, and both cDNA and genomic DNA samples were tested concurrently using the same assay to compare expression levels of B and D transcripts.

We amplified the cDNA and genomic DNA samples using GoTaq Flexi DNA polymerase (Promega Corporation, www.promega.com). PCR products were purified using ExoSap-IT (USB Corporation, www.usbweb.com) followed by SNAPshot to extend primer by a single fluorescently labeled ddNTPs. Fluorescently labeled products were purified using celf intestinal phosphatase (CIP, New England BioLabs, www.neb.com) and separated by capillary electrophoresis on ABI3130 (Applied Biosystems). Quantification was done using GeneMapper v4.0 software (Applied Biosystems), and transcript abundance was measured by peak intensities associated with each allele. Ratio of B and D allele in both cDNA and gDNA pools was computed, and t-test (one tail, unequal variance) was done to validate expression difference and polarity of parental alleles.

SNP Analysis in Multiple Crosses
GeneNetwork has compiled SNP data from different sources—Celera (http://www.celera.com), Perlegen/NIEHS (http://mouse.perlegen.com/mouse/download.html), BROAD institute (http://www.broad.mit.edu/snp/mouse), Wellcome–CTC [107], dbSNP, and Mouse Phenome Database (http://www.jax.org/phenome/SNP). SNP counts were done on the GeneNetwork SNP browser.

Partial Correlation Analysis
A partial correlation is the correlation between X and Y conditioned on one or more control variables. In this study, first
order partial correlation was used to detect the interaction between trans-regulated transcripts and cis-regulated candidate genes conditioned on the genotype (marker rs242401 at 173.050 Mb). If x, y and z are trans-regulated transcripts, cis-regulated transcript, and genotype in the QTL, respectively, then the first order partial correlation coefficient is calculated as—

$$r_{xy\mid z} = \frac{r_{xy} - r_{xz} r_{yz}}{\sqrt{(1 - r_{xz}^2)(1 - r_{yz}^2)}}$$

where $r_{xy}$ can be either Pearson correlation or Spearman’s rank correlation between x and y. We employed the Spearman’s rank correlation because the expression levels of many transcripts do not follow a normal distribution.

The significance of a partial correlation with n data points was assessed with a two-tailed t test on \( t = \sqrt{\frac{2k}{n-2-k}} \) for the first order partial correlation coefficient, and k is the number of variables on which we are conditioning.

**Immunocytochemistry**

Cultured hippocampal neurons from male B6 mice, prepared as described in Schikorski et al. [111] and cultured for 23 days, were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in HEPES buffered saline (pH 7.2) for 15 min. Cell membranes were permeabilized with 0.1% triton X-100 and unspecific binding sites were quenched with 10% BSA for 20 min at room temperature (RT). Neurons were incubated with a polyclonal anti-FMN2 antibody (Protein Tech Group, www.ptglab.com) diluted to 0.3 μg/ml at RT overnight. An anti-rabbit antibody raised in donkey (1:500, Invitrogen; http://www.invitrogen.com) conjugated with the fluorescent dye Alexa488 was used for the detection of the first antibody. All regions of interest were photographed with identical illumination and camera settings to allow for a direct comparison of the staining in labeled and control neurons.

**Fmn2**<sup>−/−</sup> and Fmn2<sup>+/+</sup> Microarray Analysis

The Fmn2<sup>−/−</sup> mice were generated using 129/SvEv (now strain 129S6/SvEvTac) derived TG-1 embryonic stem cells. Chimeric mice were backcrossed to 129/SvEv [70]. The Fmn2-null and littermate controls are therefore cisogenic. To validate the isogenicity of regions surrounding the targeted locus [112], we genotyped the Fmn2<sup>+/−</sup>, Fmn2<sup>++++</sup>, and Fmn2<sup>−−</sup> mice using ten microsatellite markers located on, and flanking Fmn2 (markers distributed from 172 Mb to 182 Mb). These markers are D1Mit455, D1Mit113, D1Mit456, D1Mit356, D1Mit206, D1Mit355, D1Mit150, D1Mit403, D1Mit615, and D1Mit426. With the exception of a marker at Fmn2 (D1Mit150), all alleles in null, heterozygote, and wildtype animals were identical.

RNA was isolated from whole brain samples of Fmn2<sup>+/+</sup> and Fmn2<sup>−−</sup> mice, and assayed on Illumina Mouse-6 array slides (six samples per slide). We compared five samples from Fmn2<sup>−−</sup> nulls, and five samples from Fmn2<sup>+/+</sup> wildtype. Equal numbers of each genotype were placed on each slide to avoid batch confounders. Microarray data were processed using both raw and rank invariant protocols provided by Illumina as part of the BeadStation software suite (www.illumina.com). We subsequently log-transformed expression values and stabilized the variance of each array. To identify genes with significant expression difference between the Fmn2<sup>−−</sup> and Fmn2<sup>+/+</sup> cases, we carried out two-tailed t-tests and applied a Bonferroni correction for multiple testing, and selected probes with a minimum adjusted p-value<0.05.

**Bioinformatics Tools**

Classical QTLs counts are based on the April 2008 version of Mouse Genome Informatics (MGI: www.informatics.jax.org) [113]. Search for tRNAs was done using tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) [65]. GO analysis was done using the analytical tool DAVID 2007 (http://david.abcc. nci.nih.gov/) [114]. Overrepresented GO terms were identified and statistical significance of enrichment was calculated using a modified Fisher’s Exact Test or EASE score [115]. We used the Allen Brain Atlas to analyze expression pattern in the brain of young C57BL/6j male mice (www.brain-map.org) [85,116].

**Control for Non-Syntenic Association and Paralogous Regions**

In RI strains, non-syntenic associations can lead to LD between distant loci [89,106]. In the BXDs, we detected such non-syntenic associations between markers in Qrr1 and markers on distal Chr 2 and proximal Chr 15. As a result of these associations, some transcripts that have strong cis- or trans-QTLs in Qrr1 tend to have weak LOD peaks, usually below the suggestive threshold, on distal Chr 2 and proximal Chr15. However, there is no bias for genes located in these intervals in LD with Qrr1 to have trans-QTLs in Qrr1.

The Qrr1 segment has been reported to have paralogues on mouse Chr 1 (proximal region), 2, 3, 6, 7, 9, and 17 [117,118]. We examined if the trans-QTLs in Qrr1 are of genes located in these paralogous regions. However, genes located in the paralogous regions are not overrepresented among the trans-QTL.

**Supporting Information**

**Table S1** Number of classical QTLs in Qrr1 and in hundred other chromosomal intervals.

Found at: doi:10.1371/journal.pgen.1000260.s001 (0.23 MB DOC)

**Table S2** Transcripts of genes associated with seizure or epilepsy that have trans-QTLs in Qrr1p near the seizure susceptibility QTL.

Found at: doi:10.1371/journal.pgen.1000260.s002 (0.05 MB DOC)

**Dataset S1** Precision of Cis-QTLs in Qrr1.

Found at: doi:10.1371/journal.pgen.1000260.s003 (0.13 MB XLS)

**Dataset S2** Gene ontology analysis of transcripts that map to Qrr1p and Qrr1d in the BXD hippocampus dataset.

Found at: doi:10.1371/journal.pgen.1000260.s004 (0.03 MB XLS)

**Dataset S3** tRNAs in Qrr1d.

Found at: doi:10.1371/journal.pgen.1000260.s005 (0.09 MB XLS)

**Dataset S4** Partial correlation analysis.

Found at: doi:10.1371/journal.pgen.1000260.s006 (0.04 MB XLS)

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Author Contributions
Conceived and designed the experiments: LR. Performed the experiments: KM DCC TS. Analyzed the data: KM DCC TS XW RWW. Contributed reagents/materials/analysis tools: LR. Wrote the paper: KM.

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