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Genetic variability in adenosine deaminase-like contributes to variation in alcohol preference in mice

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Abstract

Background

A substantial part of the risk for alcohol use disorder (AUD) is determined by genetic factors. We previously used chromosome substitution (CSS) mice, to identify a 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 chromosome substitution (CSS) mice, to four 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 three 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.

Conclusion

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

Keywords adenosine, alcohol, QTL, preference, mice

Introduction

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 48-71%.

Human genome wide association studies (GWAS) 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 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 (1) we first selected coding SNPs within the QTL, (2) 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, i.e. the PFc, AMG and NAc, and (3) 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 prefrontal cortex (PFc), amygdala (AMG) and nucleus accumbens (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, Main, USA) and bred in our department. Experimental animals were male mice, 8-10 weeks old at the onset of testing. The mice were group-housed with mice from the same genotype under controlled conditions (20 ± 2ºC and 50-70% humidity) and they were acclimatized to a 12-h 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 non-synonymous SNPs within the QTL range, i.e. chromosome 2: 112-134 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 behavioural 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, i.e. 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, cancer etc. were excluded at this stage. With this approach, the QTL range was narrowed down to 4 candidate genes: Adal (Adenosine deaminase-like), 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 qPCR

We reasoned that, if genetic variation in the identified candidate genes, i.e. 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 two 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 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, Germany). Total RNA was isolated from these samples using Trizol (Invitrogen, The Netherlands), DNAse treated (Ambion, TX, USA) and purified using the RNeasy MinElute Cleanup kit (Qiagen N.V., The Netherlands). Subsequently, cDNA was synthesized from the RNA samples using oligo-dT primers. qPCR analysis was performed using the LightCycler (Roche, The Netherlands), the Fast Start DNA Master PLUS SYBRgreen I kit (Roche) and primers listed in Table 1. After initial normalization to the housekeeping 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 four 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, 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 i.p., 2 ml/kg in saline; Tocris, Bristol, UK) (*N* = 8 per strain and treatment). The mice were randomly assigned to the treatment groups (saline or EHNA).

The procedures for ethanol consumption were similar to those used previously (Lesscher et al., 2009a; 2012). A limited access paradigm using a 15% ethanol solution was employed. In with this paradigm, mice readily consume high amounts of alcohol and show a gradual increase in alcohol consumption over the course of 2-4 weeks (Lesscher et al., 2009b; Lesscher et al., 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 et al. (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 h starting at 10:00 AM daily for three consecutive weeks. The mice received access to two drinking tubes, i.e. 10 ml polysterene pipettes fitted with a stainless steel ball-bearing sipper tube. One tube delivered tap water and the other 15% ethanol (v/v in tap water). During the initial 7 days of training, the water and ethanol 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 three consecutive weeks and average ethanol intake (g/kg), ethanol 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) was used for statistical analyses. qPCR data were analyzed per brain region (AMG, PFc, NAC) using multivariate ANOVA's, with genotype as the between-subjects factor. Ethanol consumption data were analyzed by three-way repeated measures ANOVA with genotype and treatment as the between-subjects factors 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 ± S.E.M. 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 (112-134 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 (112-134 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 (Figure 1). Thereafter, coding non-synonymous 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, i.e. 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 four genes that are expressed in reward-related brain regions (PFc, AMG and NAc) and have been implicated, directly or indirectly, in (reward-related) behavior, i.e. 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 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 three brain regions: PFc ($F_{genotype(1,11)}$ = 30.4, P < 0.001), AMG ($F_{genotype(1,11)}$ = 5.7, P < 0.05) and NAc ($F_{genotype(1,11)}$ = 7.4, P < 0.05) (Figure 2). Ubr1 was up-regulated in the AMG of CSS-2 mice ($F_{genotype(1,11)}$ = 7.2, P < 0.05) while Chrm5 levels were increased in the PFc of the CSS-2 strain ($F_{genotype(1,11)}$ = 5.3, P < 0.05). Collectively, these data provide evidence for involvement of three of 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

Since 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), in order 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 ($F_{time(2,54)}$ = 29.9, P < 0.001), indicative of escalation of alcohol intake as we have reported previously (Lesscher et al., 2009a; Lesscher et al., 2012). In agreement with previous findings, the CSS-2 mice showed lower levels of alcohol intake ($F_{genotype(1,27)}$

=29.5, P < 0.001; $F_{time\ x\ genotype\ (2,54)}$ = 18.2, P < 0.001), and lower alcohol preference when compared to C57BL/6J mice ($F_{genotype(1,27)}$ = 53.4, P < 0.001), independent of time ($F_{time\ x\ genotype\ (2,54)}$ = 2.6, N.S., Figure 3) (Lesscher et al., 2009a). There were no genotype differences in the total amount of fluid consumed ($F_{genotype(1,27)}$ = 0.48, N.S.; $F_{time\ x\ genotype(2,54)}$ = 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 (Figure 3). There was no overall effect of EHNA on alcohol preference ($F_{treatment(1,27)}$ = 1.3, N.S.). However, treatment with EHNA altered alcohol preference in a genotype-dependent manner ($F_{genotype\ x\ treatment(1,27)}$ = 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 ($F_{treatment(1,27)}$ = 0.17, N.S.), nor was there a genotype-dependent effect of this compound on alcohol intake ($F_{genotype\ x\ treatment(2,54)}$ = 0.3, N.S.). Importantly, EHNA did not affect total fluid consumption ($F_{treatment(1,26)}$ = 0.06, N.S.; $F_{genotype\ x\ treatment(1,26)}$ = 1.4, N.S.), ruling out aspecific effects on thirst or fluid ingestion. Moreover, there were no

differences in body weight across the experiment for the genotypes or treatment groups ($F_{genotype\ (1,27)}$ =0.46, N.S.; $F_{time\ x\ genotype\ (2,54)}$ = 2.1, N.S.; $F_{treatment(1,27)}$ = 0.17, N.S.; $F_{genotype\ x\ treatment(1,27)}$ = 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 non-coding SNPs or other brain regions may also contribute to alcohol preference. Moreover, using this approach, only genes with a known function, i.e. based on available literature were selected, thus ruling out potential novel candidate genes. The previously identified QTL for alcohol preference was narrowed down to four candidate genes that have, directly or indirectly, been implicated in the modulation of reward sensitivity, i.e. Adal, Chrm5, Disp2 and Ubr1. Adenosine deaminase-like (Adal) belongs to the adenosine deaminase (Ada) family, based

on phylogenetic analyses (Maier et al., 2005; Rosemberg et al., 2007). Ethanol 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, i.e. 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, i.e. 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 three 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

Adenosine deaminase-like (Adal) belongs to the adenosine deaminase (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 adenosine deaminase-like (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 receptor (A2A) null mutant mice show enhanced alcohol consumption (Houchi et al., 2008; Naassila 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 et al. (2008) and Naassila et al. (2002) is that we restricted access to alcohol to 2 hours each day, as opposed to using a 24h two-bottle choice paradigm. We report selective effects of genotype (C57BL/6J versus 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 24h two-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 whilst consuming similar levels of alcohol (Lesscher and 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 CNS, 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, adenosine 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 (Darcq et al., 2014; 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 nucleus accumbens 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, since 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, 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.

References

Abiola O, Angel JM, Avner P, Bachmanov AA, Belknap JK, Bennett B, Blankenhorn EP, Blizard DA, Bolivar V, Brockmann GA, Buck KJ, Bureau JF, Casley WL, Chesler EJ, Cheverud JM, Churchill GA, Cook M, Crabbe JC, Crusio WE, Darvasi A, de Haan G, Dermant P, Doerge RW, Elliot RW, Farber CR, Flaherty L, Flint J, Gershenfeld H, Gibson JP, Gu J, Gu W, Himmelbauer H, Hitzemann R, Hsu HC, Hunter K, Iraqi FF, Jansen RC, Johnson TE, Jones BC, Kempermann G, Lammert F, Lu L, Manly KF, Matthews DB, Medrano JF, Mehrabian M, Mittlemann G, Mock BA, Mogil JS, Montagutelli X, Morahan G, Mountz JD, Nagase H, Nowakowski RS, O'Hara BF, Osadchuk AV, Paigen B, Palmer AA, Peirce JL, Pomp D, Rosemann M, Rosen GD, Schalkwyk LC, Seltzer Z, Settle S, Shimomura K, Shou S, Sikela JM, Siracusa LD, Spearow JL, Teuscher C, Threadgill DW, Toth LA, Toye AA, Vadasz C, Van Zant G, Wakeland E, Williams RW, Zhang

HG, Zou F, Complex Trait Consortium (2003) The nature and identification of quantitative trait loci: a community's view. Nat Rev Genet 4:911-916.

Arolfo MP, Yao L, Gordon AS, Diamond I, Janak PH (2004) Ethanol operant self-administration in rats is regulated by adenosine A2 receptors. Alcohol Clin Exp Res 28:1308-1316.

Balogh SA, McDowell CS, Denenberg VH (2002) Behavioral characterization of mice lacking the ubiquitin ligase UBR1 of the N-end rule pathway. Genes Brain Behav 1:223-229.

Basile AS, Fedorova I, Zapata A, Liu X, Shippenberg T, Duttaroy A, Yamada M, Wess J (2002) Deletion of the M5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. Proc Natl Acad Sci U S A 99:11452-11457.

Brigida I, Sauer AV, Ferrua F, Giannelli S, Scaramuzza S, Pistoia V, Castiello MC, Barendregt BH, Cicalese MP, Casiraghi M, Brombin C, Puck J, Muller K, Notarangelo LD, Montin D, van Montfrans JM, Roncarolo MG, Traggiai E, van Dongen JJ, van der Burg M, Aiuti A (2014) B-cell development and functions and therapeutic options in adenosine deaminase-deficient patients. J Allergy Clin Immunol 133:799-806.e10.

Bubier JA, Jay JJ, Baker CL, Bergeson SE, Ohno H, Metten P, Crabbe JC, Chesler EJ (2014) Identification of a QTL in Mus musculus for Alcohol Preference, Withdrawal, and Ap3m2 Expression Using Integrative Functional Genomics and Precision Genetics. Genetics 197:1377-1393.

Burnstock G, Krugel U, Abbracchio MP, Illes P (2011) Purinergic signalling: from normal behaviour to pathological brain function. Prog Neurobiol 95:229-274.

Choi DS, Cascini MG, Mailliard W, Young H, Paredes P, McMahon T, Diamond I, Bonci A, Messing RO (2004) The type 1 equilibrative nucleoside transporter regulates ethanol intoxication and preference. Nat Neurosci 7:855-861.

Cozzoli DK, Courson J, Caruana AL, Miller BW, Greentree DI, Thompson AB, Wroten MG, Zhang PW, Xiao B, Hu JH, Klugmann M, Metten P, Worley PF, Crabbe JC, Szumlinski KK (2012) Nucleus accumbens mGluR5-associated signaling regulates binge alcohol drinking under drinking-in-the-dark procedures. Alcohol Clin Exp Res 36:1623-1633.

Dalley JW, Everitt BJ (2009) Dopamine receptors in the learning, memory and drug reward circuitry. Semin Cell Dev Biol 20:403-410.

Darcq E, Warnault V, Phamluong K, Besserer GM, Liu F, Ron D (2014) MicroRNA-30a-5p in the prefrontal cortex controls the transition from moderate to excessive alcohol consumption. Mol Psychiatry doi: 10.1038/mp.2014.120.

Dole VP, Gentry RT (1984) Toward an analogue of alcoholism in mice: scale factors in the model. Proc Natl Acad Sci U S A 81:3543-3546.

Everitt BJ, Robbins TW (2013) From the ventral to the dorsal striatum: Devolving views of their roles in drug addiction. Neurosci Biobehav Rev 37:1946-1954.

Funk CK, O'Dell LE, Crawford EF, Koob GF (2006) Corticotropin-Releasing Factor within the Central Nucleus of the Amygdala Mediates Enhanced Ethanol Self-Administration in Withdrawn, Ethanol-Dependent Rats. J Neurosci 26:11324-11332.

Fuxe K, Marcellino D, Borroto-Escuela DO, Guescini M, Fernandez-Duenas V, Tanganelli S, Rivera A, Ciruela F, Agnati LF (2010) Adenosine-dopamine interactions in the pathophysiology and treatment of CNS disorders. CNS Neurosci Ther 16:e18-42.

Galli S, Lopes DM, Ammari R, Kopra J, Millar SE, Gibb A, Salinas PC (2014) Deficient Wnt signalling triggers striatal synaptic degeneration and impaired motor behaviour in adult mice. Nat Commun 5:4992.

George O, Sanders C, Freiling J, Grigoryan E, Vu S, Allen CD, Crawford E, Mandyam CD, Koob GF (2012) Recruitment of medial prefrontal cortex neurons during alcohol withdrawal predicts cognitive impairment and excessive alcohol drinking. Proc Natl Acad Sci U S A 109:18156-18161.

Gill K, Boyle AE (2005) Genetic analysis of alcohol intake in recombinant inbred and congenic strains derived from A/J and C57BL/6J progenitors. Mamm Genome 16:319-331.

Gilpin NW, Misra K, Koob GF (2008) Neuropeptide Y in the central nucleus of the amygdala suppresses dependence-induced increases in alcohol drinking. Pharmacol Biochem Behav 90:475-480.

Golembiowska K, Zylewska A (2000) Effect of adenosine kinase, adenosine deaminase and transport inhibitors on striatal dopamine and stereotypy after methamphetamine administration. Neuropharmacology 39:2124-2132.

Griffin WC,3rd, Haun HL, Hazelbaker CL, Ramachandra VS, Becker HC (2014) Increased extracellular glutamate in the nucleus accumbens promotes excessive ethanol drinking in ethanol dependent mice. Neuropsychopharmacology 39:707-717.

Grunebaum E, Cohen A, Roifman CM (2013) Recent advances in understanding and managing adenosine deaminase and purine nucleoside phosphorylase deficiencies. Curr Opin Allergy Clin Immunol 13:630-638.

Hack SP, Christie MJ (2003) Adaptations in adenosine signaling in drug dependence: therapeutic implications. Crit Rev Neurobiol 15:235-274.

Hodge CW, Mehmert KK, Kelley SP, McMahon T, Haywood A, Olive MF, Wang D, Sanchez-Perez AM, Messing RO (1999) Supersensitivity to allosteric GABA(A) receptor modulators and alcohol in mice lacking PKCepsilon. Nat Neurosci 2:997-1002.

Hopf FW, Chang SJ, Sparta DR, Bowers MS, Bonci A (2010) Motivation for Alcohol Becomes Resistant to Quinine Adulteration After 3 to 4 Months of Intermittent Alcohol Self-Administration. Alcohol Clin Exp Res 34:1565-1573.

Hopf FW, Lesscher HMB (2014) Rodent models for compulsive alcohol intake. Alcohol 48:253-264.

Hopf FW, Simms JA, Chang SJ, Seif T, Bartlett SE, Bonci A (2011) Chlorzoxazone, an SK-type potassium channel activator used in humans, reduces excessive alcohol intake in rats. Biol Psychiatry 69:618-624.

Houchi H, Persyn W, Legastelois R, Naassila M (2013) The adenosine A2A receptor agonist CGS 21680 decreases ethanol self-administration in both non-dependent and dependent animals. Addict Biol 18:812-825.

Houchi H, Warnault V, Barbier E, Dubois C, Pierrefiche O, Ledent C, Daoust M, Naassila M (2008) Involvement of A2A receptors in anxiolytic, locomotor and motivational properties of ethanol in mice. Genes Brain Behav 7:887-898.

Koob GF, Volkow ND (2010) Neurocircuitry of Addiction. Neuropsychopharmacology 35:217-238.

Korstanje R, Paigen B (2002) From QTL to gene: the harvest begins. Nat Genet 31:235-236.

Lesscher HMB, Houthuijzen JM, Groot Koerkamp MJ, Holstege FC, Vanderschuren LJMJ (2012) Amygdala 14-3-3zeta as a novel modulator of escalating alcohol intake in mice. PLoS one 7(5):e37999.

Lesscher HMB, Kas MJ, van der ES, van Lith HA, Vanderschuren LJMJ (2009a) A grandparent-influenced locus for alcohol preference on mouse chromosome 2. Pharmacogenet Genomics 19:719-729.

Lesscher HMB, van Kerkhof LWM, Vanderschuren LJMJ (2010) Inflexible and indifferent alcohol drinking in male mice. Alcohol Clin Exp Res 34:1219-1225.

Lesscher HMB, Wallace MJ, Zeng L, Wang V, Deitchman JK, McMahon T, Messing RO, Newton PM (2009b) Amygdala Protein Kinase C Epsilon Controls Alcohol Consumption. Genes Brain Behav 8:493-499.

Maier SA, Galellis JR, McDermid HE (2005) Phylogenetic analysis reveals a novel protein family closely related to adenosine deaminase. J Mol Evol 61:776-794.

Milner LC, Buck KJ (2010) Identifying quantitative trait loci (QTLs) and genes (QTGs) for alcohol-related phenotypes in mice. Int Rev Neurobiol 91:173-204.

Naassila M, Ledent C, Daoust M (2002) Low ethanol sensitivity and increased ethanol consumption in mice lacking adenosine A2A receptors. J Neurosci 22:10487-10493.

Nadeau JH, Singer JB, Matin A, Lander ES (2000) Analysing complex genetic traits with chromosome substitution strains. Nat Genet 24:221-225.

Nam HW, Bruner RC, Choi DS (2013) Adenosine signaling in striatal circuits and alcohol use disorders. Mol Cells 36:195-202.

Neasta J, Ben Hamida S, Yowell QV, Carnicella S, Ron D (2011) AKT signaling pathway in the nucleus accumbens mediates excessive alcohol drinking behaviors. Biol Psychiatry 70:575-582.

Nelson AM, Battersby AS, Baghdoyan HA, Lydic R (2009) Opioid-induced decreases in rat brain adenosine levels are reversed by inhibiting adenosine deaminase. Anesthesiology 111:1327-1333.

Nie H, Rewal M, Gill TM, Ron D, Janak PH (2011) Extrasynaptic delta-containing GABAA receptors in the nucleus accumbens dorsomedial shell contribute to alcohol intake. Proc Natl Acad Sci U S A 108:4459-4464.

Nikolskiy I, Conrad DF, Chun S, Fay JC, Cheverud JM, Lawson HA (2015) Using whole-genome sequences of the LG/J and SM/J inbred mouse strains to prioritize quantitative trait genes and nucleotides. BMC Genomics 16:415-015-1592-3.

Noyes H, Brass A, Obara I, Anderson S, Archibald AL, Bradley DG, Fisher P, Freeman A, Gibson J, Gicheru M, Hall L, Hanotte O, Hulme H, McKeever D, Murray C, Oh SJ, Tate C, Smith K, Tapio M, Wambugu J, Williams DJ, Agaba M, Kemp SJ (2011) Genetic and expression analysis of cattle identifies candidate genes in pathways responding to Trypanosoma congolense infection. Proc Natl Acad Sci U S A 108:9304-9309.

Pandey SC, Zhang H, Ugale R, Prakash A, Xu T, Misra K (2008) Effector immediate-early gene arc in the amygdala plays a critical role in alcoholism. J Neurosci 28:2589-2600.

Peirce JL, Derr R, Shendure J, Kolata T, Silver LM (1998) A major influence of sex-specific loci on alcohol preference in C57Bl/6 and DBA/2 inbred mice. Mamm Genome 9:942-948.

Phillips TJ, Belknap JK, Buck KJ, Cunningham CL (1998) Genes on mouse chromosomes 2 and 9 determine variation in ethanol consumption. Mamm Genome 9:936-941.

Pomierny-Chamiolo L, Rup K, Pomierny B, Niedzielska E, Kalivas PW, Filip M (2014) Metabotropic glutamatergic receptors and their ligands in drug addiction. Pharmacol Ther 142:281-305.

Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW, McCarley RW (1997) Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. Science 276:1265-1268.

Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC (2005) Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav 84:53-63.

Rhodes JS, Ford MM, Yu CH, Brown LL, Finn DA, Garland T,Jr., Crabbe JC (2007) Mouse inbred strain differences in ethanol drinking to intoxication. Genes Brain Behav 6:1-18.

Rodriguez LA, Plomin R, Blizard DA, Jones BC, McClearn GE (1995) Alcohol acceptance, preference, and sensitivity in mice. II. Quantitative trait loci mapping analysis using BXD recombinant inbred strains. Alcohol Clin Exp Res 19:367-373.

Rosemberg DB, Rico EP, Guidoti MR, Dias RD, Souza DO, Bonan CD, Bogo MR (2007) Adenosine deaminase-related genes: molecular identification, tissue expression pattern and truncated alternative splice isoform in adult zebrafish (Danio rerio). Life Sci 81:1526-1534.

Schmidt LS, Miller AD, Lester DB, Bay-Richter C, Schulein C, Frikke-Schmidt H, Wess J, Blaha CD, Woldbye DP, Fink-Jensen A, Wortwein G (2010) Increased amphetamine-induced locomotor activity, sensitization, and accumbal dopamine release in M5 muscarinic receptor knockout mice. Psychopharmacology (Berl) 207:547-558.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.

Sebastiao AM, Ribeiro JA (2000) Fine-tuning neuromodulation by adenosine. Trends Pharmacol Sci 21:341-346.

Seif T, Simms JA, Lei K, Wegner S, Bonci A, Messing RO, Hopf FW (2015) D-Serine and D-Cycloserine Reduce Compulsive Alcohol Intake in Rats. Neuropsychopharmacology 40:2357-2367.

Sogut I, Kanbak G (2010) In vitro effects of ethanol with aspirin on rat brain synaptosomes: the potential protective role of betaine. Int J Neurosci 120:774-783.

Spoelder M, Vanderschuren LJMJ, Lesscher HMB (2015) Individual variation in alcohol intake predicts reinforcement, motivation, and compulsive alcohol use in rats. Alcoholism: Clinical and Experimental Research 39:2427-2437.

Tabakoff B, Hoffman PL (2013) The neurobiology of alcohol consumption and alcoholism: an integrative history. Pharmacol Biochem Behav 113:20-37.

Tarantino LM, McClearn GE, Rodriguez LA, Plomin R (1998) Confirmation of quantitative trait loci for alcohol preference in mice. Alcohol Clin Exp Res 22:1099-1105.

Thomsen M, Woldbye DP, Wortwein G, Fink-Jensen A, Wess J, Caine SB (2005) Reduced cocaine self-administration in muscarinic M5 acetylcholine receptor-deficient mice. J Neurosci 25:8141-8149.

Thorsell A, Johnson J, Heilig M (2007) Effect of the adenosine A2a receptor antagonist 3,7-dimethyl-propargylxanthine on anxiety-like and depression-like behavior and alcohol consumption in Wistar Rats. Alcohol Clin Exp Res 31:1302-1307.

Vadasz C, Saito M, Gyetvai B, Mikics E, Vadasz C (2000) Scanning of five chromosomes for alcohol consumption loci. Alcohol 22:25-34.

Vanderschuren LJMJ, Minnaard AM, Smeets JAS, Lesscher HMB (2017) Punishment models of addictive behavior. Current Opinion in Behavioral Sciences 13:77-84.

Whatley VJ, Johnson TE, Erwin VG (1999) Identification and confirmation of quantitative trait loci regulating alcohol consumption in congenic strains of mice. Alcohol Clin Exp Res 23:1262-1271.

WHO. Global Status Report on Alcohol and Health. 2011.

Woodson JC, Minor TR, Job RF (1998) Inhibition of adenosine deaminase by erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) mimics the effect of inescapable shock on escape learning in rats. Behav Neurosci 112:399-409.

Wurst W, Prakash N (2014) Wnt1-regulated genetic networks in midbrain dopaminergic neuron development. J Mol Cell Biol 6:34-41.

Yao L, Arolfo MP, Dohrman DP, Jiang Z, Fan P, Fuchs S, Janak PH, Gordon AS, Diamond I (2002) betagamma Dimers mediate synergy of dopamine D2 and adenosine A2 receptor-stimulated PKA signaling and regulate ethanol consumption. Cell 109:733-743.

Yoneyama N, Crabbe JC, Ford MM, Murillo A, Finn DA (2008) Voluntary ethanol consumption in 22 inbred mouse strains. Alcohol 42:149-160.

Ystrom E, Reichborn-Kjennerud T, Aggen SH, Kendler KS (2011) Alcohol dependence in men: reliability and heritability. Alcohol Clin Exp Res 35:1716-1722.

Figure Legends

Figure 1 The QTL 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.

Figure 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, AMG and NAc. 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 ± SEM. * $P < 0.05$; ** $P < 0.01$.

Figure 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 or alcohol intake and total fluid consumption. Shown are mean ± SEM. * $P < 0.05$.

Table 1. Primer Sequences for qPCR validation of candidate genes

Primer Fwd	Primer Rev
TTCTTGGCCTTGACCTCAGT	CAGAGGCGCTAAGGAATGTC
TCAGCCATCAAATGACCAAA	AGTAACCCAAGTGCCACAGG
CTGGCCTTCATCTTCCTCTG	GGAGGCTTGAGCTGTTCATC
ACTCCGTGGTTATGGCTCAC	AGGATCTTACGGGCACCTTT

Table 2. Candidate genes identified in the alcohol preference QTL 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 (S. cerevisiae)
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 (E. coli)
Chrm5	cholinergic receptor, muscarinic 5
Ctdspl2	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase like 2
Disp2	dispatched homolog 2 (Drosophila)
Dll4	delta-like 4 (Drosophila)
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