

Genetic Variability in Adenosine Deaminase-Like Contributes to Variation in Alcohol Preference in Mice

Heidi M. B. Lesscher , Alexis Bailey, and Louk J. M. J. Vanderschuren

Background: A substantial part of the risk for alcohol use disorder is determined by genetic factors. We previously used chromosome substitution (CSS) mice, to identify a quantitative trait loci (QTL) for alcohol preference on mouse chromosome 2. The aim of this study was to identify candidate genes within this QTL that confer the risk for alcohol preference.

Methods: In order to delineate the neurobiological underpinnings of alcohol consumption, we expanded on the QTL approach to identify candidate genes for high alcohol preference in mice. We narrowed down a QTL for alcohol preference on mouse chromosome 2, that we previously identified using CSS mice, to 4 candidate genes in silico. Expression levels of these candidate genes in prefrontal cortex, amygdala, and nucleus accumbens—brain regions implicated in reward and addiction—were subsequently compared for the CSS-2 and the C57BL/6J host strain.

Results: We observed increased expression of adenosine deaminase-like (Adal) in all 3 regions in CSS-2 mice. Moreover, we found that the adenosine deaminase inhibitor EHNA reduced the difference in alcohol preference between CSS-2 and C57BL/6J mice.

Conclusions: This study identifies Adal as a genetically protective factor against alcohol consumption in mice, in which elevated Adal levels contribute to low alcohol preference.

Key Words: Adenosine, Alcohol, Quantitative Trait Loci, Preference, Mice.

ALCOHOL USE DISORDERS (AUD) are an enormous public health problem, affecting over 76 million people worldwide (WHO, 2011). The risk for AUD is determined for a substantial part by genetic factors. Twin and adoption studies have demonstrated greater risk for alcohol-related disorders in individuals who have an affected monozygotic twin, as compared to individuals with an affected dizygotic twin (Ystrom et al., 2011). From these studies, the heritability for AUD has been estimated to be 48 to 71%.

Human genomewide association studies and rodent genetic mapping studies have yielded profound insight into the molecular mechanisms underlying the individual risk for AUD. For example, inbred mouse strains, which are well characterized both genetically and behaviorally, have been used to discern the genetic components underlying the vulnerability for AUD. Indeed, multiple quantitative trait loci (QTL) for alcohol intake and/or preference have been identified (Bubier et al., 2014; Gill and Boyle, 2005; Lesscher et al.,

2009a; Phillips et al., 1998; Rodriguez et al., 1995; Vadasz et al., 2000; Whatley et al., 1999). In-depth QTL analyses have revealed specific genes that contribute to the risk for alcohol consumption and AUD (Bubier et al., 2014; Milner and Buck, 2010).

We previously used chromosome substitution (CSS) mice (Nadeau et al., 2000), to identify a grandparent-dependent QTL for alcohol preference on mouse chromosome 2. Chromosome 2 was chosen as our focal point because QTLs for high alcohol consumption were previously identified on this chromosome. CSS-2 mice, in which chromosome 2 from the A/J donor strain was introduced into the genome of C57BL/6J mice, displayed a low preference for alcohol compared to C57BL/6J mice (Lesscher et al., 2009a). CSS-2 mice were however not different from C57BL/6J mice in taste preference for sweet and bitter solutions nor in the metabolism rate of alcohol. In this study, we expanded on this QTL approach to identify quantitative trait genes (QTG) that confer the risk for alcohol preference. Therefore, we narrowed down the QTL on chromosome 2 in silico to identify candidate genes. To select QTGs (i) we first selected coding single nucleotide polymorphisms (SNPs) within the QTL, (ii) we then selected genes that are expressed in the brain and subsequently filtered those genes with expression in brain regions that have been associated with reward and addictive behavior, that is, the prefrontal cortex (PFC), amygdala (AMG), and nucleus accumbens (NAc), and (iii) we further selected genes for which literature was available to support their role in (reward-related) behavior (Abiola et al., 2003; Korstanje and Paigen, 2002; Nikolskiy et al., 2015; Noyes et al., 2011). Subsequently, expression levels of these candidate genes in the

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PFc, AMG, and NAc—brain regions widely implicated in the positive subjective and addictive properties of substances of abuse (e.g., Everitt and Robbins, 2013; Koob and Volkow, 2010; Tabakoff and Hoffman, 2013)—were compared for the CSS-2 and C57BL/6J host strain. Finally, using a pharmacological approach, the functional role of the most prominent candidate gene, adenosine deaminase-like (Adal) in the regulation of alcohol consumption in CSS-2 mice was determined.

MATERIALS AND METHODS

Animals

C57BL/6J, A/J, and C57BL/6J-Chr 2^A/NaJ (referred to as CSS-2) (Nadeau et al., 2000) mice were obtained from Jackson Laboratory (Bar Harbor, ME) and bred in our department. Experimental animals were male mice, 8 to 10 weeks old at the onset of testing. The mice were group-housed with mice from the same genotype under controlled conditions (20 ± 2°C and 50 to 70% humidity), and they were acclimatized to a 12-hour light/dark cycle (lights off at 7:00 AM) for at least 2 weeks prior to testing. Food and water were available ad libitum. The experimental procedures were approved by the Animal Ethics Committee of Utrecht University and were conducted in agreement with Dutch laws (Wet op de dierproeven, 1996) and European regulations (Guideline 86/609/EEC).

Narrowing Down the QTL and Identification of Candidate Genes

To narrow down the identified grandparent-dependent QTL for alcohol preference on chromosome 2, which ranged from 112 to 134 Mbp (Lesscher et al., 2009a), haplotype blocks for the A/J and C57BL/6J host strains were identified using the Perlegen Genotype Browser (<http://mouse.cs.ucla.edu/perlegen/>) within the QTL range. This analysis revealed multiple blocks with genetic variation between A/J and C57BL/6J mice. Subsequently, coding nonsynonymous SNPs within the QTL range, that is, chromosome 2: 112 to 134 Mbp, were identified by comparing this genomic region for A/J and C57BL/6J strains using the Mouse Genome Informatics (MGI) Mouse SNP Query (<http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snpQF>). Finally, to determine which genes are expressed in brain, we aligned the genes within the QTL range with the nearly 20,000 genes with reported brain expression of the Allen Brain Atlas (<http://www.brain-map.org/>). The remaining candidate genes, identified with this approach, were further explored for their contribution to the observed strain differences in alcohol preference based on brain expression patterns and reported behavioral effects in the literature. For this purpose, we selected genes that were expressed in brain regions that have been associated with reward and addictive behavior, that is, the PFc, AMG, and NAc (using the Allen Brain Atlas). Subsequently, we selected genes for which literature was available to support their role in (reward-related) behavior (<http://www.informatics.jax.org>). Genes that were involved in other processes, such as platelet regulation, leukemia, inner ear function, and cancer, were excluded at this stage. With this approach, the QTL range was narrowed down to 4 candidate genes: Adal, Chrm5 (muscarinic 5 acetylcholine receptor), Disp2 (Dispatched homolog 2), and Ubr1 (Ubiquitin protein ligase E3 component n-recognin 1).

Expression Analysis of Candidate Genes in Host Strains by Quantitative Polymerase Chain Reaction

We reasoned that, if genetic variation in the identified candidate genes, that is, Adal, Chrm5, Disp2, and Ubr1 is relevant for the phenotypical difference in alcohol preference between C57BL/6J

and CSS-2 mice (Lesscher et al., 2009a), then the expression of these genes in reward-related brain regions would differ between the 2 strains. Therefore, to validate their potential role in regulating alcohol preference, the expression levels of these candidate genes were compared for C57BL/6J and CSS-2 mice by quantitative polymerase chain reaction (qPCR) analysis. For this purpose, alcohol-naïve mice of both genotypes (CSS-2 and C57BL/6J, *N* = 6) were sacrificed by rapid decapitation and brains were dissected, snap frozen on dry ice, and stored at -80°C. PFc, AMG, and NAc punch samples were obtained using a 20G punch needle and were immersed instantly in RNAlater (Sigma, Munich, Germany). Total RNA was isolated from these samples using TRIzol (Invitrogen, Bleijswijk, the Netherlands), DNase treated (Ambion, Austin, TX), and purified using the RNeasy MinElute Cleanup kit (Qiagen N.V., Venlo, the Netherlands). Subsequently, cDNA was synthesized from the RNA samples using oligo-dT primers. qPCR analysis was performed using the LightCycler (Roche, Almere, the Netherlands), the Fast Start DNA Master PLUS SYBRgreen I kit (Roche), and primers listed in Table 1. After initial normalization to the house-keeping gene beta-actin, gene expression was calculated as the ratio to levels of C57BL/6J mice using the comparative Ct method (Lesscher et al., 2012; Schmittgen and Livak, 2008).

Limited-Access Ethanol Consumption and Adal Inhibition

Because Adal, of the 4 identified candidate genes, showed most pronounced and consistent expression differences between CSS-2 and C57BL/6J mice, we next assessed the contribution of enhanced Adal levels to the phenotypic difference in alcohol consumption between CSS-2 and C57BL/6J strains. To that aim, the Adal inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA; Tocris, Bristol, UK) was used to counteract the augmented Adal activity in CSS-2 mice. Immediately prior to daily alcohol consumption sessions in the limited-access choice paradigm, C57BL/6J and CSS-2 mice were treated with either vehicle (saline) or EHNA (10 mg/kg intraperitoneal, 2 ml/kg in saline) (*N* = 8 per strain and treatment). The mice were randomly assigned to the treatment groups (saline or EHNA).

The procedures for ethanol (EtOH) consumption were similar to those used previously (Lesscher et al., 2009a, 2012). A limited-access paradigm using a 15% EtOH solution was employed. In line with this paradigm, mice readily consume high amounts of alcohol and show a gradual increase in alcohol consumption over the course of 2 to 4 weeks (Lesscher et al., 2009b, 2012). Moreover, using a limited-access paradigm with 20% alcohol, strain differences in alcohol consumption in mice have been demonstrated, including reduced alcohol intake in A/J compared to C57BL/6J mice (Rhodes et al., 2007). Importantly, reduced alcohol consumption by A/J mice compared to C57BL/6J mice is not dependent on the alcohol concentration nor to limited access to alcohol. For example, a study by Yoneyama and colleagues (2008) showed that A/J mice consumed less alcohol compared to C57BL/6J mice in a continuous-access 2-bottle choice task using 3, 6, and 10% alcohol.

The mice were placed in a separate test cage for 2 hours starting at 10:00 AM daily for 3 consecutive weeks. The mice received access to 2 drinking tubes, that is, 10-ml polystyrene pipettes fitted with a stainless steel ball-bearing sipper tube. One tube delivered tap water

Table 1. Primer Sequences for qPCR Validation of Candidate Genes

Primer Fwd	Primer Rev
TTCTTGGCCTTGACCTCAGT	CAGAGGCGCTAAGGAATGTC
TCAGCCATCAAATGACCAAA	AGTAACCCAAGTGCCACAGG
CTGGCCTTTCCTTCTCTG	GGAGGCTTGAGCTGTTTCATC
ACTCCGTGGTATTGCTCAC	AGGATCTTACGGGCACCTTT

and the other 15% EtOH (v/v in tap water). During the initial 7 days of training, the water and EtOH bottles were on fixed locations. Thereafter, the bottle positions were switched daily to avoid side preference. Fluid volumes were measured to the nearest 0.05 ml prior to and after each drinking session, by reading the pipette scale. Alcohol consumption was monitored during 3 consecutive weeks, and average EtOH intake (g/kg), EtOH preference (% of total fluid intake), and total fluid consumption (ml/kg) per week were calculated and compared between strains and treatment groups.

Statistical Analysis

SPSS 22.0 (Windows; IBM Corp., Armonk, NY) was used for statistical analyses. qPCR data were analyzed per brain region (AMG, PFC, NAc) using multivariate analyses of variance (ANOVAs), with genotype as the between-subjects factor. EtOH consumption data were analyzed by 3-way repeated-measures ANOVAs with genotype and treatment as the between-subjects factor and time as the repeated-measures within-subjects factor. Post hoc analysis was performed by *t*-tests where appropriate. Differences between pairs of means were considered significant at $\alpha < 0.05$. All results are shown as mean \pm SEM values.

RESULTS

Identification of Candidate Genes

To narrow down the previously identified QTL for alcohol preference on mouse chromosome 2, we first identified, using the MGI Gene Query, a total of 369 protein-coding genes within the QTL range (112 to 134 Mbp). Subsequently, haplotype mapping was performed using the Perlegen Genotype Browser to compare the haplotype blocks for the C57BL/6J and A/J host strains within the QTL range (112 to 134 Mbp). With this approach, the QTL range was reduced to 20 blocks with genetic variation between C57BL/6J and

A/J mice, leaving 168 candidate genes (Fig. 1). Thereafter, coding nonsynonymous SNPs within the QTL range were identified using the MGI Mouse SNP Query, which resulted in a further reduction of the number of candidate genes to 52. Finally, we determined which genes within the QTL are expressed in brain tissue and may constitute part of the neuromolecular mechanism that controls alcohol consumption. The list of candidate genes was aligned with the nearly 20,000 genes expressed in the brain in accordance to Allen Brain Atlas (<http://www.brain-map.org/>). This reduced the list of candidate genes to a total of 43 genes (Table 2).

To further narrow down the list of remaining candidate genes, the expression patterns of the remaining 43 genes were explored, thereby focusing on brain regions that have been associated with reward and addictive behavior, that is, the PFC, AMG, and NAc. For the majority of genes that remained based on their brain expression pattern (20 genes), there was no evidence for involvement in the modulation of behavior (<http://www.informatics.jax.org>). These genes were, for example, implicated in platelet regulation, leukemia, inner ear function, and cancer. These genes were therefore discarded from our candidate gene list, leaving us with 4 genes that are expressed in reward-related brain regions (PFC, AMG, and NAc) and have been implicated, directly or indirectly, in (reward-related) behavior, that is, *Adal* (Golembiowska and Zylewska, 2000), *Chrm5* (Basile et al., 2002; Thomsen et al., 2005), *Disp2* (Galli et al., 2014), and *Ubr1* (Balogh et al., 2002).

Enhanced *Adal* and *Chrm5* Expression in Brains of CSS-2 Mice

To assess whether *Adal*, *Chrm5*, *Disp2*, and *Ubr1* are involved in alcohol preference, mRNA expression levels for

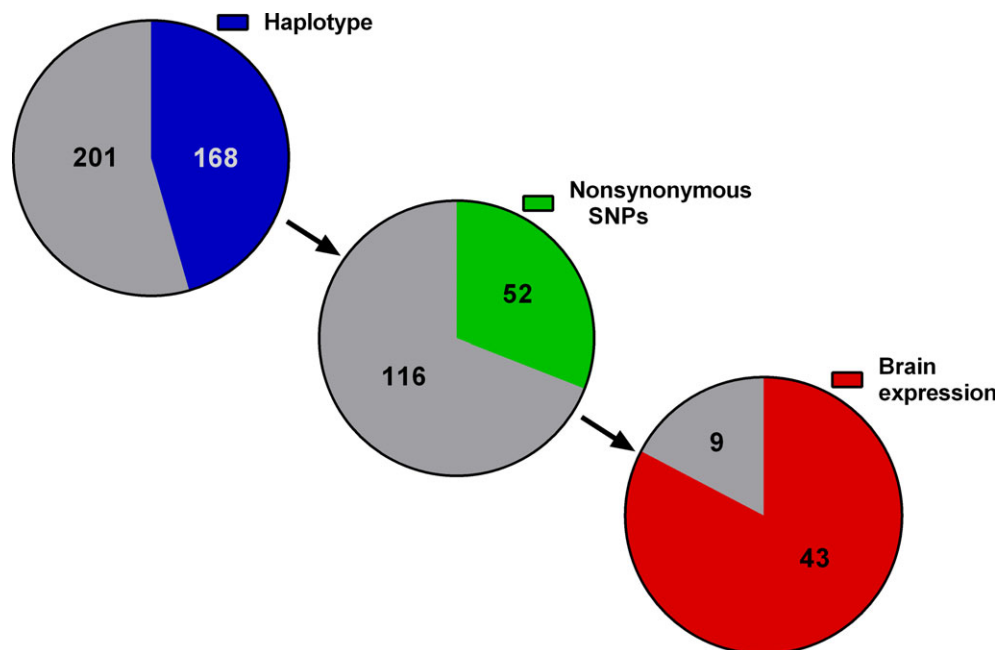


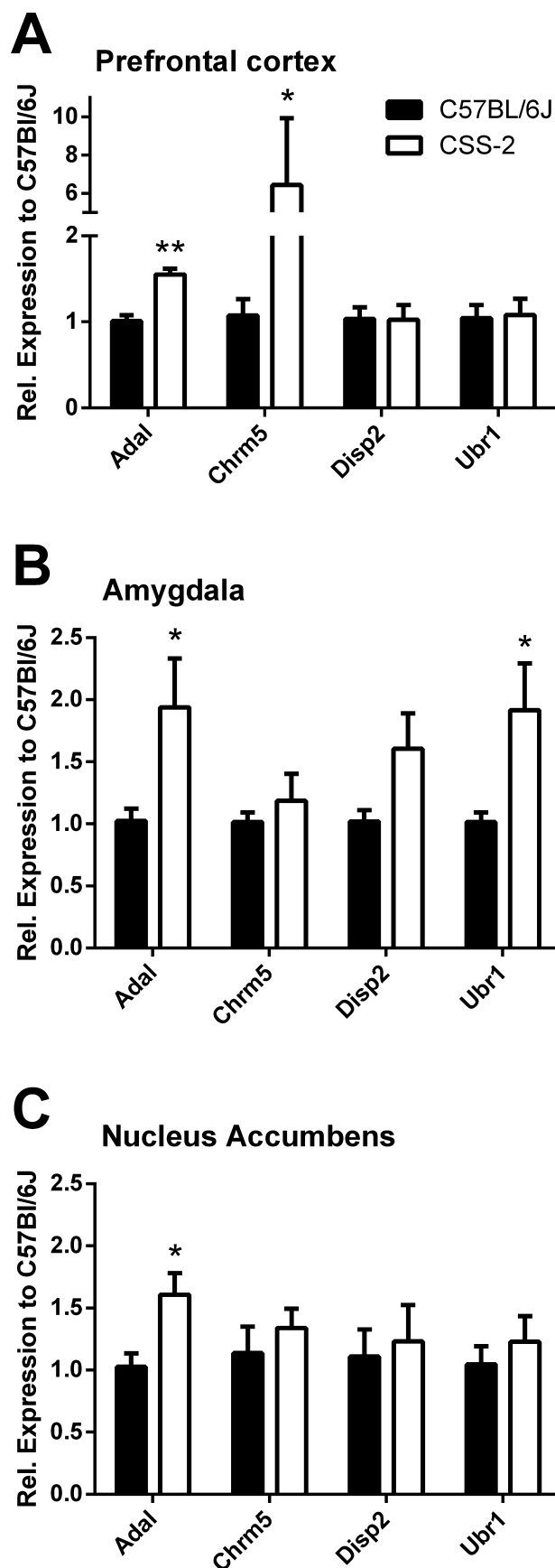
Fig. 1. The quantitative trait loci on mouse chromosome 2 for alcohol preference comprised a total of 369 genes. This number was reduced to 43 candidate genes through haplotype mapping, in silico SNP analysis and selection for brain expression.

Table 2. Candidate Genes Identified in the Alcohol Preference Quantitative Trait Locus on Mouse Chromosome 2

Adal	Adenosine deaminase-like
Ap4e1	Adaptor-related protein complex AP-4, epsilon 1
Arhgap11a	Rho GTPase-activating protein 11A
B2m	Beta-2 microglobulin
Blvra	Biliverdin reductase A
Bub1b	Budding uninhibited by benzimidazoles 1 homolog, beta (<i>Saccharomyces cerevisiae</i>)
Casc5	Cancer susceptibility candidate 5
Catsper2	Cation channel, sperm associated 2
Ccndbp1	Cyclin D-type binding protein 1
Cdan1	Congenital dyserythropoietic anemia, type I (human)
Chac1	ChaC, cation transport regulator-like 1 (<i>Escherichia coli</i>)
Chrm5	Cholinergic receptor, muscarinic 5
Ctdspl2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 2
Disp2	Dispatched homolog 2 (<i>Drosophila</i>)
Dll4	Delta-like 4 (<i>Drosophila</i>)
Dnajc17	DnaJ (Hsp40) homolog, subfamily C, member 17
Duoxa1	Dual oxidase maturation factor 1
Epb4.2	Erythrocyte protein band 4.2
Fsip1	Fibrous sheath-interacting protein 1
Hisppd2a	Histidine acid phosphatase domain containing 2A
Itpka	Inositol 1,4,5-trisphosphate 3-kinase A
Lcmt2	Leucine carboxyl methyltransferase 2
Ltk	Leukocyte tyrosine kinase
Mapkbp1	Mitogen-activated protein kinase binding protein 1
Mga	MAX gene associated
Mtap1a	Microtubule-associated protein 1 A
Ndufaf1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1
Nusap1	Nucleolar and spindle-associated protein 1
Pak6	p21 (CDKN1A)-activated kinase 6
Pla2g4b	Phospholipase A2, group IVB (cytosolic)
Plcb2	Phospholipase C, beta 2
Pldn	Pallidin
Rpap1	RNA polymerase II-associated protein 1
Slc30a4	Solute carrier family 30 (zinc transporter), member 4
Strc	Stereocilin
Trp53bp1	Transformation-related protein 53 binding protein 1
Ubr1	Ubiquitin protein ligase E3 component n-recognin 1
Zfyve19	Zinc finger, FYVE domain containing 19
2310003F16Rik	RIKEN cDNA 2310003F16 gene
6330405D24Rik	RIKEN cDNA 6330405D24 gene
A430105I19Rik	RIKEN cDNA A430105I19 gene
A530010F05Rik	RIKEN cDNA A530010F05 gene
A530057A03Rik	RIKEN cDNA A530057A03 gene

these genes were compared in PFC, AMG, and NAc of CSS-2 and C57BL/6J mice. Analysis of the data revealed an up-regulation of Adal in CSS-2 versus C57BL/6J mice in all 3 brain regions: PFC, $F_{\text{genotype}}(1, 11) = 30.4$, $p < 0.001$, AMG $F_{\text{genotype}}(1, 11) = 5.7$, $p < 0.05$, and NAc, $F_{\text{genotype}}(1, 11) = 7.4$, $p < 0.05$ (Fig. 2). Ubr1 was up-regulated in the AMG of CSS-2 mice, $F_{\text{genotype}}(1, 11) = 7.2$, $p < 0.05$, while Chrm5 levels were increased in the PFC of the CSS-2 strain, $F_{\text{genotype}}(1, 11) = 5.3$, $p < 0.05$. Collectively, these data provide evidence for involvement of 3 of

Fig. 2. qPCR analysis comparing Adal, Chrm5, Disp2, and Ubr1 expression between strains. There was an up-regulation of Adal in CSS-2 versus C57BL/6J mice in PFC (A), AMG (B), and NAc (C). In addition, Ubr1 mRNA levels were increased in CSS-2 in AMG and Chrm5 mRNA levels were higher in PFC of CSS-2 versus C57BL/6J mice. Shown are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.



the identified candidate genes, in the phenotypic differences in alcohol consumption and preference between CSS-2 and C57BL/6J mice.

Reversal of the CSS-2 Alcohol Preference Phenotype by Adal Inhibition

As of the 4 candidate genes, Adal was up-regulated in all regions examined (i.e., PFC, AMG, and NAc) of CSS-2 mice, we next explored the functional role of Adal in regulating alcohol consumption in CSS-2 mice. For that purpose, we used the adenosine deaminase inhibitor EHNA (Nelson et al., 2009; Rosemberg et al., 2007; Woodson et al., 1998), to counteract the putatively increased adenosine deaminase activity in CSS-2 mice. Animals of both strains were treated with either vehicle or EHNA prior to each daily alcohol consumption session. Alcohol intake increased over time, $F_{\text{time}}(2, 54) = 29.9$, $p < 0.001$, indicative of escalation of alcohol intake as we have reported previously (Lesscher et al., 2009a, 2012). In agreement with previous findings, the CSS-2 mice showed lower levels of alcohol intake, $F_{\text{genotype}}(1, 27) = 29.5$, $p < 0.001$, $F_{\text{time} \times \text{genotype}}(2, 54) = 18.2$, $p < 0.001$, and lower alcohol preference when compared to C57BL/6J mice, $F_{\text{genotype}}(1, 27) = 53.4$, $p < 0.001$, independent of time, $F_{\text{time} \times \text{genotype}}(2, 54) = 2.6$, N.S. (Fig. 3; Lesscher et al., 2009a). There were no genotype

differences in the total amount of fluid consumed, $F_{\text{genotype}}(1, 27) = 0.48$, N.S., $F_{\text{time} \times \text{genotype}}(2, 54) = 0.32$, N.S.

Analysis of the effects of EHNA treatment revealed a selective increase in alcohol preference in CSS-2 mice, partly reversing their preference phenotype to that of the C57BL/6J host strain (Fig. 3). There was no overall effect of EHNA on alcohol preference, $F_{\text{treatment}}(1, 27) = 1.3$, N.S. However, treatment with EHNA altered alcohol preference in a genotype-dependent manner, $F_{\text{genotype} \times \text{treatment}}(1, 27) = 5.9$, $p < 0.05$. Post hoc pairwise comparisons confirmed that EHNA increased alcohol preference in CSS-2 mice ($p = 0.019$) but did not affect alcohol preference in C57BL/6J mice ($p = 0.366$), supporting the functional contribution of Adal to the low alcohol preference phenotype of CSS-2 mice. Post hoc pairwise comparisons by week revealed that EHNA increased alcohol preference in CSS-2 mice predominantly in weeks 2 and 3 of the experiment (wk1: $p = 0.066$; wk2: $p = 0.024$; wk3: $p = 0.043$). Although EHNA increased alcohol preference in CSS-2 mice, the Adal inhibitor did not alter alcohol intake: there was no overall effect of treatment on alcohol intake, $F_{\text{treatment}}(1, 27) = 0.17$, N.S., nor was there a genotype-dependent effect of this compound on alcohol intake, $F_{\text{genotype} \times \text{treatment}}(2, 54) = 0.3$, N.S. Importantly, EHNA did not affect total fluid consumption, $F_{\text{treatment}}(1, 26) = 0.06$, N.S., $F_{\text{genotype} \times \text{treatment}}(1, 26) = 1.4$, N.S., ruling out aspecific effects on thirst or fluid ingestion.

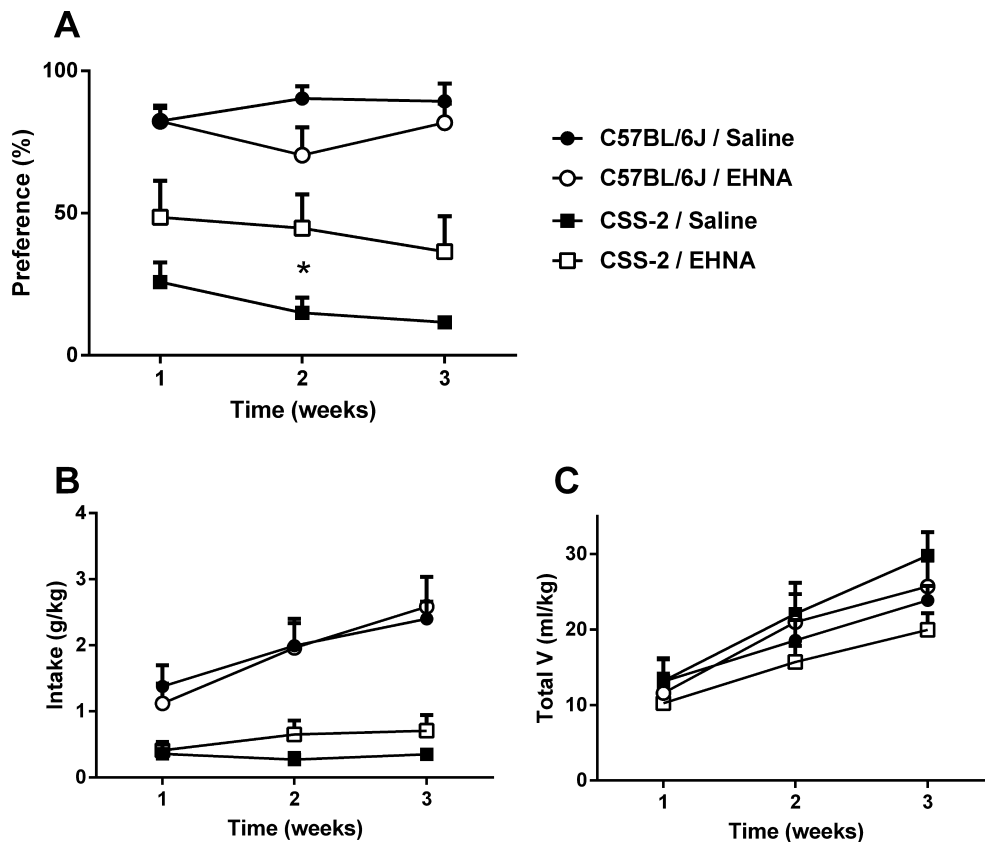


Fig. 3. Adal inhibition by systemic administration of EHNA partly reversed the low alcohol preference phenotype of CSS-2 mice without affecting alcohol preference in C57BL/6J mice (A) or alcohol intake (B) and total fluid consumption (C). Shown are mean \pm SEM. * $p < 0.05$.

Moreover, there were no differences in body weight across the experiment for the genotypes or treatment groups, $F_{\text{genotype}}(1, 27) = 0.46$, N.S., $F_{\text{time} \times \text{genotype}}(2, 54) = 2.1$, N.S., $F_{\text{treatment}}(1, 27) = 0.17$, N.S., $F_{\text{genotype} \times \text{treatment}}(1, 27) = 1.8$, N.S. (data not shown).

DISCUSSION

This study identifies *Adal* as an important candidate gene within a QTL for alcohol preference on mouse chromosome 2. Compared to C57BL/6J mice, *Adal* expression was enhanced in reward-related brain regions in the low alcohol preferring CSS-2 strain, and inhibition of *Adal* activity selectively increased alcohol preference in CSS-2 mice.

Candidate Genes Identified Within the Alcohol Preference QTL

To identify candidate genes within the alcohol preference QTL, we applied criteria that have previously been used to distinguish candidate genes within a QTL: coding SNPs, gene expression patterns, and gene function (Korstanje and Paigen, 2002; Nikolskiy et al., 2015; Noyes et al., 2011). A limitation of this approach is that potentially relevant genes are excluded because noncoding SNPs or other brain regions may also contribute to alcohol preference. Moreover, using this approach, only genes with a known function, that is, based on available literature were selected, thus ruling out potential novel candidate genes. The previously identified QTL for alcohol preference was narrowed down to 4 candidate genes that have, directly or indirectly, been implicated in the modulation of reward sensitivity, that is, *Adal*, *Chrm5*, *Disp2*, and *Ubr1*. *Adal* belongs to the adenosine deaminase (*Ada*) family, based on phylogenetic analyses (Maier et al., 2005; Rosemberg et al., 2007). EtOH has been shown to inhibit *Ada* activity in rat forebrain (Sogut and Kanbak, 2010), and *Ada* inhibition reduced methamphetamine-induced dopamine release and stereotypy (Golembiowska and Zylewska, 2000). *Chrm5* knockout mice show reduced morphine-induced conditioned place preference and cocaine self-administration (Basile et al., 2002; Thomsen et al., 2005) and altered amphetamine- and morphine-induced NAc dopamine release (Schmidt et al., 2010). *Disp2* is involved in hedgehog signaling that affects transcription of *Wnt* genes, which in turn are important for the development and maintenance of mesolimbic dopamine neurons and contribute to amphetamine-induced activity (Galli et al., 2014; Wurst and Prakash, 2014). Finally, *Ubr1* null mice show reduced motor activity and impaired spatial learning (Balogh et al., 2002) that may also have implications for reward learning and substance addiction.

Differential expression of these genes between CSS-2 and C57BL/6J mice suggests that they contribute to the alcohol phenotype of CSS-2 mice, that is, reduced alcohol consumption and preference compared to C57BL/6J mice (Lesscher et al., 2009a). Therefore, expression levels of *Adal*, *Chrm5*,

Disp2, and *Ubr1* were compared in brain regions that contribute to reward and addiction, that is, PFC, AMG, and NAc (Everitt and Robbins, 2013; Koob and Volkow, 2010; Tabakoff and Hoffman, 2013). We found that *Adal* expression was increased in all 3 regions in CSS-2 mice. In addition, the expression of *Ubr1* and *Chrm5* was increased in the AMG and PFC of CSS-2 mice, respectively.

Adenosine and Alcohol Consumption

Adal belongs to the *Ada* family (Maier et al., 2005; Rosemberg et al., 2007). Adenosine deaminases are known to cleave, through deamination, adenosine into inosine, thereby reducing adenosine levels, although physiological evidence to confirm that *Adal* converts adenosine to inosine, as *Ada* does, is at present lacking. Mice of the CSS-2 strain showed enhanced expression of *Adal* in PFC, AMG, and NAc. Enhanced *Adal* expression in CSS-2 mice therefore likely results in lower brain adenosine levels, which is associated with lower alcohol preference. Conversely, alcohol itself has been shown to inhibit *Ada* activity in rat forebrain (Sogut and Kanbak, 2010), which results in increased brain adenosine levels. Together, this suggests that increased forebrain adenosine activity stimulates alcohol intake. Indeed, inhibition of *Ada* using EHNA, thereby increasing adenosine levels, partially reversed the low alcohol preference phenotype of CSS-2 mice. In contrast, EHNA did not affect alcohol intake or preference in C57BL/6J mice, suggesting that a further increase in adenosine activity above baseline levels does not alter the effects of alcohol in this strain. Although we did not measure blood alcohol levels after the drinking sessions, the blood alcohol levels for CSS-2 mice are likely to be low. Thus, the question remains whether *Adal* influences the pharmacological effects of alcohol, or perhaps its rewarding or aversive effects. However, taste is not likely to account for the observed strain difference, and hence, the effects of EHNA on alcohol preference, because previous studies did not reveal differences in taste sensitivity between CSS-2 and C57BL/6J mice (Lesscher et al., 2009a). Together, these findings show that adenosine levels may determine the risk for or resilience to alcohol consumption and, ultimately, AUD.

The present findings somewhat contrast with previous work on adenosine signaling and alcohol consumption; that is, adenosine 2A (A2A) receptor null mutant mice show enhanced alcohol consumption (Houchi et al., 2008; Naasila et al., 2002) while treatment with an A2A agonist reduced alcohol intake (Houchi et al., 2013). In addition, alcohol consumption is enhanced in null mutants of one of the major transporters of adenosine in the brain, type 1 equilibrative nucleoside transporter (ENT) (e.g., Choi et al., 2004). There are several possible explanations for these seemingly discrepant findings. First, the increased sensitivity to alcohol reward in A2A null mice depends on the genetic background; it is only apparent on a CD1, but not a C57BL/6J background (Houchi et al., 2008). Second, because ENT is a bidirectional adenosine transporter, the adenosine

dynamics of ENT knockout mice is very much different from CSS-2 mice, with presumably lower circulating adenosine. Third, an important difference with the studies by Houchi and colleagues (2008) and Naassila and colleagues (2002) is that we restricted access to alcohol to 2 hours each day, as opposed to using a 24-hour 2-bottle choice paradigm. We report selective effects of genotype (C57BL/6J vs. CSS-2) and EHNA on alcohol preference using this limited-access paradigm. These findings agree with the selectivity of the previously identified QTL for alcohol preference (Lesscher et al., 2009a), suggesting that there is a genetic and neurobiological dissociation of alcohol preference and alcohol intake. Limited-access paradigms result in higher levels of alcohol intake, but also in a clear preference for alcohol (e.g., Lesscher et al., 2009a, 2012), which is often not evident when mice have continuous access to alcohol in 24-hour 2-bottle choice tasks (e.g., Gill and Boyle, 2005; Hodge et al., 1999; Nie et al., 2011; Peirce et al., 1998; Tarantino et al., 1998). Our current findings suggest that when access to alcohol is restricted, adenosine signaling may selectively alter alcohol preference. Indeed, A2A knockout mice also show a selective increase in alcohol preference in the limited-access paradigm while consuming similar levels of alcohol (H.M.B. Lesscher and A. Bailey, unpublished). Taken together, the current findings confirm the importance of adenosine signaling for alcohol drinking, although the precise mechanisms require further study.

Actual adenosine levels, but also the dynamics of adenosine signaling, may impact on behavior. Diurnal fluctuations in adenosine levels—low during sleep time while rising during wake time—have been proposed to contribute to day–night cycling (Porkka-Heiskanen et al., 1997). Importantly, day–night cycling is a key factor in alcohol consumption by rodents. Limited-access paradigms (Lesscher et al., 2009a, 2012; Rhodes et al., 2005) employ the natural tendency of rodents to consume most of their fluids during the active phase, providing access to alcohol in the beginning of the dark cycle, when they consume most of their fluids (Dole and Gentry, 1984). It is therefore conceivable that altered adenosine dynamics may, by altering the sleep–wake cycle, lead to the reduced alcohol preference alcohol observed in CSS-2 mice.

Neurobiological Mechanisms of Adenosine Modulation of Alcohol Reward

Adenosine acts as a modulator of neurotransmission in the central nervous system, which may influence a variety of behaviors, including addictive behavior (Burnstock et al., 2011). The effects of adenosine in the central nervous system are mediated through adenosine A1 and A2 receptors, the latter of which has been implicated in drug taking (Arolfo et al., 2004; Houchi et al., 2008; Naassila et al., 2002; Thorsell et al., 2007). These adenosine receptors have been shown to interact with multiple receptor types, which allows adenosine to impact on a wide array of neurobiological systems and behaviors. For example, A2A receptors interact with dopamine D1

and D2 receptors, but also with A1 receptors and metabotropic glutamate receptors (e.g., Nam et al., 2013; Sebastiao and Ribeiro, 2000), all of which have been implicated in addictive behavior (Dalley and Everitt, 2009; Fuxe et al., 2010; Hack and Christie, 2003; Pomierny-Chamiolo et al., 2014). Indeed, A2A and D2 receptors have been shown to synergistically regulate alcohol consumption (Yao et al., 2002).

The elevation in Adal levels in CSS-2 mice versus C57BL/6J mice was observed in the PFC, AMG, and NAC. These brain regions have been implicated in the transition to excessive alcohol use, which is a critical determinant of alcoholism (Darq et al., 2015; George et al., 2012). The AMG, and in particular its central nucleus (CeA), is known to contribute to dependence-induced drinking (e.g., Funk et al., 2006; Gilpin et al., 2008; Pandey et al., 2008). Moreover, the CeA contributes to escalation of alcohol intake and the development of quinine-resistant alcohol consumption (e.g., Lesscher et al., 2012). Finally, there is a substantial body of evidence implicating the NAC in alcohol consumption (e.g., Cozzoli et al., 2012; Griffin et al., 2014; Hopf et al., 2011; Neasta et al., 2011). Thus, the observed up-regulation of Adal in CSS-2 mice in these brain regions likely contributes to the low alcohol preference of these animals.

CONCLUSION

This study identified Adal as a genetically protective factor against alcohol preference drinking in mice, where elevated Adal levels contribute to low levels of alcohol intake. An intriguing question that remains to be addressed is whether, conversely, adenosine deaminase deficiencies increase an individuals' propensity to consume alcohol. This is conceivable, as high alcohol drinking C57BL/6J mice (Rhodes et al., 2007) are considered to be a rodent model for AUD (for review, see Hopf and Lesscher, 2014). Ada replacement therapies are used clinically, and these have, for example, been used to successfully treat immune deficient patients, who suffer from Ada insufficiency (Brigida et al., 2014; Grunebaum et al., 2013). Ada replacement may therefore represent a strategy to treat AUD and perhaps other forms of addiction. Future studies, using clinically relevant models for AUD (Hopf et al., 2010; Lesscher et al., 2010; Seif et al., 2015; Spoelder et al., 2015; Vanderschuren et al., 2017), should investigate this possibility.

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