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mGluR7 Genetics and Alcohol: Intersection Yields Clues for Addiction

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Abstract

Development of addiction to alcohol or other substances can be attributed in part to exposure-dependent modifications at synaptic efficacy leading to an organism which functions at an altered homeostatic setpoint. Genetic factors may also influence setpoints and the stability of the homeostatic system of an organism. Quantitative genetic analysis of voluntary alcohol drinking, and mapping of the involved genes in the quasi-congenic Recombinant QTL Introgression (RQI) strain system, identified *Eac2* as a Quantitative Trait Locus (QTL) on mouse chromosome 6 which explained 18% of the variance with an effect size of 2.09 g/kg/day alcohol consumption, and *Grm7* as a quantitative trait gene (QTG) underlying *Eac2* (Vadasz et al., 2007a; Vadasz et al., 2007c). In earlier studies, the product of *Grm7* mGluR7, a G protein-coupled receptor, has been implicated in stress systems (Mitsukawa et al., 2005), anxiety-like behaviors (Cryan et al., 2003), memory (Holscher et al., 2005), and psychiatric disorders (e.g., (Mick et al., 2008; Ohtsuki et al., 2008; Pergadia et al., 2008). Here, in experiments with mice, we show that (1) *Grm7* knockout mice express increased alcohol consumption, (2) sub-congenic, and congenic mice carrying a *Grm7* variant characterized by higher *Grm7* mRNA drink less alcohol, and show a tendency for higher circadian dark phase motor activity in a wheel running paradigm, respectively, and (3) there are significant genetic differences in *Grm7* mRNA abundance in the mouse brain between congenic and background mice identifying brain areas whose function is implicated in addiction related processes. We hypothesize that metabotropic glutamate receptors may function as regulators of homeostasis, and *Grm7* (mGluR7) is involved in multiple processes (including stress, circadian activity, reward control, memory, etc.) which interact with substance use and the development of addiction. In conclusion, we suggest that mGluR7 is a significant new therapeutic target in addiction and related neurobehavioral disorders.

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Keywords

QTL; QTG; RQI; genetics; knockout; gene expression; *Grm7*; mGluR7; brain; hippocampus; cortex; alcohol; addiction; circadian activity; C57BL/6; BALB/c

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Introduction

Consumption of alcohol containing beverages is a characteristic feature of the western world: Seventy-five percent of U.S. adults over the age of 18 have consumed alcohol (NCHS, 2009). A recent study, ranking 20 drugs on 16 measures of harm to users and to wider society, concluded that in terms of the cost to society, alcohol causes the biggest harm (Nutt et al., 2010). Estimates of social and economic costs of alcohol abuse for industrialized countries vary between 1 to 6% of the GDP (Gross Domestic Product) reaching, for example, \$184.6 billion in the USA (1998), or €26–66 billion in Italy (2003) (WHO, 2004).

Alcoholism is a complex, heterogeneous disorder, and no single animal model is available to reflect the entire spectrum of its characteristics. However, accepted animal models are available for various aspects of alcoholism. In the progression from occasional social drinking to alcohol dependence, alcohol preference drinking is considered as the first and required phase. Alcohol preference drinking in a laboratory choice paradigm is generally considered as a useful model of the initial phase of the process leading to addiction to alcohol.

Genetic factors significantly affect alcoholism, and alcohol consumption. Approximately 50% of the variance in alcohol dependence is accounted for by genetic factors (Goldman et al., 2005), and there is evidence for genetic contributions to alcohol consumption (Grant et al., 2009). Symptoms of alcohol use disorders are associated with the intensity of alcohol use, and integration of a quantifiable indicator of excessive consumption into diagnostic criteria has been proposed (Li, 2008). In animal models alcohol consumption is a quantitative trait, therefore, discovery of quantitative trait genes which modulate consumption have clinical implications.

Intensive search for genes which influence the development of alcoholism yielded only limited success and led to the realization that the “alcoholism” phenotype is more complex than it was hypothesized. Work with genetic animal models targeting identification of quantitative trait genes (QTGs) for alcohol related behaviors may be expected to be more promising, because mapping is more powerful in experimental crosses than in human families (Lander and Schork, 1994). However, QTG identification in experimental crosses did not fare much better in spite of progress in genomics and bioinformatics. Identifying the genes underlying quantitative trait locus (QTL) peaks by positional “cloning” has proven *elusive* in all but a handful of cases (Mackay, 2004; Weiss, 2008). One of the major factors responsible for the limited progress was the neglect of epistasis (interaction among loci or between genes and environmental factors) in complex trait studies (Carlborg and Haley, 2004).

To reduce the epistasis-induced genetic background variation (“genetic noise”) in an experimental mapping population we introduced the principle of Recombinant QTL Introgression (RQI) for identification of QTLs and QTGs on a homogeneous genetic background (Abiola et al., 2003). We used an RQI mouse strain panel for mapping QTLs for alcohol preference drinking using a two-bottle choice paradigm, and we detected several QTLs including *Eac2*, a QTL on mouse chromosome 6, which significantly influenced alcohol drinking (Vadasz et al., 2007a). In further studies we identified a cis-regulated gene (*Grm7*, glutamate receptor metabotropic subtype 7) as a candidate for underlying *Eac2* (Vadasz et al., 2007c). *Grm7* is the first identified polymorphic gene which modulates alcohol drinking and provides an excellent pharmacological target. *Eac2* is syntenic with human chromosome 3p. Genetic studies recently demonstrated that the *Grm7*^{BALB/cJ} gene variant was associated with higher abundance of *Grm7* mRNA in the brain, and lower level of alcohol preference drinking when compared with individuals carrying the *Grm7*^{B6By} variant on the same genetic background (Vadasz et al., 2007c). This observation led to the hypotheses that (1) lower expression of the *Grm7* gene, and diminished levels of the mGlu7 receptor can lead to an increase in the daily consumption of alcohol suggesting a role for *Grm7* in addiction, and (2) pleiotropic actions of *Grm7* on other phenotypes may also be significantly influenced by polymorphism of *Grm7*. Lending further support to our hypothesis that genetic variation in *Grm7* plays a significant role in addiction, recent genome wide association studies implicated *GRM7* (3p26.1-p25.1) and glutamate signaling in human alcohol use disorders (Joslyn et al., 2010).

L-glutamate, the principal excitatory neurotransmitter in the brain, interacts with both ionotropic and metabotropic glutamate receptors (mGluRs). At least eight sub-types of metabotropic receptor (mGluR1-8) have been identified in cloning studies. They have been divided into 3 groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties (*Group I*: mGluR1, and 5, *Group II*: mGluR2, and 3, and *Group III*: mGluR4, 6, 7, and 8 (see for a review (Niswender and Conn)). While *Group I* mGluRs are coupled to the polyphosphoinositide signaling pathway, *Group II* and *Group III* mGluRs are linked to the inhibition of the cyclic AMP cascade, but differ in their agonist selectivities (Okamoto et al., 1994).

mGluR7 functions as an inhibitory presynaptic autoreceptor controlling glutamate release, or as a heteroreceptor regulating release of other neurotransmitters, for example GABA (Sansig et al., 2001; Somogyi et al., 2003). It is the most conserved metabotropic glutamate receptor across a wide variety of species (Flor et al., 1997; Makoff et al., 1996). High representation of mGluR7 was observed in the periventricular zone of the hypothalamus, the majority of neurons at all levels of the olfactory circuitry (Kinzie et al., 1995), the hippocampus, locus ceruleus, and amygdala (Kinoshita et al., 1998; Masugi et al., 1999). Simonyi et al. found the highest levels of mGluR7 expression in the dentate gyrus, CA1, CA3, and piriform cortex (Simonyi et al., 2000). Mice with genetic ablation of mGluR7 show 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 (Cryan et al., 2003; Masugi et al., 1999; Mitsukawa et al., 2006; Palucha et al., 2007; Stachowicz et al., 2008). mGluR7 functions as a low-pass filter that inhibits synapses which are firing above

certain frequencies (Shigemoto et al., 1997), and as a switch controlling bidirectional plasticity (Pelkey et al., 2005). Accumulating evidence highlights a considerable pleiotropic activity: *Grm7* (mGluR7) is implicated in emotion, cognition, and addiction (Li et al., 2009; O'Connor et al., 2010; Vadasz et al., 2007c).

Today mGluRs are considered by many to be the single most promising new collection of targets for CNS drug discovery, with therapeutic potential to treat illnesses ranging from migraine (Marin and Goadsby, 2010) to Fragile X (Bear, 2005), and from schizophrenia (Homayoun and Moghaddam, 2008) to Parkinson's disease (Lopez et al., 2007; Niswender et al., 2008).

Here, to further investigate the role of *Grm7* in alcohol related behaviors we show that (1) genetic dysfunction of *Grm7* increases alcohol consumption, (2) in a new congenic model, which carries the "hyper-functioning" *Grm7*^{BALB/cJ} allelic variant, alcohol drinking is significantly reduced, and (3) the cis-regulated *Grm7*^{BALB/cJ} allele is associated with higher *Grm7* mRNA abundance in the hippocampus, cingulate cortex and motor cortex, and with a tendency for higher motor activity in the circadian dark phase.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6ByJ (B6By), and BALB/cJ (C) inbred, B6By.C6.132.54 and B6By.C6.327.54 congenic, and knockout (B6.129P2-*Grm7*^{tm1Dgen} and B6.129/Ola-*Grm7*^{tmNovartis}) mice were maintained at the Animal Facility of the Nathan S. Kline Institute for Psychiatric Research. Development of the B6By.C6.132.54 congenic strain, which carries a small segment of BALB/cJ chromosome #6 on C57BL/6ByJ background, has been described (Vadasz et al., 2007c). The new congenic strain B6By.C6.327.54 was established by multiple crosses between C57BL/6ByJ and B6By.C6.132.54, and screening for recombinants. B6By.C6.327.54 carries a homozygous segment where D6Mit327, D6Mit148, D6Mit287, and D6Mit54 markers were of BALB/c-type, while the flanking markers D6Mit105 and D6Mit134 (and the rest of the tested markers in the genome) were of C57BL/6ByJ-type.

We used mGluR7 (*Grm7*) knockout mice from two sources. First, we used knockout mice developed by Deltagen, Inc. with mixed genetic background. Deltagen mice were obtained from Mutant Mouse Regional Resource Centers (MMRRC, Stock Number: 011626-UNC; B6.129P2-*Grm7*^{tm1Dgen}). Mice recovered from a cryo-archive had health surveillance performed on the resuscitated animals. At the Animal Facility of the Nathan S. Kline Institute for Psychiatric Research the knockouts were maintained by backcrossing to C57BL/6ByJ. The knockout (*Grm7* KO/KO), heterozygote (*Grm7* WT/KO), and littermate wildtype (*Grm7* WT/WT) mice used in experiments were derived from a generation after three backcrosses to C57BL/6ByJ. Second, we used mGluR7 knockout mice obtained from Novartis Pharma AG (Basel, Switzerland). The generation of mGluR7-deficient mice (B6.129/Ola-*Grm7*^{tmNovartis}) has been described elsewhere (Sansig et al., 2001). Homozygous KO/KO and wild-type (WT/WT) B6.129/Ola-*Grm7*^{tmNovartis} mice were generated by mating F₁₅₊ B6-backcross generation mice heterozygous for the *Grm7* mutant

gene, and their gender-matched littermate offspring were used in the studies reported here. Animals were genotyped by PCR targeting the neomycin gene (Genbank/Refseq Acc. U00004.1 Ranges 1565–1583, 1626–1607, and 1585–1604). Detection of the KO allele was indirect by the presence of the neomycin marker. All procedures followed guidelines consistent with those developed by the National Institute of Health and the Institutional Animal Care and Use Committee of the Nathan S. Kline Institute.

Alcohol preference drinking in *Grm7* knockout experiments

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 one week prior to beginning the study. Mice were tested for alcohol preference and consumption according to the procedure for oral self-administration as described (Vadasz et al., 2007a). First, pilot studies were carried out on B6.129P2-*Grm7^{tm1Dgen}* mice on mixed genetic background (n=2–8). In further experiments we used fully backcrossed mGluR7 knockouts from Novartis Pharma AG.

In studies on *Grm7* knockouts the test consisted of 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 trials 1 and 2 (Day 1–6) was increased to 6% in trials 3 and 4 (Day 6–12), and to 12% for trials 5 and 6 (Day 12–18), and further increased to 18% for trials 7 and 8 (Day 19–24). Data collection focused on alcohol consumption of 12% (vol/vol) alcohol in males because these parameters were used in earlier studies which established *Grm7* as a quantitative trait gene for alcohol consumption (Vadasz, 1973; Vadasz et al., 2007b; Vadasz et al., 2007c). However, consumption at all concentrations were measured in males (3% n=2, 6% n=6–8, 12% n=10–13, 18% n=4–6). In females the same method was used but for technical reasons consumption at the 3% concentration was not measured (6%, 12%, and 18% n=3–5). 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. The position of the water and alcohol drinking tubes on the cage cover was 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. Data from two 3-day trials for each concentration were averaged for each individual.

Alcohol preference drinking in B6By.C6.327.54 sub-congenic experiments

The applied test was similar to that described for knockouts, however 18% alcohol solution was not tested. 3%, 6%, and 12% (vol/vol) alcohol solutions were tested, in two trials at each concentration using male mice of B6By.C6.327.54 sub-congenic and C57BL/6By background strains (n=9).

Circadian wheel-running baseline activity experiments on *Grm7* congenic and progenitor mice

Animals were individually housed in a light/dark cycle of 12/12 hrs (lights off at 17:30) in standard polycarbonate mouse cages with a flat stainless steel cover. This type of cover allowed positioning of water bottles on the top of the cage, thus providing space for a Mouse Low Profile RF Running Wheel (80 ENV-044) placed inside the cage. Information on revolutions of the wheel was collected by an USB Interface Hub for Wireless Running Wheels (DIG-804) attached to a computer, and RF Wheel Manager Software (SOF-860). Components of the system was obtained from Med Associates, Inc (St. Albans, VT). Food and water was available ad libitum. Data for analysis was used after at least one week of habituation to the cages equipped with low profile running wheels. In one batch of experiments eight cages equipped with low profile running wheels were used (four per strain). Data (number of wheel revolutions per hour) were collected continuously for eight days after habituation. Activity was analyzed in two 180 min intervals of peak activity (dark phase: 6–9 hr pm, shown as 19, 20, and 21 hrs; and light phase: 9–12 hr am, shown as 10, 11, and 12 hrs). The following strain comparisons were carried out on male mice. Experiment #1: BALB/cJ vs. C57BL/6ByJ (n=4), experiment #2: congenic B6By.C6.132.54 vs. C57BL/6ByJ (n=3–4). For statistical evaluation, first, average activity in the intervals in the dark or light phase was calculated for each individual using data collected during the 8 baseline days of the experiment. Next, strain differences were tested using the 8-day averages from individuals.

Quantitation of *Grm7* gene expression in serial tissue sections by *in situ* hybridization (ISH)

Tissue preparation—Whole mouse brains were frozen and stored at – 80 °C until sectioning. Cryostat cut coronal sections (12µm) were collected, dried, and stored at – 80 °C until use. Brain sections were selected through the nucleus accumbens and the dorsal hippocampus, with the appropriate anatomical levels determined according to the atlas of Franklin and Paxinos (Franklin and Paxinos, 2008). All tissues to be evaluated by a single statistical test were included in the same hybridization.

Probes—Oligonucleotides were 3' end-labeled by terminal deoxynucleotidyl transferase (Roche, Indianapolis, IN) with ³⁵S-dATP (Perkin Elmer, Boston, MA). The probe for metabotropic glutamate receptor 7 subtype was the same as in our earlier publications (Simonyi et al., 2004; Simonyi et al., 2000).

In situ hybridization—In situ hybridization was carried out as previously described (Simonyi et al., 2004; Simonyi et al., 2000). Briefly, brain sections were fixed in 4% paraformaldehyde/phosphate-buffered saline for 5 min. After treatment with acetic anhydride triethanolamine hydrochloride, slides were dehydrated through a graded series of ethanol, delipidated in chloroform, rehydrated to 95% ethanol, and air-dried. Fifty microliters of hybridization buffer was applied to each slide, and incubated at 42°C overnight. The hybridization buffer contained 50% formamide, 4 X SSC (600 mM NaCl/60 mM sodium citrate), transfer RNA (250 µg/mL), sheared, single-stranded salmon sperm DNA (100 µg/mL), 1 X Denhardt's solution (0.02% each of BSA, Ficoll, and

polyvinylpyrrolidone), 10% (w/v) dextran sulfate (MW 500,000), 100 mM DTT and 1×10^6 cpm probe. After hybridization, slides were washed in 1 X SSC (100 mM DTT) at 55°C for 4 X 15 min. Following two 30 min rinses in 1 X SSC at room temperature, the tissues were dipped in distilled water, immersed in 70% ethanol, and air-dried.

Autoradiography and signal quantitation—Slides were held against KODAK BIOMAX MR film with standards (0–2.15 $\mu\text{Ci/g}$; American Radiolabeled Chemicals Inc., St. Louis, MO, USA) in x-ray cassettes. Microdensitometry of film autoradiograms was performed on the signal over different brain regions using the BIOQUANT True Color Windows 95 software version 2.50 as previously described (Simonyi et al., 2004; Simonyi et al., 2000). [^{14}C]-microscale standards were used to construct calibration curves and quantitate signals in nCi/g tissue equivalents. The average density measured from experimental regions fell within the linear range of the standards. Background signal was subtracted from all measurements. Resulting values were averaged from four sections for each animal before being evaluated for statistical significance. The Franklin - Paxinos atlas (Franklin and Paxinos, 2008) was used for identification of brain nuclei. Two independent operators who were not aware of the identity of the brain sections performed the image analysis; only when both operators found specific effects are they reported as such.

Genotyping

B6By.C6.327.54 congenic strain was constructed by using microsatellite DNA markers polymorphic for C57BL/6ByJ and BALB/cJ. PCR products were analyzed by capillary gel electrophoresis (ABI 3100) as described (Vadasz et al., 2007a).

Statistical analysis

In all studies the values are expressed as mean $\pm$ Standard Error of Mean (S.E.M.). Statistical analysis of the data was performed by One-way ANOVA, or by Mann-Whitney U test when parametric analysis was not suitable. ANOVA was followed by Newman-Keuls Multiple Comparisons in the ISH data analysis.

RESULTS

Effects of targeted mutation of the gene coding for mGluR7 was first investigated in a pilot study using B6.129P2-*Grm7^{tm1Dgen}* mice on mixed genetic background. We found that male heterozygotes consumed more 12% alcohol than their wildtype littermates (11.21 vs. 4.91 g alcohol/kg body weight/day). However, because of the small sample size and the mixed genetic background, these data were considered only as potentially indicative ($p > 0.05$). Following up this lead, further experiments on fully backcrossed male B6.129/Ola-*Grm7^{tmNovartis}* knockouts and their wildtype littermates showed statistically significant differences in consumption of 12% (vol/vol) alcohol between homozygous knockouts (KO/KO) and their wild-type (WT/WT) littermates (Mann Whitney $U_{(1,22)} = 37$, $p < 0.05$; Fig. 1).

While higher voluntary alcohol intake in knockout mice support our working hypothesis that lower *Grm7* expression and lower mGlu7 receptor availability predispose to higher alcohol

consumption, considering inherent problems in interpretation of data obtained in studies on knockout animals due to developmental compensation, we planned to test further this hypothesis i.e. higher expression of *Grm7* in congenic animals confers “protection” against excessive drinking. Our laboratory reported the construction of B6.C6.132.54, the first congenic mouse strain carrying a *Grm7* allele on a chr. 6 segment of BALB/cJ origin on C57BL/6By background (Vadasz et al., 2007c). The objective of the current experiments was to reduce the length of the introgressed chromosome interval in congenic strain B6By.C6.132.54, which shows lower alcohol consumption than the background strain, and test the prediction that the newly constructed sub-congenic strain (B6By.C6.327.54, carrying a smaller number of “passenger” genes, and the *Grm7*^{BALB/cJ} gene variant) will still be showing lower alcohol consumption.

Heterozygous recombinants derived from crosses between B6.C6.132.54 and C57BL/6By were mated to produce homozygous offspring. The introgressed interval in the resulting B6By.C6.327.54 sub-congenic strain spanned D6Mit327 (49.99 cM, Chr6:109282670 bp) and D6Mit54 (52.14 cM; Chr6:112219951 bp) defining a 2.9 Mbp BALB/c donor segment. The closest available flanking C57BL/6By-type background microsatellite markers were D6Mit105 (49.71 cM, Chr6:107745604) and D6Mit134 (59.32 cM, Chr6:125258637). In earlier studies, a genome SNP scan of the progenitor B6.C6.132.54 detected background type flanking markers on the distal end of the chromosome at rs3023093 positioned at Chr6:122259669 (Vadasz et al., 2007c). Therefore the introgressed segment length is between 2.9 Mb and 14.6 Mb, which represents about 70% reduction of the donor segment length in the new sub-congenic strain B6.C6.327.54. Testing of D6Mit148 (110,734,287 – 110,734,425), which resided on the segment and was within *Grm7* (109,900K bp – 112,210K bp) yielded homozygous BALB/cJ genotype. All positions are from UniSTS annotation of NCBI Build 37.1. In our standard alcohol preference drinking paradigm adult male congenic mice consumed significantly less alcohol at 12% (vol/vol) than the background animals (Fig. 2, $F_{1,17}=5.33$, and $F_{1,17}=4.61$, both $p<0.05$). Alcohol intake was also significantly diminished in the second trial at 3% (Day 3–6, $F_{1,17}=5.75$, $p<0.05$) and 6% (Day 12–15, $F_{1,17}=4.56$, $p<0.05$) alcohol concentrations. These studies showed approximately 27% lower alcohol consumption in B6By.C6.327.54 in comparison to B6By, and established B6By.C6.327.54 as a new congenic strain carrying the *Grm7*^{BALB/cJ} gene variant with associated lower alcohol consumption. Earlier separate studies comparing B6By.C6.132.54 and B6By indicated somewhat greater reduction in alcohol consumption (40% and 37%, Vadasz et al., 2007c). Further studies with larger sample sizes are needed to test the differences between congenics and the hypothesis of clustering of alcohol consumption QTLs on the investigated chr. 6 region. Allele-dependent regional variation in *Grm7* gene expression in brain tissue sections can be highly informative in experiments aimed to explain the mechanism of alcohol preference drinking and other phenotypes affected by *Grm7* polymorphisms. In our ISH studies the highest expression of *Grm7* was detected in the dentate gyrus, in cornu ammonis 3 of hippocampus (CA3), followed by the piriform cortex. and the cornu ammonis 1 of hippocampus (CA1) fields (Table 1–2). Highly significant genetic strain effects were observed in CA1, cortical Cg1–2 (cingulate cortex), and M1 (motor cortex) regions. Gene expression was consistently lower in the background strain, but the difference did not reach the level of statistical significance in LPMR-LPLR

(lateral posterior thalamic nucleus -- mediorostral part of LP), amygdala, visual, sensory and auditory cortex, caudate-putamen, nucleus accumbens-core, and nucleus accumbens-shell. While the observed genetic differences support the hypothesis of *Grm7* involvement in voluntary alcohol drinking, they also indicate the need for more detailed studies to explain the lack of statistically significant differences in the striatum which has been implicated in brain reward mechanisms.

Variations in alcohol consumption are reflections of interactions between complex genetic and environmental factors. *Grm7* was identified as a QTG for alcohol preference drinking in a mapping panel whose progenitor strains were well known to significantly differ in alcohol consumption, locomotor activity, and circadian rhythm (Hofstetter et al., 1995; McClearn, 1960; McClearn and Rodgers, 1959). Thus, it was of interest to explore potential effects of *Grm7* polymorphism on circadian activity. In Experiment #1, comparison of baseline circadian wheel-running activity in C57BL/6By and BALB/cJ strains demonstrated a highly significant, nearly three-fold difference in the peak dark phase activity of 180 min (including 19, 20, and 21 hrs; $F_{1,7}=75.75$, $p<0.001$; Fig. 3, panel A). During the 8 days of the baseline period, the average number of revolutions per hour ($\pm$ S.E.M.) in C57BL/6By, and BALB/cJ strains were 5014.05 ± 337.22 , and 1735.42 ± 167.91 , respectively. Activity in the light phase (10, 11, 12 hrs) was not significantly different ($F_{1,7}=0.23$, $p>0.05$). In Experiment #2, studies on C57BL/6By background mice and their congenic partners indicated a tendency for higher wheel-running activity in the congenic mice (dark phase 19, 20, and 21 hrs) without reaching statistical significance ($F_{1,6}=5.99$, $p>0.05$; Fig 3, panel B). Repetition of this background vs. congenic comparison with another set of mice showed again higher, but non-significant wheel-running activity in the congenic mice (dark phase 19, 20, and 21 hrs; $F_{1,6}=0.106$, $p>0.05$; Fig. 3, panel C). Thus, we conclude that further studies with greater statistical power seem to be warranted.

DISCUSSION

Experiments with *Grm7* knockouts aimed to test the hypothesis that lower expression of *Grm7* mRNA is associated with higher voluntary alcohol consumption. Constitutional dysfunction of mGlu7 receptors in knockout mice resulted in significantly increased 12% (vol/vol) alcohol consumption (Fig. 1). The overall tendency observable in both males and females suggests that increase in alcohol consumption may also reach statistical significance at lower and higher alcohol concentrations with greater sample size. Although the knockout results are consistent with our hypothesis, dysfunction of this highly conserved gene during development is likely to induce considerable developmental compensation, thus the observed phenotypic change in a knockout individual does not necessarily reflect normal physiological gene function. Indeed, recent studies indicate GABAergic dysfunction in mGlu7 receptor-deficient mice (Wieronska et al., 2010). Therefore, in the next study, we investigated the effect of “knocking-up” of *Grm7* gene expression in the new sub-congenic strain, *B6By.C6.327.54*. The new sub-congenic retained only about 30% of the original segment preserved in *B6By.C6.132.54*, and showed significantly lower alcohol consumption than the *B6By* background. Thus, it provides further support to our hypothesis that *Grm7* is a QTG which affects alcohol consumption. The exact length of the segment is not known. Search in the MGI 4.4 database for the 107.745–122.259 Mbp interval limited by *B6By*

background markers returned 143 protein coding genes. Search for the range limited by BALB/cJ donor markers (between D6Mit327 and D6Mit54) returned 2 protein coding genes: *Grm7* and RIKEN cDNA 1700054K19 gene, thus the number of protein coding genes on the introgressed interval in the new sub-congenic B6By.C6.327.54 strain must be between 2 and 143. Therefore, the significantly lower alcohol consumption in B6By.C6.327.54, in comparison with B6By (Fig. 2), may considerably increase confidence in *Grm7* as a QTG, because we now have a much shorter list of genes residing on the introgressed interval. High density SNP scanning of the genome will allow informed cost/benefit analysis, and decision on the need for further reduction of the segment size. Very small (<1cM) segment size is desirable because potential interference from “passenger genes” can be eliminated, however, congenic models with larger introgressed chromosome intervals have been reported as informative (e.g., ISCS1, Fehr et al., 2002, Chen et al., 2008.).

The mGlu receptor mRNA levels are also modulated by non-genetic factors. Chronic exposure of rats to alcohol revealed decreases in mRNA expression of several mGlu receptors in different subregions of the hippocampus (Simonyi et al., 2004). In the dentate gyrus, mGlu3 and mGlu5 receptor mRNA levels were significantly lower in the ethanol-treated rats than in the control rats. In the CA3 region, the mRNA expression of mGlu1, mGlu5, and mGlu7 receptors showed substantial decreases after ethanol exposure. The mGlu7 receptor mRNA levels were also declined in the CA1 region and the polymorph layer of the dentate gyrus. No changes were found in mRNA expression of mGlu2, mGlu4, and mGlu8 receptors. These observations are consonant with the present results, and indicate that environmental effects may also reduce mGluR7 expression, and higher alcohol intake may be associated with lower mGluR7 expression. Microinjection of the mGluR5 antagonist MPEP and mGluR2/3 agonist LY379268 in the nucleus accumbens reduced ethanol self-administration at a dose that did not alter locomotor activity, or sucrose self-administration (Besheer et al., 2010).

Recent availability of the first agonist (AMN082 (Mitsukawa et al., 2005)) and antagonist (MMPiP (Suzuki et al., 2007)) specific to mGluR7 facilitated pharmacological experiments which help better explain the functional aspects of the proposed genetic link between mGluR7 and addictive substance use related behaviors (Vadasz et al., 2007c). In the rat, AMN082 dose-dependently inhibited cocaine self-administration, and reinstatement, in an operant behavioral paradigm, without affecting locomotor activity or sucrose self-administration, and these effects were blocked by MMPiP (Li et al., 2010; Li et al., 2009). Because LY341497 (a selective mGluR2/3 antagonist) could block the effect of AMN082 pretreatment on cocaine-induced reinstatement, it was suggested that that mGluR7 activation inhibits cocaine-induced reinstatement of drug-seeking behavior by a glutamate-mGluR2/3 mechanism in the nucleus accumbens (Li et al., 2010). Further experiments to clarify the role of mGluR2/3 are warranted because nonspecific reductions in locomotor activity after infusion of the mGluR2/3 agonist LY379268 was recently reported (Besheer et al., 2010).

In the mouse, AMN082 significantly inhibited self-administration of sucrose-sweetened alcohol in an operant conditioning paradigm at the dose of 10 mg/kg (Salling et al., 2008). This dose also reduced sucrose reinforcement, and locomotor activity. The causes of the

conflicting results are not clear, however, earlier studies have already pointed out that lower doses are required in mice than rats to reach equivalent AMN082 brain levels (Fendt et al., 2008; Palucha et al., 2007). Although pharmacological effects of AMN082 may not be simplistically related to genetic effects caused by *Grm7* polymorphism, we may examine some of the possible reasons for the apparent discrepancy. Considering methodological issues, it appears that sweetened alcohol was used by necessity because the authors found in a previous study that self-administration of unsweetened ethanol (10% v/v) during 1 hour sessions led to low response rates in C57BL/6J mice and inconsistent ethanol consumption (i.e., the mice lever pressed but did not drink) (Salling et al., 2008). Other differences, such as operant conditioning with FR4 schedule of reinforcement and limited access (1 h) vs. free two-bottle choice with direct and unlimited (24 h/day for several days) access to alcohol, or restriction of operant alcohol self-administration for the circadian light phase between 1400 and 1600 h (characterized by low motor and alcohol drinking activity) vs. no light phase restriction for alcohol preference drinking (i.e., the dark phase, characterized by higher motor and alcohol drinking activity, was also included) can also contribute to the discrepancies. Thus, we hypothesize that using the unlimited access two bottle-choice alcohol preference drinking paradigm results of pharmacological experiments with agonists and antagonists specific to mGluR7 will be consistent with our hypothesis that high expression (activation) of mGluR7 suppresses alcohol consumption.

Detection of *Grm7* mRNA in the brain by ISH—Although neuroanatomical distribution of *Grm7* mRNA and receptor protein has been described in several species (Corti et al., 1998; Kinoshita et al., 1998; Makoff et al., 1996; Simonyi et al., 2000) genetic differences at neuroanatomical level are not well known. This is the first study to demonstrate differential expression of *Grm7* mRNA in the brains of two genetically defined mouse strains by ISH. Since ISH allows higher spatial resolution of analysis in comparison to quantitation of dissected tissue samples, surveys of complete coronal sections of the mouse brain could be performed (Tables I-II.). The detected significant differences between the congenic and background strains in the CA1 field of the hippocampus and in cingulate cortex and motor cortex are important because they point to potential genetic variation in functions which characterize these regions, and predict new phenotypic differences between these animal models, thus contributing to the better understanding of the underlying mechanisms. In our studies the observed regional pattern of expression is consistent with those of earlier reports (Corti et al., 1998; Kinoshita et al., 1998; Makoff et al., 1996; Simonyi et al., 2000).

Functional consequences of genetic variation—Dorsal and ventral CA1 are genetically wired for different functional specializations (Dong et al., 2009). The former is selectively involved in cognitive aspects of the learning and memory associated with navigation, exploration, and locomotion (place learning (Bartsch et al., 2010), storage and retrieval of associative memory (Langston et al., 2010)), whereas in contrast the latter is part of the temporal lobe associated most directly with motivational and emotional aspects. Ventral CA1 projects to the medial amygdaloid region including the medial, intercalated, and basomedial nuclei implicated in fear conditioning and emotional behavior (Kishi et al., 2006), and to the hypothalamic periventricular region and medial zone, which integrate

neuroendocrine, autonomic, and somatic motor responses associated with motivated behaviors: ingestive (feeding and drinking), reproductive (sexual and parental), and defensive (fight or flight) (cf. (Dong et al., 2009)). Pyramidal cells of CA3 send connections to region CA1 through a set of fibers called the Schaffer collaterals (an integral part of memory formation and emotion related networks) which release glutamate that binds to mGluR7 receptors in the CA1 region. Our results suggest that one significant target of the allele-dependent variation in mGluR7 mRNA expression is the Schaffer collateral-CA1 synapse which is part of the trisynaptic circuit (perforant path→dentate gyrus→CA3→CA1), a major pathway of flow of information in the hippocampus. Because mGluR7 is a presynaptic receptor, changes in mRNA levels in the CA1 region can induce differences in receptor protein expression in many areas of the brain where CA1 neurons project. mGluR7 is predominantly expressed in presynaptic terminals of excitatory glutamatergic synapses (Schaffer collateral-CA1 synapse) where it is believed to serve as an autoreceptor to inhibit glutamate release (Shigemoto et al., 1997). Thus hippocampi of individuals with *Grm7*^{BALB/cJ} alleles are under more expressed inhibitory influence. It is noteworthy that the mGluR7-specific agonist AMN082 (Ayala et al., 2008), and the mGluR7-specific antagonist MMPIP are not effective at the Schaffer collateral-CA1 synapse, suggesting context specificity and intriguing implications for mGluR7-based drug design and development (Niswender et al., 2010).

As June et al. pointed out, hippocampal fields are interesting candidate sites for the study of alcohol-motivated behaviors because (1) projections from the CA1 and CA3 fields, via the subiculum, innervate several putative ethanol reward substrates (e.g., nucleus accumbens, amygdala, bed nucleus of the stria terminalis, hypothalamus, and olfactory tubercle), (2) intrahippocampal infusions of an alpha5 subunit-selective benzodiazepine inverse agonist RY 023 alter lever pressing maintained by concurrent presentation of ethanol (10% v/v) and (3) immunocytochemical, *in situ* hybridization, and radioligand binding studies show that the CA1, CA2, and CA3 fields are enriched in this subunit compared with other brain areas (June et al., 2001). In the “extended alcohol reward circuitry” alterations in GABA neurotransmission within the hippocampus may reduce ethanol-maintained responding by interfering with conditioned stimuli, or may interact with mesolimbic dopamine systems (June et al., 2001). A possible mechanism for such interactions is modulation of the efficacy of GABAergic neurotransmission by mGluR7. Considering that external factors may interact with genetic variation, and that in rats after chronic alcohol consumption the mGluR7 mRNA levels declined in the CA1 region (suggesting that the reduced expression of this receptor might contribute to ethanol withdrawal-induced seizures and also may play a role in cognitive deficits (Simonyi et al., 2004)), further studies on the mGluR7 congenic strain and its background partner may address the question whether genetically determined higher expression of mGluR7 confers some protection against chronic exposure to alcohol.

The anterior cingulate cortex (ACC) may function, in part, to signal the occurrence of conflicts in information processing (Botvinick et al., 2004). Another theory suggests that ACC plays a role in reward-based decision making (Bush et al., 2002). Experiments to elucidate the role of rat medial frontal cortex (MFC) (including prelimbic, infralimbic, and cingulate cortices) in effort-based decision making imply that medial frontal cortex is

important for allowing the animal to put in more work to obtain greater rewards (Walton et al., 2002). More recent results also support the hypothesis that the ACC plays a critical role in stimulus-reinforcement learning and reward-guided selection of actions (Schweimer and Hauber, 2005).

The primary motor cortex (M1) is the final cortical processing site for voluntary motor commands. It projects directly to the brain stem and spinal cord to coordinate motor activity, and it integrates input from numerous cortical and subcortical sites. Recent studies on rats show that aversive, stressful context (open-field test, fear conditioning) induced c-Fos in Cg1, and M1. Chronic corticosterone treatment enhanced this effect in the M1, and additionally it was observed in the CA1, in comparison to control animals not subjected to contextual fear test (Skorzewska et al., 2006). Some of the above functions attributed to CA1, Cg1, and M1 are relevant to the complex phenotype of addiction, and may serve as useful subjects of analysis with congenic preparations carrying *Grm7* variants.

Currently we do not know well the neural circuitry which controls the alcohol consumption phenotype, however, mGluR7 polymorphism may affect several brain regions where mGluR7 is expressed and the associated functions have been implicated in addiction. The primary candidates include the hippocampus–parvocellular-PVN–HPA axis and stress susceptibility (cf. (Kinoshita et al., 1998; Mitsukawa et al., 2006; Mitsukawa et al., 2005; Pohorecky, 1990; Roberts et al., 1995; Sillaber et al., 2002; Simonyi et al., 2000; Sinha et al., 1998)), the amygdala, locus ceruleus and susceptibility to aversion, stress, fear, anxiety (cf. (Funk and Koob, 2007; Heilig and Koob, 2007; Itoi and Sugimoto, 2010; Kinoshita et al., 1998; Koob, 1999; Masugi et al., 1999; Nie et al., 2004; Richter et al., 2000; Sarnyai et al., 2001; Van Bockstaele et al., 2010)), the orexigenic hypothalamic–basal forebrain cholinergic corticopetal system and attentional function, sleep, arousal, energy homeostasis, appetitive stimulus processing (cf. (Acuna-Goycolea et al., 2004; Fadel and Burk, 2009; Hur et al., 2009; Li et al., 2002; Martinez et al., 2005; Morganstern et al., 2010; Sarter and Bruno, 1999; Sarter et al., 2003; Zaborszky et al., 1999; Zmarowski et al., 2007)); and the nucleus accumbens, striato-pallidal, cortico-striatal pathways and reward/pleasure related functions (cf. (Kosinski et al., 1999; Li and Lumeng, 1984; Li et al., 2008; Li et al., 2010; Li et al., 2009)). Our hypothesis is that multiple foci in the brain are affected, these foci synergistically interact to produce a detectable behavioral outcome. Thus, better understanding of the mechanism of the development of addiction requires systems-level studies.

Circadian wheel running activity—In recent years it has become apparent that there is a complex network of interactions between alcohol consumption, circadian rhythm, exercise (running, wheel running), and hippocampal mechanisms (neurogenesis, learning) (Brower, 2001; Crews et al., 2004; Huang et al., 2010; Leasure and Nixon, 2010; Redila et al., 2006; Spanagel et al., 2005b; Wasielewski and Holloway, 2001). In various aspects of this complex network significant genetic variations have been observed. For example, the alcohol preferring C57BL/6J and the alcohol avoiding BALB/cJ are among the best known strains showing extreme phenotypic expression of alcohol consumption (McClearn and Rodgers, 1959; Rodgers and Mc, 1962), and they also differ in other traits whose mechanisms may be relevant to addiction, such as motor activity, and circadian rhythms:

C57BL/6J shows higher open-field locomotor activity (Vadasz et al., 1992a; Vadasz et al., 1992b), higher wheel running activity (Lightfoot et al., 2004), and longer circadian period (tau) of locomotor activity (Mayeda et al., 1996) than BALB/cJ.

We argued that if the effects of the *Grm7* gene variation on circadian activity can be isolated from the rest of the gene network interaction effects, we can get a better understanding of the relationships between neuroanatomical variation in *Grm7* expression, alcohol consumption, motor activity, exercise, and circadian rhythm. As a first step in this direction we investigated the baseline circadian wheel running activity of trained inbred B6By and BALB/cJ mice (Fig. 3). Wheel running activity could be detected almost exclusively in the dark phase in both strains, and B6By showed significantly higher dark phase activity which is consistent with other studies (Lightfoot et al., 2004). Considering the ~99% genomic identity between congenic and background strains, one would expect similar circadian activities unless the donor interval affects the phenotype. We found a small difference in dark phase wheel running activity (hrs 19, 20, 21) indicating higher activity in the congenic strain, however, it did not reach the level of statistical significance (Fig. 3, $p>0.05$). When we repeated the experiment with an independent small set of animals, we found a similar, but again nonsignificant difference, which we attribute to the low power due to limited availability of running wheels (data not shown). In our study BALB/cJ showed lower dark phase running wheel activity than B6By, and the congenic showed higher activity than B6By. We hypothesize that this unexpected result is an example of the effects of gene dispersion and background-dependent interactions which masked the relatively small effect of *Grm7* on running wheel activity in BALB/cJ. Based on these results, we suggest that mGluR7 expression in the brain may be associated with dark phase activity, however, the latter hypothesis will require further testing with larger sample sizes.

In summary, our results are consistent with the hypothesis that constitutionally low *Grm7* expression contributes to the predisposition to excessive alcohol self-administration. These data sit well with the overwhelming functional evidence implicating glutamatergic neurotransmission in psychostimulant action and drug addiction (Kalivas, 2004; Kalivas et al., 2003; Kelley, 2004; Vorel et al., 2001; Wolf, 1998), with more recent data demonstrating the role of mGluR7 in addiction (Li et al., 2008; Li et al., 2009; Li et al., 2006) (but see (Salling et al., 2008)), and merit future research aimed at elucidating underlying mechanisms and new approaches for treatment of psychiatric disorders.

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Fig. 1. Alcohol (3, 6, 12, and 18% vol/vol) preference drinking in B6.129/Ola-*Grm7^{tmNovartis}* knockout (KO/KO) and wild-type littermate (WT/WT) mice. KO/KO male mice consumed significantly more 12% (vol/vol) alcohol than wild-type male littermates (Mann-Whitney $U_{(1,22)}=37$, $p<0.05$). Although female KO/KO mice showed higher levels of alcohol consumption, especially at 18% (vol/vol) alcohol solution, this did not reach statistical significance presumably due to the small sample size of the animals.

Fig. 2.

Alcohol (3, 6, and 12 %, vol/vol) preference drinking in C57BL/6By background (n=9) and B6By.C6.327.54 congenic (n=9) male mice. The abscissa shows the days of the experiments when consumption was measured. Congenic mice with *Grm7*^{BALB/cJ/BALB/cJ} genotype consumed significantly less 12% (vol/vol) alcohol in both 3-day trials (see Days 15–18 and 18–21) than the background partner with *Grm7*^{B6By/B6By} genotype ($p<0.05$). Consumption was also significantly lower at 3% (Day 3–6) and 6% (Day 13–15)

Fig. 3.

Baseline circadian pattern of wheel-running activity in trained C57BL/6By background, B6By.C6.132.54 congenic, and BALB/cJ donor inbred mouse strains. Ordinate shows revolutions per hour, abscissa shows hours of the day. For clarity, a 24 hr period is double plotted. Hourly activities were averaged for 8 days (at least 1 week after the initiation of voluntary wheel running). Wheel-running in a dark phase interval of peak activity (shown as 19, 20, 21 hrs; 180 min) was selected for statistical analysis. Wheel-running activity was significantly higher in C57BL/6By than in BALB/cJ mice (ANOVA, $F_{1,7}=75.75$, $p<0.001$; Panel A). Comparison of C57BL/6By background and B6By.C6.132.54 congenic strains in two independent experiments indicated higher baseline dark phase activity in the congenic strain (hrs 19, 20, 21), however, it did not reach statistical significance ($p>0.05$, Panels B, and C).

Table 1

Metabotropic glutamate receptor 7 mRNA expression of in mouse brain (Bregma=−2.18)

Regions	C57BL/6ByJ	B6.C6.132.54	BALB/cJ	p values
CA1	172±5	217±9 ^a	207±6 ^b	0.0003
CA3	285±6	300±8	301±8	0.2286
Dentate gyrus - upper blade	345±14	367±13	327±10	0.1033
Dentate gyrus - lower blade	333±14	354±15	330±12	0.4197
LPMR-LPLR	69±3	71±2	69±2	0.6604
VPM-VPL	73±3	71±3	76±2	0.4086
Amygdala, BLP-BMP	58±3	62±3	63±5	0.5812
Piriform cortex	243±11	228±14	245±14	0.0771
Retrosplenial cortex, RSG	95±4	102±3	92±3	0.1035
Visual cortex, V1-V2L	75±2	80±2	81±4	0.2611
Auditory cortex	65±3	68±3	69±3	0.6231

Note: Data are expressed in nCi/g tissue ± SEM from 8–10 animals/group.

^a p<0.001 between C57BL/6ByJ and B6.C6.132.54

^b p<0.01 between C57BL/6ByJ and BALB/cJ

One-way ANOVA, Newman-Keuls Multiple Comparison Test.

Table 2

Metabotropic glutamate receptor 7 mRNA expression in mouse brain (Bregma=+0.98)

Regions	C57BL/6ByJ	B6.C6.132.54	BALB/cJ	p values
Cingulate cortex, Cg1-2	73±3	87±3 ^a	85±4 ^b	0.0040
Motor cortex, M1	68±2	74±2 ^c	81±3 ^d	0.0042
Sensory cortex, S1ULp	52±4	60±4	63±5	0.1603
Caudate-putamen	72±2	78±4	81±4	0.1397
Nucleus accumbens-core	64±3	70±4	71±3	0.2230
Nucleus accumbens-shell	70±4	76±5	74±8	0.7437

Note: Data are expressed in nCi/g tissue ± SEM from 8–10 animals/group.

^a p<0.01 between C57BL/6ByJ and B6.C6.132.54

^b p<0.05 between C57BL/6ByJ and BALB/cJ

^c p<0.05 between C57BL/6ByJ and B6.C6.132.54

^d p<0.01 between C57BL/6ByJ and BALB/cJ

One-way ANOVA, Newman-Keuls Multiple Comparison Test.