Hippocampal Gene Expression Analysis Highlights $\text{Ly6a}/\text{Sca-1}$ as Candidate Gene for Previously Mapped Novelty Induced Behaviors in Mice

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Abstract

In this study, we show that the covariation between behavior and gene expression in the brain can help further unravel the determinants of neurobehavioral traits. Previously, a QTL for novelty induced motor activity levels was identified on murine chromosome 15 using consomic strains. With the goal of narrowing down the linked region and possibly identifying the gene underlying the quantitative trait, gene expression data from this F2-population was collected and used for expression QTL analysis. While genetic variation in these mice was limited to chromosome 15, eQTL analysis of gene expression showed strong cis-effects as well as trans-effects elsewhere in the genome. Using weighted gene co-expression network analysis, we were able to identify modules of co-expressed genes related to novelty induced motor activity levels. In eQTL analyses, the expression of $\text{Ly6a}$ (a.k.a. Sca-1) was found to be cis-regulated by chromosome 15. $\text{Ly6a}$ also surfaced in a group of genes resulting from the network analysis that was correlated with behavior. Behavioral analysis of $\text{Ly6a}$ knock-out mice revealed reduced novelty induced motor activity levels when compared to wild type controls, confirming functional importance of $\text{Ly6a}$ in this behavior, possibly through regulating other genes in a pathway. This study shows that gene expression profiling can be used to narrow down a previously identified behavioral QTL in mice, providing support for $\text{Ly6a}$ as a candidate gene for functional involvement in novelty responsiveness.

Introduction

With a prevalence of 10–20% worldwide, mood disorders affect a substantial number of people and finding the genetic risk factors will aid in prevention and treatment [1]. The heritability estimates for mood disorders range from 43% for panic disorder to 28% for anxiety disorder, indicating a genetic component to these disorders [2]. In animal research, behavior and novelty responsiveness are considered to be an important endophenotype in anxiety research [3,4]. These behaviors are used to model different symptoms of mood disorders in mice, mainly fear, fatigue or loss of energy, and avoidance. These symptoms can be diminished when administering anxiolytic drugs [5,6,7]. Exploration behavior has been found to also be significantly heritable in mice [8].

Previously, a panel of mouse chromosome substitution strains (CSS) derived from host C57BL/6J and donor A/J mice [9,10] was screened in several behavioral tests, including exposure to an open field arena and an automated home cage environment [3]. Subsequent fine-mapping in an F2-population revealed quantitative trait loci (QTL) for several novelty induced motor activity parameters on chromosome 15 [11]. The QTL region at mouse chromosome 15 has been implicated in these exploration behaviors before [12,13].

The current study aims to explore the usefulness of genome-wide gene expression profiles for narrowing down quantitative trait loci (QTL) for behavioral parameters in mouse. Whole genome expression arrays were performed on hippocampal brain tissue of the same chromosome 15 F2 mouse population that was previously used for genetic mapping [11]. Because novelty induced locomotor activity is thought to reflect an endophenotype for anxiety, the hippocampus was selected because of its role in emotion and cognition [14] and locomotor behavior in rodent species [15,16].
Expression QTL (eQTL) analysis identified a number of cis- and trans-effects related to genetic variation at chromosome 15. In addition, we applied a weighted gene co-expression analysis (WGCNA) to discover networks of interconnected genes that are related to novelty induced motor activity levels [17,18,19,20,21]. Results from both the eQTL and network analysis indicated that genetic variation on chromosome 15 has genome-wide effects on expression. We also found that gene expression networks can be related to novelty induced motor activity parameters and can be used as tool in behavioral research. Most importantly, overlap between results from these methods revealed one gene, Ly6a, which we subsequently found to have a functional effect in gene knock-out mice. This study shows that gene expression data can be used to reveal relations between genomic variation, gene expression information, and behavioral parameters.

Results

Data preprocessing

After quality control, expression arrays were background corrected, transformed and normalized according to the Lumi procedure [22]. Genes were then filtered based on detection values generated by Beadstudio®. We set the detection p-value at 0.01, leaving 13,450 of the 24,620 features on the microarrays for analysis. Figure 1 shows a diagram illustrating the approach of this study.

Behavioral QTL regions

Behavior has been recorded in an automated home cage system, described before [11]. In short, the home cage environment is equipped with a home base shelter, a drinking spout, and two feeding platforms. On one feeding platform the mouse is exposed to the environment while the other platform allows sheltered feeding. The top unit contains an infrared camera and infrared light-emitting diode lights allowing continuous recording independent of lighting conditions in the test room.

Behavioral analysis was previously performed on 57 female CSS15F2 mice. A sex-specific effect of the A/J chromosome on the phenotypes of interest was observed and the behaviors were mapped for females only. In this study, a region for avoidance behavior parameters was mapped to chromosome 15 [11]. Since only one chromosome, i.e. chromosome 15, was interrogated, significance for the behavioral QTLs in a chromosome substitution strain was set at logarithm of odds (LOD)≥1.5 with a support interval of –LOD1 [11,23]. The –LOD1 support interval of the parameter DM1 (distance moved on day 1) is 66,864,123 bp–90,864,125 bp, with the peak marker (rs13482668) at position 80,750,829 bp (mouse genome build 36.1) with a LOD score of 1.61. The interval for FVSOP1 (frequency of visiting exposed platform on day 1) is 76,492,248 bp–99,864,125 bp, peaking at the same marker with a maximum LOD score of 2.18. Plots are shown in Figure 2. The behavioral parameters are significantly correlated (r = 0.75, p = 9.2e-12).

Expression QTL analysis

For expression QTL (eQTL) analysis, gene expression values are taken as phenotypes for genetic mapping. They were tested for association with genetic markers on chromosome 15, the only region variable between individual animals in our experiment. 1,000 random permutations resulted in a significance level of LOD≥3.22 for an estimated false discovery rate of 1%. This generated 136 significant eQTLs, of which 19 (14%) were located on chromosome 15. Of the top 10 probes (LOD≥4.93), 9 were located on chromosome 15. Multiple significant probes targeting genes on chromosome 15 were found to contain a SNP in the probe sequence (6 probes, of which 4 in top-10 results) and are likely to represent an artifact; these were removed from further analyses [24].

To identify possible candidate genes for the behaviors of interest, we looked at overlap between eQTLs and the region associated to behavior. 53 eQTLs (of which 10 target genes located on chromosome 15) overlap with both behavioral QTLs, while 27 more eQTLs (of which 3 target genes located on chromosome 15) only overlap with the broader peak of DM1. A list of significant eQTLs on chromosome 15 and their overlap with the behavioral QTL is given in Table 1. To confirm actual expression differences (p<0.01) between the parental strains A/J
and C57BL/6J, we consulted another gene expression dataset that was available [24] Card10 and 1700088E04Rik are over-expressed in A/J compared to C57BL/6J. Ly6a, Ly6c and Sub1 have lower gene expression levels in A/J than in C57BL/6J. Values for all the significant genes in the eQTL analysis can be found in Table S1.

Weighted gene co-expression analysis

Network construction and module detection. We used a network-based approach (weighted gene co-expression network analysis, WGCNA) to define clusters of co-expressed genes ('modules'). It has been hypothesized that these modules represent biological meaningful networks. Once reconstructed, the correlation between each of the modules and the phenotype of interest is being examined. In other words, rather than generating a list of differentially expressed genes, this approach reconstructs networks of genes with related expression profiles that are thought to represent biological meaningful correlations. A detailed description can be found in the Methods section. In short, a weighted adjacency matrix containing pair-wise connection strengths was constructed by using the soft-thresholding approach ($k=7$) on the matrix of pair-wise correlation coefficients. A connectivity measure ($k$) per gene was calculated by summing the connection strengths with other genes. Modules were defined as branches of a hierarchical clustering tree using a dissimilarity measure (1 - topological overlap matrix) [20,25]. Each module (or branch) is subsequently assigned a unique color label which is visualized in the color band underneath the cluster tree (Figure 3). Our module detection method followed the standard WGCNA approach, which has been successfully used in multiple applications [18,19,20,21,26]. We identified 18 modules ranging in size from 25 genes in the Grey60 module to 868 in the Turquoise module.

Modules related to behavioral parameters. The next step was to identify the modules that are related to the behavioral measures of interest, DM1 (distance moved on day 1) and FVSOP1 (frequency of visits to exposed platform on day 1). When modules are related to these novelty induced motor activity phenotypes, the gene content can represent a biological pathway and harbor possible candidate genes.

To define a representative module expression profile (referred to as the module eigengene value), we summarized the (standardized) gene expression profiles of the module by their first principal component. The module eigengene can be considered a weighted (quantitative) average of the gene expression profiles in the module. The correlation between the module eigengene and the sample trait of interest is referred to as 'eigengene significance'. A standard correlation test can be used to assess the statistical significance ($p$-value) of the eigengene significance.

Because DM1 and FVSOP1 both measure novelty induced motor activity levels, modules that overlap in these parameters were chosen as the focus of subsequent analyses. The Turquoise (868 probes; DM1 $r = 0.36$, $p = 0.03$; FVSOP1 $r = 0.39$, $p = 0.02$), Midnight blue (76 probes; DM1 $r = 0.38$, $p = 0.02$; FVSOP1 $r = 0.46$, $p = 0.004$), Lightcyan (72 probes; DM1 $r = 0.36$, $p = 0.03$; FVSOP1 $r = 0.42$, $p = 0.01$), Pink (177 probes; DM1 $r = -0.35$, $p = 0.03$; FVSOP1 $r = -0.39$, $p = 0.02$) and Purple (138 probes; DM1 $r = -0.38$, $p = 0.02$; FVSOP1 $r = -0.45$, $p = 0.005$) modules were shown to be most interesting for DM1 and FVSOP1. Figure S1 represents a heat map showing correlations and corresponding $p$-values for each module with DM1 and FVSOP1, as well as the genetic markers used. Values for all probes within the significant modules are listed in Table S2.

Genetic markers were most correlated with the Green (226 probes) Grey60 (25 probes), Black (184 probes), Blue (481 probes), and Grey module (30 probes). Only the Turquoise module had three probes containing a SNP in the sequence, constituting 0.35% of the total probes in that module.

To confirm actual expression differences between the parental mouse strains A/J and C57BL/6J, we consulted an independent gene expression dataset of hippocampal tissue which was available [24]. Josd1 and Ly6a are under- and Cog5 is over-expressed in A/J vs. C57BL/6J.
Overlap of WGCNA and eQTL results

Of the 136 eQTLs, 28 fell within the modules resulting from the WGCNA. Of these, 26 were trans-regulated genes and they were found only in the Brown (1 probe), Grey60 (5 probes), Grey (4 probes), Blue (3 probes), Green (3 probes) and Black (13 probes) modules, which were shown to be associated to genetic markers in WGCNA. Two genes located on and controlled by chromosome 15 appeared in the modules: Zfmp2 in Black and Ly6a in Turquoise. Ly6a is the only gene emerging in both lines of evidence related to our phenotype of interest. The expression QTL and location of the gene (74,828,318 bp–74,825,307 bp) is shown in Figure 2. The expression QTL of Ly6a covers a broad region but with a peak at the same location as the behavioral QT, rs13482668 (80,750,829 bp). At this location, expression of Ly6a shows an additive effect with higher expression for the C57BL/6J allele. The gene is located in the confidence interval of DM1 and at the border of that of FVSOP1. Limited genetic resolution interferes with precise indication of the QTLs, however, the results show that regulation of Ly6a expression peaks at the same genomic region as that of the behavioral QTL. Ly6a is found in the Turquoise module, which was found to be positively correlated to both behavioral parameters. The individual gene expression value of Ly6a was significantly positively correlated to both FVSOP1 (r = 0.44, p = 0.007) and DM1 (r = 0.34, p = 0.04). To exclude

Table 1. Genes on chromosome 15 resulting from eQTL analysis.

<table>
<thead>
<tr>
<th>Illumina Probe ID</th>
<th>Max LOD score</th>
<th>Peak Genomic position (bp)</th>
<th>Accession</th>
<th>Overlap behavioral QTLs</th>
<th>Gene in QTL</th>
</tr>
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<tr>
<td>1240142*</td>
<td>22.98</td>
<td>77864125</td>
<td>NM_199200.1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>2260593*</td>
<td>12.33</td>
<td>79864125</td>
<td>NM_024177.2</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>3940324*</td>
<td>8.47</td>
<td>59864125</td>
<td>NM_028035.2</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>6520204</td>
<td>7.67</td>
<td>72864125</td>
<td>NM_172946.1</td>
<td>dm1</td>
<td>dm1/fvsop1</td>
</tr>
<tr>
<td>6650193</td>
<td>7.06</td>
<td>61864125</td>
<td>NM_016905.1</td>
<td>dm1</td>
<td>dm1/fvsop1</td>
</tr>
<tr>
<td>5550400*</td>
<td>6.13</td>
<td>76338361</td>
<td>NM_007554.1</td>
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<td>*</td>
</tr>
<tr>
<td>1050288</td>
<td>5.4</td>
<td>85864125</td>
<td>NM_013842.2</td>
<td>dm1</td>
<td>dm1/fvsop1</td>
</tr>
</tbody>
</table>

Expression QTL analysis of CSS15F2 mouse population yielded 19 results on chromosome 15. Maximum LOD score, the peak marker position and the –LOD 1 support interval is listed. In addition, we have indicated whether the eQTL and/or the gene itself was located in the behavioral QTLs. Asterisks indicate the probes of which the sequence contains a SNP polymorphic between A/J and C57BL/6J.

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Figure 3. Network construction identifies distinct modules of co-expressed genes using hippocampus samples of CSS15F2 population (n = 37). The dendrogram was produced by average linkage hierarchical clustering of genes using topological overlap. Modules of co-expressed genes were assigned colors corresponding to the branches indicated by the horizontal bar beneath the dendrogram.

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possible non-specificity of the probe sequence causing random hybridization signals, the probe sequence was blasted against the entire mouse genome (NCBI Blast; http://blast.ncbi.nlm.nih.gov/Blast.cgi). No non-specific binding sites were detected.

**Behavioral testing Ly6a−/− mice**

In order to assess the functional relationship between novelty-induced motor activity levels and Ly6a−/−, female Ly6a−/− mice (C57BL/6J background, n = 8) and C57BL/6J wildtype mice (n = 9) were tested using an automated open field system. Total distance moved (in cm) was used as a measure of motor activity levels. Although it was impossible to test the mice in the exact same apparatus at this location, the novelty induced locomotor activity parameters in the home cage and the total distance moved in the open field arena both assess locomotor activity of the mice in the first hours they experience and explore their new environment.

A repeated measures ANOVA revealed a significant main effect of genotype on total distance moved in the six 5-minute bins (F(1,15) = 19.03, p = 0.001). Bins were tested separately using an independent sample t-test (Bonferroni corrected for multiple testing) revealing significantly lower activity of Ly6a−/− mice in bin 1 (t(15) = -3.83, p = 0.012), bin 2 (t(15) = -3.82, p = 0.012), bin 3 (t(15) = -4.14, p = 0.009) and bin 5 (t(15) = -4.11, p = 0.006). Data is shown in Figure 4.

The mice were tested again in the automated open field system in the dark phase. Again, the repeated measures ANOVA revealed a significant main effect of genotype on total distance moved on the six bins (F(1,15) = 15.01, p = 0.001) with lower activity for the Ly6a−/− mice. Independent sample t-tests (Bonferroni corrected for multiple testing) showed genotype differences for bin 2 (t(15) = -3.09, p = 0.042), bin 4 (t(15) = -3.59. p = 0.018), and bin 5 (t(15) = -3.11, p = 0.042).

To assess whether these differences are due to an effect of differences in motor function between wild type and knockout strains, a variable ‘velocity’ was computed by dividing the distance moved (in centimeters) by the duration (in seconds) over all 6 bins of 5 minutes in periphery and center. There was no significant difference in velocity between C57BL/6J and Ly6a−/− mice (t(15) = -1.11, p = 0.284). In addition, mice were subjected to a rotarod test (described in Methods section) [27]. The average latency to fall across three trials did not differ significantly between wildtype and Ly6a−/− mice (t(15) = -1.20, p = 0.25). Results are shown in Supporting Information S1.

**Figure 4. Behavioral analysis of Ly6a−/− mice confirms involvement in novelty induced phenotypes.** Ly6a−/− (n = 8) and wildtype animals (n = 9) were tested in an automated open field system for 30 minutes. The data was analyzed using a repeated measures ANOVA for 6 bins of 5 minutes, after which individual bins were tested using independent t-tests (Bonferroni corrected). Total distance moved (in cm) is depicted in the bar diagram. The line graph shows the distance moved of Ly6a−/− and wildtype mice over the six bins. The error bars represent the standard deviation (SD). Stars indicate significance (Bonferroni corrected p<0.05).

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**Discussion**

The aim of this study was to use gene expression data to narrow down a QTL found to be associated to exploration and novelty-induced behavior. This behavioral QTL was mapped to chromosome 15 in a previous study, using a female F2-population of the chromosome substitution strain [11]. We generated whole genome expression data for this population and calculated expression QTLs to identify genes of which expression is regulated by the region of interest. Next, we applied a network based approach to define clusters of co-expressed genes (‘modules’) which are related to our phenotypes of interest. Ly6a (a.k.a. Scn1) was found to be located on and regulated by the same region on chromosome 15 harboring the behavioral QTL. In addition, it belongs to a module correlated to novelty induced motor activity levels. These lines of evidence led us to test the exploration and novelty-induced behavior in Ly6a knock-out mice. The behavior was confirmed in this mouse knock-out strain, thereby providing support for Ly6a as candidate gene for functional involvement in novelty responsiveness.

This study yields insight in the relationships between genomic variation, gene expression, and behavior. By investigating a F2-population derived from a chromosome substitution strain, we know a priori that genetic variation that can be related to behavior and gene expression in this population can only stem from chromosome 15, since the rest of the genome is uniformly C57BL/6J.

Results from both the eQTL and weighted gene co-expression network analysis indicate that genetic variation at chromosome 15 has genome-wide effects on gene expression. The majority of eQTLs found were target genes located elsewhere (trans), although the cis-effects were relatively stronger. The number of cis-effects was comparable to those found on chromosome 15 in studies using recombinant inbred strains or outbred populations [28,29]. The network analysis we carried out used only the most varying gene expression values to create a gene co-expression network. When examining results from the eQTL and network analyses, we find the genes that were shown to be regulated by chromosome 15 in the eQTL analysis were also found in the modules of the network analysis correlated to the genetic markers, thereby confirming their relation with this genomic location through separate statistical methods. In addition, we were able to link networks of co-expressed genes with novelty induced motor activity levels. This
show that gene expression can be used as a tool in behavioral research.

The overlap between genes found by these methods can relate genomic variation, expression information, and behavioral parameters. The only gene that was found to be regulated by chromosome 15 and identified in a module positively related to behavior of interest was Ly6a. This gene was also found to be significantly higher expressed in C57BL/6j than A/J hippocampus in an independent dataset.

Ly6a (lymphocyte antigen 6 complex, locus A) is an 18-kDa mouse glycosyl phosphatidylinositol-anchored cell surface protein of the Ly6 gene family. This gene has been linked to stem/progenitor cell activity and cancer. The Ly6 proteins are thought to regulate or coactivate cell signaling, although the exact mechanism by which they do so is unclear. For several members of the Ly6 gene family, human orthologs have been identified. The syntenic region of Ly6a, along with other adjacent genes, is absent in rat [30]. There is also no human ortholog of Ly6a, yet other Ly6 family genes may function of Ly6a in a paralogous fashion. In addition, co-expression can indicate activation of genes belonging to a biological pathway. Ly6a could be affecting behavior indirectly through other genes in a pathway. Therefore, other genes in this module could be ‘guilty by association’ and have an effect on human behavior as well.

In order to functionally test the involvement of Ly6a in novelty induced motor activity levels, we examined behavior of the Ly6a knock-out strain. Thus far, the only phenotype studied in these mice was the altered proliferative response of their T-cells. The knock-out animals show a higher and more prolonged proliferation in response to T cell receptor-mediated activation, supporting a cell-signaling role for Ly6a [31]. No behavioral testing for the Ly6a knock-out strain had been described. The novelty-induced behavior was tested in an automated open field system. Although it was not possible to test the mice in the same home cage environment that was used before, the open field offers a close approximation of the behavior of interest and is generally used to assess novelty induced motor activity levels. In the original screen, distance moved in the open field arena (OF.DMA) was also assessed (data not shown). This parameter was positively, but not significantly, correlated to our parameters of interest (FVSOP1 & OF.DMA \( r = 0.24, p = 0.07 \), DM1 & OF.DMA \( r = 0.14, p = 0.31 \) ) However, the correlation of OF.DMA with the expression of Ly6a is significant and shows the same directionality (OF.DMA & Ly6a \( r = 0.36, p = 0.03 \) ). In addition, in the original behavioral mapping, one of the peak markers of OF.DMA is the same as that of FVSOP1 and DM1 (rs134482612), containing Ly6a. The QTL pattern and thus possibly etiology of OF.DMA is related to FVSOP1 and DM1, but somewhat more complex and this might explain the lack of significance [11]. The Ly6a \(^{-/-} \) mice showed significantly lower novelty induced ambulation in the open field recording when compared to C57BL/6j wildtype animals. This is consistent with our findings in the F2 population and therefore suggests that Ly6a is functionally involved in novelty induced motor activity levels. The C57BL/6j wildtype mice were not littersmates of the Ly6a \(^{-/-} \) mice, so separate breeding could have a confounding effect when comparing the two strains to each other. A trait-correlation of Ly6a expression was performed using the GeneNetwork website containing data of recombinant inbred strains (http://www.genenetwork.org, July 2010, sorted on correlation value). Phenotypes significantly associated with the expression of this gene contained three parameters related to novelty responsiveness in the top-6 parameters. Thus, even though the relation between the ontology of this gene in the light of novelty responsiveness related behaviors is unclear [30], the association appears to be consistent across independent datasets obtained from different mouse genetic backgrounds. Due to its involvement in stem cell activity [30], it is tempting to hypothesize that Ly6a is involved in hippocampal plasticity and influences novelty-induced behavior through this mechanism. In addition, it is possible that this gene exerts subtle influences on many other genes in its Turquoise module, or genes not detectable by microarray. Its gene family members, Ly6c and Ly6e1, appear in the results of eQTL and WGCNA analyses, respectively.

Ly6a is expressed in many tissues and it is involved in many receptor-ligand interactions. For example, it is expressed in muscle cells, where it interacts with caveolins, which in turn are implicated in forms of muscular dystrophy [32]. In addition, these mice show age-dependent signs of osteoporosis [33]. Although this effect appears in older mice (peaking at 7 months), we wanted to exclude possible motor defects by subjecting the mice to a rotarod test. The results show that the latency to fall does not differ between strains. Therefore, it is unlikely that a gross motor defect is responsible for the behavioral phenotype found in this study. Although the Ly6a \(^{-/-} \) had a significantly higher bodyweight than the wildtype animals (\( g(15) = 2.88, p = 0.011 \)) this was not correlated to latency to fall (\( r = -0.47, p = 0.055 \)).

In conclusion, our results have revealed insights inter-relating genotype variation, gene expression, and behavioral data. We explored how to link genomic information, gene expression values, and behavioral parameters by combining statistical methods (i.e. eQTL and WGCNA). The combined approach highlighted the gene Ly6a. Subsequently, this gene was found to have a functional effect in the knock-out mouse. Although the association of Ly6a appears to be consistent, the ontology of this gene in light of novelty responsiveness related behaviors warrants further investigation.

Materials and Methods

**CSS15F2 mice**

We used a female F2-population of chromosome substitution strain 15 for this experiment as described elsewhere [11]. At least two weeks prior to the start of the experiment, the animals were moved from the stables to the adaptation room next to the experimental room. The animals were then 10–14 weeks old. They were tested consecutively in the open field, automated home cage environment, elevated plus maze, and light-dark box (fixed order), with a minimum of 1.5 weeks between tests. A description of these tests can be found in [11]. In short, the automated home cage environment is equipped with a home base shelter (in which mice mainly sleep during the light phase), a drinking spout, and two feeding platforms. On one feeding platform the mouse is exposed to the environment while the other platform allows sheltered feeding. The top unit contains an infrared camera and infrared light-emitting diode lights allowing continuous recording independent of lighting conditions in the test room. For the current study, the behavioral parameters of the automated home cage environment were further studied: distance moved on day 1 (DM1) and frequency of visiting the exposed platform on day 1 (FVSOP1). These parameters measure exploration during novelty and are assessed by means of motor activity levels (horizontal distance moved) during the first day of exposure to a novel environment. Genotypes were determined using SNPs and microsatellites as described before [11] Additional genotyping of two SNPs was performed in the CSS15F2 population in the current study. Primers were designed in ENSMBL, to generate a PCR product. Sequencing was performed according to standard protocols on an ABI 3730 (Applied Biosystems) sequencer. A list of markers and positions can be found in Table S3.
Dissection procedure

Mice were sacrificed at 3–4 months of age and their brains were
quickly removed, frozen in liquid nitrogen, and stored at −80°C.
After dissection this resulted in 37 hippocampal samples. The
hippocampus has been linked to explorative behavior and is
therefore a candidate region regarding our phenotypes of interest
[34]. Brain samples (left hemisphere) were thawed from −80°C
storage to −8°C in cryostat. Coronal sections of 300 μm thickness
were taken. Frozen sections were laid down on a cooled steel plate,
covered with parafilm and immediately covered with RNAlater
(Ambion, #AM7024). Selected brain regions were punched out
using a stainless steel punch needle (1 mm in diameter) filled with
RNAlater connected to a syringe. Hippocampal tissue was taken in
6 sections starting at −2.06 mm Bregma, with two punches taken
bilaterally in the first three sections, and three in the last three,
aiming for 15 punches. These punches were pooled for each
individual mouse prior to RNA isolation.

RNA isolation and microarrays

RNAlater was pipetted off the samples. Punches were
homogenized using disposable pestels (Fisher Scientific Pellet
Stamp, #749521-1500). Phase separation was achieved using
750 μl TRizol reagent (Invitrogen, #15960-018) and 200 μl
chloroform (Merck, #R11490), after which samples were
precipitated in 500 μl isopropanol (Merck, #1.0934.2500).
DNase treatment (Qiagen Quanase-Free DNase Set, #79254)
was applied using the manufacturer’s protocol, followed by an RNA
clean-up procedure (Qiagen RNeasy MinElute columns, #74204).
Samples were stored at −80°C. Total RNA concentration was
determined using the Nanodrop ND-1000 and RNA quality was
determined using the Illumina TotalPrep kit amplification and
itTM Ribogreen,

Statistical analysis gene expression data

We used Beadstudio® software version 3.2.3. to extract raw
data and generate background, corrected gene-expression data.
Further pre-processing was done using the Lumi package for R
[22]. We applied a variance stabilizing transformation to preserve
much of the gene expression variance and normalized data using
the robust spline normalization method [35]. Chip quality and
outlier detection was done by assessing quality statistics and plots
before and after transformation and normalization. Both behav-
ioral and expression QTL analysis were performed using the r/qtl
package in R. We used the multiple imputation method,
performing interval mapping (no SNP cofactors) and imputation
of missing genotypes [36] and performed 1000 random permuta-
tions to determine the significance level for eQTL results.
Significance for the behavioral QTLs in a chromosome substitu-
tion strain (LOD≥1.5) was set before [11].

Modules of co-expressed genes were identified using the
weighted gene co-expression network analysis (WGCNA), de-
veloped by Zhang and Horvath [20,21]. First, we constructed a
 correlation matrix for these genes. This matrix was then raised to a
power (β = 7 in this study) to achieve an adjacency matrix
holding connection strengths. Connectivity is defined as the sum of
connection strengths with the other network genes. With this, a
topological overlap measure was calculated based on the number
of shared neighbors. A hierarchical clustering tree of the genes was
then constructed with these values, of which branches were cut
with a dynamic tree cut algorithm to define the modules [37]. This
method allows for testing the significance of the modules in the
network to either a continuous or dichotomous outcome measure
by assessing module eigengene significance. The module eigene-
gene is the first principal component of a module and therefore
represents a single gene expression value for all genes in a module
per sample. The importance of genes can be assessed by looking at
both gene significance and connectivity measures in the whole
network and within modules. Analyses assessing polymorphisms
between mouse inbred strains in probe sequences were performed
in silico as described before [24].

Ly6a−/− mice

Ly6a−/− mice on a C56BL/6J background were generated for
previous studies [31,38]. These mice were backcrossed to C57BL/
6J for more than 10 generations and then intercrossed to establish
+/+ and −/− mice. The Ly6a−/− mice were tested at a different
location; the Stanford laboratory in Toronto, Canada. All
procedures were approved by the animal care committee (ACC)
at the Toronto Centre for Phenogenomics. Guidelines for ethical
use of animals in research that TCP’s ACC uses are provided by
the Canadian Council on Animal Care (Protocol Permit # 2000-
8138). The mice were group housed in a 12 hour light/12 hour
dark cycle with ad libitum access to water and food. For the current
study 8 Ly6a−/− and 9 wildtype C57BL/6J mice were tested, all
female and between 4 and 4.5 months of age. All mice were test-
naive. At least two weeks prior to the start of the experiment, the
animals were moved from the stables to the adaptation room next
to the experimental room. They were tested the last two hours of
their normal light/dark cycle in a fully automated open field
system (Versamax Animal Activity Monitor, AccuScan Instru-
ments). This uses horizontal and vertical x, y, and z sensors to
detect the position and behavior of the animal in 5 minute bins for
30 minutes. Total activity in centimeters recorded in the cage was
taken to represent total distance moved. Repeated measures
ANOVA was used to assess main effects of genotype across bins,
followed by independent t-tests (Bonferroni corrected) per bin.
Mice were also subjected to a rotarod test (Economex Rota Rod,
Columbus Instruments USA), testing their motor co-ordination
and balance. Rotorod diameter was 4 centimeters and 4 rpm
( rotations per minute) constant speed. The latency to fall in
seconds was tested in three trials separated by 15 min inter-trial
intervals. An independent t-test was used to assess significance.

Supporting Information

Table S1 Genes resulting from eQTL analysis. Expression
QTL analysis of CSS15F2 mouse population yields 19 results
on chromosome 15. Maximum LOD score, the peak marker
position and the –LOD1 support interval are listed. In addition, it
is indicated whether the eQTL and/or the gene itself is located in
the behavioral QTLs. Asterisks indicate probe of which the
sequence contains a SNP polymorphic between A/J and C57BL/
6J.

Table S2 WGCNA results significant modules. Weighted
gene co-expression analysis of CSS15F2 mouse population yields
five modules related to the phenotypic parameters of interest.
Importance of genes in the whole network can be assessed by
whole network connectivity. Hubs within modules are identified using the within-module connectivity measure. Asterisks indicate probe of which the sequence contains a SNP polymorphic between A/J and C57BL/6j.

Figure S1  Heat map depicting module eigengene correlations and corresponding p-values. Module eigengenes of the gene co-expression network are correlated with behavioral measures DM1 and FVSOP1 to identify modules of interest.

Table S3  Genetic markers and their locations.

Supporting Information S1 Behavioral analysis Ly6a−/− mice. Visual representations of behavioral recordings in an automated home cage (A) and rotarod (B) of Ly6a−/− mice and C57BL/6j wildtype mice.

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Author Contributions

Conceived and designed the experiments: RAO MJHK. Performed the experiments: AGEd+m+M HO EJ SdJ IV. Analyzed the data: SdJ CRF IV. Contributed reagents/materials/analysis tools: WLS JK. Wrote the paper: SdJ.

References