The clock gene *Period2* influences the glutamatergic system and thereby modulates alcohol consumption

Rainer Spanagel[1], Gurudutt Pendyala[2], Carolina Abarca[1,3], Tarek Zghoul[1], Carles Sanchis-Segura[1], Maria Chiara Magnone[2], Jesús Lascorz[1], Martin Depner[1], David Holzberg[4], Michael Soyka[5], Stefan Schreiber[6], Fumihiko Matsuda[7], Mark Lathrop[7], Gunter Schumann[1] & Urs Albrecht[2,4]

[1] Departments of Psychopharmacology and Psychiatry, Central Institute of Mental Health, J5, 68159 Mannheim, Germany

[2] Department of Medicine, Division of Biochemistry, University of Fribourg, 1700 Fribourg, Switzerland

[3] Department of Pharmaceutical Chemistry, Experimental Neuropharmacology, Vrije Universiteit Brussel, 1090 Brussels, Belgium

[4] Max-Planck-Institute for Experimental Endocrinology, 30625 Hannover, Germany

[5] Department of Psychiatry and Psychotherapy, University of Munich, 80336 Munich, Germany

[6] Institute for Clinical Molecular Biology, Christian-Albrechts-University, UKSH Kiel, 24098 Kiel, Germany

[7] Centre National de Génotypage, 91057 Evry, France

Correspondence should be addressed to R.S. (spanagel@zi-mannheim.de) or U.A. (urs.albrecht@unifr.ch)

Period (*Per*) genes are involved in the regulation of the circadian clock and are thought to modulate several brain functions. We show that $Per2^{Brdm1}$ mutant mice display alterations in the glutamatergic system. Lowered expression of the glutamate transporter Eaat1 is observed in these animals, leading to reduced uptake of glutamate by astrocytes. As a consequence, glutamate levels increase in the extra-cellular space of $Per2^{Brdm1}$ mutant mouse brains. This is accompanied by increased alcohol intake in these animals. In humans, variations of the *PER2* gene are associated with the regulation of alcohol consumption. Acamprosate, a medication used in the prevention of craving and relapse in alcoholic patients is thought to act by dampening a hyper-glutamatergic state. This drug reduced augmented glutamate levels and normalized the drinking behavior in $Per2^{Brdm1}$ mutant mice. Collectively, these data establish glutamate as a link between the dysfunction of the circadian clock gene *Per2* and enhanced alcohol consumption.

Across a spectrum of living organisms, ranging from cyanobacteria to humans, it has been observed that biological functions follow a pattern of circadian rhythmicity. These endogenous rhythms display a periodicity close to 24 hours in the absence of environmental cues, thus reflecting the existence of an intrinsic biological clock. In mammals, circadian rhythms in different tissues are coordinated by a master clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus[1]. This circadian clock is thought to be advantageous in synchronising physiological and biochemical pathways, allowing the organism to anticipate daily changes, thus ensuring better adaptation to the environment[2].

The oscillatory mechanism of the circadian clock has been unraveled by means of genetic analysis in *Drosophila* and mammals[3-5]. In the latter, the heterodimeric complex of two transcriptional activators, CLOCK and BMAL1 (MOP3), induce the expression of several genes by interacting with the enhancer elements, termed E-boxes, of their promoters. Amongst these genes are *Per1*, *Per2*, *Cry1* and *Cry2*, whose protein products, upon entering the nucleus, inhibit the activity of the CLOCK/BMAL1 complex, and thereby generating an inhibitory feedback loop driving recurrent rhythms in mRNA and protein levels of their own genes. This molecular mechanism seems to be present in the local clocks of most tissues and brain regions. Furthermore, these different clocks may then be synchronized by the SCN via neural and endocrine outputs[6].

Several lines of evidence implicate glutamate in the activation of receptors on SCN neurons following retinal light perception[7]. This leads to the activation of several signal transduction pathways and the clock genes *Per1* and *Per2*[8]. At the behavioral level, this results in an alteration of clock phase. Interestingly, mice mutant in the *Per2* gene (*Per2^{Brdm1}*) display impaired clock resetting[9], suggesting altered

glutamate signaling. Here, we show that $Per2^{Brdm1}$ mutant mice display alterations in their glutamatergic system as the glutamate transporter Eaat1 (Excitatory Amino Acid Transporter 1, also known as Glast) is found to be reduced in these mice. Excess glutamate is cleared from the synaptic cleft by glutamate transporters[10], located on astroglial cells and transported back to the neuron via the glutamine-glutamate cycle. A deficit in the removal of glutamate from the synaptic cleft, results in a hyper-glutamatergic state and is suggested to produce alterations at the behavioral level[10,11]. Importantly, a hyper-glutamatergic state has been implicated in the aetiology of alcohol dependence[12-14]. We observe that in $Per2^{Brdm1}$ mutant mice voluntary alcohol consumption is enhanced. In humans we find an association between alcoholic patients and genetic variations in the human $PER2$ gene. Acamprosate, a medication thought to dampen a hyper-glutamatergic state in the alcohol dependent human brain[15-17] reduces augmented glutamate levels and normalizes enhanced alcohol consumption in $Per2^{Brdm1}$ mutant mice. These findings support the view that a hyper-glutamatergic state can be involved in several aspects of alcohol dependence[12-14,18-20].

RESULTS

Glutamate transporters in $Per2^{Brdm1}$ mice

Wild type and $Per2^{Brdm1}$ mutant mice differ in their behavioral response to a light pulse administered at zeitgeber time (ZT) 14[9] (ZT0 corresponds to lights on and ZT12 to lights off). Therefore, we set out to search for a difference in gene expression between wild type and $Per2^{Brdm1}$ mutant mice at ZT15. This time point has been chosen because a strong induction of $Per1$ and $Per2$ gene expression in the SCN can be observed one hour after light has been given[8]. Differential display analysis from untreated wild type and $Per2^{Brdm1}$ mutant mice should reveal the differences at the

molecular level in the SCN of these animals and guide us to the molecular cause of the observed behavioral variations of these two genotypes. Therefore, we subtracted a cDNA library derived from $Per2^{Brdm1}$ mutant mice at ZT15 from a cDNA library of wild type littermate mice. We found that $Eaat1$, an important glutamate transporter, which clears the synaptic cleft of glutamate and is solely expressed in astrocytes[10], is down-regulated at ZT15 in the brain and in the SCN of $Per2^{Brdm1}$ mutant mice (**Fig.1a,b**). Interestingly, $Eaat1$ mRNA is expressed in a diurnal manner in the SCN of wild type mice (**Fig. 1c**), suggesting that the observed difference at ZT15 between the two genotypes might represent a shift in diurnal expression. Therefore, we monitored Eaat1 protein expression in whole brain extracts of wild type and $Per2^{Brdm1}$ mutant mice (**Fig. 1d**). We found that in wild type animals, maximal expression of Eaat1 protein can be observed at ZT0 (24), while minimal expression can be seen between ZT12 and ZT18 ($P<0.0001$). In contrast, Eaat1 protein levels were significantly lower in $Per2^{Brdm1}$ mutant mice as compared to wild type ($P=0.0014$). We conclude that Eaat1 expression is reduced in $Per2^{Brdm1}$ mutant animals and therefore, these animals should exhibit elevated glutamate levels in the brain.

It is not clear how the $mPer2$ mutation leads to a reduced expression of Eaat1. One kilobase of the $Eaat1$ promoter, containing a class II E-box, is not sufficient to respond to Clock/Bmal1 (data not shown). Upstream of that E-box no additional E-boxes were found. This suggests that regulation of Eaat1 by $Per2$ is of indirect nature, through an unknown mechanism. It has been described that $Eaat1$ expression is modulated by metabotropic glutamate receptors (mGluR)[21]. In $Per2^{Brdm1}$ mutant mice, protein levels of mGluR1, mGluR2/3 and mGluR5 did not differ from those in wild type mice (**Fig. 2a,b,c**). This indicates that a change in Per2 function does not alter

Eaat1 expression via the regulation of metabotropic glutamate receptor expression[21].

However, we cannot exclude the possibility, that mGluR signaling is altered.

To keep glutamate levels in a range that is not toxic for the animals, compensation for lack of Eaat1 is expected. We found a shift in expression of the glutamate transporter Eaat2, also termed Glt-1, in *Per2*[Brdm1] mutant mice (**Fig. 2d**). The maximal expression is shifted from ZT6 in wild type animals to ZT18 in *Per2*[Brdm1] mutant mice. Two-way ANOVA revealed a significant difference between genotypes ($P=0.0051$). However, the slight elevation of Eaat2 expression in *Per2*[Brdm1] mutant mice was not significant when the two expression curves were aligned according to their maxima (ZT6 for wild type and ZT18 for *Per2*[Brdm1] mutant mice). Thus, although Eaat1 is low in *Per2*[Brdm1] mutant mice, its reduction is not efficiently compensated by up-regulation of Eaat2 to counteract a hyper-glutamatergic system in *Per2*[Brdm1] mutant mice. To further support this finding, we designed a set of experiments to test whether *Per2*[Brdm1] mutant animals exhibit elevated glutamate levels in the brain.

Per2[Brdm1] mice have a hyper-glutamatergic brain

Eaat1 is one of the main glutamate transporters in the brain, responsible for clearing glutamate accumulated in the synaptic cleft. Because Eaat1 protein is reduced in *Per2*[Brdm1] mutant mice, we expected glutamate levels to be elevated and comparable to those of Eaat1 knockout animals[11]. Therefore, we performed a glutamate transport assay with synaptosomes from total brain collected at ZT6, which yielded a similar glutamate uptake in *Per2*[Brdm1] mutant mice as compared to their wild type littermates (**Fig. 3a**). The estimated Michaelis constant (K_m) and the maximum uptake velocity (V_{max}) for wild type mice are: $K_m = 47 \pm 15$ µM, $V_{max} = 1 \pm 0.2$ nmol/min/mg, and

for $Per2^{Brdm1}$ mutant mice $K_m = 32 \pm 11$ µM, $V_{max} = 0.7 \pm 0.1$ nmol/min/mg. This finding indicates that glutamate uptake in neurons is not altered in $Per2^{Brdm1}$ mutant mice because synaptosomes represent the pinched-off pre- and post-synaptic endings of neurons. Further, we measured the intercellular amount of amino acids in the brain of wild type and $Per2^{Brdm1}$ mutant mice by assessing the amino acid concentration in the supernatant of centrifuged brain homogenates. We found that $Per2^{Brdm1}$ mutant mice had significantly higher levels of intercellular amino acids as compared to the wild type animals ($P<0.0001$) (**Fig. 3b**). A microdialysis experiment in the ventral striatum of freely moving mice revealed that $Per2^{Brdm1}$ mutant mice have glutamate levels that are almost three-fold higher than levels in wild-type littermates (**Fig. 3c**). Because Eaat1 is mainly localized on astrocytes[10], we compared glutamate uptake in these particular cells of both wild type and $Per2^{Brdm1}$ mutant littermate pups, sacrificed at ZT6. Cultured astrocytes of $Per2^{Brdm1}$ mutant animals display a strongly reduced uptake of glutamate as compared to astrocytes from wild type animals (**Fig. 3d**). The estimated K_m and V_{max} for wild type astrocytes are: $K_m = 20 \pm 12$ µM, $V_{max} = 7.8 \pm 1.4$ nmol/min/mg, and for $Per2^{Brdm1}$ astrocytes $K_m = 48 \pm 40$ µM, $V_{max} = 4.8 \pm 1.7$ nmol/min/mg (two-way ANOVA of the curves reveals a significant difference of glutamate uptake between the two genotypes, $P=0.0007$). This indicates a deficit in glutamate clearance from the synaptic cleft in $Per2^{Brdm1}$ mutant animals. We do not know, whether this defect is directly or indirectly caused by the absence of *Per2*. Whether *Per2* influences astrocyte differentiation is not known, however, astrocytes of $Per2^{Brdm1}$ mutant mice and wild type animals cannot be distinguished morphologically and both express the astrocyte marker, glial fibrillary acidic protein (GFAP) (data not shown). Taken together, the results depicted in **Figure 3**

demonstrate that glutamate levels are elevated in $Per2^{Brdm1}$ mutant mice, due to a malfunction of astrocytes, leading to a brain with a hyper-glutamatergic state.

$Per2^{Brdm1}$ mice voluntarily drink more alcohol

Enhanced glutamate levels and alterations within the glutamatergic system have been implicated in excessive alcohol consumption and dependence[12-14,18-20]. Accordingly, we proceeded to investigate voluntary alcohol consumption and preference[22]. $Per2^{Brdm1}$ mutant mice show significantly enhanced alcohol intake, when pharmacologically relevant concentrations of 8-16% ethanol are offered (P=0.001; two-way ANOVA) (**Fig. 4a**). At the highest concentration of 16%, $Per2^{Brdm1}$ mutant mice consume approximately 3-fold more ethanol than the wild type littermate control animals. A similar observation was made on alcohol preference measures. Thus, at concentrations of 8-16% ethanol, a clear genotype dependent difference is observed (P=0.001) (**Fig. 4b**).

There are different explanations why $Per2^{Brdm1}$ mutant mice drink more alcohol. One obvious explanation is that through alterations in the glutamatergic system, energy need is enhanced and, therefore, more alcohol is consumed. However, this explanation seems unlikely as no genotype dependent differences are observed at low ethanol concentrations (2-4%) (**Fig. 4a,b**). Moreover, food consumption does not differ between the two genotypes (mean daily average food intake over a period of 14 days: wild type 152 ± 6 g/kg, and $Per2^{Brdm1}$ mutant mice 167 ± 12 g/kg body weight).

Alternatively, the increase in alcohol consumption in $Per2^{Brdm1}$ mutant mice could be due to an alteration in taste sensation of these animals. Therefore, we estimated the preference ratio for a bitter tasting compound containing various concentrations of quinine[22,23]. Both genotypes behave similarly (**Fig. 4c**) and in a

sucrose preference test, where the sweet component is estimated, again no differences between genotypes, are observed (**Fig. 4d**). Thus, a difference in taste sensation probably does not account for an enhanced alcohol preference in *Per2^Brdm1^* mutant animals. Because sucrose consumption does not differ between genotypes, energy consumption is the same and hence, an increase in alcohol uptake in *Per2^Brdm1^* mice is probably not caused by higher caloric needs in these animals.

However, one could argue that alcohol metabolism and elimination is altered in *Per2^Brdm1^* mutant mice. Alcohol is mainly eliminated via oxidation, catalyzed by alcohol dehydrogenase[24]. Circadian variations in plasma ethanol levels and within alcohol oxidation pathways have been described in mice[25]. Therefore, it can be assumed that in *Per2^Brdm1^* mutant mice – the *mPer2* gene is also strongly expressed in the liver – alcohol elimination may differ and that the observed changes in alcohol drinking behavior can be simply explained by alterations in the ethanol elimination rate. Therefore, we studied blood alcohol elimination in both alcohol-naïve and alcohol-experienced *Per2^Brdm1^* mutants as well as their wild type littermates. Under none of these conditions differences in blood alcohol concentrations between genotypes were observed (**Fig. 4e,f**) excluding the possibility that altered alcohol elimination accounts for an enhanced alcohol consumption in *Per2^Brdm1^* mutant mice.

A final and likely explanation, is that a mutation in *mPer2*, influences the set point within the reinforcement system. Thus, the reinforcing value of alcohol might be differently perceived by *Per2^Brdm1^* mutant mice as compared to wild type animals. Therefore, we studied the reinforcing properties of alcohol in *Per2^Brdm1^* mutant mice, conducting operant self-administration using a two-lever paradigm. Following acquisition of lever responding using a simple fixed ratio schedule of 1 (FR1 *i.e.* one lever press resulted in the delivery of one drop of alcohol), *Per2^Brdm1^* mutant mice

exhibited approximately 50 lever responses within a 30 minute session, whereas wild type mice responded significantly less (P=0.02; approximately 25 lever responses per session (**Fig. 5a**)). Moreover, while FR1 performance is more related to the rewarding post-consumptional consequences of the delivered reinforcer, progressive effort requirements better reflect incentive-motivation processes[26]. Accordingly, we used a progressive ratio (PR) schedule to study the incentive motivation for alcohol drinking in *Per2^Brdm1* mutant mice. Using a PR2 (response requirements increased by a step size of 2), the breakpoint for alcohol responding was significantly higher in *Per2^Brdm1* mutant animals as compared to the wild type mice (P=0.02) (**Fig. 5b**).

Together, we conclude from this set of experiments that neither the caloric value, nor taste differences, nor variations in alcohol elimination can account for the enhanced alcohol intake we clearly observed in *Per2^Brdm1* mutant mice. Rather, we suggest that it is possibly due to alterations in the brain reinforcement system of *Per2^Brdm1* mutant mice, thus driving an enhanced incentive motivation to consume more alcohol than the littermate control animals. To further translate this conclusion to a neurochemical level, we propose that through a reduction of Eaat1, enhanced glutamate levels in the brain reinforcement system lead to a hyper-glutamatergic state, finally accounting for the observed "alcohol phenotype". This conclusion is further supported by a recent finding, which shows that glutamate transport is reduced in the cerebral cortex of alcohol-preferring AA rats[27].

Acamprosate treatment of *Per2^Brdm1* mice

Acamprosate is used in the clinic for relapse prevention[28]. A meta-analysis of 20 clinical trials clearly shows its effectiveness[29], however, the exact mechanism of how acamprosate diminishes alcohol consumption and reduces the likelihood of relapse is

still not clear. Different neurobiological pathways have been implicated in the aetiology of alcohol dependence and one pathway seems to involve the glutamatergic system[16,18,30,31], where chronic alcohol intake leads to compensatory changes. It is suggested that acamprosate acts mainly on a hyper-glutamatergic state, yet having only little effect on a "normal" glutamatergic state[15-17,32,33]. Due to the occurrence of a hyper-glutamatergic system in *Per2^{Brdm1}* mutant mice, we speculated that acamprosate should be more effective in reducing glutamate levels and alcohol consumption in the mutant rather than in wild type mice. Indeed, after performing further *in vivo* microdialysis experiments, we observed that 80 minutes following acamprosate treatment, extracellular glutamate levels in the nucleus accumbens in *Per2^{Brdm1}* mutant mice no longer differed from those in wild type mice (**Fig. 6a**). Furthermore, following acamprosate treatment, *Per2^{Brdm1}* mutant mice exhibited reduced levels of alcohol consumption, even below those of wild type animals (**Fig. 6b**). These experiments show that the "hyper-glutamatergic endophenotype" as well as the "alcohol phenotype" in *Per2^{Brdm1}* mutant mice can be rescued by acamprosate treatment. As acamprosate was previously shown to block enhanced extracellular dopamine levels in the nucleus accumbens after glutamate receptor stimulation[34], it is suggested that dopamine-mediated alcohol reinforcement processes[35] are influenced by acamprosate through the dampening of a hyper-glutamatergic tonus.

Genetic variations of human *PER2* and alcoholism

Based on the findings of the animal studies discussed above, we carried out an exploratory analysis of a possible association of the amount of alcohol intake and a *PER2* genotype in human alcohol dependent subjects. We performed a search for single nucleotide polymorphisms (SNPs), which was based on sequencing of the

exons, exon-intron boundaries and regulatory domains of the *PER2* gene. We identified 11 gene variations (9 SNPs, 2 deletions), one of which is in the 5'regulatory region, one in the 5'UTR, one in the 3'UTR and 8 in introns. **Figure 6c** shows the genomic organization of the *PER2* gene, sequenced regions and position of the genetic variations, as well as characteristics of each SNP.

We selected 6 informative SNPs with a minor allele frequency >0.05 for genotyping in a sample of 215 patients with a detailed assessment of alcohol intake according to the SSAGA questionnaire[36]. After performing a split to compare high (≥300 g/d) *vs.* low alcohol intake (<300 g/d)[37], we found a significant association of high *vs.* low alcohol intake with SNP 3 (p=0.02, OR 0.46), with allele G being a protective allele (**Supplementary Table 1** online). In order to assess the individual contribution of each SNP to the phenotype observed we performed a stepwise regression analysis by forwarding, which identified SNP 3 as the only relevant co-variable. To assess phase information, we then performed a haplotype analysis. Since there was an exactly similar distribution of SNPs 2 and 5 as well as SNPs 9 and 11, we excluded SNPs 2 and 11 from further analysis. The remaining haplotype consisting of SNPs 3-4-5-9 showed a significant association between high vs. low amount of alcohol intake (global *P*-value by permutation test = 0.03) (**Supplementary Table 2** online). Analysis of individual haplotypes identified a haplotype G-C-C-C, which was significantly more frequent in patients with low alcohol intake (*P*=0.0075, OR 0.42). The results of our regression analysis suggest that SNP 3 is the main contributor to a possible biological effect of the genotypes analyzed.

In order to generate hypotheses for a possible functional role of SNP 3, we first performed a phylogenetic footprint analysis, which showed that SNP 3 is

embedded in a C<u>A</u>TTTT motive, preserved in humans, chimpanzees and rats. We also found that SNP 3 is located in an enhancer-like structure in intron 3, containing transcription factor binding sites known to be expressed in the human brain. In a sequence 4 bases upstream and 14 bases downstream of the SNP, we found transcription factor binding motives for NFκB, Sp1, c-myb, E47 and IL-6 RE-BP. SNP 3 alters the binding motives for Sp1, c-myb and NFκB, suggesting a possible regulatory function of this SNP in transcriptional activation of *PER2*. Thus, our findings support the results of the studies in *Per2* mutant mice and suggest a role of the *PER2* gene in the regulation of alcohol consumption in humans.

DISCUSSION

In this study we report about the relationship between a non-functional *Per2* gene and the glutamate transporter Eaat1, which leads to elevated glutamate levels in the brain. These elevated glutamate levels cannot be compensated through adaptive up-regulation of Eaat2 and hence a hyper-glutamatergic brain in *Per2^{Brdm1}* mutant mice is the consequence. At the behavioral level, this leads to increased alcohol consumption in the mutant mice. Furthermore, we provide evidence for an analogous function of *PER2* in regulating alcohol intake in humans. Thus, the amount of alcohol consumption in alcoholic patients is associated with haplotypes of the *PER2* gene. In conclusion, we suggest that altered function of the *PER2* gene leads to changes in alcohol reinforcement processes. This is in line with other reports showing that *per* mutant flies as well as *Per1* and *Per2* mutant mice exhibit changes in cocaine and morphine sensitization and reward[38-40], suggesting that clock genes seem to be involved in common modulator mechanisms of drug abuse-related behaviors[41].

Interestingly, also methamphetamine injection causes an increase of *Per* gene expression in the caudate putamen of the mouse[42,43].

Enhanced alcohol consumption in *Per2^Brdm1* mutant mice can be rescued pharmacologically by the anti-craving and anti-relapse compound acamprosate. Thus, following acamprosate treatment, *Per2^Brdm1* mutant mice had reduced levels of alcohol consumption, which dropped even below those of wild type animals. This compound also reduced glutamate levels in the nucleus accumbens – an effect, which was not observed in wild type mice, suggesting that acamprosate acts mainly on alcohol drinking by dampening the hyper-glutamatergic tonus in *Per2^Brdm1* mutant mice. Evidence for a genetic basis of a hyper-glutamatergic system in humans is provided as seen by the differential response to acamprosate in alcoholic patients. It should be noted that compared to placebo, acamprosate treatment increases abstinence rates only by 10-20%[28,29]. However, on a diagnostic level, responders and non-responders cannot be differentiated. We hypothesize that genetic variations in the *PER2* gene may aid in the identification of alcohol dependent patients responsive to acamprosate treatment. In addition to this suggested treatment improvement, our study points to pathological consequences, when proper function of a circadian gene is disrupted. This is supported by reports that indicate enhanced alcohol consumption in shift workers and people suffering from severe jet lag, as found for example in air craft staff[44,45]. Hence, alterations in *PER2* and associated changes in the glutamatergic system may underlie those pathological consequences, suggesting one possible pathway in the aetiology of alcohol dependence.

METHODS

Animals. The mice used in this study have been characterized previously[46]. The wild

type and *Per2^{Brdm1}* mutant animals used in this study were littermates derived from

intercrosses between heterozygous *Per2^{Brdm1}* mice on a 129SvEv^{Brd}/C57BL/6-Tyr^{c-Brd}

background. Animal experiments were approved by the veterinary offices of Fribourg

and Mannheim.

Tissue preparation and cDNA synthesis. Mice held for at least two weeks in a 12h

light/12h dark cycle, were sacrificed at ZT 15. Tissue collection and cell-lysis were

carried out under dim red-light (15W). cDNA synthesis was performed using the

SMART PCR cDNA Synthesis Kit (Clontech K 1052-1) according to the

manufactures instructions.

Subtraction and amplification of differentially expressed genes. The PCR Select

cDNA Subtraction Kit (Clontech K1804-1) was used for substraction and

amplification of differentially expressed genes. PCR-Products were cloned into the

pCR II-TOPO vector with the TOPO TA Cloning Kit (Invitrogen K4600-40).

Northern blot analysis. Northern analysis was performed using denaturing

formaldehyde gels with subsequent transfer to Hybond-N membrane (Amersham).

cDNA probes had a specific activity of 10^8 cpm/µg. Signals were quantified using

Quantity One 3.0 software (Bio-Rad).

Western blot analysis. Brain proteins were resolved by SDS-PAGE and transferred

to PVDF membranes (Amersham). Antibodies used for detection were: rabbit Eaat1-

specific antibody diluted 1:1000 (Abcam, Cambridge, ab 416), Eaat2 (Alpha Diagnostics, # GLT-1 1-A), mGluR1, mGluR5 (Upstate, #06-310 and #06-451) and mGluR 2/3 (Novus Biologicals, NB 300-126). Secondary antibodies were: goat anti-rabbit IgG-HRP diluted 1:5000 for Eaat1 (Sigma, A5420) and mGluR5 and goat anti-rabbit IgG-AP 1:5000 (Pierce, # 31340).

Ninhydrin assay. 2 ml of ninhydrin solution (0.2% in ethanol) was added to 2 ml synaptosomal supernatants and the mixture was boiled for 15 min in a water bath. The tubes were cooled to room temperature and absorbance was measured at 570 nm.

Synaptosomes and glutamate uptake. Brain tissue of wild type and *Per2^{Brdm1}* mutant mice was homogenized. Aliquots of synaptosomes were added to incubation buffer containing L-[^{3}H] glutamic acid (Specific activity 51 Ci/mmol: Perkin Elmer Life Sciences Inc.). The reaction was terminated (incubation buffer in which an equimolar concentration of choline chloride was substituted for NaCl). The ^{3}H-bound radioactivity was determined by liquid scintillation counting (PACKARD liquid scintillation analyzer TRI-CARB 2200CA).

Glial cells and glutamate uptake. Glial cells were obtained from postnatal day (P) 1 brains of wild type and *Per2^{Brdm1}* mutant pups. The tissue was dissociated by trituration and the cells were plated and passaged. After the second passage, cells were counted and seeded into 24-welled plates. Glutamate uptake was performed as described in **Supplementary Methods** online.

Microdialysis and determination of glutamate levels. The CMA/7 guide cannula with a dummy was implanted into the ventral striatum. After the implantation, the mice were placed in the CMA/120 system for freely moving and connected via a collar to a swivel. The CMA/7 microdialysis probe was inserted into the guide cannula and perfused at a constant flow rate of 2 μl/min. One day after the recovery, microdialysis experiments were carried out in freely moving mice between ZT3 and ZT6. As previously described in detail[47], glutamate levels were determined from 15-μL of each dialysate sample.

Determination of blood alcohol levels. Alcohol-naïve and alcohol-experienced wild type and $Per2^{Brdm1}$ mutant mice were injected i.p. with 3.5 g/kg ethanol. Blood alcohol content was determined using the NADH enzyme spectrophotometric method (Greiner AG).

Alcohol self-administration and pharmacological treatment. After one week of habituation to the animal room, male $Per2^{Brdm1}$ mutant and control mice were given continuous free access to two bottles of tap water for 3 days, followed by tap water and 2% (v/v) ethanol solution for another 3 days (Days 1-3). Ethanol consumption was calculated in terms of g of ethanol consumed/kg of body weight/day (g/kg/day). In a second batch of mice, we tested the effects of acamprosate treatment. Two daily injections of acamprosate (200 mg/kg, every 12:00 h) were given to all mice and drinking data were compared to those of the four last days of the baseline.

Operant ethanol self-administration and PR measurements. Experiments were performed during the active phase in operant chambers (TSE Systems) equipped with

two levers, only one being active, and individual sessions lasting 30 minutes. Animals were trained to orally self-administer ethanol (10%) after having undergone a standard sucrose fading procedure under a fixed ratio 1 (FR1) paradigm. Mice were also tested on a progressive-ratio (PR) schedule for ethanol (10%) reinforcement, in which the response requirements increased by a step size of 2. The final ratio completed was defined as the breaking point.

Taste preference tests. Alcohol-naïve and alcohol-experienced mice were used for these tests. Sucrose (0.5, 2.5, and 5% w/v) and quinine (5,10, and 20 mg/dl) solution intake was measured in a two-bottle free choice test (sucrose or quinine against water). A test lasted for 9 days and bottles were weighed every 3 days along with the position of the bottles being changed.

Subjects and psychiatric assessment. 215 patients of German origin (172 males, 43 females; mean age 41.3 years, SD 8.4) were recruited by the Department of Psychiatry of the University of Munich. All patients were consecutively admitted for an in-patient alcohol withdrawal therapy and fulfilled the DSM-IV criteria for alcohol dependence. Symptoms related to alcohol taking behaviour were assessed in using the semi-structured assessment for the Genetics of Alcoholism (SSAGA)[36]. To distinguish patients with low *vs*. high alcohol intake, a cut-off was set at 300 g/d, ensuring comparability of the results with previous work on genes regulating glutamatergic neurotransmission[37]. Written informed consent was obtained from all individuals, when they were in a state of full legal capacity. The study was approved by the ethics committee of the University of Munich.

Mutation screening and promoter analysis. Identification of SNPs was performed by sequencing 32 Caucasian DNA samples. For details see **Supplementary Methods** online.

Genotype analysis. DNA was prepared from whole blood with standard salting out methods. Six Single-nucleotide polymorphisms in the *PER2* gene were genotyped using the TaqMan MGB biallelic discrimination system.

Association analysis and haplotype analysis. The *P*-values of genotypes reported refer to the two-sided trend test for 2x3 contingency tables as originally proposed by Armitage[48]. Haplotype association analyses were performed with COCAPHASE 2.35 (see web adresses).

Web addresses: COCAPHASE 2.35 www.hgmp.mrc.ac.uk

Note: Supplementary information is available on the Nature Medicine website.

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AUTHORS CONTRIBUTIONS

Studies on the glutamate system were performed by G.P., C.A., and M.C.M., differential display by

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humans by M.D., J.L., S.S. and G.S, the samples were provided by M.S., the mutation analysis was

done by F.M. and M.L., and the experimental design and data management by R.S. and U.A.

COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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Figure Legends

Figure 1 Expression of the glutamate transporter Eaat1 in brain tissue. (**a**) Northern blot analysis showing reduced levels of *Eaat1* mRNA at zeitgeber time (ZT) 15 in brain tissue of *Per2^{Brdm1}* mutant mice. Loading control: *Gapdh* mRNA. (**b**) Expression of *Eaat1* mRNA revealed by in situ hybridization is reduced at ZT15 in the SCN of *Per2^{Brdm1}* mutant mice. (**c**) Temporal profile of *Eaat1* mRNA in wild type SCN revealed by in situ hybridization. (**d**) Western blot analysis: temporal profile of Eaat1 protein relative to actin in brain of wild type (*wt*) and *Per2^{Brdm1}* mutant mice. Representative blots are shown at the bottom. Each value is the mean $\pm$ S.E.M. of 3 animals. Data of ZT0 are plotted twice (ZT0 and ZT24). * indicate significant difference from wild type mice (*P*<0.05). Scale bar = 500 µm.

Figure 2 Protein levels of mGluR1,2,3,5 and Eaat2. Western blot analysis of wild type (*wt,* solid line) and *Per2^{Brdm1}* mutant (hatched line) mouse brains over 24 hours. (**a**) mGluR1, no significant difference can be seen, (**b**) mGluR2/3, no difference is detected between the two genotypes, (**c**) mGluR5 is similar in *Per2^{Brdm1}* mutant and wild type mice. (**d**) Eaat2 expression is shifted in *Per2^{Brdm1}* mutant mice. Values are shown as mean $\pm$ S.E.M. of three animals per time point. Data of ZT0 are plotted twice (ZT0 and ZT24). Photographs below the plots depict representative Western blots with the corresponding actin controls (bottom, labeled A). * indicate significant difference from wild type mice (*P*<0.05)

Figure 3 Glutamate levels in brain tissue and glutamate uptake by astrocytes. (**a**) Glutamate transport in synaptosomes of wild type and *Per2**$Per2^{Brdm1}$* mutant mice sacrificed at ZT6. Values are mean ± S.E.M of 3-4 animals per datapoint. (**b**) Ninhydrin assay to determine amount of amino acids in extracellular space in adult brains collected at ZT6. Values are presented as mean ± S.E.M. (*P=0.001, *n*=6). (**c**) Mean basal levels of glutamate determined from extracellular dialysates of the ventral striatum (*n*=6-7 mice per group). The basal levels of glutamate are expressed in µM/15µl dialysate and the mean of 6 fractions collected was calculated. *P=0.005 compared to wild type mice. (**d**) Glutamate uptake of cultured astrocytes harvested at ZT6 of postnatal day 1 pups. Values are mean ± S.E.M of 3 animals per datapoint. Two-way ANOVA reveals a significant genotype effect, *P=0.0007.

Figure 4 Intake and preference of different alcohol solutions at increasing concentrations of *$Per2^{Brdm1}$* mutant mice and wild type (wt) littermate mice. (**a**) Drinking data are depicted as the mean ± S.E.M. ethanol intake in g/kg/day as averaged over a 3 days period for each concentration. Two-way ANOVA for alcohol intake revealed a significant interaction between the genotypes and the different concentrations of alcohol (2-16% w/v; *P=0.001). The analysis also showed a significant genotype effect (*P=0.001) (**b**) Ethanol preference over water. *$Per2^{Brdm1}$* mutant mice exhibited a significant enhanced preference for alcohol (*P=0.001) compared to wild type littermates if ethanol concentration was more than 8%. (**c**, **d**) Taste sensation was assessed using quinine (**c**) and sucrose (**d**) preference. The animals were allowed to choose in the home cage from two bottles: one of them containing tap water and the

other sucrose (0.5-5%; (w/v) and quinine (5-10 mg/dl), respectively. The free choice procedure lasted 9 days and the solutions were changed every third day. (**e**, **f**) Levels of ethanol in blood samples were determined at several times (30,60,120 and 240 min) after the i.p. injection of ethanol (20%v/v) at a dose of 3.5 g/kg body weight. No differences between genotypes at any time after the injection could be detected in alcohol-naïve (**e**) and alcohol-experienced mice (**f**). All data are mean values ± S.E.M., group sizes were *n*=8-18 depending on the experiment. * indicates significant differences between genotype (*P*<0.001).

Figure 5 Operant ethanol self-administration and progressive ratio measurements in *Per2^Brdm1^* mutant mice and wild type littermates. (**a**) Mice were trained to respond to an FR1 for alcohol (fixed ratio schedule of 1, i.e. one lever press results in the delivery of one drop of alcohol), using a sucrose fading procedure, until reaching a stable response rate for a 16% ethanol solution. A two-way ANOVA revealed an effect of genotype [$F_{(1,12)}= 6.62$; *P*<0.02] but the factor day and the group x day interaction failed to reach significance, revealing that *Per2^Brdm1^* mutant mice displayed a significantly higher number of responses at the active lever than WT mice across all sessions. (**b**) Two days later, an additional self-administration session under a progressive ratio schedule (step size=2) was conducted. A Student's t test for independent samples showed that both groups of animals differed in the number of responses at the active lever, which resulted in a significant difference [$t_{(12)}= 2.83$; *P*< 0.02] in the break point for each group.

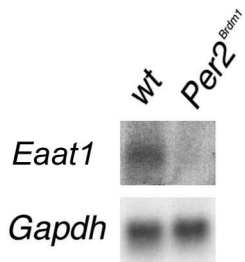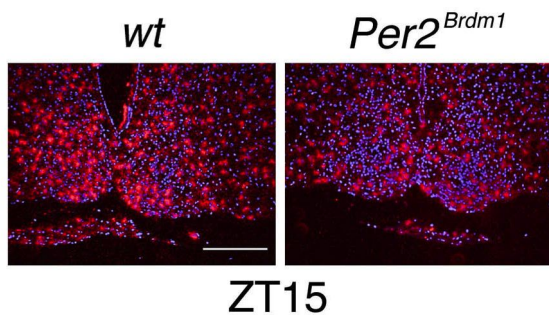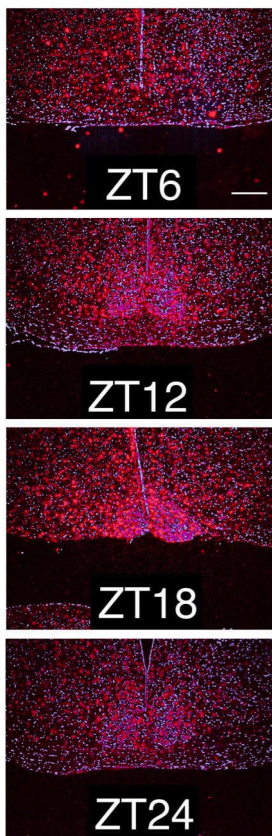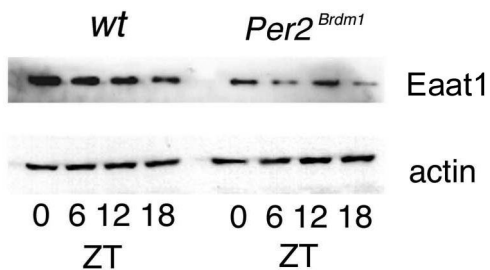**Figure 6** Effects of acamprosate on extracellular glutamate levels and on alcohol consumption in *Per2^Brdm1^* mutant mice and wild type littermates.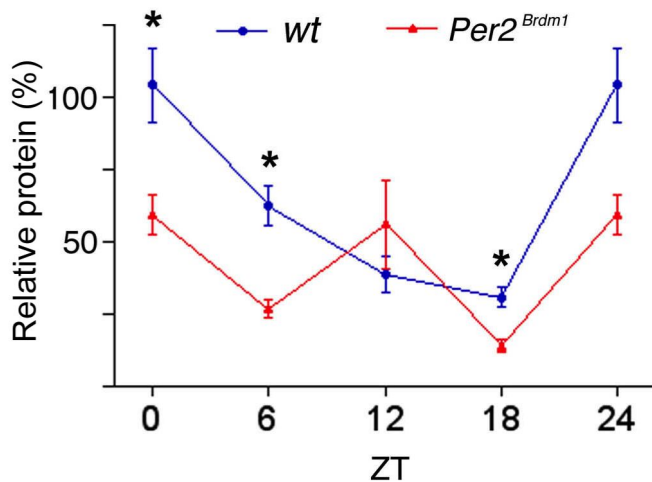 Schematic representation of the 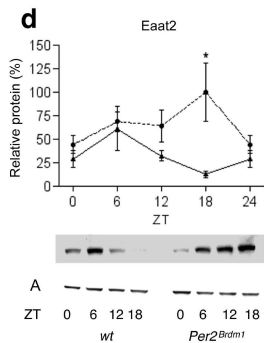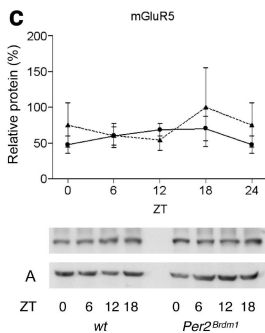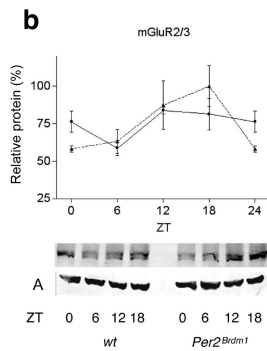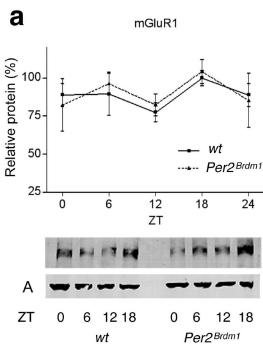human *PER2* (GI:1365471) gene. (**a**) Glutamate was determined in extracellular dialysates of the ventral striatum (*n*=6-7 mice per group). The microdialysis data obtained after acamprosate injection (200 mg/kg i.p.) are expressed as the percentage of basal values from wild type mi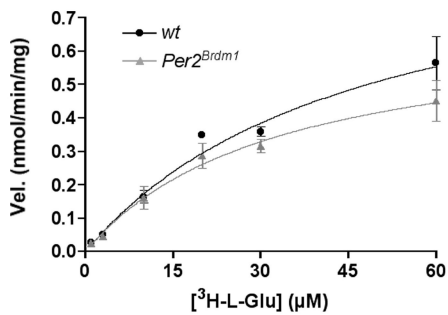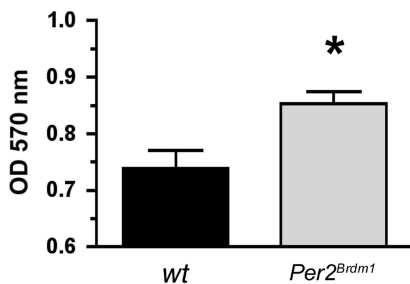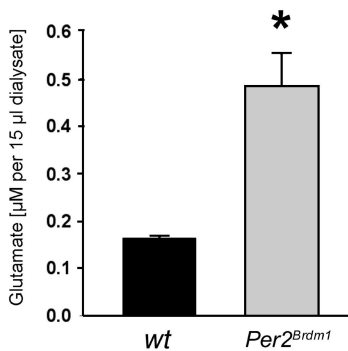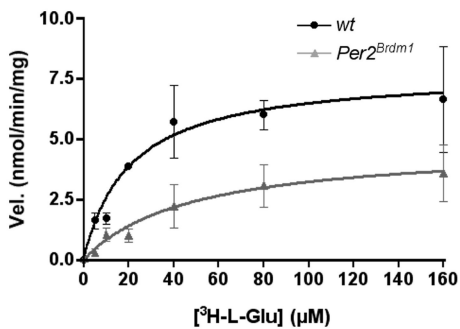ce and were analysed by ANOVA for repeated measures. (**b**) Pharmacological rescue by acamprosate of augmented drinking behaviour in *Per2^Brdm1^* mutant mice. Two daily injections of acamprosate (200 mg/kg i.p.) for 4 days (arrows) produced a complete rescue of drinking behaviour in *Per2^Brdm1^* mutant mice. To better illustrate this effect, alcohol intake data of both groups of mice were transformed into a percentage measure of group differences. The ANOVA results revealed an effect of the factor group [F=13.70, $P<0.01$] and the factor day [F= 3.59; $P<0.01$] as well as a significant interaction between these factors [F= 3.60; $P<0.01$], showing that during the acamprosate treatment period, the differences between groups disappeared. (**c**) Genomic organization of the human *PER2* gene with 23 exons (green rectangles with Roman numerals) and 22 introns is shown (horizontal lines, not drawn to scale). Single Nucleotide Polymorphisms (SNPs, continous arrows) or Insertion/Deletion (dotted arrow) are identified with Arabic numerals and described in orange (SNPs selected for genotyping) or white (SNPs not selected for genotyping) boxes. The second line in each box indicates the base pair exchange, the third line indicates the position of the SNP relative to the ATG site and the fourth line indicates the frequency of the

minor allele. Despite its informative allele frequency, SNP 8 was not analysed

due to genotyping problems.

a

wt   Per2^{Brdm1}

Eaat1

Gapdh

b

wt   *Per2^{Brdm1}*

ZT15

c

ZT6

ZT12

ZT18

ZT24

d

Relative protein (%)

• *wt*   ▲ *Per2^{Brdm1}*

100

50

0   6   12   18   24

ZT

wt   *Per2^{Brdm1}*

Eaat1

actin

0 6 12 18   0 6 12 18

ZT   ZT

a mGluR1

b mGluR2/3

c mGluR5

d Eaat2

a

Vel. (nmol/min/mg) vs [^{3}H-L-Glu] (µM)

- *wt*
- *Per2^{Brdm1}*

b

OD 570 nm

wt   *Per2^{Brdm1}*

c

Glutamate [µM per 15 µl dialysate]

wt   *Per2^{Brdm1}*

d

Vel. (nmol/min/mg) vs [^{3}H-L-Glu] (µM)

- *wt*
- *Per2^{Brdm1}*

a

b

a

Glutamate (% of mean basal level of wt)

wt
Per2^Brdm1

Time (min): basal, 20, 40, 60, 80, 100, 120, 140, 160

b

Group difference (%) in ethanol intake

wt
Per2^Brdm1

Time (days): BL1 BL2 BL3 BL4 T1 T2 T3 T4 PT1 PT2 PT3 PT4

c

PER2

ATG

I II III IV V VI VII IX X XIII XXIII

5'   3'

STOP*

1	2	3	4	5	6	7	8	9	10	11
C/-	C/G	A/G	T/C	C/T	-/GT	C/T	G/A	C/G	G/A	C/T
-10,537	-12	+1,071	+4,673	+4,717	+4,725	+6,663	+6,790	+12,672	+16,810	+32,128
0.02	0.08	0.18	0.08	0.08	0.03	0.03	0.08	0.05	0.02	0.05

SUPPLEMENTARY METHODS

Animals

The mice used in this study have been characterized previously[47]. *Per2^{Brdm1}* mutant

mice have 87 amino acids deleted in the PAS domain of the Per2 protein which leads

to a dramatic reduction of the *Per2* mRNA and protein in cells. The wild type and

Per2^{Brdm1} mutant animals used in this study were littermates derived from intercrosses

between heterozygous *Per2^{Brdm1}* mice on a 129SvEv^{Brd}/C57BL/6-Tyr^{c-Brd} background.

Animal experiments were approved by the veterinary offices of Fribourg and

Mannheim.

A. Molecular methods

Tissue preparation and cDNA synthesis

Mice held for at least two weaks in a 12h light/12h dark cycle were sacrificed at ZT

15. The SCN of 6 wild type and 6 *Per2^{Brdm1}* mutant mice were collected in 1ml of

RNAzol (Wak Chemie, WAK-CS-105) and RNA isolation was performed according

to the manufacturers instructions. Tissue collection and cell-lysis were carried out

under dim red-light (15W). cDNA synthesis was performed using the SMART PCR

cDNA Synthesis Kit (Clontech K 1052-1) according to the manufactures instructions.

In brief, 1 μg of total RNA were used for First-Strand cDNA Synthesis with 200U of

Superscript II reverse transcriptase (Gibco BRL M-3516) in a final volume of 10 μl.

cDNA was stored at –20°C and 2 μl were used for amplification by long distance

PCR (LD-PCR) using the primers supplied in the kit. The reaction was performed in a

total volume of 100 μl for 20 cycles with the following parameters : 95°C (initial

denaturation) for 2 min, denaturation at 95°C for 5 s, annealing at 65°C for 5 s and

extension at 68°C for 6 min. For each sample (wild type, mutant and control) 600 μl

of cDNA were prepared and directly purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and concentrated by n-butanol treatment to a volume of 60μl. The cDNA was further purified on a CHROMA SPIN-1000 column and finally eluted in two steps with 320 μl and 75 μl of TNE (10mM Tris-HCl, pH 8, 10mM NaCl, 0,1mM EDTA). Eluates were combined, directly digested with 10U of Rsa I for 3h, purified on an QIAquick column (Qiagen 28106) and eluted in 50 μl of TE. The digested cDNA was precipitated with ammonium acetate, washed with 80% ethanol and air dried. The pellet was dissolved in 6,7 μl of TNE and the concentration was adjusted to 300ng/μl.

Subtraction and amplification of differentially expressed genes

The PCR Select cDNA Subtraction Kit (Clontech K1804-1) was used for substraction and amplification of differentially expressed genes. In brief, 1 μl of digested cDNA from SCN tissue from wild type mice (tester population) was diluted in 5 μl of ddH$_2$0. 2μl of this dilution were ligated to either adaptor 1 or adaptor 2R provided with the Kit in a final volume of 10μl overnight at 16°C. Subtractive hybridization was performed in two steps as outlined in the manual. Briefly 1,5μl of digested cDNA from *Per2^{Brdm1}* mutant mice (driver population) where mixed with 1,5μl of tester cDNA (wild type), ligated to either adaptor 1 or adaptor 2R, in hybridization buffer and hybridized at 68°C for 8h. The second hybridization was carried out by addition of 1μl of fresh driver cDNA and subsequent incubation at 68°C overnight. The samples were diluted in 200μl of dilution buffer and stored at –20°C. Amplification of differentially expressed sequences was carried out in two steps as described in the manual. For primary PCR (27 cycles) 1μl of the diluted, subtracted sample was used. The PCR product of the primary PCR was diluted 1:10 and 1μl was used for

secondary PCR with 10 cycles. PCR-Products were cloned into the pCR II-TOPO vector with the TOPO TA Cloning Kit (Invitrogen K4600-40). Positive clones were selected by digestion with EcoRI and sequenced on a Perkin Elmer ABI PRISM 377 DNA-Sequencer.

Northern blot analysis

Northern analysis was performed using denaturing formaldehyde gels with subsequent transfer to Hybond-N membrane (Amersham). For each lane 20µg of total RNA were loaded. Labeling of cDNA probe was done using the random prime labeling kit (Pharmacia) incorporating [^{32}P]dCTP to a specific activity of 10^8 cpm/µg. Blots were exposed to X-ray films (Hyperfilm MP, Amersham) for 72 h, and signals were quantified using Quantity One 3.0 software (Bio-Rad). Signal intensities were normalized to the 28S rRNA.

Western blot analysis

Brain proteins were resolved by SDS-PAGE (EAAT1, Eaat2, 10% ; mGluR1, mGluR2/3, mGluR5, 6%) and transferred to PVDF membranes (Amersham). Membranes were blocked with 5% non-fat milk in PBS pH 7.4 and were incubated with an Eaat1 specific antibody diluted 1:1000 (Abcam, Cambridge, ab 416) for 2 h at room temperature. Eaat2 (Alpha Diagnostics, TX, # GLT-1 1-A), mGluR1, mGluR5 (Upstate, NY, #06-310 and #06-451) and mGluR 2/3 (Novus Biologicals, Littleton, CO, USA, NB 300-126) were diluted 1:1000 and incubated at 4°C overnight. The membranes were washed with PBS, followed by PBST and incubated with goat anti-rabbit IgG-HRP diluted 1:5000 for Eaat1 (Sigma, A5420) and mGluR5 and goat anti-rabbit IgG-AP 1:5000 (Pierce, Rockford, IL, # 31340) for the remaining 2h at room

temperature. The membranes were washed repeatedly and the proteins were detected using the ECL western blotting detection reagents and analysis system (Amersham, RPN 2209) for Eaat1 and mGluR5. Alkaline Phosphatase reaction with BCIP/NBT (Interchim, Montlucon, France, UP099851) was used for the remaining.

B. Biochemical methods

Ninhydrin assay

Synaptosomal supernatants from the wild type and $Per2^{Brdm1}$ mutant mice were assayed using the ninhydrin reagent to assess the intercellular amino acids levels. Briefly, 2ml of ninhydrin solution (0.2% in ethanol) was added to 2ml synaptosomal supernatants and the mixture was boiled for 15 min in a water bath. The tubes were cooled to room temperature and absorbance was measured at 570 nm.

Synaptosomes and glutamate uptake

Brain tissue of wild type and $Per2^{Brdm1}$ mutant mice was homogenized at 4°C in 25 volumes (wt/vol) of 0.32 M sucrose with a glass homogenizer (Heidolph RZR 2102 control, Germany) fitted with a Teflon pestle. The homogenate was centrifuged at 200 g for 10 min, and the supernatant was centrifuged at 20,000 g for 20 min. The pellet was resuspended in 0.32 M sucrose and centrifuged at 20,000 g for 20 min. The crude synaptosomal pellet was finally resuspended in 3 ml of 0.32 M sucrose and used for the glutamate uptake assays. 25-µl aliquots of synaptosomes were added to 250 µl of incubation buffer (5 mM Tris, 10 mM HEPES, 2.5 mM KCl, 1.4 M NaCl, 1.2 mM CaCl$_2$, 1.2 mM MgCl$_2$, 1.2 mM K$_2$HPO$_4$, and 10 mM dextrose, pH 7.4) containing L-[^{3}H] glutamic acid (Specific activity 51 Ci/mmol: Perkin Elmer Life Sciences Inc.) and incubated for 3 min at 37°C in a water bath. The reaction was terminated using 1 ml of ice-cold choline buffer (incubation buffer in which an equimolar concentration

of choline chloride was substituted for NaCl), and the samples were centrifuged at 10,000 g for 2 min to recover synaptosomes. The ^{3}H-bound radioactivity was determined by liquid scintillation counting (PACKARD liquid scintillation analyzer TRI-CARB 2200CA). Sodium dependent uptake was determined by subtracting the signal obtained in the choline chloride buffer from that obtained in the presence of sodium.

Glial cells and glutamate uptake

Glial cells were obtained from postnatal day (P) 1 brains of wild type and $Per2^{Brdm1}$ mutant pups. Cerebral hemispheres were dissected under sterile conditions and were collected in 100 mm culture dishes containing ice-cold TBS. Dissected tissue pieces were incubated for 20 min in Trypsin/EDTA at 37°C and 5% CO_2. Trypsin action was terminated by transferring the tissue pieces to DMEM (AMIMED) supplemented with 10% fetal calf serum. The tissue was dissociated gently by trituration through a sterile plastic pipette and the cell suspension was centrifuged at 400 x g for 5 min and the pellet was resuspended in DMEM supplemented with 10% fetal calf serum. Cells were plated onto 100 mm culture dishes (NUNC ™ Brand Products). On reaching confluency, the cultured cells were washed twice in TBS, trypsinized and replated. After the second passage, the cells were counted and seeded into 24-welled plates for the glutamate uptake assay.

Glutamate uptake was performed in either sodium or lithium-supplemented Tris buffer containing 5 mM Tris base, 10mM HEPES, 140 mM NaCl or LiCl, 2.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2mM K_2HPO_4, 10 mM dextrose and the glutamine synthetase inhibitor methionine sulfoximine (1mM; Sigma). Tritium-labeled glutamate (specific activity 51 Ci/mmol; Perkin Elmer Life Sciences Inc.) and

unlabeled glutamate were mixed to obtain a total glutamate concentration of 960 μM. The stock solution was further diluted with the respective uptake buffer to yield working concentrations of 160, 80, 40, 20, 10 and 5 μM glutamate. Each well received 250μl of the respective [3](H) glutamate solution and the uptake was terminated after 10 min by removing the radioactive solution and rinsing the cultures three times with ice-cold lithium-containing Tris buffer. Cells were then lysed in 0.1 M NaOH and the amount of incorporated glutamate was determined by liquid scintillation counting (PACKARD liquid scintillation analyzer TRI-CARB 2200CA) of the cell lysate. Sodium-dependent glutamate uptake was defined to be the difference of the amount of radioactivity incorporated by the glia in the presence of sodium- and lithium-containing buffer and normalized to the protein in the sister cultures.

Microdialysis and determination of glutamate levels

Mice were anaesthetised with sodium pentobarbital (70mg/kg i.p.) and mounted on a David Kopf stereotaxic frame. The body temperature was maintained during the operation via a heating pad. The skull was exposed and one hole was made for implanting the microdialysis guide cannula and another for the fixing microscrew, both of which were cemented to the skull. The CMA/7 guide cannula with a dummy was implanted into the ventral striatum (anterior-posterior, 1.1; lateral, ± 1.5; ventral, - 3.5 mm).

After the implantation, the mice were placed in the CMA/120 system for freely moving animals and connected via a collar to a swivel. The CMA/7 microdialysis probe (2-mm membrane length) was inserted into the guide cannula and perfused at a constant flow rate of 2 μl/min with a Ringer solution containing 147mM

NaCl, 4mM KCl, 2.3mM CaCl$_2$. One day after the recovery, microdialysis experiments were carried out in freely moving mice between ZT3 and ZT6. Dialysates were collected every 20 min. The first six samples were collected to provide an estimation of basal extracellular levels of glutamate. The animals were then treated with either saline or acamprosate (200mg/kg i.p.) and 8 additional samples were collected every 20 min.

As previously described in detail[48], glutamate levels were determined from 15-μL of each dialysate sample. Briefly, a gradient microbore liquid chromatography assay with fluorescence detection was used for glutamate after pre-column derivatization with o-phtalaldehyde/-mercaptoethanol.

Determination of blood alcohol levels

Alcohol-naïve and alcohol-experienced wild type and *Per2^{Brdm1}* mutant mice were injected i.p. with 3.5 g/kg ethanol. Blood alcohol levels were measured by drawing blood samples (25-30 µl) from the tip of the tail at various time points after injection (30, 60, 120, and 240 min). Blood alcohol levels were also determined in some animals following the alcohol self-administration procedure. Blood alcohol content was determined using the NADH enzyme spectrophotometric method (Greiner, Stuttgart, Germany).

C. Behavioural methods

Alcohol self-administration and pharmacological treatment

After one week of habituation to the animal room, male *Per2^{Brdm1}* mutant and control mice were given continuous free access to two bottles of tap water for 3 days,

followed by tap water and 2% (v/v) ethanol solution for another 3 days (Days 1-3). At all subsequent periods the mice had access to tap water as well as an ethanol solution with increasing concentrations: days 4-6, the mice received 4% ethanol solution; days 7-16 access to 8% ethanol solution; days 17-25 12% solution; and 16% ethanol solution from day 26 onward. Spillage and evaporation were minimized by the use of self-made glass cannulae in combination with a small plastic bottle (Techniplast, Milano, Italy). Under these conditions ethanol concentration in a given solution stayed constant for at least 1 week, when measured with an alcoholometer (GECO, Gering, Germany). Bottles were weighted daily at 10:00 a.m. and all drinking solutions were renewed every 3 days and the positions of the 2 bottles changed to avoid side preferences. Water and ethanol consumption as well as total fluid intake were calculated in terms of ml of solution consumed/day (ml/day). Ethanol consumption was calculated in terms of g of ethanol consumed/kg of body weight/day (g/kg/day). Ethanol-drinking solutions consisted of 96% ethanol, diluted with tap water to achieve the different concentrations.

In a second batch of mice, we tested the effects of acamprosate treatment. Thus, 10 wild type and 10 $Per2^{Brdm1}$ mutant mice were submitted to the alcohol drinking protocol as described in the previous paragraph. Once alcohol intake at the 16% solution was stable, two daily injections of acamprosate (200 mg/kg, every 12:00 h) were given to all mice and drinking data were compared to those of the four last days of the baseline. The drinking data were then transformed into a percentage measure of the group differences and statistically analyzed by a two-way ANOVA.

Operant ethanol self-administration and PR measurements

Experiments were performed during the active phase in operant chambers (TSE Systems, Bad Homburg, Germany) equipped with two levers, only one being active, and individual sessions lasting 30 minutes. Animals (8 wild type and 8 $Per2^{Brdm1}$ mutant littermates) were trained to orally self-administer ethanol (10%) after having undergone a standard sucrose fading procedure under a fixed ratio 1 (FR1) paradigm. In essence, a single press of the active lever resulted in a yellow light being turned on with a simultaneous delivery of 10 µl of solution exactly underneath it. The time line of operant self-administration consisted of an initial 10 days of sucrose (5%) solution, followed by 5 days of sucrose (5%) and ethanol (5%), 5 days of ethanol (5%), 5 days of sucrose (5%) and ethanol (8%), 5 days of ethanol (8%), 10 days of sucrose (5 %) and ethanol (10%). And, finally, ethanol (10%) only was introduced. Further, mice were then tested on a progressive-ratio (PR) schedule for ethanol (10%) reinforcement, in which the response requirements increased by a step size of 2. The final ratio completed was defined as the breaking point.

Taste preference tests

Alcohol-naïve and alcohol-experienced mice were used for these tests. Sucrose (0.5, 2.5, and 5% w/v) and quinine (5,10, and 20 mg/dl) solution intake was measured in a two-bottle free choice test (sucrose or quinine against water). A test lasted for 9 days and bottles were weighed every 3 days along with the position of the bottles being changed.

D. Genetic methods

Subjects and psychiatric assessment

215 patients of German origin (172 males, 43 females; mean age 41.3 years, SD 8.4) were recruited by the Department of Psychiatry of the University of Munich. All patients were consecutively admitted for an in-patient alcohol withdrawal therapy and fulfilled the DSM-IV criteria for alcohol dependence. Symptoms related to alcohol taking behaviour were assessed in using the semi-structured assessment for the Genetics of Alcoholism (SSAGA)[37]. To distinguish patients with low *vs.* high alcohol intake, a cut-off was set at 300g/d, ensuring comparability of the results with previous work on genes regulating glutamatergic neurotransmission[38]. There was no significant age difference between both groups and gender specific analysis revealed no effect of gender on genotype (data not shown). Written informed consent was obtained from all individuals, when they were in a state of full legal capacity. The study was approved by the ethics committee of the University of Munich.

Mutation screening and promoter analysis

Identification of SNPs was performed by sequencing 32 Caucasian DNA samples. 16 DNA pools consisting of an equimolar mixture of two DNA samples were prepared and used as PCR templates. For each gene, primers were chosen in order to amplify the regulatory domains, the exon-containing DNA fragments, including exon-intron boundaries. The PCRs were performed in a 15ul reaction mixture containing 25ng DNA. The list of the primers for each gene is available on the web site of the CNG (www.cng.fr). Sequencing reactions were performed using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Alignment of experimental results

and identification of SNPs were done using the Genalys software developed by the CNG.

Genotype analysis

DNA was prepared from whole blood with standard salting out methods. Six Single-nucleotide polymorphisms in the *PER2* gene were genotyped using the TaqMan MGB biallelic discrimination system. Probes and primers were ordered from and automatically designed by Applied Biosystems using the Assay-by-Design product. PCR reactions were performed in Biometra T1 thermocyclers, and fluorescence results were determined with the use of an ABI Prism 7900HT sequence-detector end-point read. Process and genotyping data were exported into an internal LIM System. Complete genotypes were obtained from 200 patients, which were included in the haplotype analysis.

Association analysis and haplotype analysis

The p-values of genotypes reported refer to the two-sided trend test for 2x3 contingency tables as originally proposed by Armitage[49]. Haplotype association analyses were performed with COCAPHASE 2.35 (www.hgmp.mrc.ac.uk). This package performs likelihood ratio tests under a log-linear model of the probability that an allele or haplotype belongs to the case rather than control group, using a standard unconditional logistic regression. In COCAPHASE the EM algorithm is used to provide maximum-likelihood estimates of frequencies. Since the EM algorithm does not accurately estimate haplotype frequencies below 1%, haplotypes <1% in either group were excluded.

Stepwise regression analysis was performed using the stepwise regression procedure by forwarding from SAS version 8.2. SNP3 was the first variable to be included. Significance level for entry was 0.05.

For the haplotype analyses, the global null hypotheses that all odds ratios are equal were also tested by permutation, owing to the fact that estimated haplotype frequencies cannot be treated as observed data. The method randomly reassigns the 'case' and 'control' labels in the actual data. The permutation procedure gives a significance level corrected for all markers or haplotypes tested. 10,000 permutations were performed for the haplotype tests (for this test p-value is assigned as global p-value). When the global test was significant, individual haplotypes were tested for association by grouping all others together and applying the $\chi 2$-test with 1 df.

Supplementary Table 1: Distribution of genotypes and results of association tests for alcohol dependent patients with high (≥300g/d) and low alcohol intake (<300g/d).

Groups for every marker	Cases genotyped	Distribution of Genotypes			p-value Armitage- trend Test (z-Values)	Odds Ratio 2.Allel vs. 1. Allel [Conf. Interv.]
2 (SNP 10553)		**CC**	**CG**	**GG**		
Low alcohol intake	114	91 (79,82%)	21 (18,42%)	2 (1,75%)	0.335	0.696
High alcohol intake	95	81 (85,26%)	13 (13,68%)	1 (1,05%)	(1.032)	[0.356-1.346]
3 (SNP 10870)		**AA**	**AG**	**GG**		
Low alcohol intake	116	82 (70,69%)	30 (25,86%)	4 (3,45%)	0.020	0.464
High alcohol intake	96	80 (83,33%)	16 (16,67%)	0 (0,00%)	(2.445)	[0.250-0.861]
4 (SNP 10562)		**CC**	**CT**	**TT**		
Low alcohol intake	112	13 (11,61%)	38 ()33,93%	61 (54,46%)	0.196	1.391
High alcohol intake	94	4 (4,26%)	34 (36,17%)	56 (59,57%)	(-1.383)	[0.887-2.179]
5 (SNP 10563)		**CC**	**CT**	**TT**		
Low alcohol intake	116	92 (79,31%)	22 (18,97%)	2 (1,72%)	0.3364	0.679
High alcohol intake	95	81 (85,26%)	13 (13,68%)	1 (1,05%)	(1.113)	[0.349-1.323]
9 (SNP 10565)		**CC**	**CG**	**GG**		
Low alcohol intake	116	114 (98,28%)	2 (1,72%)	0 (0,00%)	0.1447	3.704
High alcohol intake	96	90 (93,75%)	6 (6,25%)	0 (0,00%)	(-1.721)	[0.740-18.519]
11 (SNP 10559)		**CC**	**CT**	**TT**		
Low alcohol intake	116	114 (98,28%)	2 (1,72%)	0 (0,00%)	0.1447	3.704
High alcohol intake	96	90 (93,75%)	6 (6,25%)	0 (0,00%)	(-1.721)	[0.740-18.519]

Supplementary Table 2: Results of haplotype analysis

Haplotype	low alcohol intake freq	high alcohol intake freq	Odds Ratio[2]	95%-Confidence Interval (OR)	Global p-value = 0.0363[1] p-value individual haplotypes (Chi-Square)
G-C-C-C	0.1525	0.0710	0.42	0.22 –0.83	0.0075 (χ^2=7.141)
A-C-C-C	0.0368	0.0613	1.71	0.67 – 4.33	0.2679 (χ^2=1.228)
A-C-T-C	0.0963	0.0766	0.78	0.38 – 1.58	0.4914 (χ^2=0.4734)
A-T-C-C	0.7004	0.7463	1.26	0.81 – 1.96	0.2657 (χ^2=1.239)
All frequent	0.9861	0.9552			
All rare[3]	0.0139	0.0448			

[1] Based on 10,000 permutation
[2] Odds ratio calculated for each haplotype vs. all others.
[3] contains all haplotypes <1% in either group (alcoholics with low or high alcohol intake)