Glutamate receptor metabotropic 7 is cis-regulated in the mouse brain and modulates alcohol drinking☆

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Abstract

Alcoholism is a heritable disease that afflicts about 8% of the adult population. Its development and symptoms, such as craving, loss of control, physical dependence, and tolerance, have been linked to changes in mesolimbic, mesocortical neurotransmitter systems utilizing biogenic amines, GABA, and glutamate. Identification of genes predisposing to alcoholism, or to alcohol-related behaviors in animal models, has been elusive because of variable interactions of multiple genes with relatively small individual effect size and sensitivity of the predisposing genotype to lifestyle and environmental factors. Here, using near-isogenic advanced animal models with reduced genetic background interactions, we integrate gene mapping and gene mRNA expression data in segregating and congenic mice and identify glutamate receptor metabotropic 7 (Grm7) as a cis-regulated gene for alcohol consumption. Traditionally, the mesoaccumbal dopamine reward hypothesis of addiction and the role of the ionotropic glutamate receptors have been emphasized. Our results lend support to an emerging direction of research on the role of metabotropic glutamate receptors in alcoholism and drug addiction. These data suggest for the first time that Grm7 is a risk factor for alcohol drinking and a new target in addiction therapy.

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Genetic predisposition is a significant factor in the expression of most of the common diseases, such as high blood pressure, diabetes, cancer, depression, alcoholism, and Alzheimer disease. Although from a societal point of view common diseases are in the focus, in most common diseases progress in identifying genes has been limited to a small percentage of individuals with fully penetrant genes of large effect. Therefore, in general, most of the research efforts have focused on the biological effects of these major genes. The vast majority of a population with a common disease is characterized by a complex phenotype with the involvement of a network of multiple interacting genes, each with individually small effect size against a background of variable environmental influences. Despite intensive research efforts, progress in identifying these minor genes has been slow. In alcoholism, even in animal models of alcohol-related behaviors [1–5], the contribution of defined genetic factors seems certain in only very few cases.

Most of the preclinical gene mapping studies with animal models have been based on recombinant inbred (RI) or Mendelian segregating population designs. In RI or Mendelian designs in each strain or in each individual, respectively, the...
genetic background is unique and represents a specific source of genetic interaction. This interaction between the target gene and the background may substantially contribute to the total genetic variance and may limit detection of polygenes with small effect size reliably. Recently, to reduce genetic background noise, we developed a different design with 78 near-isogenic inbred strains of ≥97% C57BL/6ByJ (B6By) genetic background identity and mapped six quantitative trait loci (QTLs) for alcohol consumption [6,7]. Given this and the availability of near-isogenic strains as convenient precursors for rapid development of congenic strains, we tested different genetic designs to confirm linkage between alcohol consumption and one of the QTLs, ethyl alcohol consumption-2 locus (Eac2) mapped to distal Chr. 6, and used bioinformatic methods to examine the Eac2 region in a search for the underlying quantitative trait gene (QTG).

Here, we report an integrated genome and transcriptome analysis of the Eac2 region on Chr. 6, which suggests that a gene coding for metabotropic glutamate receptor subtype-7 (Grm7) is a candidate QTG for Eac2.

Results

Alcohol consumption in a congenic strain

Using microsatellite marker-assisted selection and repeated backcrosses to the alcohol-preferring B6By background strain, we developed a new congenic strain, B6By.C.6.132.54 (B6By.C6). This congenic strain carries a BALB/cJ (C) donor chromosome segment on Chr. 6 with proximal and distal background markers D6Mit275 (25.5 cM, 51.1 Mb, from UniSTS annotation of NCBI build 36) and D6Mit134 (57.5 cM, 125.3 Mb), respectively. The congenic strain in a two-bottle free-choice alcohol preference test demonstrated significantly lower consumption of 12% (v/v) alcohol in comparison to its background partner B6By, confirming a significant decreasing effect of the genetic factor(s) residing on the donor segment (Fig. 1A). In an additional independent test, using a two-bottle choice paradigm with intermittent access to alcohol, we found that consumption of 12% alcohol was significantly higher among B6By males (12.70 ± 1.03, n = 13) in comparison with B6By.C6 males [8.37 ± 0.96 (g/kg/day, mean ± SE), n = 19, average of four 3-day trials, t30 = 3.00, p = 0.005, two-tailed].

To test the hypothesis that other undetected donor chromosome segments are responsible for this effect, a genome scan with a mouse single-nucleotide polymorphism (SNP) panel of 402 markers was carried out. No additional donor segments were detected (Fig. S1). Because the genome scan showed flanking background markers at 75.9 Mb (rs4226008; NCBI mouse build 36/dbSNP build 126) and 122.3 Mb (rs3023093), and limiting donor markers at 81.9 (rs4226024) and at 91.8 Mb (rs3712161), we concluded the segment size must be between 9.9 and 46.4 Mb.

Exclusion of identical-by-descent regions

For narrowing the set of candidate genes, first, the donor segment in the B6By.C6 congenic strain was subjected to...
bioinformatic analysis. We took advantage of the successful DNA resequencing and SNP discovery efforts overseen by the National Institute of Environmental Health Sciences (NIEHS), which recently made available 8.3 million SNPs among the genomes of 15 diverse inbred strains (http://www.niehs.nih.gov/oc/news.snp2.htm). Bioinformatic analysis of the Chr. 6 segment between 75.9 and 122.3 Mb indicated 404 known genes. The mapping and congenic data suggest that one or more DNA differences in the region between B6By and C are the ultimate cause of the QTLs. Because most inbred laboratory mouse strains [8], including the progenitor strains, shared a common ancestor [9], some regions of their genome will be largely identical by descent (IBD). IBD regions, which are not considered strong positional candidates [10], can be identified by a low frequency of SNPs between two inbred strains. Candidate genes are more likely to fall in non-IBD regions; therefore positional cloning should focus on subregions where the progenitors differ. We analyzed the SNP distribution histogram between B6 and BALB/cByJ (CBy) on Chr. 6 between 75.9 and 122.7 Mb (http://www.jax.org/phenome/snp.html). Identifying 11 IBD regions (SNP frequency $<$20/0.1 Mb: 77.4–78.1, 78.6–80.2, 83.8–85.6, 86.2–86.8, 95.6–96.6, 98.6–100.9, 102.4–104.1, 107.4–107.6, 112.4–118.8, 121.8–122.3 Mb), we could reduce the number of candidate genes on the maximum segment length (46.4 Mb) to 212 genes. Application of this criterion filtered out, for example, Slc6a1 (114.85 Mb; neurotransmitter transporter, GABA, member 1), an otherwise likely candidate (Fig. 2).

Structural polymorphism

The Chr. 6 interval between 75.9 and 122.7 Mb was searched for coding-nonsynonymous SNPs (CNs) between B6, CBy, and C. Genes (AW146020, Htra2, Dguok, Frmd4b, Pdzrn3, Cntn6, Setmar, EG640530) harboring a total of nine CNs fell in a non-IBD region, while two CNs (one each in Ppp4r2 and Mug2) fell in IBD regions, though near the borders. These results are consistent with the expected functionality of the IBD filter. Effects of these CNs on protein function, interaction, and thermodynamic stability are not known.

Analysis of Gene Ontology (GO) classifications (which was not available for Ppp4r2 or predicted gene EG640530) suggested that the expressed sequence AW146020 has DNA binding activity and is involved in the regulation of DNA-dependent transcription; Htra2, serine peptidase 2, is involved in the regulation of body size, adult locomotor/walking behavior and neuron development, mitochondrion organization and biogenesis, apoptosis, and proteolysis; Dguok, deoxyguanosine kinase, is involved in a nucleic acid metabolic process and protein amino acid phosphorylation; and Frmd4b, FERM domain-containing 4B, is in the cytoplasm and in a membrane location. GO inferences based on these domains also imply that the gene product has cytoskeletal protein binding activity. Pdzrn3, PDZ domain-containing RING finger 3, has zinc ion binding activity and is involved in an intracellular signaling cascade, the gene product has protein binding activity; Cntn6, contactin 6, has transporter activity and receptor activity and is involved in transport, has wide-spectrum protease inhibitor activity, and has serine-type endopeptidase inhibitor activity. Although the available information is insufficient to exclude these genes, the known functions do not suggest obvious plausible links to alcohol drinking preference or addiction.

Fig. 2. Narrowing of a set of candidate genes. Identification of IBD regions by analysis of SNP distribution histograms (http://www.jax.org/phenome). IBD regions were identified by low frequency of SNPs between B6 and C (SNP frequency $<$20/0.1 Mb). Gray bars represent the number of SNP locations for which allele data were available for both C57BL/6J and BALB/cByJ. Red bars represent the number of SNP locations at which alleles for C57BL/6J and BALB/cByJ are different. This representative region, near the end of the donor segment in the congenic B6.C6 strain, illustrates how Slc6a1 (a likely candidate for alcohol-related behaviors, at 114.246521 Mb) was filtered out.
**Cis-regulated genes**

In several recent studies it has been recommended that a set of candidate genes can be narrowed by requiring the gene to be expressed, to be correlated with the phenotype of interest, and to have cis-regulated expression [11–17]. To filter the remaining 212 genes for expression and genotype-dependent transcript abundance we analyzed gene expression data obtained from the CXB RI strain set. Gene expression QTL mapping of the CXB RI strains is highly informative for our study because the progenitors of the RI and RQI strains are identical (C57BL/6By) or closely related (BALB/cBy vs BALB/cJ). Interval QTL mapping of mRNA abundance of the 212 genes was implemented using the Hippocampus Consortium M430v2 CXB (Dec05) Robust Multiarray Average (RMA) database (http://www.genenetwork.org/public/WebQTL.py). The lod (logarithm of the odds to the base 10) scores for expression of the 212 genes were tested for genome-wide significance (1000 permutations – 1). Peaks of the transcript abundance QTLs for these genes fell into bins that included their genomic locations, indicating the possibility of cis-regulation of these genes. In the CXB hippocampal data set the B6 alleles increased gene expression for Lrrtm4, Sema4f, Aak1, Nfu1 (aka Hirip5), Chchd4, Grm7, and Cecr5 (Table 1). Peaks of the transcript abundance QTLs for these genes fell into bins that included their genomic locations, indicating the possibility of cis-regulation of these genes. In the CXB hippocampal data set the B6 alleles increased gene expression for Lrrtm4, Sema4f, Aak1, Nfu1 (aka Hirip5), and Chchd4, while for Grm7 and Cecr5 the C alleles increased expression (Table 1).

**Polymorphism in cis-regulated genes**

Focusing on the seven candidate genes, in a search for DNA polymorphisms in NCBI dbSNP build 126 (http://www.informatics.jax.org) between C57BL/6J (B6; reference strain) and BALB/cByJ, BALB/cJ, or BALB/c within coordinates of the candidates we found 67 matching SNPs for Lrrtm4, 0 for Sema4f, 47 for Aak1 (6 mRNA-UTR, 1 coding-synonymous, 40 intron), 11 for Nfu1 (6 mRNA-UTR, 5 intron), 14 for Chchd4 (2 mRNA-UTR and 12 intron), 218 for Grm7 (intron), and 0 for Cecr5.

**Gene expression differences in congenic and background strains**

It has been suggested that by comparing gene expression in strains with well-defined small genomic differences cis-regulation of expression can be detected [7,19,20]. To test the expected congenic vs background differences in expression, in a microarray gene expression experiment we compared the seven cis-acting genes for differences in relative brain mRNA abundance between the background B6By, the congenic B6By.C6, and the donor C. As shown in Table 2, expression of Lrrtm4, Sema4f, Aak1, and Cecr5 was not significantly different among the strains, while significant genetic variation was detected for three genes: Nfu1, Chchd4, and Grm7 (p = 0.000–0.036; one-way ANOVA, SPSS version 13). For Nfu1, Chchd4, and Grm7 significantly different expression was detected between the background B6By and the congenic B6By.C6 after correction for multiple comparisons (Table 2). The direction of the allelic effects for whole brain transcript abundance between the progenitor and the congenic strains agreed with the results of the Hippocampus Consortium M430v2 CXB (Dec05) RMA database analysis for most of the candidate genes.

**Multistrain analysis**

To see if any of these genes showed genotype–alcohol consumption correlation across commercially available common inbred strains with publicly accessible data we estimated the correlation between genotypes of the four significant (Nfu1, Chchd4, and Grm7) or near-significant (Aak1) SNPs and alcohol consumption in strains B6, AKR/J, C, AJ/J, DBA/2J, 129/SvJ, and C3H/J using previously published alcohol consumption data [21–23]. We found that alcohol consumption was highly correlated with genotypes of Nfu1 (rs30605208, n = 6, r = −0.96, p = 0.001) and Aak1 (rs30912462, n = 4, r = −0.95, p = 0.02). Relatively high correlation was found for Grm7 (rs305571150, n = 8, r = −0.57), which did not quite reach the p = 0.05 level of

<table>
<thead>
<tr>
<th>Chr</th>
<th>eM</th>
<th>Genome coordinates, NCBI build 36 (strand)</th>
<th>Symbol, name</th>
<th>Probe set</th>
<th>Lod score</th>
<th>B6 allele effect</th>
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<td>6</td>
<td>Syntenic</td>
<td>79948557–80738698 (+)</td>
<td>Lrrtm4, leucine-rich repeat transmembrane neuronal 4</td>
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<td>Increase</td>
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<td>35</td>
<td>82877544–82905386 (−)</td>
<td>Sema4f, sema domain, immunoglobulin domain, TM domain, and short cytoplasmic domain</td>
<td>1419328...at</td>
<td>4.3*</td>
<td>Increase</td>
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<tr>
<td>6</td>
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<td>86815474–86957498 (+)</td>
<td>Aak1, AP-2-associated kinase 1</td>
<td>1435038...s.at</td>
<td>7.5*</td>
<td>Increase</td>
</tr>
<tr>
<td>6</td>
<td>35.5</td>
<td>86975523–86994094 (+)</td>
<td>Nfu1, NFU1 iron–sulfur cluster scaffold homolog (Saccharomyces cerevisiae), aka Hirip5</td>
<td>1418229...s.at</td>
<td>6.6*</td>
<td>Increase</td>
</tr>
<tr>
<td>6</td>
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<td>Chchd4, coiled-coil–helix–coiled-coil–helix domain-containing 4</td>
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<td>Grm7, glutamate receptor, metabotropic 7</td>
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<td>6*</td>
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<td>6</td>
<td>54</td>
<td>120475114–120496924 (−)</td>
<td>Cecr5, cat eye syndrome chromosome region, candidate 5 homolog (human)</td>
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</tbody>
</table>

* Affymetrix oligonucleotide microarray, Mouse Genome 430 2.0, Hippocampus Consortium M430v2 CXB RMA data set.

* p<0.05, genome-wide significance test (1000 permutations).
significance ($p=0.07$). Chchd4 showed the lowest correlation (rs30014728, $n=6$, $r=-0.27$, $p=0.3$). Evaluating the results of multistrain analysis, we have to keep in mind its limitations in support of QTG candidacy. In our view, it can be only tentatively useful in either supporting or refuting QTG candidacy of a gene.

GO annotation summary of the candidate genes indicated that while the biological process in which Nfu1 is involved is not known, it may have a housekeeping function; Aak1 may be involved in protein amino acid phosphorylation and endocytosis; Chchd4 appears to be involved in mitochondrial protein transport; and Grm7 has been intensively characterized as a G-protein-coupled receptor protein that is involved in synaptic transmission, behavioral fear response, sensory perception, and stress (Table S3).

**Promoter analysis**

Analysis of the genotype–alcohol consumption correlation and the GO annotation summaries left Grm7 as the primary candidate for testing the hypothesis that genetic variation in expression is associated with a mutation in a transcription factor binding site (TFBS). Analysis of Grm7 by the PromoterInspector program predicted 13 promoters and two regulatory regions (http://www.genomatix.de). PromoterInspector is a program that predicts eukaryotic Pol II promoter regions with about 85% specificity in mammalian genomic sequences. Accordingly, one may expect PromoterInspector to find good matches but not all promoters.

Comparing the positions of the 218 SNPs detected in Grm7 we found 1 SNP (rs30557150) in a promoter region (fifth promoter, 110,819,264–110,819,864 bp, Fig. 3). MatInspector, a program that detects TFBSs in nucleotide sequences using a large library of weight matrices, identified a TFBS above a good match threshold: V$SORY/SOX5.01$ (matrix similarity=0.987; core similarity=1.0) (http://www.genomatix.de). The TFBS was lost due to SNP rs30557150 (C/T) in the rs30557150_C allele of Grm7, which is consistent with the observation that C57BL/6J carrying the rs30557150_C allele showed lower transcript abundance of Grm7 than strains with the rs30557150_T allele (Fig. 3). However, experimental evidence is required to provide sufficient proof for the involvement of this TFBS in transcriptional function. In a test of different microarray gene expression probe sets in WebQTL (http://www.genenetwork.org), results of eQTL mapping suggested cis-regulation for Sox5 in the mouse hippocampus in both BXD and CXB RI strain sets. Whole genome mapping with 1000 permutation test yielded LOD=31.56 with peak location between 144.01 and 144.82 Mb in the BXD population (database: Hippocampus Consortium M430v2 (Jun06) PDNN; Trait ID: 1446461_at); however, no significant peak was found for another probe set (1432189_a_at). No significant peak was found for 1446461_at in the Hippocampus Consortium M430v2 CXB (Dec05) RMA database. We found 362 SNPs in Sox5 in a comparison of B6, DBA/2J, and BALB/cBy, including an mRNA-UTR insertion (rs6151967, http://www.informatics.jax.org), which is consistent with the hypothesis that probe set differences may reflect heterogeneity.

**Table 2**

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<th>Gene on donor segment</th>
<th>Probe set ID</th>
<th>Strain</th>
<th>Mean</th>
<th>Standard deviation</th>
<th>Standard error</th>
<th>df</th>
<th>F</th>
<th>Significancea</th>
<th>Tukey HSDb</th>
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<td>B6ByC6</td>
<td>28.91</td>
<td>2.74</td>
<td>1.58</td>
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Affymetrix oligonucleotide microarray, Mouse Genome 430 2.0. Vadasz–Saito Cerebral Gene Expression database, GEO repository series entry GSE7155.

a Between-group difference, one-way ANOVA of RMA normalized data.

b Post hoc test for B6By vs B6ByC6.
No significant “genetic” (between-strain) correlation was found between hippocampal expression of Sox5 (1432189_a_at) and of Grm7 (1459532_at) in the CXB population (Pearson’s correlation: \( r = 0.498, n = 15, p = 0.059 \), two-tailed) or between hippocampal expression of Sox5 (1446461_at) and Grm7 (1459532_at) in the BXD population (Pearson’s correlation: \( r = -0.178, n = 69, p = 0.147 \), two-tailed).

**Confirmatory analysis of cis-regulation of Grm7**

Although the hippocampus has been implicated in addiction [24] and alcohol consumption [4], traditionally, it has not been considered as a region critically involved in alcohol preference and dependence. Therefore, we performed additional expression QTL mapping on two striatal databases created for BXD and B6D2F2 populations (see Materials and methods). To test the between-strain generalizability of the CXB hippocampal gene expression results, an additional BXD hippocampal data set was analyzed. As shown in Fig. 4, the results of the confirmatory analyses provided strong support for cis-regulation of Grm7 in both brain regions.

**Confirmatory analysis of linkage in reciprocal F2 populations**

We used an alternative genetic design for testing the association between Grm7 and alcohol consumption. Reciprocal F2 populations were created by crossing F1 hybrids of alcohol-prefering (I5B25A) and alcohol-nonpreferring (C5B3) quasi-congenic RQI strains, which are on the same genetic background (both strains carry >97% B6 genome [6]). F2 mice were selectively (>1 SD above or below mean alcohol consumption) genotyped for a SNP marker (rs3723352, G/T) that resides in intron 7 of Grm7 at Chr. 6: 111,318,602 (NCBI build 36). Because consumption of 12% (v/v) alcohol (g/kg/day, average of four 3-day trials) was not significantly different between the selectively genotyped reciprocal F2 groups (\( F_{1,56}=0.73, p = 0.79 \)), data were combined. Comparison of the B6By/B6By (G:G) and BALB/cJ/BALB/cJ (T:T) homozygotes indicated that individuals with the B6By/B6By genotype consumed significantly more alcohol (independent samples \( t \) test, \( t_{27}=2.1, p = 0.048 \); Fig. 1B). These results were consistent with data obtained from RQI QTL mapping [7], detection of T:T genotypes in other inbred strains (e.g., CByJ, 129/SvJ, DBA/2J) known to show low alcohol consumption, and results of the alcohol preference tests with B6By.C6 congenic animals (Fig. 1A). Importantly, the quasi-congenic F2 experiments also provided supportive evidence for the exclusion of competing candidate genes Nfu1, Aak1, and Chchd4, because the alcohol-nonpreferring C5B3 quasi-congenic RQI strain did not carry the BALB/cJ-type alleles of Nfu1, Aak1, and Chchd4. To create the F2 on an ~97% homogeneous background, RQI strain C5B3 was chosen for the cross because it carries a BALB/cJ-derived Chr. 6 segment (with flanking background markers D6Mit230 at 98.97 Mb and D6Mit258 at 137.07 Mb), which partially overlaps the BALB/cJ-derived Chr. 6 segment in the
Fig. 4. Expression QTL mapping in four data sets. QTL mapping of mRNA abundance of candidate genes was carried out using WebQTL (http://www.genenetwork.org). Grm7 (ProbeSetID=1459532_at) had genome-wide statistically significant lod scores with peaks at (or near) its genomic location in the following data sets: CXB Hippocampus (LOD=6.0), BXD Striatum (LOD=7.3), B6D2F2 Striatum (LOD=21.1), BXD Hippocampus (LOD=20.5). For data set details see Materials and methods.
congenic B6By.C6 (75.9–122.3 Mb, Fig. 1A). In the alcohol-prefering 15B25A quasi-congenic strain, BALB/cJ-derived alleles were detected only on Chrs. 8 and 13. In C5B3, the whole BALB/cJ-derived Chr. 6 segment was positioned distal from the genomic positions of AW146020, Htra2, Dguok, and Frmd4b (genes identified with structural polymorphism) and Nfu1, Aak1, and Chchd4 (genes identified as cis-regulated).

Discussion

In our recent studies composite interval mapping identified several significant linkages to ethanol alcohol consumption on mouse Chr. 6 and indicated that the RQI donor block region for Eae2 spans 33.5–48.2 cM [7]. Eila2 (36.5 cM), Eilm3 (30 cM), Rear1 (36.5 cM), Bits2 (37 cM), and Tast5 (48 cM) are previously identified behavior and preference QTLs mapping to this interval. Interestingly, despite intensive efforts, mapping studies on QTLs for murine alcohol preference drinking behavior using the most popular BXD recombinant inbred strains and various genetic preparations derived from the progenitor strains failed to identify QTLs in this chromosome region [1]. Reasons for the failure may include, but are not limited to, the following: different mapping strategy (our quasi-congenic RQI strains have ~97% “background homogeneity,” significantly reducing epistatic interaction variation, i.e., genetic background noise, which can mask target allele signals [25–28]), different laboratories with differences in testing procedures [29,30], and differences between DBA/2J and BALB/cJ in genomic architecture (different gene dispersion patterns≠ different mode of gene action in each strain resulting in various increaser/decreaser linkages with different tightness, different interactions with the B6 genes and environmental modifiers, etc.). It is possible that the task of mapping and sequencing true polygenes has been underestimated [31]. For example, two polygenes with small effect size exhibiting antagonistic epistasis may be mapped to a single small QTL region (<1 cM), such that their phenotypic effect depends on the genetic background [32]. If similar complications are typical for behavioral traits, it may explain the inability to predict results of new crosses based on RI or classical intercross mapping results (cf. [33]).

In this study we integrated genetic, transcriptome, and bio-informatic approaches to identify quantitative trait genes for a behavioral phenotype, oral alcohol self-administration. The results suggest that Grm7, which encodes the group III metabotropic glutamate receptor 7 (mGluR7), modulates alcohol drinking: Mice carrying the genotype that predisposes for lower expression of Grm7 in the brain consume more alcohol in a preference drinking behavioral paradigm. Molecular, cellular, and biological functions of Grm7/mGluR7 strongly support this suggestion.

Studies on human mGluR7 mRNA splicing variants have so far revealed five isoforms [34,35] and suggest that structural and functional diversity of this receptor is achieved by alternative exon usage affecting the immediate carboxy- terminus. This region interacts with defined proteins, such as PICK [36], α-tubulin [37], and calmodulin [38], thus mediating various receptor functions such as intracellular signal transduction, axonal targeting, and synaptic clustering. Most variants are expressed in brain and retina, while human isoforms GRM7_v3 and GRM7_v4 are expressed in nonneuronal tissues [35]. In rats, two splice variants (mGluR7a and mGluR7b) have been reported, which are defined by different C-termini [39]. Immunohistochemical studies using antibodies raised against the C-termini of rat mGluR7a and human mGluR7b showed similar, but not identical, distributions in the brains of rats and mice [40]. Alternative splicing, in addition to probe ambiguity (targeting multiple genes), heterogeneity (targeting different transcript variants from one gene), and other factors, may explain our observation that only one of the four Affymetrix Mouse 430 2.0 probe sets for Grm7 could detect cis-regulation [41].

Sox5 (SRY-box containing gene 5) is a novel SRY-related transcription factor [42]. The genomic location of this TF (Chr. 6, 69.5 cM, 143,790,051–144,166,639 bp) is just 33.178 Mb distal from that of Grm7. One of its known functions is related to cell fate commitment and cartilage development [43]; however, its functions in the brain or interactions with Grm7 are not known, and its cis-regulated expression did not show a correlation with that of Grm7 in CXB or BXD strain sets (see Results). Thus, the hypothesis that Sox5 may play a role as a transcription factor in the regulation of expression of Grm7 in the brain needs further support.

mGluR7 is a presynaptic autoreceptor, or heteroreceptor, in non glutamatergic axon terminals, with an inhibitory action on the release of various neurotransmitters such as glutamate, GABA, and monoamines [44]. There is a considerable overlap between the neuroanatomical distribution of mGluR7 and mesolimbic brain regions, suggesting that this receptor has a particularly crucial role in processing information, which can modulate neurotransmitter release in pathways involved in addiction and reinstatement. High representation of mGluR7 was observed in the amygdala [45], the periventricular zone of the hypothalamus, and the olfactory circuitry [46]. Simonyi et al. found the highest levels of mGluR7 expression in the dentate gyrus, CA1, CA3, and piriform cortex [47]. Recently deposited microarray data indicated relatively high Grm7 expression in amygdala, hippocampus, and cerebellum in various mouse strains, including B6 and CByJ [48].

Genetic ablation of mGluR7 in mice provided further supportive data. Grm7 knockouts showed altered amygdala-dependent conditioned fear and aversion responses and reduced anxiety-related behaviors, suggesting a role for mGluR7 in the modulation of stress-related behaviors [49,50]. Indeed, a new specific agonist of mGluR7 activated the hypothalamic–pituitary–adrenal (HPA) axis and dramatically increased stress hormone levels [51]. A possible glutamatergic mechanism underlying the activation of the HPA axis, which may have relevance to alcohol drinking, involves disinhibition of corticotropin-releasing factor (CRF)-containing paraventricular nucleus (PVN) neurons, which leads to increased ACTH and corticosterone levels. Activation of the presynaptic inhibitory mGluR7 receptor can decrease L-glutamate release from the
hippocampal–hypothalamic pathway, decreasing the excitatory tone on GABAergic neurons, or agonists may activate mGluR7 heteroreceptors directly, decreasing the inhibitory tone on PVN neurons [50,52,53]. In stress-induced alcohol drinking and relapse behavior both the glutamate system and the corticotropin-releasing hormone system have been implicated [44,54,55]. The proposed role for the amygdala in the relationship among stress, CRF, and alcohol abuse [56], and the role of Grm7 in stress, is consistent with a link between the genetic variation in Grm7 and predisposition to alcohol drinking. Our present observation of increased expression of Grm7 in BALB/cJ and earlier reports proposing the BALB/cJ strain as an animal model for pathological anxiety [57,58], characterized by increased “freezing” and emotionality in open-field test [59–61] and by elevated stress-induced corticosterone and emotionality [62], are also consistent with the anxiolytic effects of targeted disruption of Grm7 [49].

Although the mesolimbic dopamine reward hypothesis of addiction has been traditionally important in psychostimulant use and abuse [63–65], there is emerging evidence that glutamate systems play a critically important role in reinforcement and addiction [66]. It has been shown that glutamatergic innervation of the VTA and the nucleus accumbens regulates locomotor activity [67]. mGluR7 may be implicated in regulation of motor activity, because administration of group III mGlu receptor agonist L-AP4 attenuates acute amphetamine [68] and cocaine [69]-stimulated locomotor activity. In studies on the link between alcohol effects and the glutamatergic neurotransmission the emphasis has been on the role of the ionotropic glutamate receptors; however, research on the role of metabotropic glutamate receptors in alcohol use is gaining impetus [70]. It has been shown that chronic exposure to alcohol reduced mGluR1 mRNA levels in cerebellar Purkinje cells in mice [71]. In rats, chronic exposure to Lieber–DeCarli liquid diet with alcohol (5% w/v) decreased mRNA levels of mGluR3 and mGluR5 in dentate gyrus, while levels of mGluR1, mGluR5, and mGluR7 were decreased in the CA3 area of the hippocampus [72]. Such adaptations in mGluR7 expression to long-term exposure to alcohol are also expected in excessive alcohol self-administration, and reduced mGluR7 expression may become a causative factor in excessive drinking, which is consistent with our results.

Use of pharmacological agents indicates that the mGluR5 antagonist MPEP decreases relapse to alcohol self-administration [70,73,74], blocks the discriminative stimulus effects of alcohol [75], and attenuates latent inhibition in conditioned taste aversion [76]. Based on the supporting psychopharmacological data and its high expression in the nucleus accumbens, cortex, and hippocampus [77], it was proposed that mGluR5 receptors might specifically regulate ethanol reinforcement [70]. Recent experiments indicated that the novel mGluR7-specific agonist AMN082 inhibited cocaine self-administration [78], and reverse dialysis studies demonstrated that group III agonist L-AP4, possibly acting on mGluR7, decreased in vivo extracellular glutamate in the nucleus accumbens by inhibiting nonvesicular glutamate release, and group III antagonist MSOP increased extracellular accumbal glutamate [79]. Thus, it appears that the postsynaptic mGluR5 receptors need to be blocked, while the presynaptic mGluR7 receptors need to be activated, for inhibition of psychostimulant self-administration. Significant downregulation of Grm7 has recently been reported in mice lacking the subunit α1 of the GABA-A receptor (Gabra1) [80]. Because Gabra1 is associated with alcohol dependence [81], there is the possibility of signaling and genetic interaction between the two receptors.

The source of tissue in our expression QTL mapping may be a source of concern, because the hippocampus is not generally considered as tightly linked to addiction. Thus, the question may be raised “Are hippocampal expression data relevant to alcohol drinking?” Although debate continues over the biological mechanisms of reward and addiction, hippocampal expression data are relevant to alcohol drinking because the hippocampus is an important part of the limbic circuitry, a neural substrate of reinforcement and drug addiction. One of the functions of the limbic cortical–ventral striatopallidal thalamo-cortical loop circuitry is processing of contextual information by the hippocampus [82]. For example, dorsal hippocampus inactivation abolishes contextual, but fails to alter explicit conditioned stimulus- or cocaine-induced, reinstatement of cocaine-seeking behavior [83]. It appears that the amygdala mediates conditioning to discrete conditioned stimuli, and the hippocampus mediates contextual or spatial stimuli and may underlie the motivational impact of contextual stimuli on drug seeking [82].

Using progenitor strain-derived mapping population and additional expression data derived from amygdala, ventral and dorsal striatum, and prefrontal cortex would have been ideal; however, currently such databases are not available. The most relevant and available expression mapping database is derived from hippocampal tissue of CXB RI mice. The progenitor strains in this RI set are BALB/cByJ and C57BL/6ByJ; thus one of the progenitors (BALB/cByJ) is a subline of BALB/cJ, the strain that was used as progenitor in our experiments. As of July 2007, the SNP-based genetic similarity between BALB/cByJ and BALB/cJ cannot be assessed very reliably because the density of SNP coverage differs. BALB/cByJ has 7,675,370 SNPs, BALB/cJ has 86,221 SNPs (http://phenome.jax.org). Selecting the entire genome, and requiring that SNP locations must have allele data present for both strains, 26,831 SNP location rows were retrieved. Selecting the entire genome, and requiring that there must be polymorphism between BALB/cByJ and BALB/cJ for a SNP location to be displayed, 41 SNP location rows were retrieved, reflecting an average 0.15% difference. A similar comparison for BALB/cByJ and C57BL/6ByJ yielded 33.28% difference. For Grm7 no SNPs could be retrieved in a BALB/cByJ vs BALB/cJ comparison. These suggest that the genomic difference between BALB/cByJ and BALB/cJ and the risk of obtaining false information due to the strain substitution are relatively small.

Taken together, the reviewed evidence establishes a significant functional relevance of mGluR7 in psychostimulant-related behaviors, including oral alcohol self-administration.

A potential source of error is introduced in the analysis by the method used to filter candidate genes residing in the donor
Development of congenic strain B6By.C6

The alcohol-nonpreference quasi-congenic RQI strain B6.Cb57-Alpha3 (C5A3) was developed by five backcross–intercross cycles using the alcohol-avoiding C strain as donor and the alcohol-prefering B6By strain as background [6,28]. Using C5A3 as a progenitor, the congenic B6.C6 strain was developed by an additional five backcrosses to B6By with concomitant selection for C alleles of Chr. 6 microsatellite markers (e.g., D6Mit105). Other relevant markers were selected from the genome-wide genotype database of RQI strains (http://rqigene.com). Congenic status was tested by a genome scan with a mouse SNP panel of 402 markers (service provided by Genetics Division, Brigham and Women’s Hospital, Harvard Medical School). Only the expected single Chr. 6 donor segment was detected as of C origin (Fig. S1).

Oral alcohol self-administration

In all experiments a “two-bottle” choice paradigm with escalating alcohol concentration was used. Male mice were individually housed in our drinking study room for at least 1 week prior to beginning the study. Mice were tested for alcohol preference and consumption according to the procedure for oral self-administration as described [6,85]. Two versions of the “two-bottle” choice paradigm were used, one with contiguous trials, and one with intermittent trials. (1) “Two-bottle” choice paradigm with contiguous trials. The test consisted of six 3-day trials, in which mice were allowed to choose between alcohol solution and tap water. To acclimate the animals to the taste of alcohol, the alcohol solution was offered in escalating concentrations: a 3% solution for trial 1 (days 1–3) was increased to 6% in trial 2 (days 3–6), and further increased to 12% for trials 3 (days 6–9), 4 (days 9–12), 5 (days 12–15), and 6 (days 15–18). This arrangement provided multiple measures of alcohol preference at 12% concentration (v/v). Because offering alcohol solutions of 3% and 6% served the primary purpose of acclimation of animals to 12% solution, we did not carry out experiments in duplicate for 3 and 6% alcohol consumption, and we did not use these data for analysis. (2) “Two-bottle” choice paradigm with intermittent trials. The test consisted of eight 3-day trials, in which mice were allowed to choose between alcohol solution and tap water. As above, the alcohol solution was offered in escalating concentrations: a 3% solution for trial 1 and trial 2 was increased to 6% in trial 3 and trial 4 and further increased to 12% for trials 5–8. After every two trials, mice had access to water only for 24 h (from Monday morning through Tuesday morning). Thus, the total experiment with intermittent access lasted for 4 weeks. For analysis only trials 5–8 were used.

The liquids were offered in custom-made drinking tubes composed of centrifuge tubes fitted with single-hole rubber stoppers into which stainless steel sippers were inserted. We also used stainless-steel springs to fasten the tubes firmly to the top of the cage covers. Two empty cages with alcohol and tap water drinking tubes were put on the racks to obtain control weights for leakage and evaporation. For each trial, the mean control values were subtracted from the alcohol and water consumption data. Food was located on the cover at the front of the cage, with one drinking tube always nearer to the food than the other. The positions of the water and alcohol drinking tubes on the cage cover were alternated in each 3-day preference trial to avoid a position effect. The weights of the drinking tubes were measured before and after a 3-day trial using an A&D electronic analytical balance connected to a computer. Data were entered automatically using A&D software and spreadsheets. After correction for dripping and evaporation, data from the four 3-day trials were averaged for each individual.

Materials and methods

Animals

Progenitors of B6By, CXBI/ByJ (I), and C inbred mouse strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The quasi-congenic RQI strains and the chromosome 6 congenic B6By.C6 mice were developed in our research colony at The Nathan Kline Institute for Psychiatric Research, Office of Mental Health, New York State (NKI) as described [6]. All other mice were bred and maintained in our colony. Animals were housed in groups (three to five per cage) under standard laboratory conditions (12 h light/12 h dark cycle) with food and water available ad libitum. At the time of testing, animals were housed individually with lab chow and water continuously available. All reported data refer to male mice. The care and use of animals met the standards and recommendations of the IACUC of NKI in accordance with U.S. Department of Agriculture and U.S. Public Health Service guidelines.

Brain dissection, RNA labeling, and microarray processing

We used adult, male B6By, B6By.C6, and C mice (n = 30; 90 mice in total). Animals were killed by cervical dislocation, brains were quickly removed and hemisectioned. and alternate left or right hemispheres were collected in RNA-Later for expression studies. After each hemisphere was processed separately, 10 hemispheres were pooled for one high-density oligonucleotide microarray (Mouse Genome 430 2.0; Affymetrix, Santa Clara, CA, USA). For each strain, three oligonucleotide microarrays were used (n = 3). RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini Kits (Qiagen, Valencia, CA, USA). The yield of total RNA from each tissue sample...
was 45.3±7.6 μg. Fragmented cRNA probes were prepared from 7 μg of total RNA according to the protocol of Affymetrix using SuperScript Choice System (Invitrogen) and a RNA Transcript Labeling Kit (Enzo, Farmingdale, NY, USA). cRNA quality analysis on a 2100 Bioanalyzer, hybridization to GeneChip, and scanning of the arrays were carried out in accordance with the manufacturer’s protocol at the Genomics Facility of the New York University Cancer Institute using Gene Chip Operating Software (GCOS).

Microarray gene expression data acquisition and normalization

Raw data with MIAME-compliant annotations of experiments were uploaded to Gene Traffic Database (http://genetraffic.med.yu.edu/; Lobion Informatics, LLC). We used CEL files produced by GCOS for probe (cell)-level RMA-based normalization of data [86]. All subsequent analyses were done on RMA-normalized data. Data from these studies are available at Gene Expression Omnibus ( GEO series entry GSE7155). All public gene expression data were based on Affymetrix Mouse Gene Expression Array 430 2.0. CXB RI hippocampal, BXD hippocampal, BXD striatal, and B6D2F2 striatal mRNA data were obtained from the Hippocampus Consortium M430v2 CXB (Dec05) RMA database, Hippocampus Consortium M430v2 BXD (Dec05) PDNN database, HBP/Rosen Striatum M430v2 (Apr05) MA55 Clean database, and OHU/VA B6D2F2 Striatum M430v2 (Sep05) PDNN database, respectively, at http://www.genenetwork.org/public/WebQTL.

Bioinformatic analysis

Ongoing resequencing efforts made available large SNP genotype data sets for numerous inbred strains, including B6 and CBy, closely related sublines of our progenitor strains B6By and C (http://www.jax.org/phenome/snp.html). Because currently our progenitor strains B6By and C are not as well characterized in bioinformatic databases as the closely related sublines B6 and CBy, in the present analysis we took advantage of the excellent bioinformatic coverage and Mouse Phenome Database priority strain status of B6 and CBy. In a comparison of C and CBy for all SNPs on Chr. 6 we found 3063 SNPs, while the number of different SNPs was 188. This suggests 188/3063=6.14% SNP differences on Chr. 6, the length of which is 149.4 Mb. Assuming even coverage and Mouse Phenome Database priority strain status of B6 and CBy. In the present analysis we took advantage of the excellent bioinformatic coverage and Mouse Phenome Database priority strain status of B6 and CBy. In a comparison of C and CBy for all SNPs on Chr. 6 we found 3063 SNPs, while the number of different SNPs was 188. This suggests 188/3063=6.14% SNP differences on Chr. 6, the length of which is 149.4 Mb. Assuming even distribution, a rough estimate of the average density of subline SNPs is 188/149.4=1.258, i.e., subline SNP density <2 SNP/Mb (http://www.informatics.jax.org/javaw2i/servlet/WIFetch?page=snpQF). This is well below the threshold applied for detection of BBD regions (20 SNPs/0.1 Mb) by us and others. In SNP database searches B6 and CBy were substituted for B6By and C if database information was limited for the progenitor strains. Promoter analysis of Grm7 was carried out with the PromoterInspector program (http://www.genomatix.de/; Eldorado 08-2006 database, NCBI build 36, extracted region: NC_000072 Y=C/T (Fig. 3).

Segregating F2 population studies

RQI quasi-congenic strains with significant differences in alcohol consumption have been described [6,7]. Reciprocal F2 populations were created by using alcohol-prefering (15B2A) and alcohol-nonprefering (CSB3) quasi-congenic progenitor strains. Both strains carry >97% B6 genome. The sample sizes of the (CSB3×15B2A)F2 and (15B2A×CSB3)F2 male mice were 159 and 163, respectively. Phenotypic “extremes” were identified as 1 SD below or above the mean alcohol consumption value and were subjected to selective genotyping. The selectively genotyped sample sizes were n=28 for (CSB3×15B2A)F2 and n=30 for (15B2A×CSB3)F2. To test the association of allelic variants of Grm7 with alcohol consumption, the phenotypically selected F2 mice were genotyped for SNP rs3723352, which resides in intron7 of Grm7 at 111,513 Mb (NCBI build 36). For allelic variation of rs3723352 in inbred strains we searched the Mouse Phenome Database (http://phenome.jax.org/pubcgi phenome/mpdcgi?trn=snp%2FRetrieve&searchreq=rs3723352).


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