

The dopamine D₃ receptor plays an essential role in alcohol-seeking and relapse

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ABSTRACT Our study aimed to identify new candidate genes, which might be involved in alcohol craving and relapse. To find changes in gene expression after long-term alcohol consumption, we studied gene expression profiles in the striatal dopamine system by using DNA microarrays of two different alcohol-preferring rat lines (HAD and P). Our data revealed an up-regulation of the dopamine D₃ receptor (D₃R) after 1 yr of voluntary alcohol consumption in the striatum of alcohol preferring rats that was confirmed by qRT-polymerase chain reaction. This finding was further supported by the finding of up-regulated striatal D₃R mRNA in nonselected Wistar rats after long-term alcohol consumption when compared with age-matched control animals. We further examined the role of the D₃R in mediating alcohol relapse behavior using the alcohol deprivation effect (ADE) model in long-term alcohol drinking Wistar rats and the model of cue-induced reinstatement of alcohol-seeking behavior using the selective D₃R antagonist SB-277011-A (0, 1, 3, and 10 mg/kg) and the partial agonist BP 897 (0, 0.1, 1, and 3 mg/kg). Both treatments caused a dose-dependent reduction of relapse-like drinking in the ADE model as well as a decrease in cue-induced ethanol-seeking behavior. We conclude that long-term alcohol consumption leads to an up-regulation of the dopamine D₃R that may contribute to alcohol-seeking and relapse. We therefore suggest that selective antagonists of this pharmacological target provide a specific treatment approach to reduce alcohol craving and relapse behavior.—Vengeliene, V., Leonardi-Essmann, F., Perreau-Lenz, S., Gebicke-Haerter, P., Drescher, K., Gross, G., Spanagel, R. The dopamine D₃ receptor plays an essential role in alcohol-seeking and relapse. *FASEB J.* 20, 2223–2233 (2006)

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ADDICTED BEHAVIOR IN ALCOHOLIC patients is characterized by compulsive alcohol seeking and relapse. These pathological drug-related behaviors can also be observed in rats undergoing long-term voluntary alcohol consumption (1, 2). Besides environmental and social factors, clearly, genetic factors contribute to

compulsive alcohol consumption and relapse. Advanced genetic approaches such as gene expression profiling are helpful in identifying candidate genes involved in compulsive alcohol consumption. A new body of research implies that a change from normal alcohol intake to more habitual and compulsive use is accompanied by a transition of synaptic plasticity from ventral to more dorsal domains of the striatum (3, 4).

In the present study, we set forth to identify alterations in gene expression in the striatal dopamine system after long-term voluntary alcohol consumption in different alcohol-preferring rat strains. For this purpose, we used the most commonly used lines, namely the Indiana University alcohol-preferring (P) and the high-alcohol-drinking (HAD) rat lines (5). These two different lines were subjected to a long-term alcohol drinking procedure as has recently been illustrated (6). In a subsequent step, gene expression profiling using Affymetrix technology was applied. Among several changes in gene expression, the most consistent alteration we observed was an up-regulation of dopamine D₃ receptors (D₃R) in the striatum. To confirm our finding, a nonselected colony of Wistar rats was used. Again, a significant up-regulation of D₃RmRNA could be detected in the striatum in long-term drinking Wistar rats *vs.* age-matched nonalcohol drinking ones. In line with the hypothesis that long-term voluntary alcohol consumption produces neuroadaptive changes within the striatal dopaminergic system, which in turn may contribute to alcohol craving and relapse (7, 8), our observation of enhanced expression of D₃R, fits into this theoretical framework. Therefore, we proceeded to study the effects of two highly selective pharmacological tools, the partial agonist BP 897 and the antagonist SB-277011-A, on alcohol craving and relapse.

For this purpose, we used two different animal models for alcohol-seeking and relapse behavior. The most commonly used procedure in studying drug-seeking

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behavior is the reinstatement model (9). This model refers to the resumption of extinguished lever-pressing behavior after noncontingent exposure to drug or nondrug stimuli. More specifically, an animal is trained to self-administer alcohol and is then subjected to extinction. When the animal reaches a criterion of unresponsiveness, various stimuli are presented. A stimulus is said to reinstate the alcohol-seeking behavior if it causes renewed responding, i.e., lever pressing, without any further response-contingent drug reward. Reinstatement of alcohol-seeking can be used to study the neurobiological and molecular basis of craving, since there appears to be a good correspondence between the events that induce drug-seeking in laboratory animals and those that provoke craving in humans (9). An animal model to study relapse behavior is the deprivation model (1, 2). Alcohol deprivation is one factor that is known to considerably affect voluntary alcohol intake. Thus, alcohol-experienced animals show a transient increase in alcohol intake after a period of forced abstinence, which is termed the “alcohol deprivation effect” (ADE). After repeated deprivation phases, the ADE is characterized by an increased demand for the drug and is clearly dissociated from normal eating or drinking behavior (1, 2) and, therefore, resembles a lapse or relapse situation in alcoholic patients. Although we do not fully understand the nature of a deprivation effect, the fact that the clinically effective antirelapse drugs acamprosate and naltrexone reduce the alcohol deprivation effect (10) lends predictive value to this animal model for the development of new and better drugs in the treatment of relapse.

MATERIALS AND METHODS

Animals

Two groups of 2- to 3-month-old alcohol preferring rats were used for long-term alcohol consumption and gene expression profiling: male P rats ($n=15$; Indiana University, Indianapolis, IN) and male HAD rats ($n=13$; Indiana University). For further validation of the observed changes in gene expression we also studied male Wistar rats ($n=12$; from our breeding colony at the Central Institute of Mental Health, Mannheim, Germany). In addition, at 2 months old, 40 male Wistar rats were used for the cue-induced reinstatement experiments and 48 male Wistar rats were used for the ADE experiments. All animals were housed individually in standard rat cages (Ehret, Emmendingen, Germany) under a 12 h artificial light-dark cycle (lights on at 7:00 a.m.). Room temperature was kept constant (temperature: $22\pm 1^\circ\text{C}$, humidity: $55\pm 5\%$). Standard laboratory rat food (Ssniff, Soest, Germany) and tap water were provided *ad libitum* throughout the experimental period. Body weights were measured weekly. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe) and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

Alcohol drinking solutions were prepared from 96% ethanol (Merck, Darmstadt, Germany) and then diluted with tap

water. Both BP 897 (Abbott, Ludwigshafen, Germany) and SB-277011-A (Abbott, Ludwigshafen, Germany and GSK, Harlow, UK) were dissolved in water (*aqua ad iniectionem*, Braun, Melsungen AG, Germany) slightly acidified with HCl. BP 897 and SB-277011-A solutions were freshly prepared and injected as a volume of 3 ml/kg intraperitoneally. Control experiments were performed after administration of slightly acidified water.

Gene expression profiling in alcohol-preferring rat strains

Alcohol-drinking procedure

According to the protocol by Vengeliene *et al.* (6) 8 P rats, 7 HAD rats, and 6 Wistar rats were given *ad libitum* access to tap water and to 5% and 20% ethanol solution (v/v). All rats underwent a 2 wk deprivation cycle after 8 wk of continuous alcohol availability. After the deprivation period, rats were given access to alcohol again and 3 more 2 wk deprivation periods were introduced in a random manner (the duration between deprivation periods varied between 4 and 16 wk). The long-term voluntary alcohol drinking procedure including all deprivation phases lasted a total of 52 wk. Total ethanol intake (g/kg of body wt/day) was calculated as the daily average across 7 measuring days. For comparison, three age- and wt-matched control groups consisting of seven P rats, six HAD rats, and six Wistar rats underwent the exact same handling procedures for the entire time of the experiment but did not receive alcohol.

For comparison, two additional Wistar rat groups (6 animals per group) were given *ad libitum* access to both tap water and different ethanol solutions [5 and 20% ethanol solution (v/v)] or to tap water only (control group) for 2 wk (short-term alcohol exposure).

Establishment of a brain/organ bank

On the last day of the drinking procedure all animals were deprived from food and liquids for 6 h to ensure that blood ethanol concentrations were zero (to avoid direct acute alcohol effects on gene expression); then between 10 a.m. to 2 p.m. animals were sacrificed by exposing them to CO_2 and decapitated. The brains were quickly removed and put into a glass with isopentane (Sigma-Aldrich, St. Louis, MO) and kept on dry ice for 5 min. Afterward, the brains were wrapped with Parafilm and aluminum foil and stored at -80°C . Other organs, which are also known to be affected by chronic alcohol intake such as the liver, heart, and pancreas, were also quickly removed and stored in liquid nitrogen. The establishment of this brain/organ bank has been described in more detail and is open to the public domain (<http://www.zi-mannheim.de/1173.html>).

For further examination, rat brains were placed at -20°C overnight and mounted in a Leica CM3000 Cryostat (Leica, Bensheim, Germany). Brains were sliced in coronal sections 150 μm width. Different regions were extracted by punching with a set of self-constructed needles of several diameters ranging from 0.75 to 1.5 mm (FMI, Seeheim, Germany) and collected into vials. The identification of regions was based on landmarks from the stereotaxical descriptions of The Rat Brain Atlas (Paxinos and Watson). To assess the punching precision, Nissl-stained punched slices were checked by microscopy. The following brain sites were collected and stored in -80°C : prefrontal cortex, nucleus accumbens, bed nucleus of the stria terminalis, caudate putamen, hippocampus, amygdala, ventral tegmental area, and cerebellum of alcohol-treated and alcohol-naive rats. In the present study, only tissue from the caudate putamen (bregma: +2.20 till -2.30

mm) and nucleus accumbens (bregma: +2.70 till 0.70 mm) was used.

Total RNA isolation and quality control

Punched tissue was lysed in TRIzol reagent (Invitrogen, Karlsruhe, Germany) and homogenized by passing the suspension 30 times through a 22 gauge needle. Total RNA was extracted by adding chloroform. To achieve better separation of organic and aqueous phases, Phase Lock Gel Heavy tubes (Eppendorf, Hamburg, Germany) were used. Upper phases were carefully removed by pipetting and total RNA was purified using RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA quality was evaluated by optical density (OD) measurements (260 nm/280 nm) in a GeneQuant (Pharmacia, Freiburg, Germany) in 10 mM Tris-HCl, pH 7.6 and its integrity was determined by measuring ribosomal 28S/18S ratios using RNA 6000 Nano Assay RNA chips run in an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Ratios of 1.9–2.2 (optical density 260/280) and >1.6 (28S/18S rRNA) as well as an absence of a peak of DNA contamination in electropherograms at 29S were chosen as inclusion criteria.

Target labeling, GeneChip Rat Genome U34A hybridization, scanning, and quality control

Doublestranded (ds) cDNA synthesis, *in vitro* transcription (IVT) of ds cDNA into cRNA and GeneChip RG U34A (Affymetrix, Santa Clara, CA) hybridizations were carried out according to the manufacturer's protocol. Briefly, 5 µg total RNA were reverse transcribed using T7-Oligo(dT)24 primer (GeneSet Oligos, Evry, France) and SuperScriptII Reverse Transcriptase (Invitrogen, Karlsruhe, Germany). Second strand cDNA was synthesized by adding DNA polymerase I (*E. coli*), ribonuclease H, *E. coli* DNA ligase, and T4 DNA polymerase. After ds cDNA precipitation and resuspension, IVT was performed using the BioArray HighYield RNA Transcript Labeling Kit (Enzo, Farmingdale, NY). Biotin labeled cRNA was purified with RNeasy Mini Kit (Qiagen) and fragmented with RNA fragmentation buffer. IVT and cRNA fragmentation quality controls were carried out by running an mRNA Nano assay in the Agilent 2100 Bioanalyzer. cRNA electropherograms showed a single broad peak beginning at ≈22 s and ending at 74 s. Fragmented cRNAs were resolved in a single peak starting at 19 s and declining at 26 s. GeneChip RG U34A arrays were filled with hybridization cocktails containing 10 µg of fragmented cRNAs (after correction for total RNA carryover). After 16 h of hybridization in a GeneChip Hybridization Oven (Affymetrix), chips were stained with streptavidin/R-phycoerythrin conjugate (Molecular Probes, Eugene, OR) in a GeneChip Fluidics Station (Affymetrix) and immediately scanned in a GeneArray Scanner (Agilent Technologies). Chips were checked for performance using five evaluation criteria: 1) comparable scaling factors among the chips, 2) single chip average background <100 and similar among the chips, 3) Signal (3'/5') of rat housekeeping genes GAPDH, hexokinase and β-actin <2, 4) increasing signals of hybridization controls within a chip and similar intensities for each hybridization control among the chips and (5) visual assessment of scanned image (DAT files) and grid alignment.

Data mining

Micro Array Suite 5.0 (Affymetrix) derived cell intensity files (CEL) were processed in R 2.1.1 language and environment (11) by using Bioconductor 1.6 (12) packages. Among them,

package Affy 1.6.7 (13) for chip performance quality control and for Robust-Multichip-Average (RMA) global normalization was used. Genes differentially expressed between alcohol-treated and alcohol-naive groups were identified by the following series of filtering steps: 1) a probability of differential expression of $P < 0.05$ from a *t* test, 2) a 100% of Present Call in at least one group, and 3) a fold change (FC) in gene expression $-1.3 > FC > 1.3$. For the analysis of dopaminergic markers, genes that contain the word "dopamine" as Gene Ontology (GO) term (14) were selected out of the list of differentially expressed genes.

Quantification by real-time polymerase chain reaction

Relative quantification by real-time polymerase chain reaction (PCR; qRT-polymerase chain reaction) was carried out for confirmation of differentially expressed genes found with chip experiments and to assess the relative abundance of those transcripts in brain regions other than the ones that had undergone gene expression profiling. qRT-polymerase chain reaction was carried out in a total reaction volume of 20 µl using AbsoluteQPCR SYBR Green Fluorescein Mix (AB-gene, Hamburg, Germany) and run in an i-Cycler (Bio-Rad, München, Germany). Rat ribosomal protein rS12 cDNA was used as an internal standard, since it showed highly stable expression between samples. Cycling conditions were 95°C for 15s, 60°C for 30 s (56°C for rS12), 72°C for 30 s, 82–86°C for 15 s. Relative quantification was done according to the $\Delta\Delta C_t$ method. Differences in transcript abundance between alcohol-treated and alcohol-naive groups were analyzed using a *t* test for independent samples for each brain region separately.

Functional validation in rats undergoing long-term alcohol self-administration with repeated deprivation phases

For this set of experiments, the same alcohol drinking procedure was applied as the one for the studies in alcohol-preferring rats. According to the protocol by Vengeliene et al. (15) the pharmacological investigations were performed at the end of the fourth alcohol deprivation phase. To study the effects of BP 897 and SB-277011-A, rats were divided into six groups ($n=8$ per group) in such a way that the mean baseline total alcohol intake was approximately the same in each group (*i.e.*, ≈3 g/kg/day). Baseline drinking was monitored for at least 3 days. After the last day of baseline measurement, the alcohol bottles were removed from the cages leaving the animals with free access to food and water for 20 days. Thereafter, each animal was subjected to a total of 5 IP injections (starting at 7 p.m. with 12 h intervals) of BP 897 (G1: vehicle, G2: 1 mg/kg and G3: 3 mg/kg) and SB-277011-A (G4: vehicle, G5: 3 mg/kg, and G6: 10 mg/kg; see Drugs for vehicle). The drug injection schedule was based on the half-life of the drugs and several pilot studies. The alcohol bottles were reintroduced after the second injection (at ≈9 a.m. on the 21st day of alcohol deprivation) and the occurrence of an ADE was determined. Total ethanol (g/kg of body wt/day) and water intake (ml/kg of body wt/day) were measured daily at ≈9 a.m. for a subsequent week. Each body weight of the rat was recorded 24 h before the first injection and 12 h after the last injection.

Home cage locomotor activity measurements by the E-motion system

To test for any sedative effects resulting from the drug treatment or drug/alcohol interaction, we monitored home cage locomotor activity by using a novel infrared sensor

connected to a recording and data storing system (Mouse-E-Motion by Infra-e-motion, Henstedt-Ulzburg, Germany). A Mouse-E-Motion device was placed above each cage (30 cm from the bottom) so that the rat could be detected at any position inside the cage. The device was sampling every second whether the rat was moving or not. The sensor could detect body movement of the rat of at least 1.5 cm from one sample point to the successive one. The data measured by each Mouse-E-Motion device were downloaded into a personal computer and processed with Microsoft Excel.

The effect of treatment on basic locomotor activity was monitored in vehicle treated (G1 and G4), 3 mg/kg of BP 897 (G3), and 10 mg/kg of SB-277011-A (G6) treated animals. Monitoring of locomotor activity was done during the active phase of the rat (7 p.m.–7 a.m.), starting 3 days before drug treatment procedures. The monitoring was continued throughout all treatment days and for 2 more posttreatment days. The percentage of each locomotor activity of the rat during and after treatment days was calculated by using the “before treatment” activity data as a reference.

Functional validation in rats undergoing cue-induced reinstatement of alcohol-seeking behavior

Operant alcohol self-administration apparatus

All alcohol-seeking experiments were carried out in operant chambers (MED Associates Inc., St. Albans, VT) enclosed in ventilated sound-attenuating cubicles. The chambers were equipped with a response lever on each side panel of the chamber. Responses at the appropriate lever activated a syringe pump that delivered a 25–30 μ l drop of fluid into a liquid receptacle next to it. A light stimulus (house light) was mounted above the right response lever and a loudspeaker (65 dB “beep”) was positioned above the left response lever of the self-administration chamber. An IBM compatible computer controlled the delivery of fluids, presentation of stimuli, and data recording.

Alcohol self-administration training

Animals were trained to self-administer 10% (v/v) ethanol in a 30 min daily sessions using a fixed-ratio 1 (FR 1) schedule. During the first 3 days of training, responses at the left lever were reinforced by the delivery of 0.2% (w/v) saccharin solution. After acquisition of saccharin-reinforced responding, rats were trained to self-administer ethanol. Thus, rats had access to 0.2% saccharin with 5% ethanol for 1 day, 5% ethanol for 1 day, 0.2% saccharin with 8% ethanol for 1 day, 8% ethanol for 1 day, 0.2% saccharin with 10% ethanol for 1 day and 10% ethanol for 1 day. Throughout the training phase, responses at the right lever resulted in the delivery of a drop of water.

Conditioning and extinction phase

The purpose of the conditioning phase was to train the animals to discriminate between the availability of ethanol (reward) and water (nonreward). This phase started after the completion of the saccharin-fading procedure. Discriminative stimuli predicting 10% ethanol or water availability were presented during each ethanol or water self-administration session (one 30-min session/day). An orange flavor extract served as the S+ for ethanol, whereas water availability was signaled by an anise extract (S–). These olfactory stimuli were generated by depositing six drops of the respective extract into the bedding of the operant chamber before each session. In addition, each lever press resulting in alcohol

delivery was accompanied by a 5 s auditory stimulus (“beep,” CS+), whereas a 5 s light stimulus (CS–) was presented with water delivery. The 5 s period served as a “time-out,” during which responses were recorded but not reinforced. At the end of each session, the bedding of the chamber was changed and trays were thoroughly cleaned. During the first 3 days of this phase, rats were given alcohol sessions only. Subsequently, alcohol and water sessions were conducted in a random manner until the animals received a total of 10 alcohol and 10 water sessions.

After completing the conditioning phase, the rats were subjected to daily 30-min extinction sessions for 15 consecutive days, which in total was sufficient to reach the extinction criterion of <10 lever responses/session. Extinction sessions began by extending the levers without presenting olfactory discriminative stimuli. Responses at the previously active or inactive lever activated the syringe pump, without resulting in the delivery of either alcohol or water or the presentation of response-contingent cues (stimulus light or “beep”).

Reinstatement testing

Reinstatement tests began one day after the final extinction session. In these tests, rats were exposed to the same conditions as during the conditioning phase, except that the liquids (alcohol or water) were not made available. Sessions were initiated by the extension of both levers and the presentation of either the alcohol- (S+) or water- (S–) associated discriminative stimuli. Responses at each lever were followed by the activation of the syringe pump and the presentation of the CS+ (“beep”) in the S+ condition or the CS– (house light) in the S– condition. Half of the animals were tested under the S+/CS+ condition on day 1 and under the S–/CS– condition on day 2. Conditions were reversed for the other half of the animals. The number of responses on both the active (i.e., alcohol-associated lever for S+/CS+ condition and water-associated lever for S–/CS– condition) and inactive lever (i.e., water-associated lever for S+/CS+ condition and alcohol-associated lever for S–/CS– condition) was recorded throughout the experiment.

Pharmacological studies

To test the effect of BP 897 and SB-277011-A on cue-induced reinstatement, animals underwent two reinstatement procedures separated by 5 days. Rats were divided into groups on the basis of their performance during the last four conditioning sessions; each group consisted of eight animals. Thus, for the reinstatement test, animals were injected IP with either vehicle (G1, G5) or BP 897 (G2: 0.1 mg/kg, G3: 1 mg/kg and G4: 3 mg/kg) and SB-277011-A (G6: 1 mg/kg, G7: 3 mg/kg and G8: 10 mg/kg). Drug administration was performed 30 min before the test procedure. The number of responses on both the active and inactive lever was recorded throughout the experiment. Each body weight of the rat was recorded 1 day before and 1 day after the experimental procedure. Locomotor activity of control, 3 mg/kg BP 897 treated, and 10 mg/kg of SB-277011-A treated animals was measured during experimental period using E-motion system.

Statistics for the behavioral studies

Data obtained from home-cage drinking (total alcohol intake, water intake and locomotor activity) was analyzed using a two-way ANOVA with repeated measures (factors were: between subjects – treatment group, and within subjects – day) and from alcohol-seeking experiments using a three-way ANOVA (factors were: treatment, lever (active *vs.* inactive),

and experimental condition (extinction *vs.* reinstatement). Data analysis regarding the effects of treatment on the change in the animals' body wt was performed using a one-way ANOVA (factor – treatment group). Whenever significant differences were found, post hoc Student Newman Keul's tests were performed. The chosen level of significance was $P < 0.05$.

RESULTS

Gene expression profiling studies reveal an up-regulation of striatal dopamine D3R after long-term alcohol consumption

Gene expression profiling was performed in HAD and P rats after long-term alcohol consumption with repeated deprivation phases. After the first alcohol deprivation phase, both alcohol-preferring animal lines slightly increased basal total alcohol intake, which stayed stable for the remainder of the alcohol exposure. Total basal alcohol intake for the rat lines was as follows: HAD rats 4.6 ± 0.1 g/kg and P rats 5.8 ± 0.1 g/kg. It should be noted that an increase in alcohol intake after a deprivation phase, an ADE, has not been observed in the HAD rat line (see also ref 6). All animals were compared with age-matched control HAD and P rats, which had undergone the exact same experimental procedure, however, without alcohol. The number of alterations of gene expression we detected: out of 8741 targets represented in the Gene-ChipRG U34 A array 266 well annotated genes were differentially expressed in the striatum of alcohol-experienced P rats when compared with alcohol-naïve P rats; particularly there were 250 down- and 16 up-regulated genes found. In HAD rats there were 32 down-regulated and 108 up-regulated genes with generally lower FCs as compared with P rats. However, in the present study we focused on dopaminergic markers, which are listed in **Table 1**. As it is seen from the table, these genes are related to the dopamine turnover and signaling, also involving dopamine receptors. The most consistent alteration we observed was an up-

regulation of dopamine D3Rs in the striatum of alcohol-preferring rat lines which was later confirmed using Wistar rats (these animals had 2.1 ± 0.1 g/kg basal alcohol intake). Reverse transcriptase-polymerase chain reaction (RT-PCR) data revealed a significantly different D3R transcript abundance in the caudate putamen of alcohol-treated alcohol-preferring rat groups as compared with alcohol-naïve rat groups that was also seen in Wistar rats (**Fig. 1A**). The increase of dopamine D3R transcripts was statistically significant in P [t , $13 = -4.0$, <0.01] and Wistar rats [t , $10 = -2.5$, $P < 0.05$]. Although not statistically significant, a tendency of augmented transcript abundance was seen in HAD ($P = 0.28$) rats. No significant changes in D3R expression were found in the nucleus accumbens of any rat group (**Fig. 1B**).

For comparison, we also applied a short-term alcohol consumption procedure where Wistar rats had the free choice between water and different ethanol solutions for only two weeks. Our qRT-polymerase chain reaction data show that short-term alcohol exposure led to a nonsignificant reduction (19%) in D3R expression in the striatum ($P = 0.06$) and did not produce any changes in the nucleus accumbens ($P = 0.83$; data not shown).

Effect of the administration of BP 897 and SB-277011-A on ADE

The pharmacological studies were performed at the end of the last alcohol deprivation phase in Wistar rats. After the reintroduction of alcohol solutions, both vehicle treated groups (G1 and G4) showed a typical increase in alcohol consumption, indicating the occurrence of an ADE. This increase was not different from that observed during the first three deprivation periods (data not shown). With respect to the pharmacological treatment, a two-way ANOVA for repeated measures revealed a significant increase in alcohol intake after a deprivation phase in BP 897 treated animal groups (G1: vehicle, G2: 1 mg/kg and G3: 3 mg/kg) as compared with basal drinking [factor day: $F(4,84) = 113.7$, $P < 0.0001$]. This increase was mainly caused by the

TABLE 1. Alterations of dopaminergic markers in the striatum of P and HAD rats

Gene Symbol	Gene Title	P rats		HAD rats	
		P value	FC	P value	FC
Drd1a	dopamine receptor 1A	0.00170	-1.89	0.53201	1.03
Drd2	dopamine receptor 2	0.00078	-1.68	0.56691	1.03
Drd3	dopamine receptor D3	0.02220	1.37	0.04633	1.05
Drd4	dopamine receptor D4	0.01320	1.28	0.26380	1.00
Drd5	dopamine receptor D5	0.01590	1.26	0.11200	1.00
Gnao	guanine nucleotide binding protein, alpha o	0.00999	-1.56	0.11761	1.12
Nsg1	neuron specific gene family member 1	0.00264	-1.40	0.35356	1.04
Maoa	monoamine oxidase A	0.00499	-1.33	0.02039	1.08
Th	Tyrosine hydroxylase	0.00620	1.32	0.65200	-1.01
Nr4a2	nuclear receptor subfamily 4, group A, member 2	0.05031	1.34	0.24508	-1.07

List of targets that 1) contained the word “dopamine” as gene ontology term and 2) showed statistically significant differences between alcohol-naïve and alcohol-experienced rats. FC = fold change.

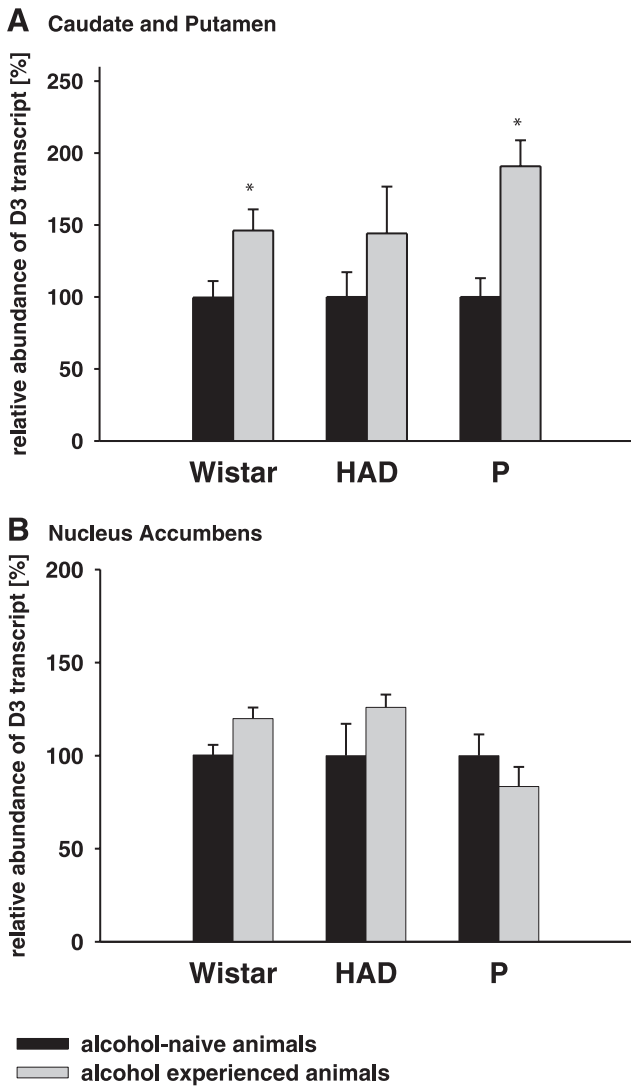


Figure 1. Relative quantification of qRT-polymerase chain reaction data in the dorsal striatum (A) and nucleus accumbens (B) of age-matched alcohol-treated and alcohol-naive Wistar, HAD and P rat lines. Percentage of transcript abundance was calculated in relation to the housekeeping gene transcription. Data are means \pm SE. *Significant differences from the alcohol-naive control group, $P < 0.05$.

vehicle (G1) and 1 mg/kg of BP 897 (G2) treated animals (Fig. 2A). The 3 mg/kg treated rat group (G3) displayed an increased alcohol intake only on the first postdeprivation day. Consequently, our data analysis showed a significant group \times day interaction effect [$F(8,84)=6.4$, $P<0.0001$], suggesting the differential expression of ADE between the groups. Finally, a two-way ANOVA displayed a significantly different alcohol intake between BP 897 treated animal groups [factor group: $F(2,21)=10.5$, $P<0.001$], showing that the treatment of rats with BP 897 was capable in reducing the expression of an ADE. Thus, although treatment with 1 mg/kg of BP 897 (G2) did not have any obvious effects on alcohol intake during the ADE, 3 mg/kg of BP 897 (G3) reduced alcohol intake considerably, which even dropped below baseline levels on postdeprivation days 2 and 3 (Fig. 2A). In contrast,

water intake (data not shown) was significantly increased during these days, suggesting that the effect of treatment was selective for alcohol (group \times day interaction effect [$F(4,42)=3.1$, $P<0.05$]).

Similarly, after the last deprivation phase, an increased alcohol intake was seen in SB-277011-A treated (G4: vehicle, G5: 3 mg/kg, and G6: 10 mg/kg) animal groups [factor day: $F(4,84)=76.8$, $P<0.0001$], which was generally caused by G4 and G5 animals (Fig. 2B). Furthermore, treatment with SB-277011-A, dose dependently reduced the expression of an ADE, however, an increased alcohol intake was again seen on the first postdeprivation day. Thus, a two-way ANOVA revealed a significant group \times day interaction effect [$F(8,84)=9.4$, $P<0.0001$] and significantly different alcohol consumption between treatment groups [factor group: $F(2,21)=8.9$, $P<0.0001$]. Treatment with 10 mg/kg of SB-277011-A reduced alcohol intake below baseline drinking on postdeprivation day 2, which was accom-

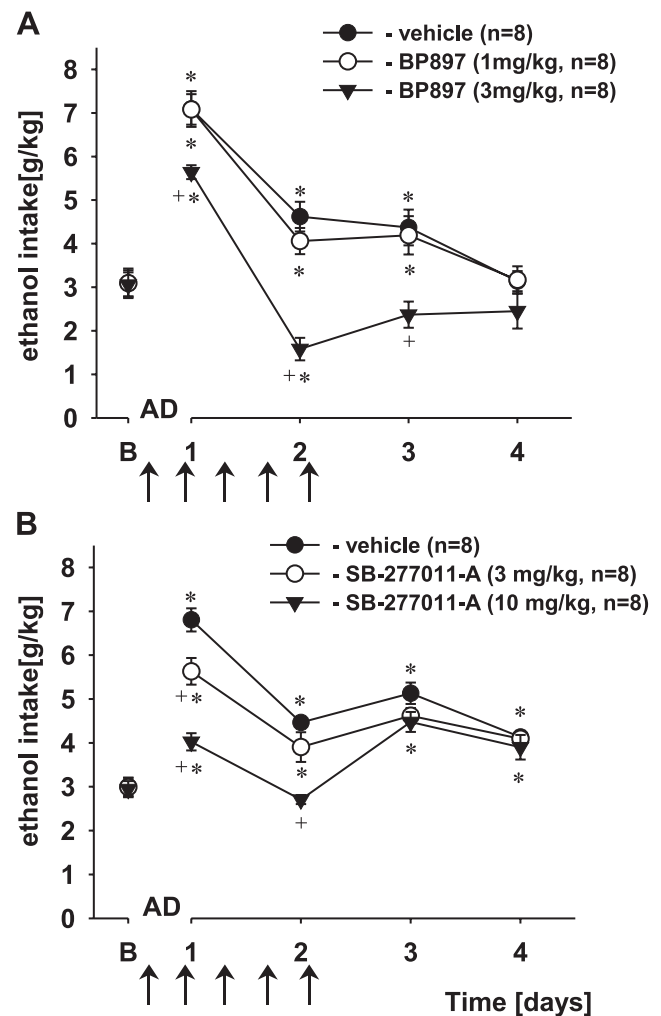


Figure 2. Total ethanol intake (g/kg/day) before and after an alcohol deprivation (AD) period of 3 wk. Arrows indicate administration of either vehicle or BP 897 (A) and SB-277011-A (B). Average of 3 day measurements of ethanol intake is given as baseline drinking – “B.” Data are means \pm SE. *Significant differences to baseline drinking; +Significant differences from the vehicle control group, $P < 0.05$.

panied with an increase in water intake on that day (data not shown; factor group: $F(2,21)=3.2, P<0.05$).

Locomotor activity measurements in both vehicle (G1) and 3 mg/kg of BP 897 (G3) treated animals did not reveal any significant differences in rodent home-cage activity either during the first hour interval postinjection or during the whole animal's active phase (12 h interval), excluding the possibility of sedation caused by the BP 897 treatment (Fig. 3A). However, a significant decrease in locomotor activity was seen in the 10 mg/kg SB-277011-A treated group (G6), when compared with the vehicle treated rats (G4) [group \times day interaction effect: $F(4,56)=7.3, P<0.0001$ and $F(4,56)=3.8, P<0.01$ for the first hour and for the whole active phase, respectively]. Interestingly, the immediate sedative effect of treatment with 10 mg/kg SB-277011-A was seen only after the first injection, while longer lasting changes in locomotor activity occurred only after the re-exposure of animals to alcohol (Fig. 3B), suggesting that the administration of SB-277011-A together with alcohol ingestion may lead to slight sedation. It should be mentioned that none of the pharmacological treatments led to significant changes in the body weight of the animals (data not shown), showing that food intake and metabolism were not altered during the treatment days.

Effect of the administration of BP 897 and SB-277011-A on cue-induced reinstatement of alcohol-seeking behavior

At the end of the conditioning phase, rats exhibited 208.3 ± 9.9 and 38.6 ± 1.5 alcohol-associated and water-associated lever presses, respectively. The number of operant responses progressively faded away across 15 extinction sessions. Thus, during the last

extinction sessions, lever presses dropped down to 4.6 ± 0.3 and 4.7 ± 0.3 for the previously alcohol-associated and water-associated lever, respectively.

During the reinstatement test, BP 897 treated animal groups (*i.e.*, G1, G2, G3, and G4) increased the number of responses on the respective lever under the S+/CS+ condition as compared with the last extinction sessions, which was confirmed by a three-way ANOVA [factor experimental condition: $F(1,56)=48.9, P<0.0001$]. Post hoc comparisons revealed that this increase derived from the increased number of responses on the ethanol-paired (active) lever by rats treated with vehicle (G1; Fig. 4A). Furthermore, treatment with BP 897 caused a significant dose-dependent reduction of the lever presses during the S+/CS+ session [factor treatment group: $F(3,56)=8.0, P<0.001$], so that responding on the active and inactive levers was significantly different [factor lever: $F(1,56)=9.5, P<0.01$] in the vehicle treated animal group, yet not differing in any of the BP 897 treated groups (Fig. 4A). Responding on the inactive lever was not considerably reduced in the BP 897 treated animal groups as compared with the vehicle treated group, which indicates the absence of unspecific reduction of lever-pressing behavior. The number of responses during the S-/CS- session was also found to be higher than that observed during the last extinction sessions [factor experimental condition: $F(1,56)=85.7, P<0.0001$]. There was also a significant effect of treatment on the lever responding during S-/CS- session [factor treatment group: $F(3,56)=7.9, P<0.001$]; however, there were no difference found between the active and inactive lever presses ($P=0.45$), neither in the vehicle treated animals nor in the BP 897 treatment groups, suggesting that the reinstatement test was specific for the alcohol-associated cue (note: data of S-/CS- condition are not shown). It should be

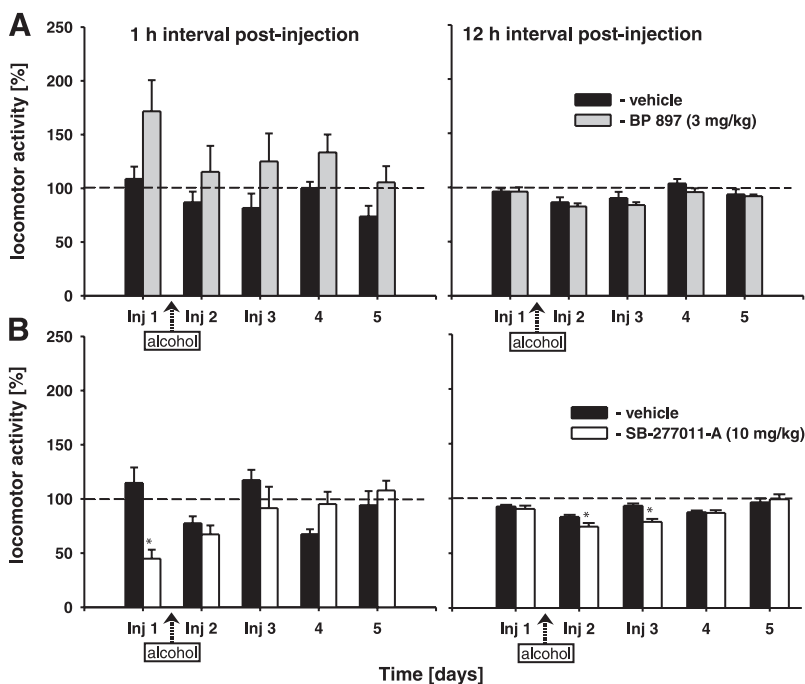


Figure 3. Differences in locomotor activity between vehicle and the highest dose of BP 897 (3 mg/kg; A) or 10 mg/kg of SB-277011-A (B) treatment during the first hour interval postinjection (left panel) and the 12-hours interval postinjection (=active phase; right panel). Injection days are marked as "Inj." Percentage of each rat's locomotor activity during and after treatment days was calculated with respect to basal activity before treatment (dotted line). Data are means \pm SE. *Significant differences from the vehicle control group, $P < 0.05$.

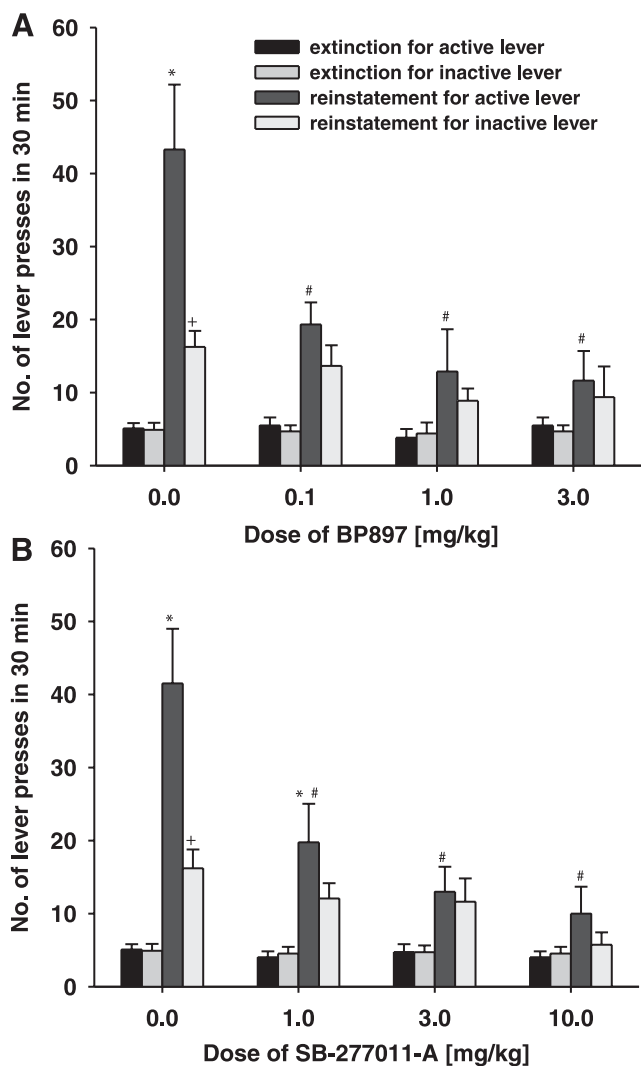


Figure 4. Effect of different doses of BP 897 (0, 0.1, 1, and 3 mg/kg; *A*) and SB-277011-A (0, 1, 3, and 10 mg/kg; *B*) on cue-induced reinstatement under S+/CS+ condition. Data are shown as average number of lever presses of the last 4 extinction sessions and as number of responses on alcohol-associated (active) and water-associated (inactive) levers after presentation of a stimulus previously paired with ethanol. Data are means \pm SE. *Significant differences to extinction lever responses; #significant differences from vehicle control group and +significant differences between active and inactive levers, $P < 0.05$.

mentioned, however, that 3 mg/kg dose of BP 897 (G4) caused a significant reduction in lever responses during S−/CS− session, which suggests that this dose was not selective for alcohol-associated cue in this paradigm.

The effect of treatment with SB-277011-A (*i.e.*, G5, G6, G7, and G8) under both S+/CS+ and S−/CS− conditions was found to be relatively similar to that seen in BP 897 treated animals (Fig. 4*B*). The number of responses on the respective cue-paired lever under the S+/CS+ condition was found to be significantly higher, when compared with the last extinction sessions [factor experimental condition: $F(1,56)=59.1$, $P<0.0001$]. Consequently, post hoc comparisons revealed that during the S+/CS+ condition, rats treated with vehicle (G5) and 1

mg/kg of SB-277011-A treated group (G6) exhibited a significantly higher number of responses on the previously ethanol-paired lever than that during the last extinction sessions (Fig. 4*B*). Furthermore, the SB-277011-A treatment groups displayed a differential number of lever responses under the S+/CS+ condition [factor treatment group: $F(3,56)=10.5$, $P<0.0001$]. Although responding on the active and inactive levers was significantly different [factor lever: $F(1,56)=10.8$, $P<0.01$], only in the vehicle-treated animal group. Thus, all three SB-277011-A doses tested, significantly reduced the number of lever presses during the reinstatement testing as compared with the vehicle group without suppressing the responding on the inactive lever. The number of lever presses during the S−/CS− session was also significantly higher from that observed in the last extinction sessions [factor experimental condition: $F(1,56)=73.2$, $P<0.0001$], however, and similar to the BP 897 treatment, there were no difference found between the active and inactive lever presses in both vehicle and SB-277011-A treated animal groups showing the selectivity of reinstatement for the ethanol associated lever.

The measurement of locomotor activity during the reinstatement using the E-motion system demonstrated a nonsignificant increase in locomotor activity to $172 \pm 52\%$ ($P=0.28$) in 3 mg/kg of BP 897 treated animals when compared with their basal locomotor activity (=100%) and a nonsignificant reduction of locomotor activity to $70 \pm 13\%$ ($P=0.31$) in 10 mg/kg of SB-277011-A treated animals (data not shown). It should be mentioned that none of the drug treatments caused a reduction in the animals' body wt (data not shown).

DISCUSSION

In the present study, we aimed to identify and verify alterations in gene expression after long-term voluntary alcohol consumption. With the use of Affymetrix technology and subsequent qRT-polymerase chain reaction validation, the most consistent alteration in different alcohol-preferring lines and nonselected Wistar rats observed was an up-regulation of dopamine D3R in the striatum. This finding was functionally validated by pharmacological means. Thus, repeated administration of the partial D3R agonist BP 897 and the antagonist SB-277011-A, caused a significant dose-dependent decrease in the expression of an ADE in Wistar rats. During ADE measurements, no influence on home-cage locomotor activity was observed with the BP 897 treatment but a slight reduction of locomotor activity during treatment days was seen in animals receiving the highest dose of SB-277011-A. In another animal model, both compounds dose dependently reduced cue-induced reinstatement of alcohol-seeking behavior without affecting responses on the inactive lever. In summary, these data suggest that after long-term alcohol consumption, dopamine D3Rs are up-regulated and that the blockade of this receptor subtype can reduce alcohol-seeking and relapse behavior.

The up-regulation of dopamine D3Rs in the striatum which has also been observed in mice after long-term alcohol exposure (16) implies that among other brain systems the nigrostriatal pathway might be involved in neurobehavioral effects of alcohol. Indeed, alcohol and other drugs of abuse produce strong habits that might involve the nigrostriatal pathway known to mediate those behaviors (1, 3, 4, 17). Thus, a marked increase in dopamine release was seen in the dorsal striatum during drug-seeking behavior (18) and blockade of dopamine receptors within the dorsal striatum decreased drug seeking under a second-order schedule of reinforcement (19) indicating a possible involvement of the dopaminergic innervation of the dorsal striatum in well-established, in other words habitual, drug-related behaviors. In this respect, up-regulation of D3Rs after long-term home-cage alcohol exposure might not be related to the alcohol intake *per se* but rather to the stimulus-response habit.

The finding of enhanced dopamine D3R expression after long-term alcohol consumption is consistent with previous reports showing that in human cocaine overdose victims, the number of binding sites of dopamine D3R was increased in the caudate putamen and nucleus accumbens (20). Dopamine D3R expression was also found to be increased after nicotine and cocaine exposure in the nucleus accumbens of rats (21, 22) as well as in the ventrolateral and ventromedial caudate putamen (23). Although both alcohol preferring rat lines (P and HAD) have an altered innervation of dopaminergic afferents in several limbic structures when compared with their nonpreferring counterparts, there is no difference in dopamine D3R binding in alcohol-naive P and HAD rats (24, 25). Thus, it is unlikely that the dopamine D3R contributes to the innate preference to alcohol and influences the acquisition of alcohol intake behavior. However, there is some indication from the literature that the dopamine D3R contributes to the maintenance of an acquired voluntary alcohol intake behavior as the dopamine D3R antagonist SB-277011-A reduced alcohol intake in a two-bottle free choice paradigm in rats (26).

In general, it is thought that dopamine neurotransmission does not play a crucial role in the maintenance of alcohol drinking but it is activated during reward expectation and under conditions of deprivation and novelty (27). Thus, in our study we examined the effect of both the D3R partial agonist BP 897, which displays a 70-fold higher affinity at the dopamine D3R over the D2 receptor (28), and the antagonist SB-277011-A, which is known to have a 100-fold higher affinity at the dopamine D3R over the D2 receptor (29), on ADE and cue-induced alcohol-seeking behavior. Both compounds dose-dependently reduced alcohol-seeking and relapse-like behavior. Our data are in good agreement with previous studies demonstrating that agents acting at the dopamine D3R are effective in reducing drug craving and vulnerability to relapse. Particularly, the administration of the partial dopamine D3R agonist BP 897 was able to reduce cue-induced cocaine- (28, 30),

amphetamine- (31) and nicotine-seeking behavior (32). BP 897 was also effective in preventing cocaine-induced conditioned place preference (33). Similar effects were found using the D3R antagonist SB-277011-A. Thus, cue-induced cocaine- (34, 35) and nicotine-seeking (32, 36) behavior as well as cocaine- (34) and heroin-induced (37) conditioned place preference was attenuated after the administration of SB-277011-A. In our study, both compounds produced similar effects on ADE and cue-induced alcohol seeking. This leads to the suggestion that at least in the presence of environmental cues previously associated with alcohol, which are known to induce mesolimbic dopamine release, BP 897 might be acting as a dopamine D3R antagonist, which is in line with the previous assumption that BP 897 is generally lacking an agonistic effect on the dopamine D3R (38).

It should be noted that any of the treatments applied in our study did not cause a reduction in the body weight of the animals. Furthermore, water intake during ADE measurements was increased in drug-treated animals as compensation to decreased ethanol intake, indicating that the effect of the dopamine D3R ligands was selective for alcohol. Importantly, neither BP 897 nor SB-277011-A is known to significantly influence cue-induced sucrose-seeking behavior (34, 39). However, even though it appears that these compounds had no sedative side effects in the 24 h home cage measurements, one cannot rule out the possibility that transient sedative effects might occur. For this reason, and for the first time, we applied an on-line E-motion system, allowing us to monitor the activity of the animals in parallel to their drinking behavior. Data from the E-motion system show that the repeated administration of BP 897 had no sedative effects during the ADE in the home-cage. However, even though previous studies with alcohol naive animals have shown that SB-277011-A does not affect spontaneous locomotor activity at the doses used in the previous study (27), a slight sedation was seen with 10 mg/kg of SB-277011-A during the active phase of the animals when administered concurrently with alcohol ingestion.

In conclusion, evidence has accumulated in recent years implicating the dopaminergic systems in a range of behavioral and neurobiological processes relevant to alcohol craving and relapse (27, 40). However, the outcome of clinical trials that aimed to target dopamine D1 and D2 receptors to reduce relapse rates in alcoholic patients was rather disappointing. Thus, dopaminergic compounds such as lisuride, tiapride, bromocriptine, or flupenthixol were tested in randomized, double-blind, placebo-controlled trials and revealed either no difference between verum and placebo group or displayed enhanced relapse rates under verum conditions (for review see ref 41). Therefore, we assume that neither an interference with dopamine D1 nor D2 receptors provides a good clinical rationale for the treatment of alcoholic patients. However, it appears to be that the crucial receptor in mediating dopamine-dependent processes related to alcohol craving and

relapse is the dopamine D3R (see also 42). Compounds, which have a clear selectivity for the dopamine D3R over the D2 receptors, are now readily available and may undergo clinical testing. **FJ**

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