

# The clock gene *Per2* influences the glutamatergic system and modulates alcohol consumption

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**Period (*Per*) genes are involved in regulation of the circadian clock and are thought to modulate several brain functions. We demonstrate that *Per2<sup>Brdm1</sup>* mutant mice, which have a deletion in the PAS domain of the *Per2* protein, show 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 extracellular space of *Per2<sup>Brdm1</sup>* mutant mouse brains. This is accompanied by increased alcohol intake in these animals. In humans, variations of the *PER2* gene are associated with regulation of alcohol consumption. Acamprosate, a drug used to prevent craving and relapse in alcoholic patients is thought to act by dampening a hyper-glutamatergic state. This drug reduced augmented glutamate levels and normalized increased alcohol consumption in *Per2<sup>Brdm1</sup>* mutant mice. Collectively, these data establish glutamate as a link between dysfunction of the circadian clock gene *Per2* and enhanced alcohol intake.**

Across a spectrum of living organisms, ranging from cyanobacteria to humans, biological functions follow a pattern of circadian rhythmicity. These endogenous rhythms display a periodicity close to 24 h 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<sup>1</sup>.

This circadian clock is thought to be advantageous in synchronizing physiological and biochemical pathways, allowing the organism to anticipate daily changes, thus ensuring better adaptation to the environment<sup>2</sup>. The oscillatory mechanism of the circadian clock has been unraveled by means of genetic analysis in *Drosophila* and mammals<sup>3–5</sup>. 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 generate an inhibitory feedback loop that drives 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 through neural and endocrine outputs<sup>6</sup>.

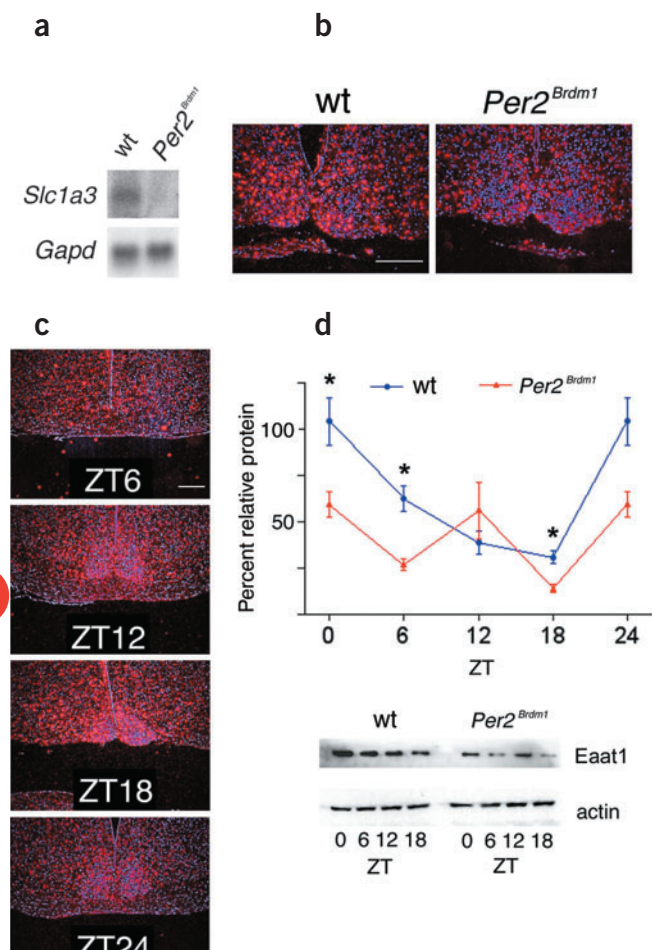
Several lines of evidence implicate glutamate in the activation of receptors on SCN neurons following retinal light perception<sup>7</sup>. This leads to the activation of several signal transduction pathways and the clock genes *Per1* and *Per2* (ref. 8). At the behavioral level, this results in an alteration of clock phase. Notably, *Per2<sup>Brdm1</sup>* mutant mice display impaired clock resetting<sup>9</sup>, suggesting altered glutamate signaling. Here, we show that *Per2<sup>Brdm1</sup>* mutant mice display alterations in their glutamatergic system. The glutamate transporter *Eaat1* (excitatory amino acid transporter 1, also known as *Glast* and encoded by the gene *Slc1a3*) is reduced in these mice. Excess glutamate is cleared from the synaptic cleft by glutamate transporters<sup>10</sup>, located on astroglial cells and transported back to the neuron through the glutamine-glutamate cycle. A deficit in the removal of glutamate from the synaptic cleft results in a hyper-glutamatergic state and may cause alterations at the behavioral level<sup>10,11</sup>. Notably, a hyper-glutamatergic state has been implicated in the etiology of alcohol dependence<sup>12–14</sup>. We observe that voluntary alcohol consumption is enhanced in *Per2<sup>Brdm1</sup>* mutant mice. In humans we find an association between alcoholic individuals and genetic variations in the human *PER2* gene. Acamprosate, a drug thought to dampen a hyper-glutamatergic state in the alcohol-dependent brain<sup>15–17</sup>, reduces augmented glutamate levels and normalizes enhanced alcohol consumption in *Per2<sup>Brdm1</sup>* mutant mice. These findings support the view that a hyper-glutamatergic state can be involved in several aspects of alcohol dependence<sup>12–14,18–20</sup>.

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## RESULTS

Glutamate transporters in *Per2<sup>Brdm1</sup>* mice

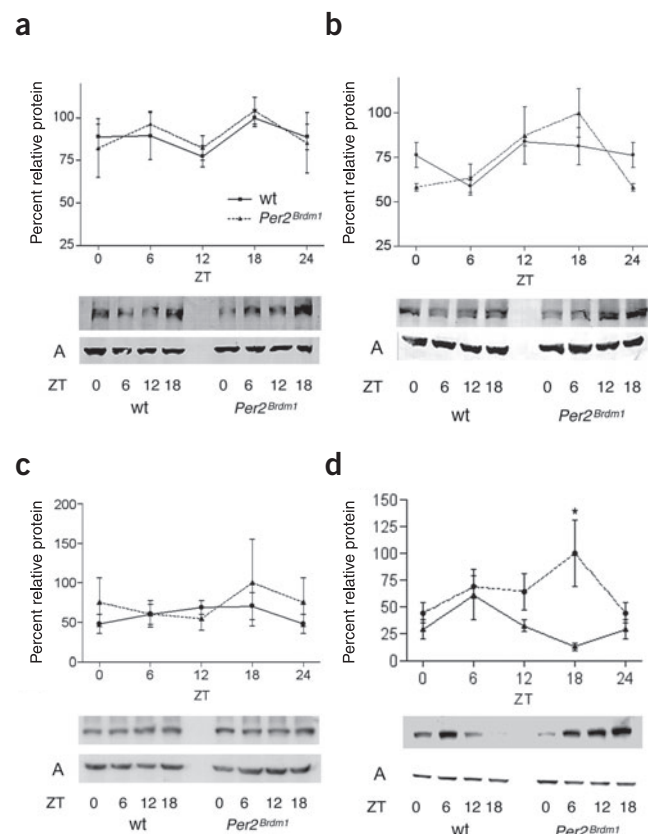
Wild-type and *Per2<sup>Brdm1</sup>* mutant mice differ in their behavioral response to a light pulse administered at zeitgeber time (ZT) 14 (ref. 9; ZT0 corresponds to lights on and ZT12 to lights off). Therefore, we searched for a difference in gene expression between wild-type and *Per2<sup>Brdm1</sup>* mutant mice at ZT15. This time point was chosen because a strong induction of *Per1* and *Per2* gene expression in the SCN can be observed 1 h after light has been given<sup>8</sup>. Differential display analysis of data from untreated wild-type and *Per2<sup>Brdm1</sup>* mutant mice should show 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<sup>Brdm1</sup>* mutant mice at ZT15 from a cDNA library of wild-type littermate mice. We found that *Eaat1*, a glutamate transporter that clears glutamate from the synaptic cleft and is solely expressed in astrocytes<sup>10</sup>, is downregulated at ZT15 in the brain and SCN of *Per2<sup>Brdm1</sup>* mutant mice (Fig. 1a,b). Notably, *Slc1a3* mRNA, which



**Figure 1** Expression of the glutamate transporter *Eaat1* in brain tissue. (a) Northern blot analysis showing reduced levels of *Slc1a3* mRNA at ZT15 in brain tissue of *Per2<sup>Brdm1</sup>* mutant mice. Loading control, *Gapd* mRNA, Wt, wild-type. (b) Expression of *Slc1a3* mRNA revealed by *in situ* hybridization is reduced at ZT15 in the SCN of *Per2<sup>Brdm1</sup>* mutant mice. Scale bar, 500  $\mu$ m. (c) Temporal profile of *Slc1a3* mRNA in wild-type SCN shown by *in situ* hybridization. Scale bar, 500  $\mu$ m. (d) Western blot analysis of temporal profile of *Eaat1* protein relative to actin in brain of wild-type (wt) and *Per2<sup>Brdm1</sup>* mutant mice. Representative blots are shown at the bottom. Each value is the mean  $\pm$  s.e.m. of three animals. Data of ZT0 are plotted twice (ZT0 and ZT24). \* $P < 0.05$  compared to wild-type mice.

encodes *Eaat1*, 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<sup>Brdm1</sup>* mutant mice (Fig. 1d). We found that in wild-type animals, maximal expression of *Eaat1* protein can be observed at ZT0 or ZT24, whereas minimal expression can be seen between ZT12 and ZT18 ( $P < 0.0001$ ). In contrast, *Eaat1* protein levels were significantly lower in *Per2<sup>Brdm1</sup>* mutant as compared to wild-type mice ( $P = 0.0014$ ). We conclude that *Eaat1* expression is reduced in *Per2<sup>Brdm1</sup>* mutant animals and therefore, these animals may show elevated glutamate levels in the brain.

It is not clear how the mutation in *Per2* leads to reduced expression of *Eaat1*. One kilobase of the *Slc1a3* 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 indirect, through an unknown mechanism. It has been described that *Slc1a3* expression is modulated by metabotropic glutamate receptors (mGluR)<sup>21</sup>. In *Per2<sup>Brdm1</sup>* mutant mice, protein levels of mGluR1, mGluR2/3 and mGluR5 did not differ from those in wild-type mice (Fig. 2a–c). This indicates that



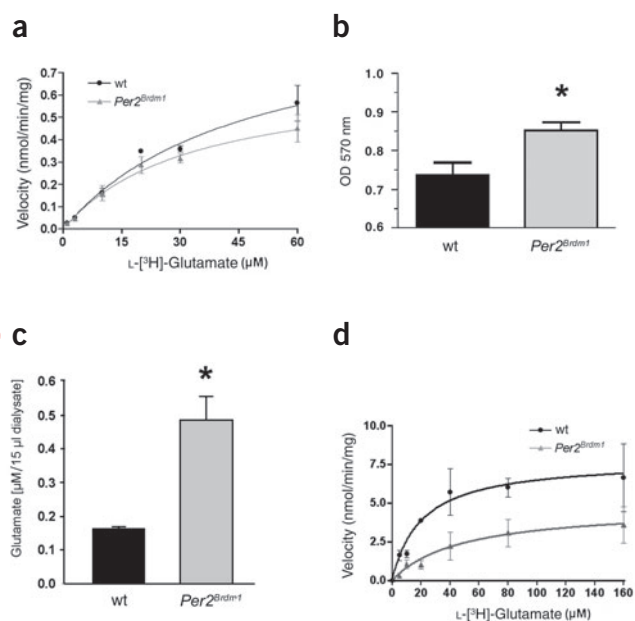
**Figure 2** Protein levels of mGluRs1, 2, 3, 5 and *Eaat2*. Western blot analysis of wild type (wt, solid line) and *Per2<sup>Brdm1</sup>* mutant (hatched line) mouse brains over 24 h. (a) mGluR1, no significant difference observed. (b) mGluR2/3, no difference detected between the two genotypes. (c) mGluR5 is similar in *Per2<sup>Brdm1</sup>* mutant and wild-type mice. (d) *Eaat2* expression is shifted in *Per2<sup>Brdm1</sup>* mutant mice. Values are shown as mean  $\pm$  s.e.m. of three animals per time point. Data at ZT0 are plotted twice (ZT0 and ZT24). Photographs below the plots depict representative western blots with the corresponding actin controls (bottom, labeled A). \* $P < 0.05$  compared to wild-type mice.

a change in Per2 function does not alter Eaata1 expression via regulation of mGluR expression<sup>21</sup>. But 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 Eaata1 is expected. We found a shift in expression of the glutamate transporter Eaata2, also termed Glt-1 or Slc1a2, in *Per2<sup>Brdm1</sup>* mutant mice (Fig. 2d). The maximal expression is shifted from ZT6 in wild-type animals to ZT18 in *Per2<sup>Brdm1</sup>* mutant mice. Two-way ANOVA showed a significant difference between genotypes ( $P = 0.0051$ ). But the slight elevation of Eaata2 expression in *Per2<sup>Brdm1</sup>* mutant mice was not significant when the two expression curves were aligned according to their maxima (ZT6 for wild-type and ZT18 for *Per2<sup>Brdm1</sup>* mutant mice). Thus, although Eaata1 is low in *Per2<sup>Brdm1</sup>* mutant mice, its reduction is not efficiently compensated by upregulation of Eaata2 to counteract a hyper-glutamatergic system in *Per2<sup>Brdm1</sup>* mutant mice. To further support this finding, we designed a set of experiments to test whether *Per2<sup>Brdm1</sup>* mutant animals show elevated glutamate levels in the brain.

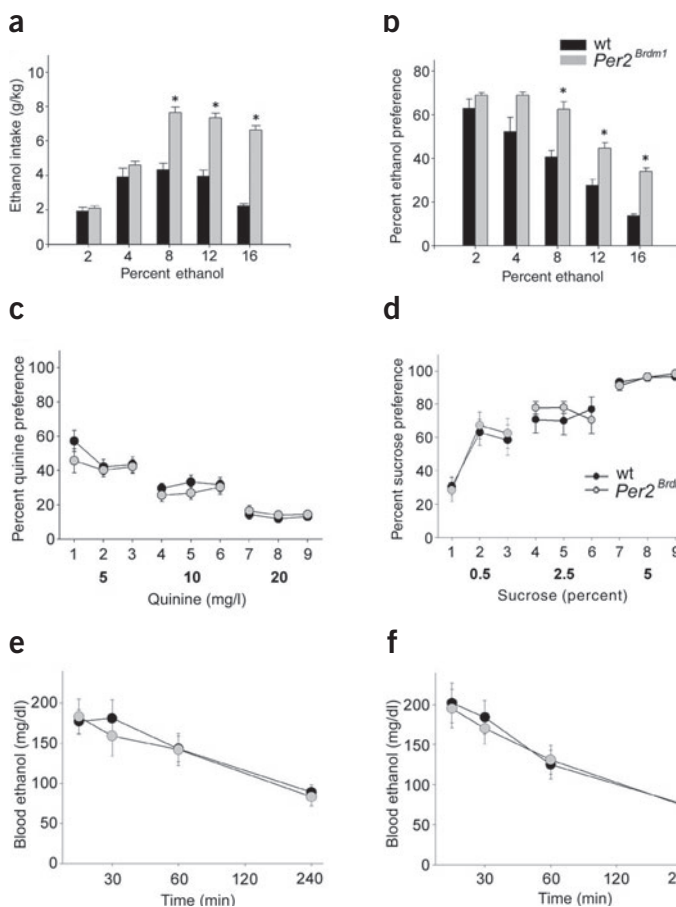
### *Per2<sup>Brdm1</sup>* mice have a hyper-glutamatergic brain

Eaata1 is one of the main glutamate transporters in the brain, clearing glutamate accumulated in the synaptic cleft. Because Eaata1 protein is reduced in *Per2<sup>Brdm1</sup>* mutant mice, we expected glutamate levels to be elevated and comparable to those of Eaata1 knockout animals<sup>11</sup>. Therefore, we performed a glutamate transport assay



**Figure 3** Glutamate levels in brain tissue and glutamate uptake by astrocytes. **(a)** Glutamate transport in synaptosomes of wild-type and *Per2<sup>Brdm1</sup>* mutant mice killed at ZT6. Values are mean  $\pm$  s.e.m. of 3–4 animals per data point. **(b)** Ninhydrin assay to determine amount of amino acids in extracellular space in adult brains collected at ZT6. Values are presented as mean  $\pm$  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  $\mu\text{M}/15 \mu\text{l}$  dialysate and the mean of six 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  $\pm$  s.e.m. of three animals per data point. Two-way ANOVA shows a significant genotype effect,  $P = 0.0007$ .

with synaptosomes from total brain collected at ZT6, which yielded a similar glutamate uptake in *Per2<sup>Brdm1</sup>* 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:  $K_m = 47 \pm 15 \mu\text{M}$ ,  $V_{max} = 1 \pm 0.2 \text{ nmol/min/mg}$ , and for *Per2<sup>Brdm1</sup>* mutant mice  $K_m = 32 \pm 11 \mu\text{M}$ ,  $V_{max} = 0.7 \pm 0.1 \text{ nmol/min/mg}$ . This indicates that glutamate uptake in neurons is not altered in *Per2<sup>Brdm1</sup>* mutant mice because synaptosomes represent the pinched-off presynaptic and postsynaptic endings of neurons. Further, we measured the intercellular amount of amino acids in the brain of wild-type and *Per2<sup>Brdm1</sup>* mutant mice by assessing the amino-acid concentration in the supernatant of centrifuged brain



**Figure 4** Intake and preference of different alcohol solutions at increasing concentrations of *Per2<sup>Brdm1</sup>* mutant mice and wild-type (wt) littermate mice. **(a)** Drinking data are depicted as the mean  $\pm$  s.e.m. ethanol intake in g/kg/d as averaged over a 3-d period for each concentration. Two-way ANOVA for alcohol intake showed a significant interaction between the genotypes and the different concentrations of alcohol (2–16% wt/vol;  $P = 0.001$ ). The analysis also showed a significant genotype effect ( $P = 0.001$ ). **(b)** Ethanol preference over water. *Per2<sup>Brdm1</sup>* mutant mice showed a significantly 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. Mice were allowed to choose in the home cage from bottles containing either tap water or sucrose (0.5–5% (wt/vol) and quinine (5–10 mg/dl), respectively). **(e,f)** Levels of ethanol in blood samples were determined at several time points (30, 60, 120 and 240 min) after the intraperitoneal injection of ethanol (20% vol/vol) at a dose of 3.5 g/kg body weight. No differences between genotypes at any time after the injection were detected in alcohol-naïve **(e)** and alcohol-experienced mice **(f)**. All data are mean values  $\pm$  s.e.m., group sizes were  $n = 8$ –18 depending on the experiment. \* $P < 0.001$  indicates significant differences between genotype.

homogenates. We found that *Per2<sup>Brdm1</sup>* mutant mice had significantly higher levels of intercellular amino acids compared to wild-type animals ( $P < 0.0001$ ; **Fig. 3b**). A microdialysis experiment in the ventral striatum of freely moving mice showed that *Per2<sup>Brdm1</sup>* mutant mice have glutamate levels that are almost threefold higher than wild-type littermates (**Fig. 3c**). Because *Eaat1* is mainly localized on astrocytes<sup>10</sup>, we compared glutamate uptake in these cells of both wild-type and *Per2<sup>Brdm1</sup>* mutant littermate pups, killed at ZT6. Cultured astrocytes of *Per2<sup>Brdm1</sup>* mutant animals show reduced uptake of glutamate compared to astrocytes from wild-type animals (**Fig. 3d**). The estimated *K<sub>m</sub>* and *V<sub>max</sub>* for wild-type astrocytes are  $K_m = 20 \pm 12 \mu\text{M}$ ,  $V_{max} = 7.8 \pm 1.4 \text{ nmol/min/mg}$ ; and for *Per2<sup>Brdm1</sup>* astrocytes  $K_m = 48 \pm 40 \mu\text{M}$ ,  $V_{max} = 4.8 \pm 1.7 \text{ nmol/min/mg}$  (two-way ANOVA of the curves shows 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<sup>Brdm1</sup>* 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<sup>Brdm1</sup>* mutant mice and wild-type animals cannot be distinguished morphologically and both express the astrocyte marker glial fibrillary acidic protein (data not shown). Taken together, the results depicted in **Figure 3** show that glutamate levels are elevated in *Per2<sup>Brdm1</sup>* mutant mice because of a malfunction of astrocytes, leading to a hyper-glutamatergic state in the brain.

### *Per2<sup>Brdm1</sup>* mice voluntarily consume more alcohol

Enhanced glutamate levels and alterations within the glutamatergic system have been implicated in excessive alcohol consumption and dependence<sup>12–14,18–20</sup>. Accordingly, we investigated voluntary alcohol consumption and preference<sup>22</sup>. *Per2<sup>Brdm1</sup>* 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<sup>Brdm1</sup>* mutant mice consume approximately threefold more ethanol than wild-type littermates. A similar observation was made regarding alcohol preference

measures. Thus, at concentrations of 8–16% ethanol, a clear genotype-dependent difference was observed ( $P = 0.001$ ; **Fig. 4b**).

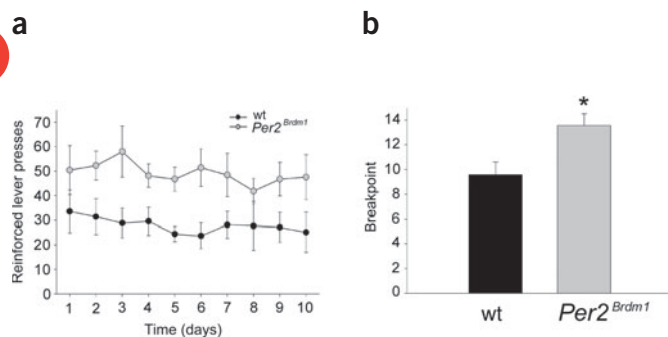
There are different explanations for why *Per2<sup>Brdm1</sup>* mutant mice drink more alcohol. One is that through alterations in the glutamatergic system, energy need is enhanced and, therefore, more alcohol is consumed. But this explanation seems unlikely, as no genotype-dependent differences are 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 d: wild-type,  $152 \pm 6 \text{ g/kg}$ ; *Per2<sup>Brdm1</sup>* mutant mice  $167 \pm 12 \text{ g/kg}$  body weight).

Alternatively, the increased alcohol consumption in *Per2<sup>Brdm1</sup>* mutant mice could be the result of altered taste sensation in these animals. Therefore, we estimated the preference ratio for a bitter-tasting compound containing various concentrations of quinine<sup>22,23</sup>. Both genotypes behave similarly (**Fig. 4c**), and in a sucrose preference test in which the sweet component is estimated, no differences between genotypes were observed (**Fig. 4d**). Thus, a difference in taste sensation probably does not account for an enhanced alcohol preference in *Per2<sup>Brdm1</sup>* mutant animals. Because sucrose consumption does not differ between genotypes, energy consumption is the same, and hence, an increase in alcohol uptake in *Per2<sup>Brdm1</sup>* mice is likely not caused by higher caloric needs in these animals.

One could argue that alcohol metabolism and elimination is altered in *Per2<sup>Brdm1</sup>* mutant mice. Alcohol is mainly eliminated through oxidation, catalyzed by alcohol dehydrogenase<sup>24</sup>. Circadian variations in plasma ethanol levels and within alcohol oxidation pathways have been described in mice<sup>25</sup>. Therefore, it can be assumed that in *Per2<sup>Brdm1</sup>* mutant mice—*Per2* is also strongly expressed in the liver—alcohol elimination may differ and the observed changes in alcohol-drinking behavior can be explained by alterations in the ethanol elimination rate. Therefore, we studied blood alcohol elimination in both alcohol-naive and alcohol-experienced *Per2<sup>Brdm1</sup>* mutants as well as their wild-type littermates. We did not observe any differences in blood alcohol concentrations under any of these conditions (**Fig. 4e,f**), excluding the possibility that altered alcohol elimination accounts for enhanced alcohol consumption in *Per2<sup>Brdm1</sup>* mutant mice.

A final and probable explanation is that a mutation in *Per2* influences the set point within the reinforcement system. Thus, the reinforcing value of alcohol might be differently perceived by *Per2<sup>Brdm1</sup>* mutant mice compared to wild-type animals. Therefore, we studied the reinforcing properties of alcohol in *Per2<sup>Brdm1</sup>* 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<sup>Brdm1</sup>* mutant mice exhibited approximately 50 lever responses within a 30-min session, whereas wild-type mice responded significantly less ( $P = 0.02$ ; approximately 25 lever responses per session; **Fig. 5a**). Moreover, whereas FR1 performance is more related to the rewarding post-consumptional consequences of the delivered reinforcer, progressive effort requirements better reflect incentive-motivation processes<sup>26</sup>. Accordingly, we used a progressive ratio schedule to study the incentive motivation for alcohol drinking in *Per2<sup>Brdm1</sup>* mutant mice. Using a progressive ratio 2 (response requirements increased by a step size of 2), the breakpoint for alcohol responding was significantly higher in *Per2<sup>Brdm1</sup>* mutant animals compared to the wild-type mice ( $P = 0.02$ ; **Fig. 5b**).

Together, we conclude from this set of experiments that neither the caloric value, taste differences nor variations in alcohol elimination can account for the enhanced alcohol intake observed in *Per2<sup>Brdm1</sup>* mutant mice. Rather, we suggest that it may be the result of alterations in the brain reinforcement system of *Per2<sup>Brdm1</sup>* mutant mice, thus driving an enhanced incentive motivation to consume more alcohol than the



**Figure 5** Operant ethanol self-administration and progressive ratio measurements in *Per2<sup>Brdm1</sup>* mutant mice and wild-type littermates. **(a)** Mice were trained to respond to an FR1 for alcohol, using a sucrose-fading procedure, until reaching a stable response rate for a 16% ethanol solution. A two-way ANOVA showed an effect of genotype ( $F(1,12) = 6.62$ ;  $P < 0.02$ ) but the factor day and the group  $\times$  day interaction was not significant, indicating that *Per2<sup>Brdm1</sup>* mutant mice showed a significantly higher number of responses at the active lever than wild-type 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 breakpoint for each group.

control animals. To further translate this conclusion at 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<sup>27</sup>.

### Acamprosate treatment of *Per2<sup>Brdm1</sup>* mice

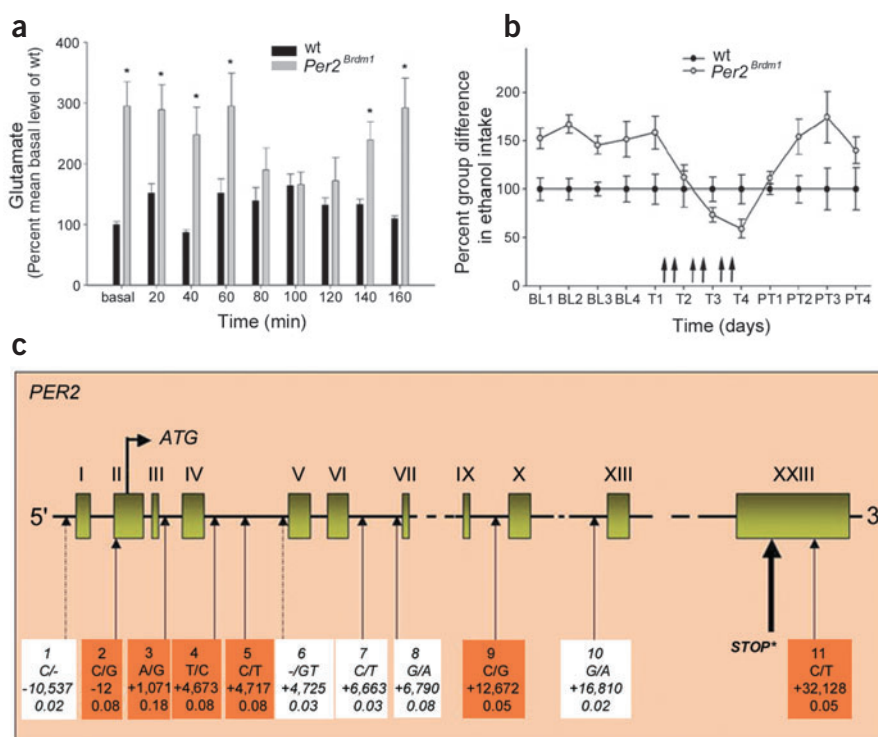
Acamprosate is used in the clinic for relapse prevention<sup>28</sup>. A meta-analysis of 20 clinical trials clearly shows its effectiveness<sup>29</sup>; however, the exact mechanism detailing how acamprosate diminishes alcohol consumption and reduces the likelihood of relapse is still not clear. Different neurobiological pathways have been implicated in the etiology of alcohol dependence, and one pathway seems to involve the glutamatergic system<sup>16,18,30,31</sup>, in which chronic alcohol intake leads to compensatory changes. It is suggested that acamprosate acts on a hyper-glutamatergic state, having only a small effect on a ‘normal’ glutamatergic state<sup>15–17,32,33</sup>. Because of the occurrence of a hyper-glutamatergic system in *Per2<sup>Brdm1</sup>* mutant mice, we speculated that acamprosate should be more effective in reducing glutamate levels and alcohol consumption in the mutant than in wild-type mice. Indeed, after performing further *in vivo* microdialysis experiments, we observed that 80 min after acamprosate treatment, extracellular glutamate levels in the nucleus accumbens in *Per2<sup>Brdm1</sup>* mutant mice no longer differed from those in wild-type mice (Fig. 6a). Furthermore, following acamprosate treatment, *Per2<sup>Brdm1</sup>* mutant mice showed 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<sup>Brdm1</sup>* 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<sup>34</sup>, it is suggested that dopamine-mediated alcohol reinforcement processes<sup>35</sup> are influenced by acamprosate through the dampening of a hyper-glutamatergic tonus.

### Genetic variations of human *PER2* and alcohol consumption

Based on findings of the animal studies, 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 (nine SNPs, two deletions), one of which is in the 5’ regulatory region, one in the 5’UTR, one in the 3’UTR and eight 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 six informative SNPs with a minor allele frequency of >0.05 for genotyping in a sample of 215 individuals with a detailed assessment of alcohol intake according to the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) questionnaire<sup>36</sup>. After performing a split to compare high ( $\geq 300$  g/d) versus low alcohol intake (<300 g/d)<sup>37</sup>, we found a significant association of high versus low alcohol intake with SNP 3 ( $P = 0.02$ , OR 0.46), with allele G being a protective allele (see **Supplementary Table 1 online**). 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 covariable. To assess phase information, we then performed a haplotype analysis.



**Figure 6** Effects of acamprosate on extracellular glutamate levels and alcohol consumption in *Per2<sup>Brdm1</sup>* mutant mice and wild-type littermates. **(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 intraperitoneally) are expressed as the percentage of basal values from wild-type mice and were analyzed by ANOVA for repeated measures. **(b)** Pharmacological rescue by acamprosate of augmented drinking behavior in *Per2<sup>Brdm1</sup>* mutant mice. Two daily injections of acamprosate (200 mg/kg intraperitoneally) for 4 d (arrows) produced a complete rescue of drinking behavior in *Per2<sup>Brdm1</sup>* 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 showed 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)** Schematic representation of the human *PER2* (GI:1365471) gene. 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). SNPs (continuous 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 analyzed because of genotyping problems.

Because there was an exactly similar distribution of SNP 2 and SNP 5 as well as SNP 9 and SNP 11, we excluded SNP 2 and SNP 11 from further analysis. The remaining haplotype consisting of SNP 3, SNP 4, SNP 5 and SNP 9 showed a significant association between high versus low amount of alcohol intake (global  $P$ -value by permutation test = 0.03; **Supplementary Table 2 online**). Analysis of individual haplotypes identified a haplotype GCCC, 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 performed a phylogenetic footprint analysis, which showed that SNP 3 is embedded in a CATTTC motif, which is 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 motifs for NF- $\kappa$ B, Sp1, c-myb, E47 and IL-6 RE-BP. SNP 3 alters the binding motifs for Sp1, c-myb and NF- $\kappa$ B, 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 for the *PER2* gene in the regulation of alcohol consumption in humans.

## DISCUSSION

Here we report the relationship between *Per2* and the glutamate transporter *Eaat1*. A nonfunctional *Per2* gene leads to a reduction of *Eaat1* expression and as a consequence, glutamate accumulates in the brain. These elevated glutamate levels cannot be compensated through adaptive upregulation of *Eaat2*, and hence, a hyper-glutamatergic brain in *Per2<sup>Brdm1</sup>* 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<sup>38–40</sup>, suggesting that clock genes seem to be involved in common modulator mechanisms of drug abuse-related behaviors<sup>41</sup>. Notably, methamphetamine injection also causes an increase of *Per* gene expression in the caudate putamen of the mouse<sup>42,43</sup>.

Enhanced alcohol consumption in *Per2<sup>Brdm1</sup>* mutant mice can be rescued pharmacologically by the anticraving and antirelapse compound acamprostate. Thus, following acamprostate treatment, *Per2<sup>Brdm1</sup>* mutant mice had reduced alcohol consumption, which dropped below those of wild-type animals. This compound also reduced glutamate levels in the nucleus accumbens—an effect that was not observed in wild-type mice, suggesting that acamprostate acts mainly on alcohol consumption by dampening the hyper-glutamatergic tonus in *Per2<sup>Brdm1</sup>* mutant mice. Evidence for a genetic basis of a hyper-glutamatergic system in humans is provided as seen by the differential response to acamprostate in alcoholic patients. It should be noted that compared to placebo, acamprostate treatment increases abstinence rates only by 10–20%<sup>28,29</sup>. On a diagnostic level, responders and nonresponders cannot be differentiated. We hypothesize that genetic variations in the *PER2* gene and characterization of a hyper-glutamatergic state may aid in the identification of alcohol-dependent individuals responsive to acamprostate 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 show

enhanced alcohol consumption in shift workers and people suffering from severe jet lag, as found in aircraft staff, for example<sup>44,45</sup>. Hence, alterations in *PER2* and associated changes in the glutamatergic system may underlie those pathological consequences, suggesting one possible pathway in the etiology of alcohol dependence.

## METHODS

**Animals.** The mice used in this study were characterized previously<sup>46</sup>. The wild-type and *Per2<sup>Brdm1</sup>* mutant animals used in this study were littermates derived from intercrosses between heterozygous *Per2<sup>Brdm1</sup>* mice on a 129SvEv<sup>Brd</sup>/C57BL/6-Tyr<sup>c-Brd</sup> background. Animal experiments were approved by the veterinary offices of Fribourg and the Regierungspräsidium Ka.

**Tissue preparation and cDNA synthesis.** Mice kept for at least 2 weeks in a 12 h light/12 h dark cycle were killed at ZT15. Tissue collection and cell lysis were carried out under dim red light (15 W). We synthesized cDNA using the SMART PCR cDNA Synthesis Kit (Clontech K 1052-1) according to the manufacturer's instructions.

**Subtraction and amplification of differentially expressed genes.** We used the PCR Select cDNA Subtraction Kit (Clontech K1804-1) for subtraction 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.** We performed northern analysis using denaturing formaldehyde gels with subsequent transfer to Hybond-N membrane (Amersham). cDNA probes had a specific activity of 10<sup>8</sup> cpm/ $\mu$ g. Signals were quantified using Quantity One 3.0 software (Bio-Rad).

**Western blot analysis.** We resolved brain proteins by SDS-PAGE and transferred them to PVDF membranes (Amersham). Antibodies used for detection were: rabbit *Eaat1*-specific antibody diluted 1:1,000 (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 1:5,000 for *Eaat1* (Sigma, A5420) and mGluR5 and goat anti-rabbit IgG-AP 1:5,000 (Pierce, #31340).

**Ninhydrin assay.** We added 2 ml of ninhydrin solution (0.2% in ethanol) to 2 ml synaptosomal supernatants and boiled the mixture 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<sup>Brdm1</sup>* mutant mice was homogenized. We added aliquots of synaptosomes to incubation buffer containing L-[<sup>3</sup>H]-glutamic acid (specific activity = 51 Ci/mmol; PerkinElmer Life Sciences). The reaction was terminated (incubation buffer in which an equimolar concentration of choline chloride was substituted for NaCl). We determined the <sup>3</sup>H-bound radioactivity by liquid scintillation counting (PACKARD liquid scintillation analyzer TRI-CARB 2200CA).

**Glial cells and glutamate uptake.** Glial cells were obtained from postnatal day 1 brains of wild-type and *Per2<sup>Brdm1</sup>* mutant pups. The tissue was dissociated by trituration and the cells were plated and passaged. After the second passage, we counted cells and seeded them into 24-well 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, we placed the mice in the CMA/120 system for freely moving animals. The CMA/7 microdialysis probe was inserted into the guide cannula and perfused at a constant flow rate of 2  $\mu$ l/min with a Ringer solution. One day after the recovery, microdialysis experiments were carried out in freely moving mice between ZT3 and ZT6. As previously described in detail<sup>47</sup>, glutamate levels were determined from 15  $\mu$ l of each dialysate sample by use of a gradient microbore liquid chromatography assay with fluorescence detection.

**Determination of blood alcohol levels.** Alcohol-naïve and alcohol-experienced wild-type and *Per2<sup>Brdm1</sup>* mutant mice were injected intraperitoneally with 3.5 g/kg ethanol. We determined blood alcohol content using the NADH enzyme spectrophotometric method (Greiner AG).

**Alcohol self-administration and pharmacological treatment.** After 1 week of habituation to the animal room, male *Per2<sup>Brim1</sup>* mutant and control mice were given continuous free access to two bottles of tap water for 3 d, followed by tap water and 2% (vol/vol) ethanol solution for another 3 d (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 a 4% ethanol solution; days 7–16 access to 8% ethanol solution; days 17–25 12% ethanol; and 16% ethanol solution from day 26 onward. Ethanol consumption was calculated in terms of g of ethanol consumed/kg of body weight/d (g/kg/d). In a second batch of mice, we tested the effects of acamprosate treatment. Once alcohol intake at the 16% solution was stable, two daily injections of acamprosate (200 mg/kg, every 12 h) were given to all mice and drinking data were compared to those of the last 4 d of the baseline.

**Operant ethanol self-administration and progressive ratio 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 min. We trained animals to orally self-administer ethanol (10%) after having undergone a standard sucrose fading procedure under a FR1 paradigm. Mice were also tested on a progressive ratio 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% wt/vol) 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 d and bottles were weighed every 3 d along with the position of the bottles being changed.

**Subjects and psychiatric assessment.** Individuals of German origin (172 males, 43 females; mean age 41.3 years, s.d. 8.4) were recruited by the Department of Psychiatry of the University of Munich. All patients were consecutively admitted for an inpatient alcohol withdrawal therapy and fulfilled the DSM-IV criteria for alcohol dependence. Symptoms related to alcohol-taking behavior were assessed in using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA)<sup>36</sup>. To distinguish patients with low versus high alcohol intake, a cutoff was set at 300 g/d, ensuring comparability of the results with previous work on genes regulating glutamatergic neurotransmission<sup>37</sup>. We obtained written informed consent 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.** We identified SNPs 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. We genotyped six single nucleotide polymorphisms in the *PER2* gene 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 2 × 3 contingency tables as originally proposed by Armitage<sup>48</sup>. Haplotype association analyses were performed with COCAPHASE 2.35.

**URL.** COCAPHASE 2.35 <http://www.hgmp.mrc.ac.uk>

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

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#### COMPETING INTEREST STATEMENT

The authors declare that they have no competing financial interests.

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