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The Metabotropic Glutamate Receptor Subtype 5 (*mGluR5*) Mediates Sensitivity to the Sedative Properties of Ethanol

Chris Downing^{1,3,4}, Michael J. Marks¹, Colin Larson¹, and Thomas E. Johnson^{1,2}

¹University of Colorado, Institute for Behavioral Genetics, Campus Box 447, Boulder, CO, 80309-0447

²University of Colorado, Department of Integrative Physiology, Campus Box 354, Boulder, CO, 80309-0354

Abstract

Objective—Inbred Long-Sleep and Short-Sleep mice (ILS and ISS) were selectively bred for differential sensitivity to the sedative effects of ethanol. Lines of mice derived from these progenitors have been used to identify several Quantitative Trait Loci (QTLs) mediating Loss Of the Righting reflex due to Ethanol (LORE). The present study investigated *mGluR5* as a candidate gene underlying *Lore7*, a QTL mediating differential LORE sensitivity.

Methods—We used knockout mice, a quantitative complementation test, pharmacological antagonism of mGluR5, real-time quantitative PCR, radioligand binding, DNA sequencing and bioinformatics to examine the role of *mGluR5* in ethanol-induced sedation.

Results—*mGluR5* knockout mice had a significantly longer LORE duration than wild-type controls. Administration of the mGluR5 antagonist 2-methyl-6-(phenylethyl)-pyridine (MPEP) had differential effects on LORE in ILS and ISS mice. A quantitative complementation test also supported *mGluR5* mediating LORE. Two intronic single-nucleotide polymorphisms in *mGluR5* were highly correlated with LORE in recombinant inbred mice derived from a cross between ILS and ISS (LXS RIs). Differences in mGluR5 mRNA level and receptor density were observed between ILS and ISS in distinct brain regions. Finally, data from WebQTL showed that *mGluR5* expression was highly correlated with several LORE phenotypes in the LXS RIs.

Conclusions—Taken together, this data provides convincing evidence that *mGluR5* mediates differential sensitivity to the sedative effects of ethanol. Studies from the human literature have also identified *MGLUR5* as a potential candidate gene for ethanol sensitivity.

Keywords

Alcohol; Loss of Righting Reflex; mGluR5; MPEP; Quantitative Complementation

³To whom correspondence should be addressed: cdowning@pharmacy.isu.edu Phone: 208-282-3837.

⁴Present Address: Idaho State University College of Pharmacy Department of Biomedical and Pharmaceutical Sciences 970 South 5th Avenue, Stop 8334 Pocatello, ID 83209-8334

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Introduction

Long-Sleep (LS) and Short-Sleep (SS) mice were selectively bred for sensitivity and resistance to the soporific effects of ethanol [1] and have been used extensively to study the neurobiological mechanisms underlying ethanol-induced sedation. In addition to high-dose ethanol sensitivity, LS and SS mice, and inbred strains derived from these progenitors (ILS and ISS), differ in sensitivity to other anesthetic agents [2-5]. Using these selectively bred mice, recombinant inbred (RI) strains derived from a cross between LS and SS (LSXSS RI), RI strains derived from ILS and ISS (LXS RIs), F₂ generation mice derived from crosses between ILS and ISS, and congenic strains constructed from these progenitors, our laboratory has identified and confirmed several quantitative trait loci (QTL) mediating loss of righting reflex (LORR) induced by ethanol and other anesthetic agents. Interestingly, QTL mediating ethanol-induced LORR (or LORE, for Loss Of Righting due to Ethanol) [6,7] and LORR induced by the anesthetic agents propofol (*Lorpl*) [4], and etomidate [8] have been mapped to what appears to be the same region on mouse chromosome 7. While the *Lore7* QTL and the QTL mediating etomidate sensitivity had rather broad confidence intervals, the *Lorpl* QTL, which explained about 80% of the genetic variance in propofol sensitivity, was localized to a narrow 2.5 centimorgan (cM) interval, tightly linked to the albino locus (tyrosinase; *Tyr*). The *Tyr* locus has been ruled out as the *Lore7* QTL [9]. One intriguing candidate gene immediately distal to *Tyr* is *mGluR5*, which codes for the metabotropic glutamate receptor subtype 5.

Over the course of the past decade, *mGluR5* has been shown to play a role in many behavioral and physiological processes, including learning, memory and long-term potentiation [10-14]. Studies have also shown that *mGluR5* can mediate responses to drugs of abuse such as phencyclidine, cocaine, amphetamine/methamphetamine, nicotine and morphine [15,16]. *mGluR5* has also been implicated in the actions of ethanol. Minami et al. [17], using a *Xenopus laevis* oocyte expression system, showed that ethanol inhibited mGluR5-induced, Ca²⁺-dependent Cl⁻ currents via protein kinase C (PKC) mediated phosphorylation. Bäckström and colleagues [18] found that, in rats, the mGluR5-specific antagonist 2-methyl-6-(phenylethyl)-pyridine (MPEP) dose-dependently attenuated ethanol seeking and dose-dependently reduced the alcohol deprivation effect (ADE). Using both Alcohol-Preferring rats (P) and inbred C57BL/6J (B6) mice, the Hodge laboratory has shown that MPEP decreases or inhibits the onset and maintenance of operant ethanol self-administration, decreases ADE, inhibits the discriminative stimulus effects of ethanol, reduces the ethanol break point in operant paradigms, increases LORE and reduces ethanol-induced locomotor activation; they reported no effect of MPEP on ethanol conditioned place preference [19-25]. In contrast, Lominac et al. [26] reported that in B6 mice, MPEP blocked the expression of ethanol-induced place conditioning, but had no effect on ethanol-induced locomotor activation Bird et al. [27] reported that *mGluR5* knockout mice displayed a place preference for ethanol when conditioned with 1.0 g/kg ethanol, while wildtype mice did not.

In the present study, we used several different methods, including the use of knockout mice, a quantitative complementation test, antagonism of *mGluR5* in ILS and ISS, gene expression analysis, radioligand binding, DNA sequencing and bioinformatics to test whether *mGluR5* was the gene underlying the *Lore7* QTL. Results support a role for *mGluR5* contributing to differential LORE sensitivity.

Materials and Methods

Animals and Husbandry

All mice were housed in the Specific Pathogen Free (SPF) facility at the Institute for Behavioral Genetics, Boulder, Colorado; they were maintained on a 12hr light/dark cycle (0700 to 1900). The colony was kept at approximately 22° C ± 1°, and mice had access to food (Teklad,

Madison, Wisconsin) and water *ad libitum*. All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee, in accordance with the National Institute of Health guidelines. ISS and ILS mice were created and are maintained at IBG. Homozygous *mGluR5* knockout (KO) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. The *mGluR5* KOs were created in the laboratory of Dr. John Roder, using embryonic stem (ES) cells from 129/Sv X 129/Sv-CP mice, implanted into CD1 blastocysts [28]. The targeting vector, a neomycin resistance cassette, deleted a 400 bp fragment containing part of exon 1 and part of intron 1. Chimeric mice were identified and homozygous mice carrying the deletion were crossed to 129, CD1, and C57BL/6J (B6) mice [11,13]. The Jackson Laboratory obtained mice crossed to B6 and backcrossed the mice twice before cryopreserving them. In our colony, to maintain the lines, homozygote KO females were crossed with B6 males. Thus, mice underwent 4 generations of backcrossing to B6 and their genetic background should be ~ 94% B6. Five males and five females of the resulting F₁ mice, which were all heterozygous for *mGluR5*, were then intercrossed, creating 5 breeder pairs. Offspring from these breeders were tested for one phenotype, sacrificed and DNA was extracted from tail for genotyping. Mice were genotyped at least twice for two markers, one for *mGluR5* and one for the neomycin resistance cassette (NEO), indicating they had a nonfunctional copy of *mGluR5*.

LORR Testing in mGluR5 Mice

Male and female mice (WT, HET, KO) were injected retro-orbitally with propofol (20 mg/kg, 10% w:v). Following injection, the animal was observed for signs of sedation and LORR occurred when the animal was unable to right itself within 15 seconds after being placed on its back in a V-shaped acrylic trough. The criterion for recovery was the mouse's ability to right itself three times within 30 seconds. Our dependent variable was duration of LORR, defined as the time (secs) between loss and recovery of the righting reflex. Similarly, for LORE, mice were injected intraperitoneally (ip) with 4.1 g/kg ethanol (20% w:v) and observed for signs of sedation as described above. LORE occurred when an animal was unable to right itself within 60 seconds, and recovery was defined as the mouse's ability to right itself 3 times within 60 seconds. The dependent variable was LORE duration in minutes. Data were analyzed using analysis of variance (ANOVA), with genotype and sex as the independent variables.

LORE Testing in ILS and ISS

We first administered saline or MPEP (Tocris; Ellisville, MO, USA), 10 mg/kg ip, to ILS and ISS mice. Ten minutes later animals were injected ip with ethanol, 2.5 g/kg for ILS mice and 4.1 g/kg for ISS mice, and LORE was measured as described above. We administered 2.5 g/kg ethanol to ILS instead of 4.1 g/kg because the 2.5 g/kg dose gives a mean LORE duration similar to that of ISS at 4.1 g/kg [29]. The 2.5 g/kg dose is routinely used in our laboratory to test ILS mice or congenic mice on an ILS background. When we observed a differential effect of MPEP pretreatment on LORE in ILS and ISS, we conducted a dose-response study. ILS and ISS mice were injected ip with either saline or 5, 10, 20, or 30 mg/kg MPEP; 10 minutes later mice were injected ip with ethanol, 4.1 g/kg for both ILS and ISS, and LORE was measured as described above.

Quantitative Complementation Test

One of the best strategies for determining if a candidate gene is a QTL is a quantitative complementation test [30-32]. This test requires strains containing a mutated (m) and a wild-type (wt) allele of the candidate gene, ideally on a coisogenic background. These strains are then crossed to strains containing the alternative QTL alleles (high and low), and the four F₁ progeny classes (high/m, low/m, high/wt, low/wt) are characterized for the trait. Failure of the mutation to complement the QTL allele is inferred if the difference in mean phenotype between

the high/m and low/m genotypes is greater than that between high/wt and low/wt genotypes. Statistically, this is detected by a significant cross (m or wt) by line (high or low) interaction from ANOVA, and such failure to complement provides good evidence for the candidate gene being the QTL. To obtain these four classes of progeny we crossed heterozygous knockouts (*mGluR5*^{+/-}) with ILS and ISS (Figure 1). Within each cross, this produced two classes of progeny: those with two functional copies of *mGluR5* (ILS^{*mGluR5*+/+} and ISS^{*mGluR5*+/+}), and those with one functional and one nonfunctional copy (ILS^{*mGluR5*+/-} and ISS^{*mGluR5*+/-}). Mice were administered 4.1 g/kg ethanol and LORE was measured as described above. Data were analyzed using ANOVA, with line (ILS or ISS) and genotype (+/+ or +/-) as independent variables.

DNA Sequencing

mGluR5 is a large gene, covering more than 540,000 bp of genomic DNA (Figure 2: <http://www.celera.com>) [33]. The human, rat and mouse genes are highly conserved; one notable difference is an additional exon (II) in mouse and rat, not found in human [33]. There are several known splice variants, including *mGluR5a* and *mGluR5b*; *mGluR5b* contains an additional 96 bp exon just downstream from the exon containing the 7 transmembrane domain. In addition, there are three alternatively spliced first exons, IA, IB and II, and multiple promoters (<http://www.celera.com>) [33]. We sequenced all exons of *mGluR5* in ILS and ISS mice to determine if there were polymorphisms in coding regions.

Genomic DNA was isolated from spleen of male mice using a DNeasy kit (Qiagen Inc., Valencia, CA) according to manufacturers' instructions. We used Primer3 (<http://frodo.wi.mit.edu/cgi-bin/primer3>) to design primers that flanked exons, amplifying products approximately 350-450 basepairs (bp); to the extent that exons were larger than this, overlapping primers were used to amplify the entire exon. DNA was amplified and products were viewed on ethidium bromide-stained agarose gels under UV light. Products were excised and purified using a QIAquick Gel Extraction Kit (Qiagen Inc.). Purified PCR products were sequenced using an Applied Biosystems (ABI) BigDye terminator (v3.1) cycle sequencing kit. Samples were loaded onto an ABI Prism 3100 Genetic Analyzer automated capillary sequencer. Sequence data were exported into the National Center for Biotechnology Information website (NCBI; www.ncbi.nih.gov) where they were compared against the Celera consensus sequence, using the "Align two sequences" function under the BLAST option.

Quantitative, Real-Time PCR

We examined expression of *mGluR5* using quantitative, real-time PCR (q-RT-PCR). Whole brain, cerebellum, hypothalamus, striatum and hippocampus were excised from male and female ILS and ISS mice (3 per strain and sex, tissue was pooled within strain and sex for RNA extraction). Tissue was homogenized and RNA was extracted using a Qiagen RNeasy Lipid Tissue kit, following manufacturer's instructions. Total RNA was then reverse transcribed to yield single stranded cDNA using a Promega ImProm II Reverse Transcription System, following manufacturer's instructions. Primers were designed for the 96 bp exon contained in *mGluR5b* using Primer Express software (Applied Biosystems; ABI). Quantitative RT-PCR was performed on cDNAs using SYBR green chemistry and an ABI Prism 7000 system. Relative quantification of mRNA levels was determined by normalizing against a control gene, *Gapdh*, using the $2^{-\Delta\Delta C_t}$ method [34]. All determinations were replicated three times.

Radioligand Binding

Whole brain, cerebellum, striatum, hypothalamus and hippocampus were excised from naïve mice (six per strain and sex). To prepare tissue for binding assays, dissected regions were placed in 10 volumes of ice-cold hypotonic buffer (NaCl, 14 mM; KCl, 0.15 mM; CaCl₂, 0.2 mM; MgSO₄, 0.1 mM; HEPES, 2.5 mM; pH 7.5) and homogenized using a motor-driven pestle.

Homogenized samples were centrifuged at $12,000 \times g$ for 20 minutes. The pellet was re-suspended in hypotonic buffer and again centrifuged. The re-suspension/centrifugation cycle was repeated three times. The resulting pellet was stored frozen in hypotonic buffer until assayed. Protein was measured using the method of Lowry [35], with bovine serum albumin as the standard. We used 25 mM [^3H] MPEP (specific activity, 46.85 Ci/mmol; American Radiolabeled Chemicals Inc., Saint Louis, MO) in a radioligand binding assay to measure ligand binding density (β_{max}) of mGluR5. Binding assays were done using 50 μg protein in a 100 μl reaction volume. After incubation for one hour at room temperature, tissues were rapidly filtered using an Inotech cell harvester (Inotech, Rockville, MD) and two filters: top - GFB (MFS, Dublin, CA) and bottom - Type A/E (Gelman Sciences, Ann Arbor, MI). Radioactivity remaining on the filters was detected by scintillation counting. Nonspecific binding was determined using 100 μM nonradioactive MPEP. Each binding experiment ($n = 6$ mice) was performed with three replicates for each strain, sex and brain region. Data were analyzed using ANOVA, with strain and sex as independent variables and amount of bound [^3H] MPEP (fmol/mg protein) as the dependent variable.

Results

LORR Testing

mGluR5 mice (WT, HET, KO) were injected with 20 mg/kg propofol and assessed for LORR. Data was analyzed using ANOVA with LORR duration as the dependent variable and genotype and sex as the independent variables. Results showed no significant effect of genotype or sex (data not shown), which fails to support the hypothesis that *mGluR5* mediates propofol sensitivity. To test for ethanol sensitivity (LORE), mice were injected ip with 4.1 g/kg ethanol. There was a significant effect of genotype ($p < .03$) but no effect of sex and no significant interaction. *Post-hoc* tests (Bonferroni-corrected *t*-tests) showed that KO mice had a significantly longer LORE duration than WT (110 ± 8 minutes vs. 82 ± 6 minutes, $p < .01$; Figure 3).

As another test of the hypothesis that *mGluR5* underlies the *Lore7* QTL, we looked at the effects of the mGluR5 antagonist 2-methyl-6-(phenylethyl)-pyridine (MPEP) on LORE in ILS and ISS mice. A preliminary study (data not shown) found that MPEP administration by itself (5, 10, or 20 mg/kg) had no activating or sedative effects in ILS or ISS. Separate groups of mice were then injected ip with either saline or 10 mg/kg MPEP, dissolved in 0.9% saline. Ten minutes later mice were injected ip with ethanol, either 2.5 g/kg (ILS) or 4.1 g/kg (ISS), and assessed for LORE. LORE duration was the dependent variable and strain, treatment (saline or MPEP) and sex were independent variables in the ANOVA. There were significant main effects of strain ($p < .001$) and treatment ($p < .01$), and significant strain by treatment ($p < .01$) and sex by treatment ($p < .05$) interactions. In ISS mice, MPEP significantly decreased LORE duration by approximately 14 minutes, in both males and females (Bonferroni-corrected *t*-tests, p 's $< .01$ and $.04$, respectively; Figure 4). A somewhat different picture emerged in ILS mice. Among saline-pretreated mice, ILS males had a longer LORE duration than ILS females (53 vs. 40 minutes, $p < .10$; Figure 4). This is consistent with previous studies showing a longer LORE duration in LS males compared to females [1,36-38]. While pretreatment with 10 mg/kg MPEP had no effect on LORE duration in male ILS mice, MPEP significantly increased LORE duration in female ILS mice ($p < .01$).

The differential effect of MPEP pretreatment on LORE in ILS and ISS led us to conduct a full dose-response study. ILS and ISS mice were injected ip with saline, 5, 10, 20, or 30 mg/kg MPEP. Ten minutes later, mice were injected ip with 4.1 g/kg ethanol (ILS and ISS) and assessed for LORE. LORE duration was the dependent variable and genotype, treatment and sex were independent variables; *post-hoc* analyses consisted of Bonferroni corrected *t*-tests. We found significant main effects of genotype, treatment and sex (p 's $< .01$), and significant

genotype by sex ($p < .04$) and treatment by sex ($p < .02$) interactions. Decomposition of the interactions shows several things (Figures 5A and 5B). In ISS mice receiving saline, males and females had identical LORE durations. Following pretreatment with MPEP, there was a V-shaped dose-response curve. At lower doses of MPEP (5 and 10 mg/kg), LORE duration was significantly decreased (p 's $< .02$), while at higher doses of MPEP, LORE duration was not significantly different from saline treated mice. There was no effect of sex on LORE duration. While the decrease in LORE duration at the lower doses of MPEP was significant, ISS have a short LORE duration to begin with and the decreases were only around 5 minutes. In contrast, in ILS mice pretreated with saline, males had a significantly longer LORE duration than females ($p < .01$). All doses of MPEP increased LORE duration in ILS females ($p < .02$ for 5 and 10 mg/kg, $p < .01$ for 20 and 30 mg/kg), while only the 30 mg/kg dose increased LORE in males ($p < .01$). This differential effect of MPEP on LORE duration in ILS males and females may be due to the fact that ILS males have a greater basal LORE duration.

Quantitative Complementation

In order to test for quantitative complementation, *mGluR5* heterozygous KOs (*mGluR5*^{+/-}) were crossed to ILS and ISS (Figure 1). This resulted in mice with one or two functional copies of *mGluR5* on both ILS (ILS^{*mGluR5*+/+} and ILS^{*mGluR5*+/-}) and ISS (ISS^{*mGluR5*+/+} and ISS^{*mGluR5*+/-}) backgrounds. LORE duration was the dependent variable and line (ILS or ISS), genotype (+/+ or +/-) and sex were the independent variables. ANOVA showed a significant line by genotype interaction ($p < .02$), which demonstrates a failure of the *mGluR5* mutation to complement the *Lore7* QTL. In the cross to ISS, +/+ and +/- mice had identical LORE durations, while in the cross to ILS, +/- mice had a significantly longer LORE than +/+ mice ($p < .01$; Figure 6). This provides additional support for *mGluR5* underlying the *Lore7* QTL. We also observed an effect of sex that was strain dependent ($p < .01$). There were no differences in LORE duration in male and female offspring from the cross to ISS. However, in the cross to ILS, while both male and female ILS^{*mGluR5*+/-} mice had significantly longer LORE durations than ILS^{*mGluR5*+/+} mice (p 's $< .03$ and $.01$), the effect was larger in females. There was an 18 minute difference in LORE duration between +/+ and +/- males, while there was a 34 minute difference between +/+ and +/- females, $p < .05$. Thus, in addition to mediating differential sensitivity to LORE in ILS and ISS, *mGluR5* also contributes to ILS male/female differences in LORE.

DNA Sequencing and SNP Analysis

DNA sequencing revealed no polymorphisms between ILS and ISS in the ~ 7500 bp of coding region of *mGluR5*, which included both 5' and 3' UTRs. However, it is possible that polymorphisms exist in noncoding or regulatory regions. Several databases have recently been created that incorporate single nucleotide polymorphism (SNP) data for many inbred and recombinant inbred (RI) strains of mice. Szatkiewicz and colleagues [39] have created a database that contains ~ 8 million SNPs by combining SNP data from public databases and imputing missing genotypes for over 50 inbred mouse strains, including ILS and ISS (<http://cgd.jax.org>). In addition, the WebQTL database (<http://www.genenetwork.org>) has SNP data on ILS and ISS. Between these two databases (counting only directly assayed SNPs and not imputed SNPs from the JAX database), 54 independent SNPs have been assayed in ILS and ISS in *mGluR5*. ILS and ISS are polymorphic for 42 of the 54 SNPs (Table 1). Forty-one of the 42 polymorphic SNPs are in the two large introns between exons 3 and 4, and exons 4 and 5 (Figure 2). While it seems unlikely that any of these intronic SNPs would be in regulatory regions, we examined all 42 polymorphic SNP sequences using the TRANSFAC database. None of the SNPs resided in a putative transcription factor binding site.

Seventy-five recombinant inbred lines derived from a cross between ILS and ISS (LXS RIs) were also assayed for a number of SNPs throughout the genome by the Wellcome Trust Center

(www.well.ox.ac.uk/mouse/INBREDS). Three of these SNPs (two polymorphic) were in introns of *mGluR5* (Table 1). We grouped the LXS RIs as ILS-like (20) or ISS-like (51) at these two SNPs and haplotype was correlated with LORE. Four RI strains had recombinations in this region and were not included in the analysis. RI lines that were ILS-like had a mean LORE duration of 91 ± 8 minutes, while RI lines that were ISS-like had a mean LORE duration of 73 ± 6 minutes (independent samples *t* test, $p < .02$). This provides additional evidence that *mGluR5* mediates sensitivity to the sedative properties of ethanol.

Quantitative PCR

We used q-RT-PCR to examine expression of *mGluR5* in ILS and ISS. Relative quantification was determined using the method of Livak and Schmittgen [34], with *Gapdh* mRNA as the endogenous reference. We examined mRNA levels in cerebellum, hypothalamus, striatum and hippocampus because these regions likely play a role in mediating LORE. Whole brain was used as the calibrator for relative expression measures. Each sample was assayed three times, in triplicate for each of the three assays. Data was analyzed using ANOVA, with strain and sex (within a brain region) as independent variables and $2^{-\Delta\Delta C_t}$ as the dependent variable. *mGluR5b* is functionally/pharmacologically the same as *mGluR5a* and it is the predominant transcript in most adult brain region [40], so we performed q-PCR only on the *mGluR5b* transcript.

As expected, *mGluR5b* was not highly expressed in cerebellum (Figure 7). The only effect we saw was that ILS males had greater relative expression than ILS females ($p < .01$). In striatum, analyses showed no significant main effects or interactions (Figure 7). In hypothalamus, ILS had greater relative expression than ISS ($p < .01$), while in hippocampus, we observed the opposite, where ISS had greater relative expression than ILS ($p < .02$; Figure 7). In addition to SNP data, WebQTL contains extensive gene expression and behavioral data for a number of inbred and recombinant inbred strains of mice, including ILS, ISS and the LXS RIs. Gene expression was assayed in hippocampal tissue in the LXS RIs using Illumina Sentrix Mouse-6 v 1.0 BeadArrays; data were normalized and analyzed using 7 different methods. The Illumina bead contained 5 probesets for *mGluR5*; three were in exons, one in an intron and one in the 3' UTR. As expected, significance of correlations between gene expression and LORE phenotypes depended on the method of normalization and analysis, each method representing a different dataset in WebQTL. Probesets 100610181 (exon 2) and 60528 (3' UTR) showed significant correlations with LORE phenotypes in 5 out of 7 and 7 out of 7 datasets, respectively. A representative output file is shown in Table 2 for the probeset 60528 (3' UTR), using the LOESS with Variance Stabilizing and Background Correction method (www.bioconductor.org). These significant correlations between *mGluR5* expression and LORE phenotypes in the LXS RIs provide further evidence that *mGluR5* mediates LORE.

Radioligand Binding

We examined ligand binding density ($\beta_{\max}$) of *mGluR5* in cerebellum, hypothalamus, striatum and hippocampus of naive ILS and ISS males and females. We conducted a preliminary study, using whole brain from ILS and ISS males and females, where we examined binding over a concentration range of 0.05-15.0 nM; nonspecific binding was determined using 10 μ M MPEP. ILS and ISS males and females reached saturation at similar concentrations (data not shown). Based upon these results, we chose a concentration of 15 nmol [3 H] MPEP to examine $\beta_{\max}$ in four brain regions. There was no effect of sex on $\beta_{\max}$ in any brain region. In hippocampus and striatum, ISS mice had significantly more MPEP bound than ILS (p 's $< .05$) (Figure 8).

Discussion

Loss of the Righting Reflex due to Ethanol (LORE) is a measure of sensitivity to the sedative effects of ethanol. The first LORE QTL identified was *Lore7* on mouse chromosome 7. DeFries et al. [6] found that in a set of LSxSS RIs derived from a cross between LS and SS, pigmented (LS-like) strains had a 19 minute longer LORE duration than albino (SS-like) strains; coat color accounted for 8.6% of the additive genetic variance in LORE. This suggested that the albino locus (tyrosinase; *Tyr*) on mouse chromosome 7, or a gene closely linked to *Tyr*, contributed to differential LORE sensitivity. While this QTL was confirmed in an F₂ population derived from a cross between ILS and ISS ($p < .01$), coat color accounted for only 1% of the phenotypic variance [7]. The *Tyr* locus, located on chromosome 7 at 94,577,327 - 94,641,899 bp (www.ensembl.org), has been ruled out as the *Lore7* QTL [9]. *mGluR5* lies immediately distal at 94,750,952 – 95,282,640 bp (www.ensembl.org), which makes it a good candidate gene for the *Lore7*, *Lorpl* and etomidate QTLs. We found no difference in propofol-induced LORE between *mGluR5* WT and KO mice, which suggests that *mGluR5* is not the *Lorpl* QTL. This conclusion is supported by a recent study showing that agonism or antagonism of mGluR5 had no effect on propofol-induced anesthesia [41]. However, several lines of evidence in the present study strongly implicate a role for *mGluR5* in sensitivity to the soporific effects of ethanol and provide good evidence that *mGluR5* is a gene that underlies the *Lore7* QTL.

The gold standard for “proving” a candidate gene underlies a QTL is an allele-swapping strategy in which the candidate allele from a low-scoring strain is swapped for an allele from a high-scoring strain, and vice versa. If phenotype changes with allele swapping, that provides convincing evidence that the allele is the QTL. This technique has not yet been perfected in mice. Instead, a number of different strategies can be used in rodents to demonstrate that a gene underlies a QTL, including investigation of transgenic mice that have a gene rendered nonfunctional or transgenic mice that over-express a gene, a quantitative complementation test, use of RNA interference to reduce mRNA level of a gene, pharmacological manipulation of a receptor/protein, detection of altered gene expression using q-PCR or microarrays, detection of altered protein levels using western blot or radioligand binding assays and detection of DNA sequence polymorphisms [42].

We found that mice in which *mGluR5* has been rendered nonfunctional exhibit a significant increase in LORE duration compared to WT littermates. These results support recent findings showing that *mGluR5* KO mice have a longer LORE duration than WT [27,43]. One caveat in interpreting these studies is that only 3-6 generations of backcrossing occurred in the KOs; thus, a gene flanking *mGluR5* may have been the gene influencing LORE and not *mGluR5*. Results with the KOs prompted us to conduct a quantitative complementation test. This breeding strategy, and variations thereof, has been used in drosophila and other model organisms for some time, but has only recently been used in mice [44-47]. We crossed *mGluR5* heterozygous KOs (*mGluR5*^{+/-}) with both ILS and ISS. Within each cross this produced two types of offspring, those with two functional copies of *mGluR5* and those with one functional and one nonfunctional copy (ILS^{*mGluR5*+/+}, ILS^{*mGluR5*+/-}, ISS^{*mGluR5*+/+} and ISS^{*mGluR5*+/-}). If the candidate gene is the QTL, the expectation is that there will be an effect of one vs. two functional copies of *mGluR5* in one of the two crosses but not the other, or there will be opposite effects in the two crosses; the mutant allele fails to complement the wildtype allele and this provides evidence that the candidate gene underlies the QTL. Alternatively, if the effect of substituting a mutant allele for a wildtype allele is the same in both crosses (to high and low scoring strains), then the mutant is “complemented” by both high and low scoring strains and this provides evidence that the candidate gene is not the QTL [30].

In the cross to ISS, there was no effect of the mutant allele: ISS^{*mGluR5*+/+} and ISS^{*mGluR5*+/-} offspring had the same LORE duration. In contrast, in the cross to ILS, ILS^{*mGluR5*+/+} offspring

had a LORE duration 22 minutes longer than ILS^{mGluR5^{+/+}} offspring. This failure to complement provides good evidence for *mGluR5* being the *Lore7* QTL. One of the drawbacks to this strategy is that, within each cross, offspring will be approximately 50% B6 and 50% ILS or ISS. It would be ideal to have the *mGluR5* null mutation on a background that is 100% ILS or ISS. Therefore, we mated *mGluR5*^{-/-} females with male ILS and ISS mice. Offspring were all heterozygous for *mGluR5*. We then mated female offspring back to ILS and ISS males. In subsequent generations, female offspring were genotyped for the presence of the neo cassette, which indicates they have one nonfunctional copy of *mGluR5*. We then mated these heterozygous females back to ILS and ISS with the intention of doing so for 10 generations. This would produce mice with one functional and one nonfunctional copy of *mGluR5* on backgrounds that would have been ~99.8% ILS or ISS. Unfortunately, our colony experienced an extreme reproduction problem and we lost all of these lines in generations 5-7 of backcrossing. An additional caveat to this strategy, as discussed by Mackay [31,48], is that failure to complement indicates a genetic interaction, but cannot discriminate whether the interaction is allelic or epistatic. Therefore, failure to complement does not constitute absolute proof that the candidate gene and QTL are the same and additional, corroborating evidence is needed.

We have also shown a differential effect of the mGluR5 antagonist/inverse agonist, MPEP, in ILS and ISS mice. Low doses of MPEP (5 and 10 mg/kg) decrease LORE duration in ISS mice, while higher doses of MPEP have no effect on LORE. In contrast, while only the highest dose of MPEP (30 mg/kg) increased LORE in ILS males, all doses of MPEP tested significantly increased LORE in ILS females. These findings with ILS support previous research showing that MPEP administration increases LORE duration in B6 and DBA/2J mice [24,43]. A picture seems to be emerging in which having less than two functional copies of *mGluR5*, or antagonizing the mGluR5 receptor, inhibits mGluR5 activity and *enhances* the acute sedative properties of ethanol. These conclusions are further supported by the finding that ISS mice, which are less sensitive to LORE, have higher mGluR5 receptor density in hippocampus and striatum, compared to the sensitive ILS mice. In contrast, inhibition of mGluR5 by either having less than two functional copies or antagonism, *decreases* ethanol consumption, seeking and self-administration, reduces the alcohol deprivation effect, inhibits the discriminative stimulus effects of ethanol and reduces the breakpoint in ethanol operant conditioning paradigms; effects on ethanol-induced locomotor activation and ethanol place preference are equivocal [18,20, 21 25,27,43].

The effects of MPEP on LORE in ISS mice are difficult to explain. As noted above, at lower doses, MPEP actually decreased LORE in ISS mice, while higher doses had no effect on LORE. These results are in contrast with findings in ILS, B6 and D2 mice, where MPEP increases LORE. This makes sense in that antagonism of receptors in the glutamate system, the primary excitatory transmitter in the CNS, would increase sedation induced by alcohol. Thus, it appears as if MPEP is acting differently in ISS mice, but how this occurs is not clear. At higher doses/concentrations, MPEP has been shown to act as an antagonist at recombinant NMDA receptors; MPEP has also been shown to inhibit the norepinephrine transporter (NET) [49,50]. Inhibition of NMDA receptors should produce effects similar to inhibition of mGluR5 receptors – inhibit the actions of the excitatory neurotransmitter glutamate. It is possible that in ISS mice, at higher doses MPEP inhibits the NMDA (or mGluR5) receptor, which may counteract the decreased LORE observed in ISS mice at lower doses of MPEP. How lower doses of MPEP decrease LORE in ISS mice is not known. One possibility is through inhibition of the NET. Inhibition of the NET would allow norepinephrine to remain in synapses of ISS mice longer and exert its' excitatory effect, which could lead to decreased LORE. Interestingly, *Net* is the only gene, other than *mGluR5*, for which there is good evidence to support a role in differential sensitivity to the sedative effects of ethanol [51]. There is 30% lower NET mRNA and protein levels in hippocampus of ILS compared to ISS mice; there is less NET for MPEP to bind in ILS [51].

However, pretreatment with the NET inhibitors nisooxetine or desipramine actually increased LORE in ILS mice but had no effect on LORE in ISS, which does not support this hypothesis. The mechanism of action leading to decreased LORE in ISS mice following pretreatment with low doses of MPEP remains to be determined.

DNA sequencing revealed no coding region polymorphisms, so it is unlikely that the structural mGluR5 protein is different between ILS and ISS. *mGluR5* is a large gene with just over 540,000 bp of genomic DNA. It has two extremely large introns, one about 196,000 bp and one about 171,000 bp. While we found no coding region SNPs, there are 42 polymorphic ILS/ISS SNPs in intronic regions, almost all of which reside in these two large introns. Two of these SNPs have been assayed in the LXS RIs, rs6181499 and rs13479389. LXS RI strains that are ILS-like at these SNPs have an 18 minute longer LORE duration than mice that are ISS-like at these SNPs. These two SNPs, or others in the same haplotype, may contribute to differential LORE sensitivity. While none of the 42 polymorphic SNPs is in a transcription factor binding site as predicted by TRANSFAC, it is still possible that one or more of these SNPs is in a region critical for transcriptional or posttranscriptional control. It is interesting to note that genes encoding other metabotropic receptors, including *GRM8*, have large introns, similar to *mGluR5/GRM5* (www.ensembl.org). One of the large introns in *Grm8* contains a microRNA (miRNA) [52]. miRNAs are short, noncoding RNAs that usually bind the 3' end of target genes and posttranscriptionally mediate gene repression. Recently, it has been shown that many, if not most, miRNAs reside in large introns of coding or noncoding transcripts [52,53]. It is tempting to speculate that one of the two large introns in *mGluR5* contains a miRNA, but none have been reported in the literature. We ran these intronic sequences through several miRNA prediction programs and found little evidence that either of these introns contains a miRNA.

An intriguing finding in the present study is that mGluR5 has different effects on LORE in ILS males and females. Studies have consistently reported that in LS and ILS mice, males have a significantly longer LORE duration than females [1,36-38], a finding supported in the present study. The quantitative complementation test showed that in the cross to ILS, male ILS^{mGluR5+/-} mice had an 18 minute longer LORE duration than ILS^{mGluR5+/+} mice, while in females the difference was 34 minutes. In addition, while MPEP significantly increased LORE duration in ILS males only at the highest dose (30 mg/kg), it increased LORE in females at all doses tested. Finally, we found that compared to ILS females, ILS males had greater relative expression of *mGluR5b* in cerebellum and hypothalamus, although the significance of these transcript differences is unclear because ILS males and females had no differences in receptor density in these regions. Interestingly, while *mGluR5* KO mice had a significantly longer LORE duration than WT littermates, we found no sex differences in LORE duration in *mGluR5* KO mice.

Results from human studies also suggest that *mGluR5* may be involved in differential ethanol sensitivity. Schuckit and colleagues have shown that a person's initial sensitivity or level of response (LR) to alcohol can be a good predictor for problems with alcohol later in life [54, 55]. LORE is a phenotype similar to LR. Earlier studies from the Collaborative Study on the Genetics of Alcoholism (COGA) have reported linkage for LR to a region on human chromosome 11 which contains *GRM5*, the human homolog of *mGluR5*, and have cited *GRM5* as a candidate gene for the LR phenotype [56-58]. What is particularly intriguing is that this region of linkage on human chromosome 11 is specific for the LR response; genome scans for other alcohol phenotypes (i.e., alcohol dependence) did not report linkage in this region. While a recent study looked at several SNPs in *GRM5* and found a significant association with two of the SNPs and alcohol dependence in a German population, the association was not confirmed in a separate German population [59].

We have provided several lines of evidence that *mGluR5* mediates differential sensitivity to LORE. Results from the complementation test support *mGluR5* being the *Lore7* QTL. *mGluR5* KO mice had a significantly longer LORE duration than WT mice; antagonism of mGluR5 had opposite effects on LORE in ILS and ISS. While we found no coding region polymorphisms between ILS and ISS, there were many intronic SNPs. Two of these SNPs were highly correlated with LORE duration in the LXS RIs. Compared to ILS, ISS had greater relative expression of *mGluR5* in the hippocampus and expression of *mGluR5* in hippocampus was highly correlated with LORE phenotypes in the LXS RIs. ISS had a greater receptor density in both hippocampus and striatum, compared to ILS. Taken together, this is the best evidence to date for a gene underlying any LORE QTL. Evidence from the human literature also supports a role for *Grm5* in mediating initial sensitivity to ethanol.

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Figure 1.

A quantitative complementation test for determining if a candidate gene underlies a QTL. Strains containing mutated and wild-type alleles (*mGluR5*^{+/-}) are crossed with strains containing alternate QTL alleles (ILS and ISS). Failure of the mutation to complement the QTL is inferred if the mean phenotypic difference between the ILS^{*mGluR5*^{+/-}} and ISS^{*mGluR5*^{+/-}} is greater than that between ILS^{*mGluR5*^{+/+}} and ISS^{*mGluR5*^{+/+}}. The interaction term in ANOVA (Line: ILS or ISS and Genotype: +/+ and +/-) is used to test for quantitative complementation.

Figure 2.

Structure of the *mGluR5* gene (www.celera.com; Corti et al. 2003). Black boxes and roman numerals indicate exons. At least two transcripts have been identified in human, mouse and rat, one containing 96 basepair exon X, and one without exon X. In addition, two alternate first exons have been reported. The rat and mouse copies of the gene also contain an extra exon, II, not found in the human gene. The rather large exon IX contains the 7 transmembrane spanning domain.

Figure 3. Loss Of Righting due to Ethanol (LORE). The asterisk indicates that KO mice had a significantly longer LORE duration than WT ($p < .01$). Sample sizes: WT ♀ = 14, ♂ = 17; HET ♀ = 21, ♂ = 20; KO ♀ = 12, ♂ = 9.

Figure 4.

Initial MPEP testing in ILS and ISS. Animals received an ip injection of either saline or 10 mg/kg MPEP. Ten minutes later mice were injected with ethanol (2.5 g/kg for ILS and 4.1 g/kg for ISS) and tested for LORE. * indicates MPEP increased LORE relative to saline, $p < .03$; ** indicates MPEP decreased LORE relative to saline, $p < .04$; *** indicates MPEP decreased LORE relative to saline, $p < .01$. Sample sizes: ILS ♀ = 12, ♂ = 10; ISS ♀ = 14, ♂ = 12.

Figure 5a.

LORE following several doses of MPEP in ISS mice. While lower doses of MPEP (5 and 10 mg/kg) significantly decreased LORE in males and females (p 's < .02 *), higher doses had no effect. Sample sizes: ♀ saline = 19; 5 mg/kg MPEP = 11; 10 mg/kg MPEP = 11; 20 mg/kg MPEP = 10; 30 mg/kg MPEP = 12. ♂ saline = 14; 5 mg/kg MPEP = 11; 10 mg/kg MPEP = 11; 20 mg/kg MPEP = 10; 30 mg/kg MPEP = 11.

Figure 5b.

LORE following several doses of MPEP in ILS mice. In contrast to ISS mice, all doses of MPEP significantly increased LORE in ILS females (p 's $< .01$ * or $.02$ **), while only the 30 mg/kg dose increased LORE in males ($p < .01$ *). Sample sizes: ♀ saline = 13; 5 mg/kg MPEP = 12; 10 mg/kg MPEP = 14; 20 mg/kg MPEP = 13; 30 mg/kg MPEP = 11. ♂ saline = 15; 5 mg/kg MPEP = 13; 10 mg/kg MPEP = 13; 20 mg/kg MPEP = 14; 30 mg/kg MPEP = 12.

Figure 6.

Results of the quantitative complementation test. Mice heterozygous for the *mGluR5* null mutation (*mGluR5*^{+/-}) were crossed with both ILS and ISS. This produced two classes of progeny within each cross; those with one functional copy of *mGluR5* and those with two functional copies. ANOVA showed a significant line (ILS or ISS) by genotype (+/+ or +/-) interaction ($p < .02$). As can be seen, in the cross to ISS, there were no differences in LORE between mice with one (+/-) and mice with two (++) functional copies of *mGluR5*. In contrast, in the cross to ILS, +/- mice had a significantly longer LORE duration than +/+ mice ($p < .01$ *). This failure to complement provides further support for *mGluR5* underlying the *Lore7* QTL. In addition to a significant Line by Cross interaction, ANOVA also revealed a significant Line by Sex interaction ($p < .01$). This interaction is readily apparent in the cross of *mGluR5*^{+/-} mice with ILS. While +/- mice had significantly longer LORE duration than (+/+), the difference is much larger in female mice compared to males ($p < .05$ **). Sample sizes: ILS +/+ ♀ = 13, ♂ = 8; ILS +/- ♀ = 13, ♂ = 9; ISS +/+ ♀ = 14, ♂ = 11; ISS +/- ♀ = 9, ♂ = 10.

Figure 7.

Quantitative PCR for *mGluR5* in cerebellum, striatum, hypothalamus and hippocampus. The amount of *mGluR5b* mRNA was determined in ILS and ISS mice, normalized to *Gapdh* and relative to whole brain, using the $2^{-\Delta\Delta CT}$ method. Significance of fold changes was examined using ANOVA, with $2^{-\Delta\Delta CT}$ values as the dependent variable and strain and sex (within a brain region) as independent variables. There were no differences in relative expression between ISS males and females, so ISS data are presented collapsed on sex. Because we did observe a male/female difference in ILS, data are presented both collapsed on sex and for each sex separately. *indicates that ILS males had greater relative expression than ILS females, $p < .01$. **indicates that ILS had greater relative expression than ISS, $p < .01$. ***indicates that ISS had greater relative expression than ILS, $p < .02$. Each sample was assayed 3 times; for each assay, samples were run in triplicate. Thus, each Ct represents the average of 9 values.

Figure 8.

Radioligand binding in ILS and ISS mice. * indicates that ISS mice had significantly greater binding of [³H] MPEP in hippocampus and striatum compared to ILS mice (p 's < .03). Means are based on twelve mice (6 males and 6 females) per strain. Sample size = 6 per strain (3 male and 3 female) and brain region.

Table 1

Single nucleotide polymorphisms (SNPs) in the *mGluR5* gene in Inbred Long-Sleep and Inbred Short-Sleep mice. Megabase positions are taken from the ensemble website, March 2009 (www.ensembl.org). Bolded SNPs indicate they were genotyped in the LXS RIs as well.

Perlegen SNP ID	refSNP ID	Megabase	ILS	ISS
	rs13479387	94578015	T	C
NES14934290	rs31836123	94583030	C	A
NES14934214	rs31241583	94586047	A	G
NES14934068	rs31757497	94594847	A	G
NES14934071	rs31808916	94595048	G	A
NES14933728	rs31929479	94615933	A	G
NES14933680	rs31965260	94619140	T	G
	rs31480317	94621246	C	T
NES14933224	rs31191169	94641553	C	G
NES14932418	rs31539196	94715257	C	T
NES16096603	rs45695412	94743459	T	C
NES16096607	rs51656034	94743875	G	A
NES16096613	rs46273793	94744323	T	C
NES16096617	rs32172220	94744373	T	C
NES16096422	rs32210874	94758884	T	G
NES16096423	rs49721682	94759084	C	T
NES16096416	rs51309083	94759624	G	A
NES16096403	rs46027436	94760966	C	T
NES16096404	rs51850154	94761362	G	A
NES16096406	rs49389493	94761411	G	T
NES16096001	rs48793025	94793919	C	A
NES16095976	rs48301046	94796205	T	C
NES16095978	rs49732139	94796319	T	C
NES16095980	rs51611898	94796670	A	C
NES16095946	rs48910102	94797873	A	G
NES16095947	rs49346278	94798022	A	G
NES16095909	rs46278976	94798755	A	G
NES16095913	rs32311480	94799195	C	G
NES16095914	rs31479529	94799294	T	C
NES16095916	rs45813501	94799472	T	C
NES16095864	rs49977281	94800047	C	T
NES16095865	rs49985127	94800074	T	C
NES16095869	rs49539602	94800415	C	T
NES16095870	rs48443033	94800478	A	G
NES16095859	rs50549068	94801571	A	G

Perlegen SNP ID	refSNP ID	Megabase	ILS	ISS
	rs32105080	94811879	G	A
NES16095763	rs50765775	94817200	C	T
	rs32530740	94821414	T	C
NES16095714	rs32410969	94821444	A	T
NES16095519	rs31845860	94837191	G	A
NES16095521	rs31094508	94837283	G	T
NES16095471	rs31057530	94838395	T	C
NES16095334	rs6181499	94853434	A	C
NES14932094	rs32402357	94863634	G	A
NES14932095	rs37424339	94863660	T	A
NES14932101	rs38401377	94864636	T	C
NES14932044	rs36654768	94865804	C	T
NES14932045	rs36963687	94865912	G	T
NES14932009	rs38059009	94866995	T	C
NES14932010	rs36423415	94867583	T	G
NES14931909	rs32090707	94880439	C	C
	rs31385981	94884788	G	G
NES14931811	rs31492839	94889125	A	T
NES14931732	rs31403109	94894097	G	G
	rs32110279	94895052	G	G
	rs31595865	94917607	C	C
NES14930843	rs31087771	94963993	G	G
	rs31248147	94964335	T	T
NES14930740	rs31574832	94967803	T	T
NES14930660	rs13479389	94974789	C	G
NES14929875	rs6381934	95032942	G	G
	rs3672782	95155271	C	C
NES14927824	rs6214573	95230998	T	T
NES14927828	rs6229390	95231350	A	A
NES14926894	rs13479390	95309450	C	T

Table 2

Correlations between *mGluR5* expression in hippocampus and measures of LORE in the LXS RIs. All data are taken from WebQTL (www.genenetwork.org). Data here represent correlations with the probeset ILM60528 (3' UTR), using the Illumina Sentrix Mouse 6 v.1 microarray system. For more detailed information on the LORE phenotypes see references.

Record ID	Phenotype	Reference	Correlation	P-value
10067	Cohort2 male sleep time average	Bennet & Carosone-Link 2006	.2855	.01706
10090	Loss of righting reflex	Haughey et al. 2005	.2368	.04198
10068	LXS male sleep time	Bennett & Carosone-Link 2006	.2216	.05584
10071	LXS female sleep time	Bennett & Carosone-Link 2006	.2044	.08065
10069	Cohort1 female sleep time average	Bennett & Carosone-Link 2006	.1983	.09268