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Acamprosate Produces Its Anti-Relapse Effects Via Calcium

Rainer Spanagel*[,1,5], Valentina Vengeliene[1,5], Bernd Jandeleit[2,5], Wolf-Nicolas Fischer[2], Kent Grindstaff[2], Xuexiang Zhang[2], Mark A Gallop[2], Elena V Krstew[3], Andrew J Lawrence[3] and Falk Kiefer[4]

[1]Institute of Psychopharmacology, Central Institute of Mental Health, University of Heidelberg, Medical Faculty Mannheim, Mannheim, Germany; [2]XenoPort, Inc., Santa Clara, CA, USA; [3]Florey Institute of Neuroscience & Mental Health, University of Melbourne, Melbourne, Australia; [4]Department of Addictive Behavior and Addiction Medicine, Central Institute for Mental Health, Mannheim, Germany

Alcoholism is one of the most prevalent neuropsychiatric diseases, having an enormous health and socioeconomic impact. Along with a few other medications, acamprosate (Campral—calcium-bis (*N*-acetylhomotaurinate)) is clinically used in many countries for relapse prevention. Although there is accumulated evidence suggesting that acamprosate interferes with the glutamate system, the molecular mode of action still remains undefined. Here we show that acamprosate does not interact with proposed glutamate receptor mechanisms. In particular, acamprosate does not interact with NMDA receptors or metabotropic glutamate receptor group I. In three different preclinical animal models of either excessive alcohol drinking, alcohol-seeking, or relapse-like drinking behavior, we demonstrate that *N*-acetylhomotaurinate by itself is not an active psychotropic molecule. Hence, the sodium salt of *N*-acetylhomotaurinate (i) is ineffective in alcohol-preferring rats to reduce operant responding for ethanol, (ii) is ineffective in alcohol-seeking rats in a cue-induced reinstatement paradigm, (iii) and is ineffective in rats with an alcohol deprivation effect. Surprisingly, calcium salts produce acamprosate-like effects in all three animal models. We conclude that calcium is the active moiety of acamprosate. Indeed, when translating these findings to the human situation, we found that patients with high plasma calcium levels due to acamprosate treatment showed better primary efficacy parameters such as time to relapse and cumulative abstinence. We conclude that *N*-acetylhomotaurinate is a biologically inactive molecule and that the effects of acamprosate described in more than 450 published original investigations and clinical trials and 1.5 million treated patients can possibly be attributed to calcium.

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INTRODUCTION

Alcohol use and abuse account for a tremendous burden of disease and injury and economic costs worldwide. Excessive alcohol drinking is a leading risk factor for chronic non-communicable diseases and in fact, is linked to more than 60 diseases, including cancers, cardiovascular diseases, liver cirrhosis, neuropsychiatric disorders, and fetal alcohol syndrome (Rehm *et al*, 2009). Consequently, alcohol use and abuse bring considerable costs to society; on a global scale, the annual costs are estimated to be 760 billion Euros. One further consequence of excessive alcohol use is that 76 million adults worldwide are alcohol dependent (Rehm *et al*, 2009). Currently, there are four medications approved by the U.S. Food and Drug Administration (FDA) to treat alcohol dependence: disulfiram, oral naltrexone, a long-lasting injectable naltrexone, and acamprosate (Litten *et al*, 2012). In Europe, nalmefene has been also approved in 2013 (Mann *et al*, 2013).

Almost 30 years ago, the inhibiting effect of acamprosate (calcium-bis(*N*-acetylhomotaurinate)—in the entire text abbreviated as Ca-AOTA) on alcohol consumption in laboratory animals was described (Boismare *et al*, 1984). This initial observation led to the clinical development of acamprosate (Campral) and nowadays this drug is currently used in many countries for relapse prevention in abstinent alcohol-dependent patients. Acamprosate is a safe and well-tolerated drug that does not affect craving (Umhau *et al*, 2011) but the risk to relapse. In a recent Cochrane Review Rösner *et al* (2010) summarized 24 randomized controlled trials (RCTs) on acamprosate, and concluded that acamprosate significantly reduces the risk of any drinking with a relative risk (RR) of 0.86. A RR of 1 means that there is no difference between placebo and treatment, whereas a RR <1 means that relapse occurs less frequently in the treatment group. Acamprosate has been used to treat alcohol dependence in over 1.5 million patients since its introduction in Europe in 1989 and is currently available in most European and Latin American countries, Australia, parts of Asia, and Africa (Mason and Heyser, 2010). In 2004, it was approved by the FDA for the maintenance of abstinence from alcohol in detoxified alcohol-dependent

*Correspondence: Professor R Spanagel, Institute of Psychopharmacology, Central Institute for Mental Health, University of Heidelberg, Medical Faculty Mannheim, Square J5, Mannheim D-68159, Germany, Tel: +49 621 1703 6251, Fax: +49 621 1703 6255,
E-mail: rainer.spanagel@zi-mannheim.de
[5]These authors contributed equally to this work.
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patients. A recent survey found that acamprosate is now the most widely prescribed medication for the treatment of alcoholism in the United States of America (Mark *et al*, 2009).

In the past three decades, huge research efforts sought to elucidate the molecular mode of action of acamprosate. Many molecular candidate targets have been described (Spanagel and Vengeliene, 2013; De Witte *et al*, 2005; Mann *et al*, 2008) but the only accumulated evidence was found with respect to an interaction with the glutamate system. In concert, these studies suggest that acamprosate attenuates hyperglutamatergic states that occur during early and protracted abstinence, possibly involving *N*-methyl-D-aspartate (NMDA) receptors and metabotropic glutamate receptor 5 (mGluR5) (Spanagel and Vengeliene, 2013; De Witte *et al*, 2005; Mann *et al*, 2008; Rammes *et al*, 2001; Harris *et al*, 2002). A prominent theory in the alcohol research field posits that chronic alcohol consumption leads to glutamatergic dysfunction. As a consequence, exaggerated glutamate activity is observed during alcohol withdrawal and conditioned withdrawal responses. This hyperglutamatergic state may then drive alcohol-seeking and relapse behavior (Tsai *et al* 1995; Spanagel and Kiefer 2008). Acamprosate dampens hyperglutamatergic activity in excessively ethanol drinking mice, thereby reducing alcohol intake (Mann *et al* 2008; Spanagel *et al*, 2005). In a recent double-blind, placebo-controlled study, which applied magnetic resonance spectroscopy acamprosate also reduced glutamate levels in the brains of detoxified alcohol-dependent patients (Umhau *et al*, 2010). Although the effects of acamprosate on glutamate levels are well documented, the molecular mode of action of this drug and the putative interaction between acamprosate and glutamate receptors remains unclear.

Here we describe a series of experiments and come to the surprising conclusion that *N*-acetylhomotaurinate by itself is not an active psychotropic molecule. Instead, calcium is the active moiety of acamprosate! In the first set of experiments, we tested the putative interactions of acamprosate with NMDA receptors and mGluR5 (Mann *et al*, 2008; Harris *et al*, 2002; Madamba *et al*, 1996). From these experiments, we have to conclude that the proposed glutamate receptor interactions of acamprosate cannot sufficiently explain the anti-relapse action of this drug. In comparative experiments, we then studied the effects of Ca-AOTA *vs* sodium-*N*-acetylhomotaurinate (Na-AOTA) in three preclinical models of either excessive alcohol drinking, alcohol-seeking, or relapse-like drinking behavior. The rationale for doing these comparative experiments stems from US patent 4,355,043 (1982) where the initial investigators of acamprosate described that various salts of *N*-acetylhomotaurinate produce their effects according to the nature of the counter ion. This assertion has so far not been tested. From these animal experiments, we conclude that calcium exhibits anti-relapse effects and seems to be the major active ingredient of acamprosate. We further translated these findings at the clinical level. Using a clinical sample of placebo *vs* acamprosate-treated abstinent alcohol-dependent patients, we measured calcium plasma concentrations and show that patients with high plasma calcium levels due to acamprosate treatment exhibit better primary efficacy parameters.

MATERIALS AND METHODS

Six different studies were performed. All information is provided in the Supplementary Information.

Study 1: Screening panel for the mode of action of acamprosate.

Study 2: Testing different salt formulations of acamprosate in the ADE model.

Study 3: Testing different salt forms of acamprosate in alcohol-seeking rats in the cue-induced reinstatement model.

Study 4: Testing different salt forms of acamprosate in alcohol-preferring iP-rats.

Study 5: Pharmacokinetic (PK) profiles of different salt forms of acamprosate.

Study 6: Calcium plasma levels in placebo and acamprosate-treated patients.

RESULTS

Acamprosate (Ca-AOTA) does not Interact with NMDA Receptors or mGluR1/5

Several possible modes of action of acamprosate have been described (Mann *et al*, 2008); however, a body of evidence suggests that acamprosate interacts with NMDA receptors and/or mGluR5 (Mann *et al*, 2008; Harris *et al*, 2002; Madamba *et al*, 1996). To test these putative interactions, we applied an extensive screening panel. By expressing human NR1 and NR2B subunits in *Xenopus laevis* oocytes, we first tested agonist activity of acamprosate on the glycine and glutamate-binding site of the NMDA receptor. The two subunit types expressed for our purposes (hNR1A and hNR2B) provide a combination that is thought to be predominantly present in the human forebrain (Scherzer *et al*, 1998; Kosinski *et al*, 1998). To determine NMDA receptor agonist activity, glycine and glutamate were substituted with Ca-AOTA. Multiple concentrations of Ca-AOTA were tested up to a maximal concentration of 1 mM. Elicited currents were measured and compared with currents seen with saturating concentrations of glycine and glutamate. Even at a maximal concentration, no activation of the receptor was seen indicating that acamprosate does not appear to have any effect at the glycine agonist binding site nor at the glutamate-binding site (Figure 1a and b). To determine NMDA receptor antagonist activity, standard Schild-plot analyses were performed to assess whether or not acamprosate could shift the affinity of glycine and glutamate. Concentration response curves of glycine and glutamate were again prepared with varying concentrations of Ca-AOTA (100, 500, and 1000 μM). No shift in affinity was seen in concentration response curves for glycine or glutamate, indicating no apparent antagonist activity in our assay (Figure 1c and d). In addition, using acute brain slices from rats, electrically evoked NMDA receptor dependent excitatory post-synaptic potentials (EPSPs) were measured in the nucleus accumbens. Modest changes in EPSP amplitude were observed in ~50% of the cells following addition of Ca-AOTA (Supplementary Figure 1) suggesting that acamprosate may have some effect on the quantum release of presynaptic glutamate.

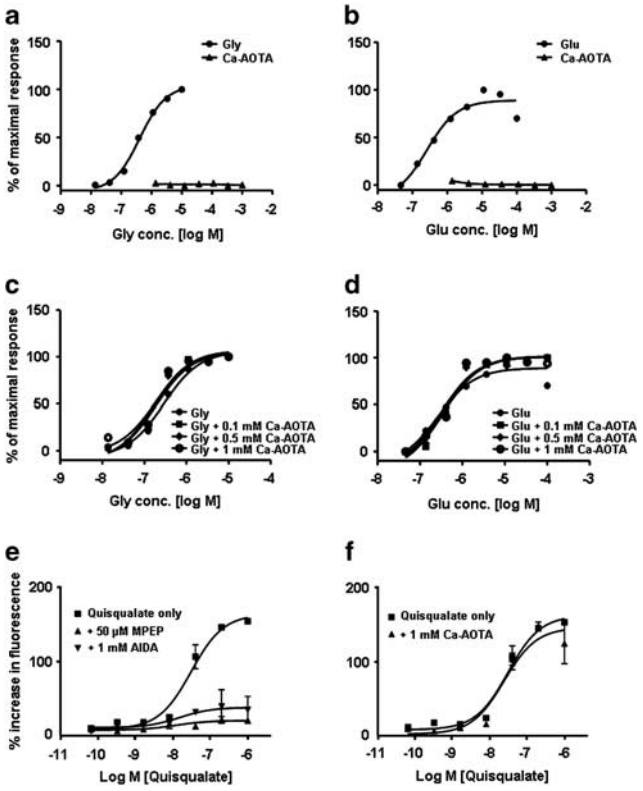

Figure 1 (a) Glycine-site agonist screen. Glycine was substituted with acamprosate in the presence of excess co-agonist glutamate. No activation of the receptor was seen indicating that acamprosate (Ca-AOTA) does not appear to have any effect at the glycine agonist binding site up to a 1 mM concentration. (b) Glutamate-site agonist screen. Glutamate was substituted with acamprosate in the presence of excess co-agonist glycine. No activation of the receptor was seen indicating that acamprosate does not appear to have any effect at the glutamate agonist binding site up to a 1 mM concentration. (c) Glycine-site antagonist screen of acamprosate. Concentration response curves of glycine show no shift in affinity when increasing concentrations of acamprosate is introduced, indicating no apparent glycine-site antagonist activity. (d) Glutamate-site antagonist screen of acamprosate. Concentration response curves of glutamate show no shift in affinity when increasing concentrations of acamprosate is introduced, indicating no apparent glutamate-site antagonist activity. (e) Functional Ca^{2+} flux assays illustrate dose-dependent mGluR1/5 activation by quisqualate and antagonism of this response by AIDA and MPEP, respectively. (f) Shows the lack of effect of Ca-AOTA (acamprosate) on the response to quisqualate.

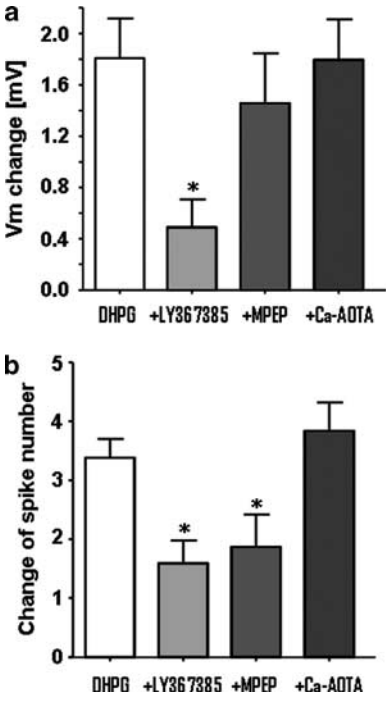

Figure 2 (a) Effects of DHPG on resting membrane potential (Vm change) in CA1 neurons of the hippocampal region in the presence of mGluR1/5 antagonists or acamprosate. Application of 100 µM DHPG (mGluR1/5 agonist) depolarized cells by ~1.8 mV ($n=33$). Addition of 300 µM LY367385 (mGluR 1 antagonist) significantly reduced DHPG-induced depolarization ($n=11$) ($P<0.05$). DHPG + 10 µM MPEP (mGluR 5 antagonist) resulted in a modest but not significant decrease in the depolarization ($n=9$). Acamprosate (Ca-AOTA; 300 µM) had no effect on DHPG-induced depolarization ($n=13$) (b) Effects of DHPG on spike number change in the presence of mGluR1/5 antagonists or acamprosate. DHPG alone ($n=33$) increased the spike number by more than 3. Addition of LY367385 ($n=10$) or MPEP ($n=9$) significantly reduced the spike number in the presence of DHPG ($P<0.05$). Ca-AOTA ($n=12$) had no effect on the number of spikes. The data are presented as means ± SEM
* Indicates significant differences from vehicle control group, $P<0.05$. Note: In the condition DHPG + LY367385 and DHPG + acamprosate we lost in each experiment one recording during spike induction.

Next, calcium flux assays were used to study acamprosate's interaction with mGluR1/5. For these experiments, human mGluR1 and 5 were expressed in HEK 293 cells. The mGluR1/5 agonist quisqualate produced a robust dose-dependent increase in the fluorescence signal that was completely inhibited by the mGluR1/5 antagonist AIDA as well as by MPEP (Figure 1e). Ca-AOTA had no effect in this assay (Figure 1f). Then we performed patch clamp electrophysiology to measure effects of mGluR1/5 on resting membrane potential and spike number. Again, brain slices were prepared from 6–10-week-old male rats. Using whole-cell patch clamp, the effects of the mGluR1/5 agonist DHPG on resting membrane potential and spike frequency were measured. DHPG-induced changes to the membrane potential were inhibited by the mGluR1 antagonist LY367385 whereas DHPG-induced increases in action potentials were blunted by addition of the mGluR1 and 5 antagonists, LY367385 and MPEP, respectively. In contrast, Ca-AOTA had no effect under these conditions on membrane potential or the number of action potentials (Figure 2). From these studies, we conclude that the proposed glutamate receptor interactions of acamprosate, especially with NMDA- and mGluR5 receptors cannot sufficiently explain the anti-relapse action of this drug.

Na-AOTA Is Ineffective in Preclinical Animal Models but Calcium Salts Produce Acamprosate-Like Effects

In US patent 4,355,043 (1982), the initial investigators of acamprosate described that various salts of *N*-acetylhomotaurinate produce their effects according to the nature of the counter ion. This assertion has so far not been tested. Therefore, in a comparative experiment, we studied the effects of Ca-AOTA *vs* Na-AOTA in the alcohol deprivation effect (ADE) model. This is a standard preclinical rat model that measures relapse-like drinking behavior by monitoring the ADE (Spanagel, 2009). This model provides excellent

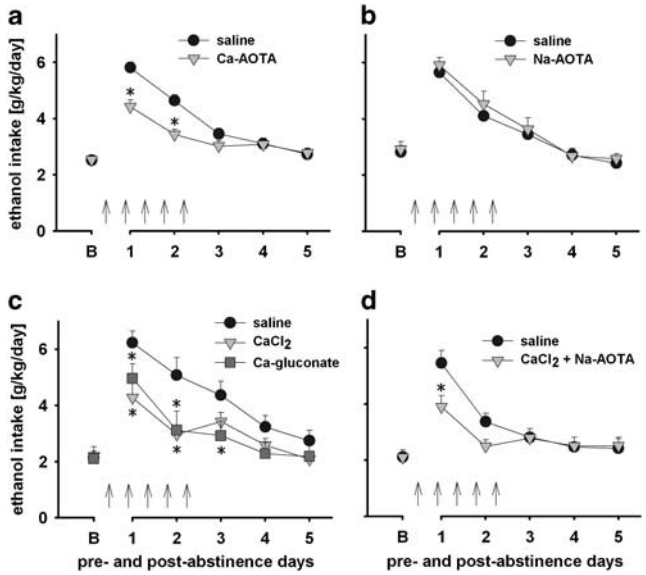

Figure 3 Total ethanol intake (g/kg/day) before and after an alcohol deprivation period of 3 weeks in male Wistar rats. The average of the last 3 pre-abstinence days measurement of ethanol intake is given as baseline drinking 'B'. Arrows indicate the i.p. administration of either saline ($n=7$–26) or compounds: 200 mg/kg of Ca-AOTA ($n=26$) (a), 200 mg/kg of Na-AOTA ($n=9$) (b), 73.4 mg/kg of $CaCl_2 \times 2\,H_2O$ ($n=8$) and 215 mg/kg of Ca-gluconate ($n=7$) (c), and 200 mg/kg of Na-AOTA combined with 73.4 mg/kg of $CaCl_2$ ($n=7$) (d). Note 1: 73.4 mg/kg of $CaCl_2$, 215 mg/kg of Ca-gluconate and 200 mg/kg of Ca-AOTA contain equivalent amounts of Ca^{2+} ions (0.499 mmol/kg). Note 2: 200 mg/kg Na-AOTA and 200 mg/kg Ca-AOTA contain almost equal amounts of *N*-acetylhomotaurinate (0.984 mmol/kg *vs* 0.999 mmol/kg; corresponding to 1.5% difference). All data are presented as means ± SEM * indicates significant differences from vehicle control group, $P<0.05$.

face and construct validity (Vengeliene *et al*, 2009), and has shown predictive validity as well (Spanagel and Kiefer, 2008). In particular, acamprosate was found in previous studies to reduce the ADE (Spanagel *et al*, 1996a; Heyser *et al*, 1998; Lidö *et al*, 2012). As described in these previous studies, the calcium salt of *N*-acetylhomotaurinate (acamprosate) decreased the ADE in the present experiment. Hence, a two-way repeated measures ANOVA displayed a significantly different alcohol intake during ADE days between vehicle and Ca-AOTA-treated animal groups ($F_{(5,250)}=10.327$, $P<0.0001$). In contrast, an equimolar concentration of the corresponding sodium salt had no effect on the ADE ($P=0.950$) (Figure 3a and b). This surprising finding suggests that calcium is a critical component for the efficacy of acamprosate, and we hypothesized that calcium salts should produce acamprosate-like effects on the ADE. Indeed, equimolar concentrations of calcium derived either from calcium chloride or calcium gluconate reduced the ADE in a similar way as acamprosate two-way repeated measures ANOVA revealed significantly lower alcohol intake during ADE days in both calcium chloride- and calcium gluconate-treated animal groups when compared with the control animal group ($F_{(10,105)}=2.860$, $P<0.003$) (Figure 3c). We then tested a mixture of Na-AOTA and calcium chloride, which restored the effect of acamprosate on the ADE ($F_{(5,60)}=3.685$, $P<0.006$) (Figure 3d). Locomotor activity was monitored throughout the ADE measurements by the home cage E-motion system. Ca-AOTA, as observed in previous experiments by us, (Spanagel *et al*, 1996b) reduced home cage activity following the first injection. This effect was, however, absent in NA-AOTA (Supplementary Figure 2). This set of experiments suggests that *N*-acetylhomotaurinate is an inactive molecule and that calcium is the active moiety of acamprosate.

To further confirm these surprising findings, we used yet another standard preclinical rat model—the reinstatement model (Shaham *et al*, 2003; Sanchis-Segura and Spanagel, 2006). This model refers to the resumption of extinguished lever-pressing behavior after exposure of an animal to alcohol-conditioned stimuli. Reinstatement of alcohol-seeking is used to study the neurobiological and molecular basis of craving, as there appears to be a good correspondence between the events that induce alcohol-seeking in laboratory animals and those that provoke craving in humans (Shaham *et al*, 2003). In a previous study, it was shown that acamprosate completely abolishes the cue-induced reinstatement response (Bachteler *et al*, 2005). As described in this previous report, the calcium salt of *N*-acetylhomotaurinate (acamprosate) abolished the reinstatement response but equimolar concentration of the corresponding sodium salt formulation of acamprosate had no effect (Figure 4). Hence administration of Ca-AOTA and Na-AOTA as well as calcium chloride significantly changed lever responding during the cue-induced reinstatement sessions (factor drug $F(3,87)=12.7$, $P<0.0001$; factor drug × lever interaction: $F(6,87)=4.9$, $P<0.001$ and factor drug × lever × session interaction: $F(6,87)=2.3$, $P<0.05$). *Post hoc* comparisons revealed that administration of both Ca-AOTA and calcium chloride significantly reduced responses on the ethanol-associated lever during S+/CS+ session (Figure 4a) and had no effect on lever responding during S−/CS− session (Figure 4b). No significant effect on lever responding during the cue-induced reinstatement sessions was observed in the Na-AOTA-treated group (Figure 4a and b). Responding on the inactive lever was not affected by either drug during both reinstatement sessions, indicating the absence of a nonspecific reduction in lever-pressing behavior (Figure 4a and b). In summary, these results clearly support our conclusion from the ADE model that calcium is the active moiety of acamprosate.

In light of the major implications of this study, we initiated an independent blinded replication of our findings in another laboratory (Lawrence group at Florey Institute of Neuroscience & Mental Health, University of Melbourne). Alcohol-preferring rats have been used for decades to study excessive alcohol consumption and the efficacy of putative pharmacological interventions (Spanagel and Kiefer, 2008; Bell *et al*, 2006). In particular, iP rats self-administering alcohol show sensitivity to acamprosate treatment (Cowen *et al*, 2005). Accordingly, Ca-AOTA and Na-AOTA were tested on operant behavior under a fixed ratio schedule 3 (FR3) (Supplementary Figure 3). As reported previously, (Cowen *et al*, 2005) Ca-AOTA (200 mg/kg i.p.) reduced significantly the number of ethanol reinforcers and calcium chloride produced a very similar response. A repeated measure one-way ANOVA indicated a main effect of treatment ($F_{(3,31)}=11.18$, $P<0.001$). *Post hoc* pairwise multiple comparison revealed for saline *vs* Ca-AOTA $P<0.001$ and for saline *vs* calcium chloride $P<0.007$. In a second group of animals, saline treatment was compared with Na-AOTA and calcium chloride. Although statistics

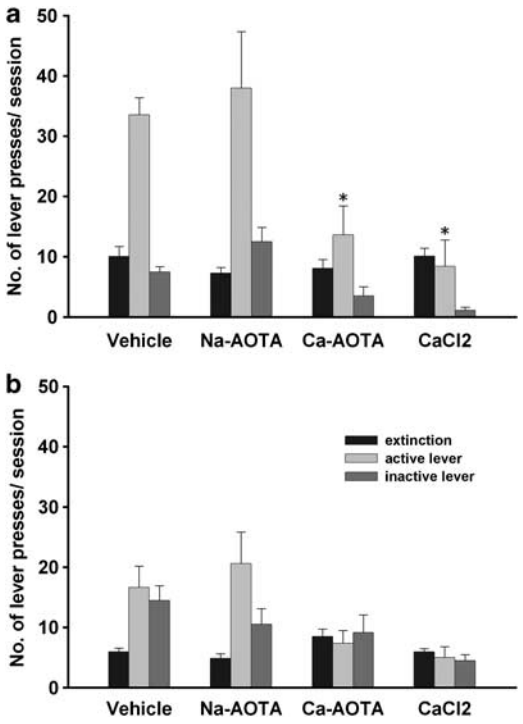

Figure 4 The effect of either vehicle ($n=9$) or 200 mg/kg of Na-AOTA ($n=8$), 200 mg/kg of Ca-AOTA ($n=8$) and 73.4 mg/kg of $CaCl_2$ ($n=8$) on cue-induced reinstatement under S+/CS+ (a) and S−/CS− (b) conditions. Data are shown as the average number of lever presses on the active lever during the last four extinction sessions (extinction) and as the number of responses on the active lever (active lever) after the presentation of a stimulus previously paired with either ethanol (S+/CS+ session) (a) or water (S−/CS− session) (b). Inactive lever (inactive lever) was present throughout the experiment and was used as a measure of a sedative effect of the treatment. All animals were injected with either of the compounds 12 h and 2 h before reinstatement sessions. Data are presented as means ± SEM * indicates significant differences from the control vehicle group, $P<0.05$.

indicated a treatment effect ($F_{(3,28)}=4.59$, $P=0.025$) *post hoc* analysis indicated that Na-AOTA did not significantly reduce the numbers of ethanol rewards (saline *vs* Na-AOTA, $P=0.083$), whereas again calcium chloride produced a significant reduction in earned ethanol rewards (saline *vs* $CaCl_2$, $P=0.02$). This independent experiment further supports our conclusion that *N*-acetylhomotaurinate by itself is not an active psychotropic molecule. Instead, calcium seems to be the active moiety of acamprosate.

To ensure that both salt formulations of *N*-acetylhomotaurinate lead to similar blood concentrations over time, we established pharmacokinetic (PK) profiles after i.p. injections and found no significant differences in assessed PK values for Na-AOTA and Ca-AOTA (Supplementary Table 1, Supplementary Figure 4). In addition, we measured PK parameters in the brain 30 min after i.p. injections of equimolar doses of 200 mg/kg Na-AOTA and Ca-AOTA. Amounts of *N*-acetylhomotaurinate detected in brain tissue were 3.88 ± 0.28 μg/g for Na-AOTA and 4.08 ± 0.58 μg/g for Ca-AOTA, respectively. We also calculated the brain:plasma ratio and found a ratio of 0.034 ± 0.004 for Na-AOTA *vs* 0.026 ± 0.004 for Ca-AOTA. We conclude that Na-AOTA and Ca-AOTA have a similar PK profile in blood and that similar amounts of each compound reach the brain. Given these similarities in PK profile and previous data demonstrating that salts of *N*-acetylhomotaurinate become totally dissociated in hydrophilic media (Chabenat *et al*, 1988), we can exclude the possibility that differences of bioavailability for these two salt forms of *N*-acetylhomotaurinate can account for the observed behavioral effects and therefore conclude that calcium acts as the active moiety of acamprosate in rats.

Plasma Calcium Levels Correlate with Acamprosate Response in Alcohol-Dependent Patients

We further translated these animal findings at the clinical level. Using a clinical sample of placebo *vs* acamprosate-treated abstinent alcohol-dependent patients, we measured calcium plasma concentrations. Before treatment, the same physiological calcium concentration of about 2.4 mmol/l were found in the placebo group and acamprosate group (Supplementary Figure 5). Additionally, blood was drawn after 1, 2, and 3 months of treatment. Although calcium levels are extremely tightly controlled by the kidneys and parathyroid hormone and are more or less constant in each individual, we found a tendency ($P<0.1$) of enhanced calcium plasma levels in acamprosate *vs* placebo treated patients (Supplementary Figure 5). When correlating the primary efficacy parameters of first drink, severe relapse, and cumulative abstinence with calcium plasma levels, we did not find any correlation in the placebo group, whereas significant correlations were found in the acamprosate group (Figure 5). Thus, patients with high plasma calcium levels due to acamprosate treatment showed better primary efficacy parameters.

DISCUSSION

In summary, our combined *in vitro*, animal and human data demonstrate that *N*-acetylhomotaurinate by itself is not an active psychotropic molecule and therefore it is not surprising that a molecular mode of action could not be established for acamprosate. Although previous studies have suggested that acamprosate might interact with NMDA receptors and mGluR5 (Mann *et al* 2008; Harris *et al* 2002; Madamba *et al*, 1996) our *in vitro* screening panel does not support this assumption. Instead, our findings in three independent preclinical animal studies suggest that calcium produces anti-drinking, anti-alcohol-seeking and anti-relapse effects. Furthermore, when translating these findings to the human condition, we demonstrated that calcium plasma levels in acamprosate-treated alcohol-dependent patients correlate with primary efficacy parameters such as time to first drink, time to severe relapse, and cumulative abstinence duration. Collectively, these data lead to the conclusion that calcium is the active moiety of acamprosate.

This conclusion may be somewhat surprising; however, previous studies showed that (i) calcium sensitivity of the synapse is important for alcohol tolerance development (Lynch and Littleton, 1983), (ii) calcium given intraventricularly significantly enhances alcohol intoxication in a dose-dependent manner (Erickson *et al*, 1978), (iii) activity of calcium-dependent ion channels modulate alcohol drinking and recently small conductance calcium-activated potassium type 2 channels (SK2) were shown to modulate

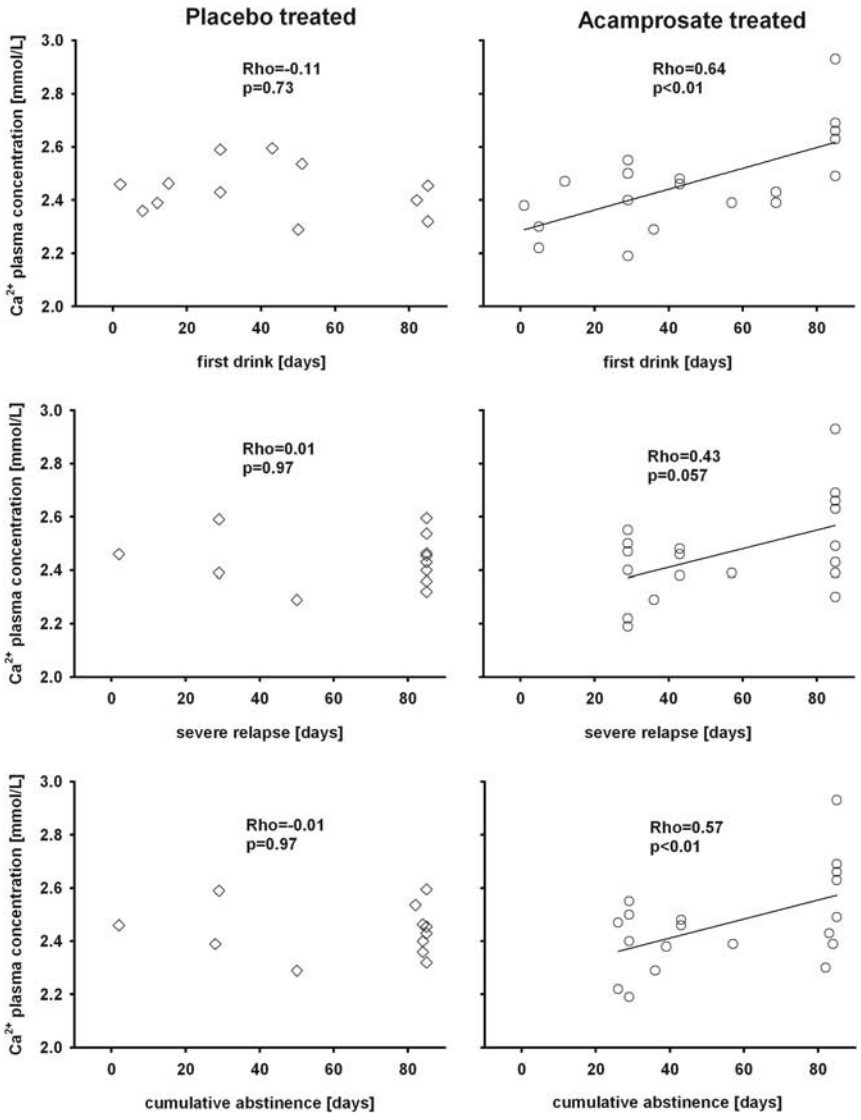

Figure 5 Plasma Ca^{2+} concentrations in patients receiving placebo ($n = 12$) or acamprosate ($n = 19$) treatment (1998 mg/day) in correlation to abstinence duration. Blood was drawn 1, 2, and 3 months after treatment begun and mean calcium concentrations were correlated with the primary efficacy parameters first drink, severe relapse, and cumulative abstinence.

hyperglutamatergic activity and alcohol consumption in rats (Mulholland *et al*, 2011; Hopf *et al*, 2011), finally and most importantly, (iv) clinical studies from the early 1950s proposed calcium therapy (at that time called calmonose) for the treatment of alcoholism (O'Brien, 1952; O'Brien 1964). Our data suggest that acamprosate may also act as a calcium therapy and indeed the application of a daily recommended dose of 1998 mg of Campral leads to enhanced calcium plasma levels in alcohol-dependent patients. Furthermore, enhanced calcium plasma levels are associated with better primary efficacy parameters such as time to relapse and cumulative abstinence. What could be a putative mode of action of a calcium bolus on alcohol drinking, seeking, and relapse considering that plasma and brain calcium are tightly regulated? First, given that a calcium bolus can enhance plasma calcium levels, it may thereby also enhance brain calcium levels and affect brain function. This suggestion is supported by population-based studies, which show that calcium in the drinking water correlates with cognitive functions (Emsley *et al*, 2000) and that a low content of calcium in drinking water is a risk factor in Alzheimer dementia (Stutzmann, 2007). Thus, although tightly regulated exogenously applied calcium seems to affect brain function and disease. Therefore, we speculate that acamprosate treatment may enhance brain calcium levels and thereby the activity of calcium-dependent ion channels including the SK2 channels, which may reduce hyperglutamatergic activity and alcohol consumption (Mulholland *et al*, 2011; Hopf *et al*, 2011). Second, in alcohol-dependent patients, impairment in electrolyte regulation is often observed and a calcium bolus might counteract this impairment leading to well being and less alcohol consumption. Whatever the putative mode of action of a calcium bolus may be, our screening panel for the mode of action of the calcium salt of *N*-acetylhomotaurinate argues against a direct effect on glutamate receptors.

Indeed our electrophysiological results obtained in *Xenopus* oocytes where we expressed hNR1A and hNR2B subunits—a subunit composition, which is thought to be predominantly present in the human forebrain (Scherzer *et al*, 1998; Kosinski *et al*, 1998)—show that acamprosate acts not via NMDA receptors. Thus, glycine and glutamate-mediated activation of the receptor could not be substituted even by 1 mM of acamprosate. Further, no shift in affinity was noted in concentration response curves for glycine or glutamate, indicating no apparent antagonist activity in our assay. Rammes *et al* (2001) reported that acamprosate acts as a weak antagonist against NMDA-induced currents in *Xenopus* oocytes expressing NR1A/2B receptors. Testing acamprosate as the calcium salt also adds excess calcium to the preparation. Therefore, Rammes *et al* (2001) tested in addition the effects of equimolar calcium concentrations and found that in four out of the eight cells tested, calcium also decreased NMDA-induced currents and a pronounced effect of acamprosate was only seen in these four cells. Thus, it seems that even the effects of high concentrations of acamprosate on NMDA receptors are not due to acamprosate itself, but rather due to nonspecific effects of calcium ions added (Rammes *et al*, 2001) Altogether, a detailed review of published reports and our data demonstrate that acamprosate exhibits neither agonistic nor antagonistic effects on NMDA receptors. However, there is a limitation to this conclusion, namely that in our *Xenopus* oocyte test system, we expressed only one possible combination (hNR1A/hNR2B) and cannot exclude the possibility that other NMDA receptor subunit compositions would yield different results. Furthermore, our experiments on accumbal slices indicate that NMDA receptor dependent post-synaptic potentials seem to be affected by very high acamprosate concentrations (1 mM). Thus, acamprosate produced a modest nonsignificant decrease in EPSP amplitude in approximately half of the recorded cells. A similar observation was made in an earlier *in vitro* recording study in rat neocortical neurons (Zeise *et al*, 1993).

Harris *et al* (2002) showed that acamprosate has binding and functional characteristics similar to group I mGluR antagonists, in particular to mGluR5. However, in those brain-binding studies in rats, trans-ACPD was used, which is a non-selective ligand at mGluRs. In *Xenopus* oocytes injected with mGluR1 or mGluR5 cRNA, acamprosate did not affect receptor function directly, nor did it alter glutamate responses during co-application (Reilly *et al*, 2008). Here we expressed human mGluR1 and 5 in HEK 293 cells and performed functional calcium flux assays. We then performed patch clamp electrophysiology in rat brain slices to measure effects of mGluR1/5 on resting membrane potential and spike number. Acamprosate had no effect on calcium flux, membrane potential, or the number of action potentials. In summary, neither in expression systems nor in slices from rat brain did acamprosate interfere with mGluR1/5 binding or receptor function.

Taking all of these results into consideration, one can conclude that despite substantive investigation it is not possible to characterize a binding site for acamprosate in the CNS. However, recently it was reported that acamprosate may work via direct or indirect interference with the inhibitory glycine receptor (Chau *et al*, 2010a,b). We have not tested this putative binding site in the present study but earlier electrophysiological experiments in *Xenopus* oocytes expressing different homomeric and heteromeric glycine receptors excluded the possibility that acamprosate is directly binding at inhibitory glycine receptors (Reilly *et al*, 2008). Therefore, we asked ourselves whether acamprosate was actually a biologically active molecule and so we reviewed in detail the US patent 4,355,043 (1982). In this patent, the initial investigators of acamprosate described that various salts of *N*-acetylhomotaurinate produced their effects according to the nature of the counter ion. This would imply that the counter ion could be the biologically active molecule. Therefore, we set out to test the sodium *vs* calcium salt of *N*-acetylhomotaurinate on relapse-like drinking behavior in rats. In rats that had long-term voluntary access to alcohol followed by deprivation for several weeks, the re-presentation of alcohol leads to relapse-like drinking, a temporal increase in alcohol intake over baseline drinking. This robust phenomenon is called the ADE. In recent years, this model has become widely used for examining the efficacy of pharmacological agents in preventing compulsive alcohol consumption and relapse (Spanagel, 2009; Vengeliene *et al*, 2009). Moreover, acamprosate produces a reliable reduction of the ADE (Spanagel *et al*, 1996a; Heyser *et al*, 1998; Lidö *et al*, 2012). Critically, this effect is only observed if the calcium salt of *N*-acetylhomotaurinate or any other calcium salt is applied, which strongly suggests that the calcium counter ion of the acamprosate molecule is the biologically active species. Similar results were obtained in the reinstatement test (Shaham *et al*, 2003; Sanchis-Segura and Spanagel, 2006) where we could show that a cue-induced alcohol-seeking response is only decreased by calcium. Our conclusion was further supported by an independent series of experiments in iP rats. This latter experiment was performed in a blind fashion in yet another laboratory (Lawrence lab); *ie*, the experimenters did not know which substances they applied to their animals. Again the results of these experiments further suggest that calcium loads given by i.p. bolus injections can cause a reduction in excessive alcohol consumption. From a translational perspective, we found a correlation of calcium levels and primary efficacy parameters of acamprosate treatment in alcohol-dependent patients. This analysis was, however, done in a retrospective manner from the German COMBINE study (Kiefer *et al*, 2003) were only a limited number of plasma samples were still available. Clearly, a prospective study should be done to confirm these findings.

Accordingly, we provide convincing evidence that *N*-acetylhomotaurinate is in fact a biologically *inactive* molecule and that the effects of acamprosate described in more than 450 published original investigations and clinical trials can be attributed to calcium. There is now a strong need for a complete independent replication of our findings before withdrawal of acamprosate from the market. In particular, a clinical investigation should be initiated to study the effects of calcium salts in terms of relapse prevention. However, a historical perspective is also important in this respect. Clinical studies from the early 1950s proposed intensive calcium therapy (at that time called calmonose) for the treatment of alcoholism (O'Brien 1952; O'Brien 1964). Although this therapy disappeared from the treatment landscape for unknown reasons, in light

of our new findings, calcium supplements could be easily re-introduced into treatment programs. Furthermore, epidemiological data show that the calcium level in drinking water significantly affects cognitive functions (Emsley *et al*, 2000). For that reason, one could speculate that calcium levels in drinking water and food might also influence excessive alcohol drinking on a population wide scale.

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RS acted as a consultant for XenoPort, Inc. All other authors declare no competing financial interests.

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AUTHOR CONTRIBUTIONS

RS designed experiments, analyzed data, and wrote the manuscript; VV conducted the ADE experiments and analyzed data; BJ, WNF, and MAG led the overall project at XenoPort, Inc., provided Na-AOTA and coordinated all *in vitro* and *in vivo* studies and PK analyses; KG and XZ conducted electrophysiological studies; EVK and AJL did the behavioral work in iP rats; AJL assisted in manuscript writing; FK conducted the clinical study.

REFERENCES

Bachteler D, Economidou D, Danysz W, Ciccocioppo R, Spanagel R (2005). The effects of acamprosate and neramexane on cue-induced reinstatement of ethanol-seeking behavior in rat. *Neuropsychopharmacology* **30**: 1104–1110.

Bell RL, Rodd ZA, Lumeng L, Murphy JM, McBride WJ (2006). The alcohol-preferring P rat and animal models of excessive alcohol drinking. *Addict Biol* **11**: 270–288.

Boismare F, Daoust M, Moore N, Saligaut C, Lhuintre JP, Chretien P *et al* (1984). A homotaurine derivative reduces the voluntary intake of ethanol by rats: are cerebral GABA receptors involved? *Pharmacol Biochem Behav* **21**: 787–789.

Chabenat C, Chretien P, Daoust M, Moore N, Andre D, Lhuintre JP *et al* (1988). Physicochemical, pharmacological and pharmacokinetic study of a new GABAergic compound, calcium acetylhomotaurinate. *Methods Find Exp Clin Pharmacol* **10**: 311–317.

Chau P, Höifödt-Lidö H, Löf E, Söderpalm B, Ericson M (2010a). Glycine receptors in the nucleus accumbens involved in the ethanol intake-reducing effect of acamprosate. *Alcohol Clin Exp Res* **34**: 39–45.

Chau P, Stomberg R, Fagerberg A, Söderpalm B, Ericson M (2010b). Glycine receptors involved in acamprosate's modulation of accumbal dopamine levels: an *in vivo* microdialysis study. *Alcohol Clin Exp Res* **34**: 32–38.

Cowen MS, Adams C, Kraehenbuehl T, Vengeliene V, Lawrence AJ (2005). The acute anti-craving effect of acamprosate in alcohol-preferring rats is associated with modulation of the mesolimbic dopamine system. *Addict Biol* **10**: 233–242.

De Witte P, Littleton J, Parot P, Koob G (2005). Neuroprotective and abstinence-promoting effects of acamprosate: elucidating the mechanism of action. *CNS Drugs* **19**: 517–537.

Emsley CL, Gao S, Li Y, Liang C, Ji R, Hall KS *et al* (2000). Trace element levels in drinking water and cognitive function among elderly Chinese. *Am J Epidemiol* **151**: 913–920.

Erickson CK, Tyler TD, Harris RA (1978). Ethanol: modifications of acute intoxication by divalent cations. *Science* **199**: 1219–1221.

Harris BR, Prendergast MA, Gibson DA, Rogers DT, Blanchard JA, Holley RC *et al* (2002). Acamprosate inhibits the binding and neurotoxic effects of trans-ACPD, suggesting a novel site of action at metabotropic glutamate receptors. *Alcohol Clin Exp Res* **26**: 1779–1793.

Heyser CJ, Schulteis G, Durbin P, Koob GF (1998). Chronic acamprosate eliminates the alcohol deprivation effect while having limited effects on baseline responding for ethanol in rats. *Neuropsychopharmacology* **18**: 125–133.

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

Kiefer F, Jahn H, Tarnaske T, Helwig H, Briken P, Holzbach R *et al* (2003). Comparing and combining naltrexone and acamprosate in relapse prevention of alcoholism: a double-blind, placebo-controlled study. *Arch Gen Psychiatry* **60**: 92–99.

Kosinski CM, Standaert DG, Counihan TJ, Scherzer CR, Kerner JA, Daggett LP *et al* (1998). Expression of N-methyl-D-aspartate receptor subunit mRNAs in the human brain: striatum and globus pallidus. *J Comp Neurol* **390**: 63–74.

Lidö HH, Marston H, Ericson M, Söderpalm B (2012). The glycine reuptake inhibitor Org24598 and acamprosate reduce ethanol intake in the rat; tolerance development to acamprosate but not to Org24598. *Addict Biol* **17**: 897–907.

Litten RZ, Egli M, Heilig M, Cui C, Fertig JB, Ryan ML *et al* (2012). Medications development to treat alcohol dependence: a vision for the next decade. *Addict Biol* **17**: 513–527.

Lynch MA, Littleton JM (1983). Possible association of alcohol tolerance with increased synaptic Ca2+ sensitivity. *Nature* **303**: 175–176.

Madamba SG, Schweitzer P, Zieglgänsberger W, Siggins GR (1996). *Alcohol Clin Exp Res* **20**: 651–658.

Mann K, Kiefer F, Spanagel R, Littleton J (2008). Acamprosate: recent findings and future research directions. *Alcohol Clin Exp Res* **32**: 1105–1110.

Mann K, Bladström A, Torup L, Gual A, van den Brink W (2013). Extending the treatment options in alcohol dependence: a randomized controlled study of as-needed nalmefene. *Biol Psychiatry* **73**: 706–713.

Mark TL, Kassed CA, Vandivort-Warren R, Levit KR, Kranzler HR (2009). Alcohol and opioid dependence medications: prescription trends, overall and by physician specialty. *Drug Alcohol Depend* **99**: 345–349.

Mason BJ, Heyser CJ (2010). Acamprosate: a prototypic neuromodulator in the treatment of alcohol dependence. *CNS Neurol Disord Drug Targets* **9**: 23–32.

Mulholland PJ, Becker HC, Woodward JJ, Chandler LJ (2011). Small conductance calcium-activated potassium type 2 channels regulate alcohol-associated plasticity of glutamatergic synapses. *Biol Psychiatry* **69**: 625–632.

O'Brien CC (1952). Experimental evidence in the treatment of alcoholism by intensive calcium therapy. *J Am Osteopath Assoc* **51**: 393–394.

O'Brien CC (1964). Intensive calcium therapy as an initial approach to the psychotherapeutic relationship in the rehabilitation of the compulsive drinker. *J Psychol* **57**: 125–129.

Rammes G, Mahal B, Putzke J, Parsons C, Spielmanns P, Pestel E *et al* (2001). The anti-craving compound acamprosate acts as a weak NMDA-receptor antagonist, but modulates NMDA-receptor subunit expression similar to memantine and MK-801. *Neuropharmacology* **40**: 749–760.

Reilly MT, Lobo IA, McCracken LM, Borghese CM, Gong D, Horishita T *et al* (2008). Effects of acamprosate on neuronal receptors and ion channels expressed in Xenopus oocytes. *Alcohol Clin Exp Res* **32**: 188–196.

Rehm J, Mathers C, Popova S, Thavorncharoensap M, Teerawattananon Y, Patra J (2009). Global burden of disease and injury and economic cost attributable to alcohol use and alcohol-use disorders. *Lancet* **373**: 2223–2233.

Rösner S, Hackl-Herrwerth A, Leucht S, Lehert P, Vecchi S, Soyka M (2010). Acamprosate for alcohol dependence. *Cochrane Database Syst Rev* **8**: CD004332.

Sanchis-Segura C, Spanagel R (2006). Behavioural assessment of drug-reinforcement and addictive features in rodents: an overview. *Addict Biol* **11**: 2–38.

Scherzer CR, Landwehrmeyer GB, Kerner JA, Counihan TJ, Kosinski CM, Standaert DG *et al* (1998). Expression of N-methyl-D-aspartate receptor subunit mRNAs in the human brain: hippocampus and cortex. *J Comp Neurol* **390**: 75–90.

Shaham Y, Shalev U, Lu L, De Wit H, Stewart J (2003). The reinstatement model of drug relapse: history, methodology and major findings. *Psychopharmacology (Berl)* **168**: 3–20.

Spanagel R, Hölter SM, Allingham K, Landgraf R, Zieglgänsberger W (1996a). Acamprosate and alcohol: I. Effects on alcohol intake following alcohol deprivation in the rat. *Eur J Pharmacol* **305**: 39–44.

Spanagel R, Putzke J, Zieglgänsberger W, Stefferl A, Schöbitz B (1996b). Acamprosate and alcohol: II. Effects on alcohol withdrawal in the rat. *Eur J Pharmacol* **305**: 45–50.

Spanagel R, Pendyala G, Abarca C, Zghoul T, Sanchis-Segura C, Magnone MC *et al* (2005). The clock gene Per2 influences the glutamatergic system and modulates alcohol consumption. *Nat Med* **11**: 35–42.

Spanagel R, Kiefer F (2008). Drugs for relapse prevention of alcoholism: ten years of progress. *Trends Pharmacol Sci* **29**: 109–115.

Spanagel R (2009). Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiol Rev* **89**: 649–705.

Spanagel R, Bartsch D, Brors B, Dahmen N, Deussing J, Eils R *et al* (2010). An integrated genome research network for studying the genetics of alcohol addiction. *Addict Biol* **15**: 369–379.

Spanagel R, Vengeliene V (2013). New pharmacological treatment strategies for relapse prevention. *Curr Top Behav Neurosci* **13**: 583–609.

Stutzmann GE (2007). The pathogenesis of Alzheimers disease is it a lifelong 'calciumopathy'? *Neuroscientist* **13**: 546–559.

Tsai G, Gastfriend DR, Coyle JT (1995). The glutamatergic basis of human alcoholism. Drugs for relapse prevention of alcoholism: ten years of progress. *Am J Psychiatry* **152**: 332–340.

Umhau JC, Momenan R, Schwandt ML, Singley E, Lifshitz M, Doty L *et al* (2010). Effect of acamprosate on magnetic resonance spectroscopy measures of central glutamate in detoxified alcohol-dependent individuals: a randomized controlled experimental medicine study. *Arch Gen Psychiatry* **67**: 1069–1077.

Umhau JC, Schwandt ML, Usala J, Geyer C, Singley E, George DT *et al* (2011). Pharmacologically induced alcohol craving in treatment seeking alcoholics correlates with alcoholism severity, but is insensitive to acamprosate. *Neuropsychopharmacology* **36**: 1178–1186.

Vengeliene V, Celerier E, Chaskiel L, Penzo F, Spanagel R (2009). Compulsive alcohol drinking in rodents. *Addict Biol* **14**: 384–396.

Zeise ML, Kasparov S, Capogna M, Zieglgänsberger W (1993). Acamprosate (calciumacetylhomotaurinate) decreases postsynaptic potentials in the rat neocortex: possible involvement of excitatory amino acid receptors. *Eur J Pharmacol* **231**: 47–52.

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