

## ARCHIVAL REPORT

# Glycine Transporter-1 Blockade Leads to Persistently Reduced Relapse-like Alcohol Drinking in Rats

Valentina Vengeliene, Fernando Leonardi-Essmann, Wolfgang H. Sommer, Hugh M. Marston, and Rainer Spanagel

**Background:** Residual dysfunction of multiple neurotransmitter systems due to chronic alcohol use is likely responsible for the occurrence of compulsive alcohol seeking during abstinence and relapse behavior. There is increasing evidence that glycine, which activates both glycine and *N*-methyl-D-aspartate receptors, contributes to excessive alcohol consumption. We therefore hypothesized that the blockade of glycine transporter 1 might interfere with compulsive alcohol consumption and relapse behavior.

**Methods:** We used our animal model of alcoholism—long-term alcohol consumption with repeated deprivation phases in rats—to study the effects of a selective blocker of glycine transporter 1 Org25935. The abstinence-promoting drug acamprosate was used as a reference compound. Subsequently, we examined alterations in dorsal striatal gene expression caused by chronic ethanol (EtOH) consumption, focusing on glycinergic and glutamatergic signaling-related genes. Gene expression profiles of Org25935-treated EtOH-drinking rats were compared with vehicle-treated EtOH-drinking versus age-matched EtOH-naive rats.

**Results:** We found that repeated treatment with Org25935 reduced compulsive relapse-like drinking without the development of tolerance. Importantly, these antirelapse properties were maintained for at least 6 weeks in a treatment-free period. This persistent effect was paralleled by a reversal of altered expression levels of a set of glycinergic and glutamatergic signaling-related genes to the levels found in EtOH-naive control rats.

**Conclusions:** This study shows that treatment of rats with Org25935 leads to a reduction of compulsive alcohol consumption and relapse-like drinking behavior—an effect that persists into treatment-free periods. This long-term antirelapse effect might result from a restoration of normal glycinergic and glutamatergic signaling function.

**Key Words:** Alcohol addiction, alcohol deprivation effect, gene expression profiling, glycine transporter 1, relapse

The World Health Organization ranks alcohol use as one of the primary causes of the global burden of disease in industrialized countries. In many cases, chronic alcohol use can lead to addictive behaviors. Addiction is characterized as a behavioral syndrome with compulsive alcohol use and chronic relapses that can occur even after years of abstinence. In recent years, a variety of new behavioral and pharmacological-based treatment strategies have been developed to reduce compulsive alcohol drinking and relapse (1,2).

One new concept in the field of treatment development is to interfere with the glycinergic system. Activity of the dopaminergic reinforcement system is largely under the control of several neurotransmitter systems that are targeted by alcohol directly, including strychnine sensitive glycine receptors (GlyRs) and *N*-methyl-D-aspartate (NMDA) receptors (NMDARs), which also have a glycine-binding site (3–5). Thus, ethanol acts on specific residues in the transmembrane domains (6), as well as on the extracellular domain of GlyRs (7). The GlyRs in the nucleus accumbens have been defined as an access point for ethanol to the brain reward system (3) and it has been demonstrated in alcohol-drinking rats that bilateral ac-

cumbal glycine perfusion via a microdialysis probe produced a significant increase in accumbal dopamine overflow and a decrease in alcohol preference and intake (8). Conversely, bilateral infusion of the competitive GlyR antagonist strychnine into the nucleus accumbens significantly decreased accumbal dopamine levels and significantly increased alcohol preference and intake (8). These results suggest that the availability of extracellular glycine is crucial for regulating alcohol consumption—an effect that seems to be mediated via accumbal GlyRs but possibly also by NMDARs. In fact, the blockade of the glycine binding site on NMDARs with L-701.324 showed a complete substitution for ethanol in a discrimination task (9,10), produced a dose-dependent inhibition of alcohol withdrawal signs (11,12), prevented the acquisition of the rewarding properties of alcohol as measured in the conditioned place preference procedure (13), and dose-dependently reduced ethanol-seeking behavior (14) and relapse-like drinking behavior (15). These findings led to the assumption that regulating the extracellular glycine pool by, for example, selective transporter blockade should affect alcohol consumption via GlyRs and NMDARs.

Two glycine transporters—glycine transporter 1 (GlyT1) and glycine transporter 2 (GlyT2)—have been identified and characterized. The glial transporter GlyT1 catalyzes the removal of glycine from the synaptic cleft, whereas GlyT2 is required for the reuptake of glycine into nerve terminals, thereby allowing for neurotransmitter reloading of synaptic vesicles (16). At glycinergic synapses, GlyT1 shortens the duration of the postsynaptic response by lowering glycine concentrations at inhibitory GlyRs, and its genetic inactivation or pharmacological blockade results in glycinergic overinhibition due to the sustained activation of GlyRs (16). Thus, the extracellular glycine pool is directly regulated by GlyT1 activity. Hence, GlyT1 is a key regulator of both glycinergic inhibitory and glutamatergic excitatory neurotransmission and blockade of this transporter should therefore affect alcohol consumption. Indeed, application of a selective GlyT1 inhibitor in alcohol-drinking rats

From the Department of Psychopharmacology (VV, FL-E, WHS, RS), Central Institute of Mental Health, University of Heidelberg, Mannheim, Germany; and Department of Pharmacology (HMM), Merck Sharp & Dohme, Newhouse, Lanarkshire, United Kingdom.

Authors VV and FL-E contributed equally to this work.

Address correspondence to Valentina Vengeliene, Ph.D., University of Heidelberg, Central Institute of Mental Health, Department of Psychopharmacology, J5, 68159 Mannheim, Germany; E-mail: [valentina.vengeliene@zi-mannheim.de](mailto:valentina.vengeliene@zi-mannheim.de).

Received Apr 1, 2010; revised May 17, 2010; accepted May 19, 2010.

resulted in reduced alcohol intake and preference (17). In the aforementioned study, *cis*-N-methyl-N-(6-methoxy-1-phenyl-1,2,3,4-tetrahydronaphthalen-2-ylmethyl)amino-methylcarboxylic acid hydrochloride (Org25935), a compound that has been developed for clinical purposes that easily passes the blood-brain barrier, was used. The Org25935 exerts its main action on GlyT1 with negligible action on GlyT2 and raises extracellular glycine levels after systemic administration (18). Taking previous results (3), we hypothesized that Org25935 might also interfere with compulsive alcohol drinking and relapse behavior.

To this end, we investigated the effects of the GlyT1 inhibitor Org25935 in an animal model that is used for target definition for putative anticraving/antirelapse compounds, namely long-term alcohol consumption with repeated deprivation phases (19,20). As a positive control treatment, we used acamprosate, known as an abstinence-promoting drug that is widely used in the treatment of alcohol addiction (1,2). Acamprosate reduces relapse-like drinking behavior in our animal model (21) and it dampens a hyperglutamatergic state in the alcohol dependent brain and thereby reduces the risk of relapse (2,22). The model of long-term alcohol consumption with repeated deprivation phases reflects compulsive alcohol drinking and relapse-like behavior (20). Relapse-like drinking behavior in animals is characterized by the alcohol deprivation effect (ADE). For example, following a period of alcohol abstinence, animals considerably but temporally increase voluntary alcohol intake compared with basal consumption levels. Following repeated deprivation phases, the ADE is characterized by an increased and compulsive demand for alcohol that clearly dissociates from normal drinking behavior (19,20). This model has good predictive validity and, as such, has been repeatedly used to identify new putative antirelapse compounds (2,5).

In the course of the present study, we observed a pronounced and persistent antirelapse effect of Org25395 in our rat model of long-term alcohol consumption with repeated deprivation phases. Hence, we hypothesized that residual dysfunction of multiple neurotransmitter systems induced by long-term alcohol exposure might, in part, be reversed by Org25395 treatment. Given that a selective glycine transporter blockade should affect alcohol consumption via GlyRs and NMDARs, we performed a hypothesis-driven gene expression profiling. For this purpose, we designed a custom made microarray containing glutamate and glycine transmission-related genes. With this chip, we were able to screen for about 200 selected genes, including sets of 1) presynaptic genes (vesicles, docking, and exocytosis associated genes); 2) postsynaptic genes (receptors, anchoring, signal transduction, and transcription associated genes); and 3) perisynaptic genes (glial transporters and cofactors associated genes). This approach of a hypothesis-driven gene expression profiling dramatically reduces the number of statistical tests compared with a whole transcriptome analysis and thereby provides increased statistical power (23). The targeted gene expression profiling was conducted in brain tissue derived from the dorsal striatum of three groups of rats: an ethanol (EtOH)-naive age-matched group (EtOH-naive) versus two EtOH-drinking groups treated with either vehicle (EtOH-drinking + vehicle) or Org25935 (EtOH-drinking + Org25935). The dorsal striatum plays a critical role in compulsive alcohol consumption (20), and in long-term alcohol-drinking rats with repeated deprivation phases, more pronounced changes in gene expression are seen in this brain site compared with other areas such as the nucleus accumbens core and shell and prefrontal cortex (5,24,25).

## Methods and Materials

### Animals

Fifty-eight 2-month-old male Wistar rats (from our own breeding colony at the Central Institute of Mental Health, Mannheim, Germany) were used for the ADE and gene expression experiments. For further information, see Supplementary Methods and Materials in Supplement 1.

### Long-Term Alcohol Self-Administration with Repeated Deprivation Phases

The long-term voluntary alcohol-drinking procedure excluding all deprivation phases lasted a total of 52 weeks (see Supplementary Methods and Materials in Supplement 1 for detailed information).

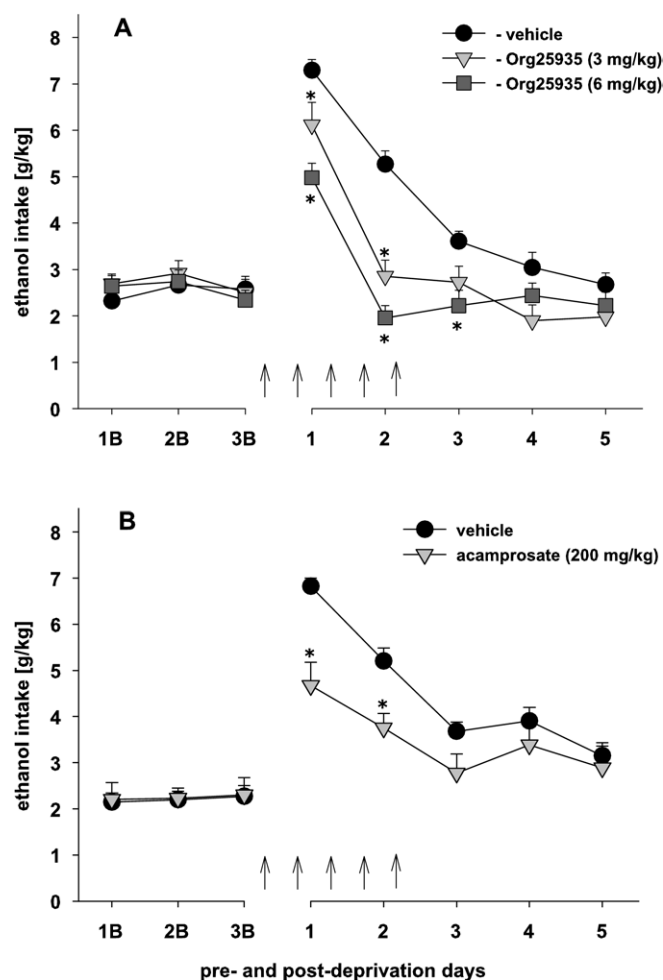
The pharmacological studies were introduced at the end of the eighth alcohol deprivation procedure during the 53rd week of voluntary access to alcohol. To study the effects of Org25935 and acamprosate, rats were divided into four groups ( $n = 7-15$ , see Figure 1A,B legend for the exact number of rats used in each group) in such a way that the mean baseline total alcohol intake was approximately the same in each group (i.e.,  $\sim 2.3-3.0$  g/kg per day). Baseline drinking was measured daily for 1 week. After the last day of baseline measurement, the alcohol bottles were removed from the cages leaving the rats with free access to food and water for 20 days. Thereafter, each animal was subjected to a total of five intraperitoneal injections (starting at 7:00 PM with 12-hour intervals) of either vehicle or Org25935 (3 mg/kg and 6 mg/kg). The fourth group of rats received acamprosate (200 mg/kg) as a reference compound (21). Acamprosate intraperitoneal injections were given in the same way as Org25935 injections. The alcohol bottles were reintroduced after the second injection (at  $\sim 9:00$  AM on the 21st day of alcohol deprivation) and the occurrence of an ADE was determined. Total ethanol (grams per kilogram of body weight per day) and water intake (milliliters per kilogram of body weight per day) were measured daily at  $\sim 9:00$  AM for a subsequent week. Each rat's body weight was recorded 24 hours before the first injection and 12 hours after the last injection.

Subsequent ADE measurements were continued with three rat groups (eight control rats, seven rats treated with 6 mg/kg of Org25935, and seven rats treated with 200 mg/kg of acamprosate) to test for the development of tolerance and for studying persistent treatment effects in a drug-free period. For this purpose, all rats were deprived from alcohol two additional times following the 56th and 60th weeks of access to alcohol. At the end of these two subsequent abstinence phases, rats were subjected to repeated vehicle, 6 mg/kg of Org25935 or 200 mg/kg of acamprosate, injections. Drug administration was performed in the same manner as described before. One further final deprivation phase was then introduced after the 64th week of access to alcohol. This final deprivation phase lasted for 2 weeks and the alcohol bottles were then reintroduced without concurrent drug application (week 65 treatment-free ADE), to see possible long-lasting effects of drug treatment on relapse-like drinking (see Figure 2A for the experimental design).

See Supplementary Methods and Materials in Supplement 1 for detailed information on drugs and home cage locomotor activity measurements by the Mouse-E-motion system (Infra-e-motion, Henstedt-Ulzburg, Germany).

### Statistics for the Behavioral Studies

For data analysis, the statistical package Statistica was used (StatSoft, Tulsa, Oklahoma). Data obtained from ADE measurements (total alcohol and fluid intake, water intake) and locomotor



**Figure 1.** Total ethanol intake (g/kg/day) before and after an alcohol deprivation period of 3 weeks. Arrows indicate the administration of either (A) vehicle ( $n = 15$ ), 3 mg/kg of Org25935 ( $n = 7$ ), and 6 mg/kg of Org25935 ( $n = 14$ ); or (B) vehicle ( $n = 8$ ) and 200 mg/kg ( $n = 8$ ) of acamprosate. The last 3 days measurements of ethanol intake are given as baseline drinking. Data are presented as means  $\pm$  SEM. \*Indicates significant differences from the control vehicle group,  $p < .05$ . B, baseline drinking.

activity were analyzed using a two-way analysis of variance (ANOVA) with repeated measures (factors were treatment and time). Data analysis regarding the effects of treatment on the change in the rat body weight was performed using either independent two-tailed  $t$  test or a one-way ANOVA (factor was treatment). Whenever significant differences were found, post hoc student Newman-Keul tests were performed. The chosen level of significance was  $p < .05$ .

### Gene Expression Profiling

**Rat Brain Collection and Dissection.** Gene expression profiling was performed in male Wistar rats following the 65th week of access to alcohol following a long-lasting treatment-free period (which means that the last vehicle or Org25935 treatment was 7 weeks ago). On the last day of the drinking procedure, all rats were deprived from alcohol for 48 hours (late withdrawal phase) to ensure that blood ethanol concentrations were zero (to avoid direct acute alcohol effects on gene expression). For comparison, six age-matched Wistar rats underwent the exact same handling procedures for the entire time of the experiment. Between 10:00 AM and

11:00 AM, all rats were sacrificed. Brains were quickly removed, submerged for 3 minutes in  $-40^{\circ}\text{C}$  isopentane (Sigma-Aldrich Co., St. Louis, Missouri) and stored at  $-80^{\circ}\text{C}$ . For dissection, brains were sliced in coronal sections of 120  $\mu\text{m}$  in a Leica CM3000 Cryostat (Leica, Bensheim, Germany). The caudate putamen was punched out and collected into vials and stored at  $-80^{\circ}\text{C}$ .

See Supplementary Methods and Materials in Supplement 1 for detailed information on total RNA isolation and quality control; chip design, target labeling, array hybridization, scanning, and quality control; data mining; in silico pathway analysis; and relative quantification by real-time reverse transcription polymerase chain reaction (qRT-PCR).

## Results

### Effect of the Administration of Org25935 on ADE Measurements

Following the reintroduction of alcohol solutions after the eighth deprivation phase, the vehicle-treated group showed a typical increase in alcohol consumption, indicating the occurrence of an ADE (Figure 1A,B). This increase was not different from that observed during the first seven deprivation phases (data not shown). With respect to the pharmacological treatment, a two-way ANOVA for repeated measures revealed significantly different alcohol intake after a deprivation phase in all animal groups compared with basal drinking [factor day:  $F(7,231) = 75.1$ ,  $p < .0001$  and  $F(7,98) = 50.8$ ,  $p < .0001$  for Org25935 and acamprosate treatment groups, respectively] (Figure 1A,B). The Org25935 treatment dose-dependently reduced expression of the ADE, while alcohol intake was still increased during the first postdeprivation day (although to a significantly lower extent than the vehicle control group); it dropped to baseline levels starting from the second day on. Hence, a two-way ANOVA displayed a significantly different alcohol intake between vehicle- and Org25935-treated animal groups [factor treatment group  $\times$  day interaction effect:  $F(14,231) = 9.3$ ,  $p < .0001$ ], showing that the treatment of rats with Org25935 decreased ADE (Figure 1A). Similar results were obtained with acamprosate treatment. The acamprosate-treated animal group still increased their alcohol consumption; however, it was significantly lower than that in the control animal group [factor treatment group  $\times$  day interaction effect:  $F(7,98) = 5.2$ ,  $p < .0001$ ] (Figure 1B). In contrast, water intake during the first 5 postabstinence days was significantly higher in both acamprosate- and Org25935-treated animal groups compared with vehicle-treated rats [factor treatment group  $\times$  day interaction effect:  $F(10,165) = 2.5$ ,  $p < .01$  and  $F(5,70) = 3.0$ ,  $p < .05$  for Org25935 and acamprosate treatment groups, respectively], suggesting that the effect of treatment was selective for alcohol (Figure S1A,B in Supplement 1). Detailed statistical information on total fluid intake during all ADE measurements is provided under Supplementary Results in Supplement 1. It should be mentioned that 6 mg/kg of Org25935 treatment led to a nonsignificant loss of 1% of body weight, showing that food intake or metabolism was not considerably altered during the treatment days ( $p = .09$ ). Contrary, treatment with acamprosate significantly reduced rats' body weight by 2.7% [factor treatment group:  $t(14) = 4.9$ ,  $p < .001$ ].

Locomotor activity data were analyzed using recordings of 12-hour postinjection intervals that corresponded to the rats' active phase. Overall, there was a general reduction in home-cage activity seen in all animal groups, which was likely caused by alcohol intoxication. Two-way ANOVA revealed a significant change in activity of Org25935-treated rats when compared with the vehicle-treated rats [factor treatment group:  $F(2,33) = 4.2$ ,  $p < .05$ ; treatment group  $\times$  day interaction effect:  $F(10,165) = 4.6$ ,  $p < .0001$ ]. Post hoc



× treatment for locomotor activity observed with repeated ADE measurements [ADE after the 53rd week of access to alcohol:  $F(10,95) = 11.2, p < .0001$ ; ADE after the 56th week of access to alcohol:  $F(10,95) = 1.1, p = .32$ ; and ADE after the 61st week of access to alcohol:  $F(10,95) = 1.8, p = .07$ ].

### Expression Profiling of Glutamate and Glycine

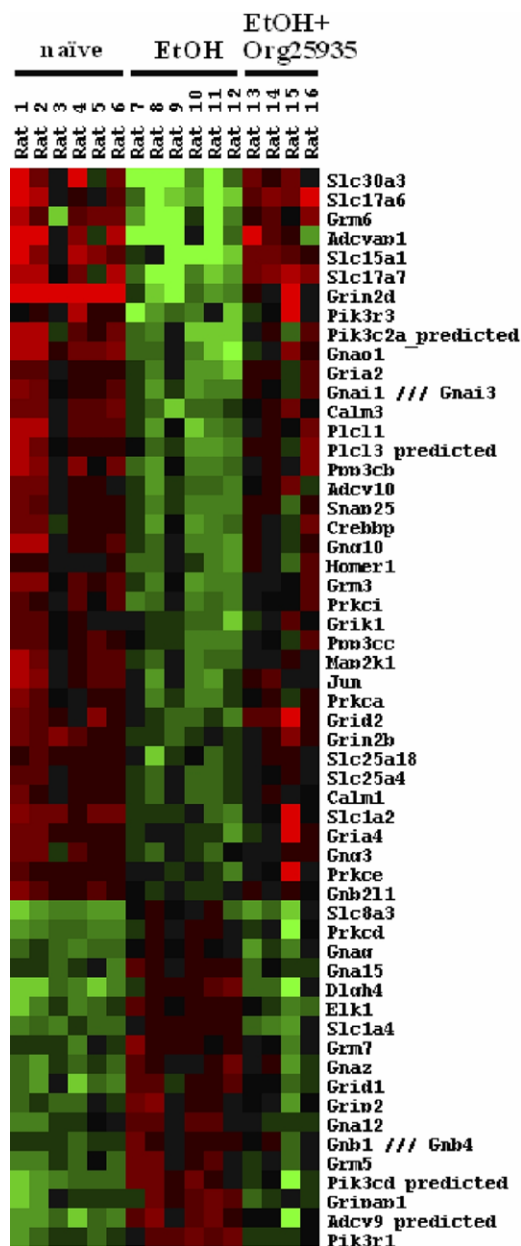
#### Transmission-Related Genes

Three age-matched groups of rats were analyzed: EtOH-naive versus EtOH-drinking + vehicle versus EtOH-drinking + Org25935 treatment. Out of 202 glutamate and glycine transmission-related genes in the caudate putamen, 168 genes showed significant alteration after Benjamini-Hochberg correction for multiple testing in the EtOH-drinking + vehicle group versus EtOH-naive control group (Table S3 in Supplement 1). Out of these 168 genes, 56 genes were also significantly altered and reversed to levels seen in the EtOH-naive control group by Org25935 treatment (Figure 3). In comparison with the EtOH-naive control group and EtOH-drinking + Org25935 groups, 38 genes were downregulated in the EtOH-drinking + vehicle group, with fold change (FC) down to  $-7.8$ . The remaining 18 genes were upregulated in the EtOH-drinking group when compared with EtOH-naive and EtOH-drinking + Org25935 groups with FCs up to 1.9. Ten genes were chosen for further confirmation by qRT-PCR. Two out of these 10 genes—*N*-methyl-D-aspartate 2D (*Grin2d*) and phosphatidylinositol 3-kinase catalytic delta polypeptide (predicted) (*Pik3c2a-predicted*)—could not be assessed due to technical problems with the qRT-PCR. However, the remaining eight genes—one of them shows a significant upregulation in the EtOH-drinking group, whereas the remaining seven genes show a significant downregulation (Figure 4; for *p* values, see Table S5 in Supplement 1)—could be confirmed and there were remarkable similarities between the FC values obtained by qRT-PCR and microarray screening (Table S5 in Supplement 1).

In the EtOH-drinking + Org25935 treated group, the altered gene expression profile could be reversed to levels seen in the EtOH-naive age-matched control group. As presented in the heat map in Figure 3, the expression profiles of the 56 genes that were either upregulated or downregulated in the EtOH-drinking + vehicle treated group were reversed to the levels seen in the EtOH-naive age-matched control group. This overall picture was again confirmed by qRT-PCR. Thus, the altered expression levels of the chosen eight genes were completely reversed to those seen in the EtOH-naive age-matched control group (Figure 4).

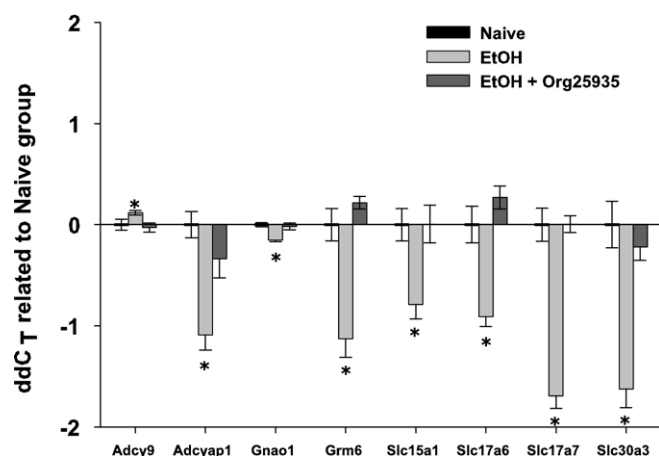
### Discussion

In the present study, we demonstrated that administration of the glycine transporter GlyT1 blocker Org25935 caused a significant dose-dependent reduction of relapse-like alcohol consumption in male Wistar rats. This effect was comparable with that seen in acamprosate-treated rats. Following both acamprosate and Org25935 treatment, water intake during ADE measurement was significantly increased, indicating the selectivity of these treatments toward alcohol consumption. However, the effect of the reference compound acamprosate diminished with repeated application. This phenomenon of tolerance development to the effects of acamprosate has already previously been reported in two other rat models of excessive alcohol consumption (26). In contrast, repeated treatment of Org25935 was able to maintain reduced post-abstinence drinking without the development of tolerance. The antirelapse effects of Org25935 even persisted for at least 6 weeks into a treatment-free period, suggesting that a subchronic treatment regimen is sufficient to induce long-lasting remission with



**Figure 3.** Heat map of microarray intensities of genes that were differentially regulated by long-term alcohol consumption and that were reversed to the levels seen in ethanol (EtOH) age-matched control rats by Org25935 treatment. The EtOH-naive group consists of rats numbers 1 to 6, EtOH-drinking + vehicle group consists of rats numbers 7 to 12, and EtOH-drinking + Org25935 group consists of rats numbers 13 to 16. All gene expression levels shown in the figure are statistically different when compared EtOH-naive versus EtOH-drinking + vehicle group, and EtOH-drinking + vehicle group versus EtOH-drinking + Org25935 group. Color scale represents level of expression for a particular gene with green for lower levels and red for higher levels of expression. EtOH, ethanol.

respect to compulsive alcohol consumption. Such a persistent treatment effect is usually not observed with other antirelapse compounds (2) and can only be explained by long-lasting restoration of molecular changes that have been induced by chronic alcohol consumption. In fact, assuming that the primary action of Org25935 is on glycinergic and glutamatergic neurotransmission, we conducted a hypothesis-driven gene expression profiling and



**Figure 4.** Relative quantification by real-time reverse transcription polymerase chain reaction confirmations of some gene expression levels that were significantly altered by alcohol drinking and reversed by Org25935 treatment. The listed genes are adenylate cyclase 9 (predicted) (*Adcy9\_predicted*); adenylate cyclase activating polypeptide 1 (*Adcyap1*); guanine nucleotide binding protein, alpha o (*Gnao1*); metabotropic glutamate receptor 6 (*Grm6*); oligopeptide transporter, member 1 (*Slc15a1*); sodium-dependent inorganic phosphate cotransporter, member 6 (*Slc17a6*); sodium-dependent inorganic phosphate cotransporter, member 7 (*Slc17a7*); and zinc transporter, member 3 (*Slc30a3*). Asterisks indicate significant differences from both the ethanol-naive age-matched control group and the ethanol-drinking Org25935 treated group. ddCT, relative difference of cycle thresholds; EtOH, ethanol.

were able to demonstrate that long-lasting altered expression levels of 56 genes in alcohol drinking rats were reversed by Org25935 treatment to the same levels as those seen in alcohol-naive age-matched control rats. In conclusion, Org25935 has long-lasting antirelapse properties after subchronic application and this effect can be explained by a restoration of molecular changes induced by chronic alcohol consumption. It should be noted that both compounds Org25935 and acamprostate exhibited a short-lasting locomotor-reducing effect that diminished with repeated drug use, suggesting only a minor unspecific influence on alcohol consumption.

In the central nervous system, glycine is cleared from the extracellular space by two distinct glycine transporters, GlyT1 and GlyT2. Glycine transporter 2 is found in caudal brain regions that receive inhibitory glycinergic innervation, such as the spinal cord, brainstem, and cerebellum. In contrast, GlyT1 is detected in both caudal and rostral regions of the brain, such as cortex, basal ganglia, hippocampus, and thalamus (27). These rostral areas are known as having none to moderate expression of strychnine-sensitive GlyRs (28) but exhibit rich expression of NMDARs (29). Glycine and D-serine are known as obligatory co-agonists at the NMDAR, occupying the strychnine-insensitive site on the NR1 subunit (30,31). This led to the assumption that there is an association of GlyT1 with NMDAR-mediated glutamatergic neurotransmission (32). Thus, behavioral effects caused by the administration of GlyT1 blockers might be induced by increased activity of both GlyRs and NMDARs; the antirelapse-like effects of Org25935 observed in the present study are likely mediated via both receptors. Several previous studies performed by Söderpalm *et al.* (33) and Chau *et al.* (34) have suggested a significant role of GlyRs within the mesocorticolimbic dopaminergic system as crucial to the regulation of excessive alcohol consumption. Both mesocorticolimbic and nigrostriatal dopaminergic systems are under tight control of NMDAR activity (35). An important role of both dopamine and NMDARs in mediating the ADE has repeatedly been demonstrated as well (5,15,24).

Given that both GlyRs and NMDARs are primary targets of alcohol (4,6,7,36), are important in mediating excessive alcohol intake and relapse-like behavior (5,15,33,34), and are affected by glycine transporter blockade (16), we hypothesized dramatic alterations in gene expression profiles related to glycinergic and glutamatergic signaling. We were especially interested in gene expression profiles derived from the dorsal striatum, as our previous work has indicated that following long-term alcohol consumption with repeated deprivation phases, more pronounced transcriptional changes occur in the dorsal striatum in comparison with other brain areas (5,24,25). In addition, given that the dorsal striatum plays a critical role in mediating compulsive behavior (37,38), this brain area has been also suggested to mediate the compulsatory component of excessive alcohol drinking and relapse behavior (20). Our gene expression profiling experiment showed that among 202 genes related to glycine and glutamatergic signaling, 168 of them were affected by chronic alcohol consumption, and 56 of those returned to expression levels seen in alcohol-naive rats following repeated administration of Org25935. To further examine the relationship of Org25935 treatment-responsive genes, an *in silico* analysis was performed, which revealed a functional network pointing to a critical role of adenylate cyclase activating polypeptide 1 (*Adcyap1*) in mediating the effect of Org25935 (Figure S4 in Supplement 1). The *Adcyap1* encodes a neuropeptide, also known as pituitary adenylate cyclase-activating polypeptide (PACAP), with highly pleiotropic actions, including modulation of the glutamatergic system (39). Thus, PACAP activates the adenylate cyclase and protein kinase C signaling pathways and extracellular signal-regulated kinases 1 and 2 cascades, thereby inducing expression of many transcription factors and Homer protein homolog 1, which, in turn, interact with various glutamate receptors (39). Furthermore, neuronal-derived PACAP regulates glutamate turnover via expression of glial glutamate transporters, including solute carrier family 1 member 2 or glutamate transporter subtype 1 (40). Furthermore, PACAP directly affects the signaling properties of the NMDAR, and this effect is likely mediated through the glycine binding site, as the interaction takes place at nearly 20 times lower concentrations of glycine than of NMDA (41). Given these effects of PACAP on glutamatergic signaling, it is not surprising that PACAP-knockout mice show increased alcohol consumption (42), a finding consistent with our data demonstrating a strong downregulation of striatal *Adcyap1* in long-term alcohol-drinking rats. Although the PACAP/glutamate link might be just one important element in mediating the antirelapse properties of Org25935, our molecular findings give general support to the hypothesis that NMDAR signaling triggered by extracellular glycine levels is altered following long-term alcohol consumption and that a partial restoration of this neurotransmitter system may underlie the antirelapse effects of Org25935.

In conclusion, our study has demonstrated long-lasting antirelapse properties of Org25935. Together with previous results obtained in selected high-alcohol-drinking rats that showed reduced alcohol intake and preference following Org25935 treatment (17), a prospective, double-blind, placebo-controlled trial investigating the efficacy and safety of Org25935 in relapse prevention in subjects with alcohol dependence is justified.

*Our work is supported by the German Federal Ministry of Education through the Research National Genome Research Network initiative (FKZ 01GS08152) and the European Commission (FP6, PHECOMP #LHSM-CT-2007-037669). For more information, see <http://www.phecomp.com>.*

*VV was involved in the study design, performed the behavioral studies, did the data analysis, and wrote a first version of the manu-*

script. FL-E was involved in the study design of the molecular work, performed the molecular studies, did the data mining and data analysis of the gene expression results, and helped in data interpretation. WS did the *in silico* analysis. HMM initiated the study on behalf of Organon, was involved in planning and study design as well as final interpretation and data presentation. RS was involved in study design, data analysis, and manuscript writing.

We thank Sabrina Koch and Elisabeth Röbel for excellent technical assistance and Anna Molander for assistance with drug administration. We also thank Rick E. Bernardi for proofreading this manuscript.

RS disclosed having received a research grant from Organon on studying the effects of Org25395 on alcohol craving and relapse. VV, FL-E, and WHS reported no biomedical financial interests or potential conflicts of interest. HMM was a full time employee of Organon laboratories Ltd (Current position: Section Head - Psychopharmacology at Merck Sharp & Dohme).

[ClinicalTrials.gov](http://clinicaltrials.gov): A Prospective, Double-Blind, Placebo-Controlled Trial Investigating the Efficacy and Safety of Org 25935 in Relapse Prevention in Subjects With Alcohol Dependence; <http://clinicaltrials.gov/show/NCT00764660>; NCT00764660.

Supplementary material cited in this article is available online.

- Heilig M, Egli M (2006): Pharmacological treatment of alcohol dependence: Target symptoms and target mechanisms. *Pharmacol Ther* 111: 855–876.
- Spanagel R, Kiefer F (2008): Drugs for relapse prevention of alcoholism—10 years of progress. *Trends Pharmacol Sci* 29:109–115.
- Molander A, Söderpalm B (2005): Accumbal strychnine-sensitive glycine receptors: An access point for ethanol to the brain reward system. *Alcohol Clin Exp Res* 29:27–37.
- Vengeliene V, Bilbao A, Molander A, Spanagel R (2008): Neuropharmacology of alcohol addiction. *Br J Pharmacol* 154:299–315.
- Spanagel R (2009): Alcoholism—a systems approach from molecular physiology to addictive behaviour. *Physiol Rev* 89:649–705.
- Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, *et al.* (1997): Sites of alcohol and volatile anaesthetic action on GABA(A) and glycine receptors. *Nature* 389:385–389.
- Crawford DK, Trudell JR, Bertaccini EJ, Li K, Davies DL, Alkana RL (2007): Evidence that ethanol acts on a target in loop 2 of the extracellular domain of alpha1 glycine receptors. *J Neurochem* 102:2097–2109.
- Molander A, Löf E, Stomberg R, Ericson M, Söderpalm B (2005): Involvement of accumbal glycine receptors in the regulation of voluntary ethanol intake in the rat. *Alcohol Clin Exp Res* 29:38–45.
- Kotlinska J, Liljequist S (1997): The NMDA/glycine receptor antagonist, L-701.324, produces discriminative stimuli similar to those of ethanol. *Eur J Pharmacol* 332:1–8.
- Bienkowski P, Danysz W, Kostowski W (1998): Study on the role of glycine, strychnine-insensitive receptors (glycineB sites) in the discriminative stimulus effects of ethanol in the rat. *Alcohol* 15:87–91.
- Kotlinska J, Liljequist S (1996): Oral administration of glycine and polyamine receptor antagonists blocks ethanol withdrawal seizures. *Psychopharmacology (Berl)* 127:238–244.
- Kotlinska J (2001): NMDA antagonists inhibit the development of ethanol dependence in rats. *Pol J Pharmacol* 53:47–50.
- Biala G, Kotlinska J (1999): Blockade of the acquisition of ethanol-induced conditioned place preference by N-methyl-D-aspartate receptor antagonists. *Alcohol Alcohol* 34:175–182.
- Backström P, Hyytia P (2004): Ionotropic glutamate receptor antagonists modulate cue-induced reinstatement of ethanol-seeking behavior. *Alcohol Clin Exp Res* 28:558–565.
- Vengeliene V, Bachteler D, Wojciech D, Spanagel R (2005): The role of the NMDA receptor complex in alcohol relapse: A pharmacological mapping study using the alcohol deprivation effect. *Neuropharmacology* 48:822–829.
- Gomez J, Armsen W, Betz H, Eulenburg V (2006): Lessons from the knocked-out glycine transporters. *Handb Exp Pharmacol* 175:457–483.
- Molander A, Lidö HH, Löf E, Ericson M, Söderpalm B (2007): The glycine reuptake inhibitor Org 25935 decreases ethanol intake and preference in male Wistar rats. *Alcohol Alcohol* 42:11–18.
- Lidö HH, Stomberg R, Fagerberg A, Ericson M, Söderpalm B (2009): The glycine reuptake inhibitor org 25935 interacts with basal and ethanol-induced dopamine release in rat nucleus accumbens. *Alcohol Clin Exp Res* 33:1151–1157.
- Spanagel R, Hölter SM (1999): Long-term alcohol self-administration with repeated alcohol deprivation phases: An animal model of alcoholism? *Alcohol Alcohol* 34:231–243.
- Vengeliene V, Celerier E, Chaskiel L, Penzo F, Spanagel R (2009): Compulsive alcohol drinking in rodents. *Addict Biol* 14:384–396.
- Spanagel R, Hölter S, Allingham K, Landgraf R, Ziegglängsberger W (1996): Acamprosate and alcohol: I. Effects on alcohol intake following alcohol deprivation in the rat. *Eur J Pharmacol* 305:39–44.
- Spanagel R, Pendyala G, Abarca C, Zghoul T, Sanchis-Segura C, Magrone MC, *et al.* (2005): The circadian clock gene *Period 2* alters the glutamatergic system and thereby modulates alcohol consumption. *Nat Med* 11:35–42.
- Gebicke-Haerter P (2005): Expression profiling methods used in drug abuse research. *Addict Biol* 10:37–46.
- Vengeliene V, Leonardi-Essmann F, Perreau-Lenz S, Gebicke-Haerter P, Drescher K, Gross G, Spanagel R (2006): The dopamine D3 receptor plays an essential role in alcohol-seeking and relapse. *FASEB J* 20:2223–2233.
- Matthäus F, Smith VA, Fogtman A, Sommer WH, Leonardi-Essmann F, Lourdusamy A, *et al.* (2009): Interactive molecular networks obtained by computer-aided conversion of microarray data from brains of alcohol-drinking rats. *Pharmacopsychiatry* 42(suppl 1):S118–S128.
- Cowen MS, Adams C, Kraehenbuehl T, Vengeliene V, Lawrence AJ (2005): The acute anti-craving effect of acamprosate in alcohol-prefering rats is associated with modulation of the mesolimbic dopamine system. *Addict Biol* 10:233–242.
- Zafra F, Aragón C, Olivares L, Danbolt NC, Giménez C, Storm-Mathisen J (1995): Glycine transporters are differentially expressed among CNS cells. *J Neurosci* 15:3952–3969.
- Malosio ML, Marquèze-Pouey B, Kuhse J, Betz H (1991): Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J* 10:2401–2409.
- Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH (1994): Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12:529–540.
- Johnson JW, Ascher P (1987): Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325:529–531.
- Mothet JP, Parent AT, Wolosker H, Brady RO Jr, Linden DJ, Ferris CD, *et al.* (2000): D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci U S A* 97:4926–4931.
- Cubelos B, Giménez C, Zafra F (2005): Localization of the GLYT1 glycine transporter at glutamatergic synapses in the rat brain. *Cereb Cortex* 15:448–459.
- Söderpalm B, Löf E, Ericson M (2009): Mechanistic studies of ethanol's interaction with the mesolimbic dopamine reward system. *Pharmacopsychiatry* 42:587–594.
- Chau P, Höfödt-Lidö H, Löf E, Söderpalm B, Ericson M (2010): Glycine receptors in the nucleus accumbens involved in the ethanol intake-reducing effect of acamprosate. *Alcohol Clin Exp Res* 34:39–45.
- Gass JT, Olive MF (2008): Glutamatergic substrates of drug addiction and alcoholism. *Biochem Pharmacol* 75:218–265.
- Lovinger DM, White G, Weight FF (1989): Ethanol inhibits NMDA-activated ion current in hippocampal neurons. *Science* 243:1721–1724.
- Everitt BJ, Dickinson A, Robbins TW (2001): The neuropsychological basis of addictive behaviour. *Brain Res Rev* 36:129–138.
- Vanderschuren LJ, Everitt BJ (2005): Behavioral and neural mechanisms of compulsive drug seeking. *Eur J Pharmacol* 526:77–88.
- Vaudry D, Falluel-Morel A, Bourgault S, Basille M, Burel D, Wurtz O, *et al.* (2009): Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. *Pharmacol Rev* 61:283–357.
- Figiel M, Engele J (2000): Pituitary adenylate cyclase-activating polypeptide (PACAP), a neuron-derived peptide regulating glial glutamate transport and metabolism. *J Neurosci* 20:3596–3605.
- Liu GJ, Madsen BW (1997): PACAP38 modulates activity of NMDA receptors in cultured chick cortical neurons. *J Neurophysiol* 78:2231–2234.
- Tanaka K, Kunishige-Yamamoto A, Hashimoto H, Shintani N, Hayata A, Baba A (2010): Increased ethanol preference and serotonin 1A receptor-dependent attenuation of ethanol-induced hypothermia in PACAP-deficient mice. *Biochem Biophys Res Commun* 391:773–777.