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Involvement of nucleus accumbens dopamine D1 receptors in ethanol drinking, ethanol-induced conditioned place preference, and ethanol-induced psychomotor sensitization in mice

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Abstract
Rationale Dopamine D1 receptor (D1R) signaling has been associated to ethanol consumption and reward in laboratory animals.
Objectives Here, we hypothesize that this receptor, which is located within the nucleus accumbens (NAc) neurons, modulates alcohol reward mechanisms.
Methods To test this hypothesis, we measured alcohol consumption and ethanol-induced psychomotor sensitization and conditioned place preference (CPP) in mice that received bilateral microinjections of small interference RNA (siRNA)-expressing lentiviral vectors (LV-siD1R) producing D1R knock-down. The other group received control (LV-Mock) viral vectors into the NAc.
Results There were no differences in the total fluid consumed and also no differences in the amount of ethanol consumed between groups prior to surgery. However, after surgery, the LV-siD1R group consumed less ethanol than the control group. This difference was not associated to taste neophobia. In addition, results have shown that down-regulation of endogenous D1R using viral-mediated siRNA in the NAc significantly decreased ethanol-induced behavioral sensitization as well as acquisition, but not expression, of ethanol-induced place preference.
Conclusions We conclude that decreased D1R expression into the NAc led to reduced ethanol rewarding properties, thereby leading to lower voluntary ethanol consumption. Together, these findings demonstrate that the D1 receptor pathway within the NAc controls ethanol reward and intake.

Keywords D1 receptor · Ethanol · Reward · Place preference · Behavioral sensitization · SCH-39166 · siRNA · Lentivitus

Abbreviations

CPP	Conditioned place preference
D1R	Dopamine D1 receptor
GPCR	G protein-coupled receptors
EtOH	Ethanol
LV	Lentiviral vector
NAc	Nucleus accumbens
siRNA	Small interference RNA
VTA	Ventral tegmental area

Introduction

Alcohol is widely consumed for its desirable effects, but unfortunately has strong addictive properties. In fact, some individuals control their alcohol consumption throughout life, but others escalate their drinking to levels that increase the risk for addiction. Nevertheless, the neurobiological and molecular bases for ethanol action on the brain and addiction processes are poorly understood. Dopamine is widely recognized as a key neurotransmitter signal involved in drugs of abuse reward, and the dopaminergic system is believed to play a crucial role in the reinforcing properties of ethanol consumption (Badanich et al. 2007; Bassareo

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et al. 2003; Di Chiara 1997; Grace 2000; Herz 1997; Koob and Weiss 1992; Oreland et al. 2011; Phillips and Shen 1996). However, the dopamine system is very complex and the role of the various dopaminergic receptor subtypes has been difficult to tease apart. The dopamine receptors can subdivided in two families: D1 family (dopamine D1 receptor (D1R) and D5R) and D2 family (D2R, D3R, and D4R) (Bordet 2004; Le Foll et al. 2009; Nieoullon and Amalric 2002; Sibley 1999; Velasco et al. 2002; Velasco and Luchsinger 1998; Verheij and Cools 2008). Most importantly, genetic studies have found associations between the dopaminergic system and alcoholism, including the identification of gene polymorphisms for some dopamine receptors (Ball and Murray 1994; Kohnke 2008; Li 2000; Tiihonen et al. 1995; Tyndale 2003), but not all reports are in agreement (Bolos et al. 1990). Thus, there is a putative genetic and physiological link between dopaminergic neurotransmission and ethanol abuse.

Several reports from research on laboratory animals suggest that the dopaminergic neurotransmission affects ethanol reward. For example, low doses of ethanol (0.25–0.5 g/kg, i.p.) stimulated dopamine release specifically in the nucleus accumbens (NAc) and elicited pure behavioral stimulation. This finding was abolished by pretreatment with gamma-butyrolactone, an agent which blocks dopamine firing and dopamine release (Di Chiara and Imperato 1988; Imperato and Di Chiara 1986). In addition, voluntary oral ethanol self-administration in alcohol-preferring Wistar rats has shown a robust increase of extracellular dopamine levels in the NAc with maximal effects at approximately 15–30 min after peak intake (Blanchard et al. 1993; Bustamante et al. 2008; Jerlhag et al. 2011; Kiianmaa et al. 1995; Nurmi et al. 1998; Weiss et al. 1993), suggesting that dopaminergic neurotransmission in the NAc may be an important factor in alcohol reinforcement. Also, the dopamine transporter together with dopamine innervation density, as determined by tyrosine hydroxylase immunostaining, was found to be lower in the NAc of the ethanol-preferring compared with that of non-preferring rats (Casu et al. 2002a, b; Zhou et al. 1995). Together, these observations suggest that alterations in dopamine transmission may drive ethanol consumption and preference.

Several dopaminergic receptors have been implicated in ethanol consumption, including D1R. Chronic ethanol intake led to bidirectional changes in the maximum number of [3H]SCH-23390 binding to striatal D1R measured 10 h after termination of the ethanol intake (Hamdi and Prasad 1993). In non-human primates, genetic variation in D1R influences alcohol consumption in rhesus macaques (Newman et al. 2009). Also, in rodents, chronic ethanol consumption induced a significant increase in the number of D1R sites in the caudate putamen (Lograno et al. 1993). Report with D1R knock-out mice has shown that constitutive absence of this receptor causes decreased ethanol intake as compared to their wild-type littermates (El-Ghundi et al. 1998). When infused into the NAc core or shell, the D1R antagonist (*R*-(+)-7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepine-8-ol) "SCH-23390" dose-dependently reduced reinstatement of ethanol self-administration in rats (Chaudhri et al. 2009). In addition, in high alcohol drinking line of rats, SCH-23390 (3–30 μg/kg) dose-dependently decreased ethanol drinking during the first hour of access (Chaudhri et al. 2009; Cohen et al. 1997; Dyr et al. 1993; Eiler et al. 2003; Hodge et al. 1997). However, other studies with the same antagonist have shown no effect on ethanol consumption (Melendez et al. 2005; Phillips et al. 1998; Silvestre et al. 1996). Surprisingly, the D1R partial agonist ((+/−)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7, 8-diol hydrochloride) (SKF-38393) also decreased alcohol intake in both rats (Cohen et al. 1999; Dyr et al. 1993; Silvestre et al. 1996) and mice (Ng and George 1994). We are focusing on D1 receptors expressed in medium spiny neurons, because they are expressed at high levels in the ventral striatum (Caille et al. 1996; Matamales et al. 2009; Muly et al. 2010; Podda et al. 2010) and are thought to be major regulators of dopaminergic neurons originating from the ventral tegmental area (VTA) that provide innervation to the NAc (Altier and Stewart 1999; Arias-Carrion et al. 2010; Cooper 2002; Gonzales et al. 2004; Herz 1997; McBride et al. 1999).

Given the links between dopamine, ethanol intake, and dopamine D1 receptor, we hypothesize in the current study that knocking-down the D1R mRNA in the nucleus accumbens will alter motivational aspects of ethanol drinking, behavioral sensitization, and conditioned place preference (CPP) during both the initiation and maintenance of ethanol reward.

Materials and methods

Animals

C57BL/6 male mice (25 to 35 g) were housed in groups of five per cage for at least 7 days before use and were maintained on a 12:00/12:00 hour light/dark cycle (lights off at 7:00 am) with food and water available ad libitum. All animal care and use were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All experimental procedures were approved by the local research ethics committee.

Drugs

Ethanol (2 g/kg, 20% *v*/*v*) was obtained by dilution of absolute ethanol in isotonic saline (0.9% NaCl). The specific

dopamine D1 receptor antagonist (6aS-*trans*)-11-chloro-6,6a,7,8,9,13b-hexahydro-7-methyl-5Hbenzo[d]naphth[2,1-b]azepin-12-ol hydrobromide "SCH-39166" (12.5 and 50 μg/kg) (Sigma) was dissolved in isotonic saline and administered s.c. (10 ml/kg) 15 min before ethanol.

Virus preparation of D1R small interference RNA-expressing vectors and viral delivery

The cloning of the dopamine D1 receptor small interference RNA (siRNA)-expressing lentiviruses has been described previously in detail (Ortiz et al. 2010). Briefly, three 19-nucleotide D1R siRNA sequences were added to the U6 promoter by PCR. The amplicon was then purified, digested with *Bam*HI/*Xho*I and ligated into the pTK431 previously digested with the same enzymes. Positive clones were confirmed by digestion and sequencing. Preparation of lentiviral vectors was initiated by triple transfection of HEK293T cells by calcium phosphate method using pTK431 together with pDeltaNRF and pMDG-VSV. Cells were harvested 72 h later and viruses were concentrated from the supernatant by ultracentrifugation. Vectors were resuspended in PBS–BSA and stored at −80°C till use (Bahi et al. 2004a, b, 2005a, b, 2006, 2008a, b, c; Bahi and Dreyer 2008).

To deliver the viruses into the NAc, we used stereotaxic injection. Briefly, mice first anesthetized with a ketamine/xylazine mixture and installed in a stereotaxic frame. Using a precision Hamilton micro-syringe with a 26 G needle, mice were bilaterally infused with viral solution using the following coordinates: +1.6 mm antero-posterior, ±0.8 mm lateral from the bregma, and 4 mm ventral from the skull (Franklin and Paxinos 1996). Viruses were infused at a rate of 0.1 μl/min for 5 min (final volume 0.5 μl/site) and the Hamilton micro-syringe was held in place for an additional 10 min before being withdrawn slowly. Following surgery, mice were allowed to recover for 7 days before undergoing the behavioral assays. Placement of the injections was determined by RT-PCR in punches from mice used to evaluate the efficacy of the shRNA-mediated knock-down.

Cell culture

HEK293T cells transfected with D1R-expressing plasmid were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were then transfected using 10 mg/ml Polybrene (Sigma) with lentiviruses expressing small interference RNA (siRNA)-expressing lentiviral vectors (LV-siD1R) or LV-Mock for 3 days before analysis of D1 receptor mRNA level.

Total RNA isolation and RT-PCR quantification

Total RNA from cells and brain tissue were isolated using Trizol (Invitrogen) and reverse-transcribed using Super-Script III reverse transcription (Invitrogen). RNA expression was analyzed by Q-PCR using SyberGreen with temperature cycling parameters consisting of initial denaturation at 95°C for 4 min followed by 40 cycles of denaturation at 94°C for 30 s, annealing and extension at 64°C for 45 s. PCR for the control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was run with the same cycling parameters. PCR products were normalized to GAPDH as indicated in the figure legends.

Two-bottle choice drinking test and preference for non-ethanol tastants

For two-bottle choice tests, mice were singly housed, and one 10-ml pipette containing tap water and one containing increasing ethanol concentration (3%, 6%, 10%, 15%, and 20%, *v/v*) were placed on each cage. The positions of the pipettes on the cage were random and inverted every day to avoid side preference. Mice were allowed free choice of these drinking solutions for 24-h periods with simultaneous free access to food. Ethanol concentration was raised every 5 days. Ethanol intake was calculated on the basis of ethanol volume consumed after each 24-h period, and body weights were used to calculate grams per kilogram of ethanol consumed (Bahi et al. 2011). One week after the completion of the ethanol drinking experiment, LV-Mock and LV-siD1R mice were also tested for saccharin and quinine consumption. One 10-ml pipette always contained water and the other contained the tastant solution. Mice were serially offered saccharin (0.04% and 0.08%) and quinine hemisulfate (0.02 and 0.04 mM) and intakes were calculated. Each concentration was offered for 5 days, with tube position changed every day. For each tastant, the low concentration was always presented first, followed by the higher concentration. Between tastants, mice had two bottles both containing water for 2 weeks (Bahi 2011; Bahi and Dreyer 2011; Bahi et al. 2011).

Ethanol-induced psychomotor sensitization

The acquisition and expression of psychomotor sensitization to alcohol was investigated using a 21-day protocol. On days 1–2, all mice received saline before being placed in the activity chambers for 15 min; this served to habituate the mice with the test procedures and provided a measure of baseline activity. On days 3–15, mice were injected with saline or 2 g/kg ethanol. On days 3, 7, 11, and 15, mice were placed in the activity chamber, immediately following the injection, and locomotor activity was monitored for 20 min.

These tests provided measures of psychomotor sensitization development. From days 13 to 19, animals were kept in their home cages and were not injected. On day 20, all animals received 2 g/kg, i.p. ethanol to assess the expression of sensitization. Finally, on day 21, all animals were evaluated for locomotor activity after saline to permit comparison of drug-free activity levels before and after repeated ethanol.

Conditioned place preference: apparatus, procedure, and experimental designs

The methodology was previously described (Bahi 2011; Bahi and Dreyer 2011). The apparatus consisted of two rectangular boxes (30 L×30 W×30 Hcm each) separated by a guillotine door. Different visual and tactile cues distinguished the two compartments: black walls and large grid floor for one compartment (A), and white walls and fine grid floor for the other compartment (B). Each experiment consisted of three phases. During the first phase (day 0, pre-conditioning phase), the guillotine door was kept lifted and each mouse was placed randomly in one or the other compartment and was given access to both compartments of the apparatus for 15 min (900 s). The time spent by each mouse in one compartment was recorded. During the second phase, conditioning phase (days 1 to 12), the mice were administered ethanol (2 g/kg) and immediately placed for 30 min in a given compartment. On alternate days, mice were administered with saline and immediately placed in the opposite compartment. During the last phase, post-conditioning test, 24 h after the last treatment, the guillotine door was kept opened and the time spent by each mouse in the drug-paired compartment was recorded during 15 min of observation. The conditions of the post-conditioning test were identical to those of the pre-conditioning test.

Effect of SCH-39166 on the acquisition of ethanol-induced CPP In this experiment, we examined the effect of SCH-39166 on the acquisition of ethanol (EtOH)-CPP. Mice were first tested for their baseline preference and alternately conditioned to ethanol in one compartment and saline in the other compartment. To determine the effects of SCH-39166 on the acquisition of ethanol memory, groups of mice received injections of different doses of SCH-39166 (12.5 and 50 μg/kg, s.c.) 15 min before each ethanol conditioning session. After the 12-day conditioning sessions, mice were tested for ethanol-induced CPP as described above. SCH-39166 working doses were chosen based on previously published work (Elliot et al. 2003; McCreary and Handley 1999; Witkin et al. 1999).

Effect of LV-siD1R on the acquisition of ethanol-induced CPP In this experiment, mice were tested for their baseline preference as described above. Mice were then stereotaxically injected with either LV-Mock or LV-siD1R in the NAc. After recovery (7 days), mice were conditioned with saline or ethanol as described above and tested for ethanol-induced CPP on day 13.

Effect of LV-siD1R on the expression of ethanol-induced CPP Mice were tested for their baseline preference and received 12 days of conditioning sessions. Mice were then stereotaxically injected with either LV-Mock or LV-siD1R in the NAc. After recovery (7 days), mice were tested for ethanol-induced CPP.

Statistical analysis

The statistics software package SPSS (version 19.0) was used throughout the analysis. Locomotor activity data were analyzed by a mixed three-way analysis of variance (ANOVA), with time as the repeated-measures factor (i.e., day of locomotor activity assessment for behavioral sensitization studies) and treatment (i.e., ethanol or saline administration on days 3–15) and virus group (i.e., LV-Mock or LV-siD1R) as the between-subjects factor. For ethanol drinking analysis, raw data (change in volume) were converted to grams of alcohol, ingested per kilogram of body weight for every data point. This allowed us to determine the exact dose of alcohol consumed by each animal. The data were analyzed with mixed two-way ANOVA using virus group (i.e., LV-Mock or LV-siD1R) as between-subjects factors. The within-subjects factor was the ethanol (i.e., concentration). For ethanol-induced CPP, data are expressed as mean±SEM of time spent during 900 s of observation in the ethanol-paired compartment during the post-conditioning phase with respect to the time spent during the pre-conditioning phase. To determine the effect of ethanol on CPP, the effects of SCH-39166 on ethanol-induced CPP acquisition and the effect of LV-siD1R on EtOH-CPP acquisition and expression data were analyzed by three-way ANOVA with pretreatment and treatment as independent factors (between-subjects), and pre- and post-conditioning as dependent factors (within-subjects, repeated measures). Post hoc analyses were undertaken if a significant effect of the interaction was found ($p<0.05$). Comparisons were carried out by Bonferroni post hoc evaluation.

Results

To study the possible role of endogenous D1R in the NAc in the regulation of ethanol reward, we utilized the lentivirus-mediated delivery of siRNA to knock-down the level of the receptor. This technique was extensively used in our laboratory to manipulate the number of genes in the central nervous system (Bahi et al. 2004a, b, 2005a, b, 2006, 2008a, b, c; Bahi

and Dreyer 2008). Three distinct D1R siRNA sequences were cloned into a lentiviral transfer vector containing that packaged into a lentiviral vector. These viruses were successfully used to knock-down the expression D1R mRNA in the NAc and proteins in the hippocampus (Ortiz et al. 2010).

As shown in Fig. 1a, D1R siRNA lentiviruses (LV-siD1R) significantly reduced the expression level of D1R mRNA in HEK293T cells transfected with D1R overexpressing vector as compared with uninfected cells and cells infected with a control non-specific RNA sequence lentivirus (LV-Mock). Next, we measured the level of D1R mRNA in the NAc after the administration of LV-siD1R and observed a decrease in D1R expression 7 days post-infusion of LV-siD1R, which was still observed 15 and 30 days later (Fig. 1b). A two-way ANOVA revealed a significant effect of virus injection [main effect of virus: $F_{(1, 35)}=11.155$, $p<0.01$; interaction virus × time points $F_{(2, 35)}=5.844$, $p<0.05$]. Furthermore, the down-regulation of D1R mRNA levels was specific, since infection of the NAc did not result in a decrease in the mRNA levels of the related dopamine

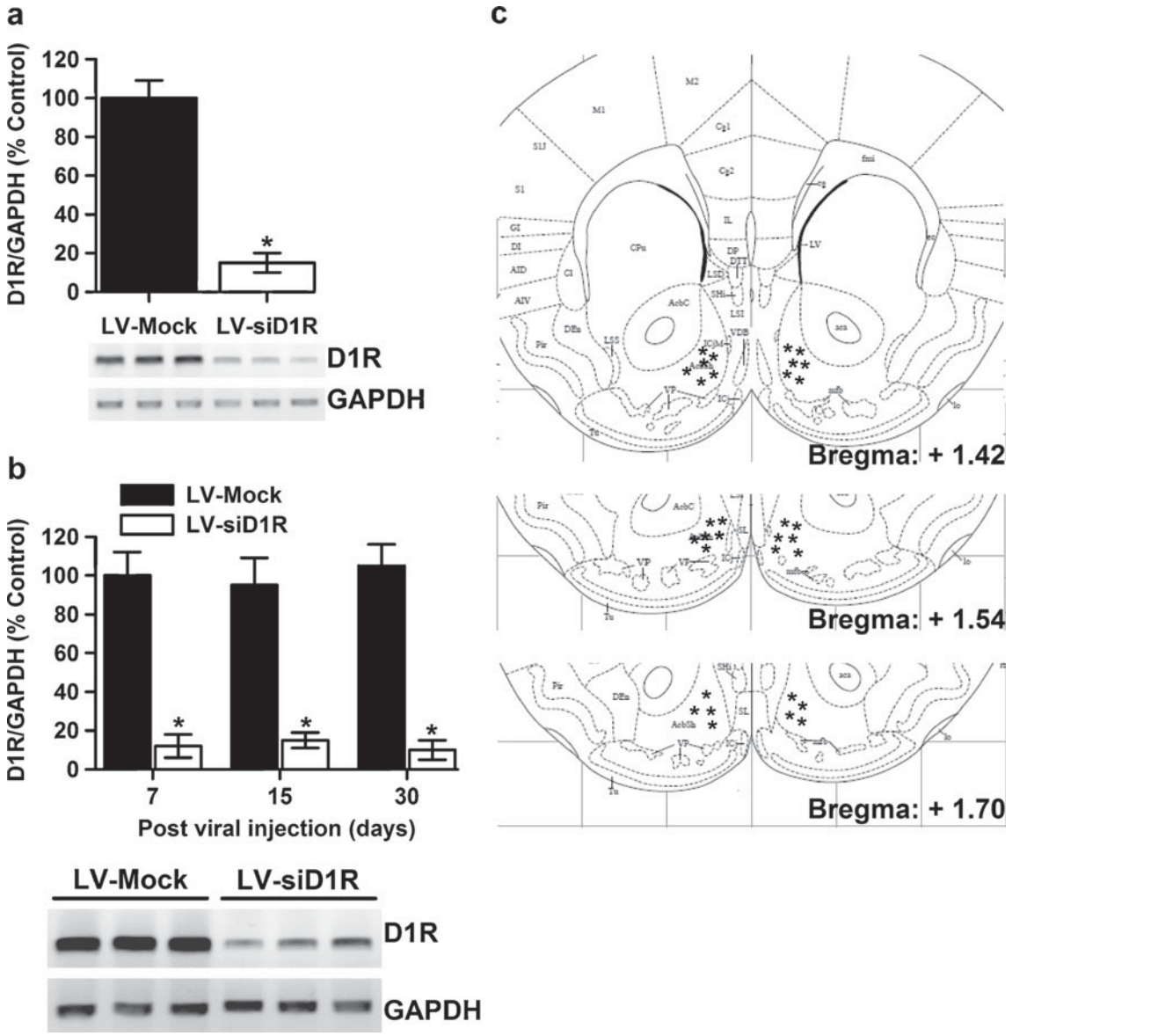

Fig. 1 Knock-down of D1R expression both in vitro and in vivo. **a** LV-siD1R-siRNA decreases D1R mRNA expression in vitro. D1R overexpressing HEK293T cells were infected with LV-siD1R or LV-Mock. Cells were collected and used for RT-PCR analysis of D1R expression. Histogram depicts the mean ratios of D1R/GAPDH±SEM ($n=5$). **b** Mice were stereotaxically infused with LV-siD1R into the NAc. Seven, 15, and 30 days after the microinjection, NAc were dissected out and used for RT-PCR analysis. LV-siD1R decreased D1R expression in the NAc. Histograms depict the mean ratios of D1R/GAPDH±SEM normalized to D1R levels obtained from LV-Mock animals. The *insert* is a representative image of D1R mRNA levels from samples collected 30 days after infection by the Mock or siD1R-expressing virus ($n=7$), *$p<0.01$. **c** Schematic representation of the sites of LV-D1R injection into the NAc

transporters D2R and D3R (data not shown). Together, these results show that lentivirus-mediated D1R siRNAs decrease receptor expression, which corresponds with a decrease in D1R mRNA level. The placement of injection sites is shown in Fig. 1c.

Dopamine D1 receptor knock-down reduced ethanol consumption

Two-bottle choice drinking paradigm allows measurement of ethanol preference and intake under conditions of voluntary consumption. As shown in Fig. 2a, in a two-bottle choice test, in which mice could drink either water or an ascending series of ethanol concentrations (3%, 6%, 9%, 15%, and 20%), mice injected with LV-siD1R displayed decreased ethanol consumption [main effect of virus: $F_{(1,\ 95)}=5.611$, $p<0.05$; main effect of ethanol concentration: $F_{(4,\ 95)}=12.084$, $p<0.01$; two-way AVOVA with repeated measures, factors were virus and ethanol concentration]. Results have also shown that blocking D1 receptor reduced ethanol preference as depicted in Fig. 2b [main effect of virus: $F_{(1,\ 95)}=7.152$, $p<0.05$; main effect of ethanol concentration: $F_{(4,\ 95)}=12.084$, $p<0.01$]. For both ethanol consumption and preference, a virus×ethanol concentration interaction was found. There were no differences in total intake of fluid between LV-Mock and LV-D1R mice as shown in Fig. 2c [main effect of virus: $F_{(1,\ 95)}=0.574$, $p>0.05$; main effect of ethanol concentration: $F_{(4,\ 95)}=1.095$, $p>0.05$].

When tested for taste neophobia using non-ethanol tastants, mice lacking D1R did not differ from control mice in preference for saccharin as shown in Fig. 3a [main effect of virus: $F_{(1,\ 36)}=0.781$, $p>0.05$; main effect of saccharin concentration: $F_{(1,\ 36)}=0.377$, $p>0.05$; two-way AVOVA with repeated measures, factors were virus and saccharin concentration]. Also D1R knock-down had no effect on quinine preference in mice as depicted in Fig. 3c [main effect of virus: $F_{(1,\ 38)}=0.914$, $p>0.05$; main effect of quinine concentration: $F_{(1,\ 38)}=6.488$, $p<0.05$]. There were no significant differences in total fluid intake (gram per kilogram per day) between LV-Mock and LV-D1R mice for saccharin [main effect of virus: $F_{(1,\ 36)}=0.187$, $p>0.05$; main effect of saccharin concentration: $F_{(1,\ 36)}=12.945$, $p<0.05$] or quinine [main effect of virus: $F_{(1,\ 38)}=0.294$, $p>0.05$; main effect of quinine concentration: $F_{(1,\ 38)}=0.715$, $p>0.05$] as shown in panels b and d of Fig. 3, respectively.

Effect of D1 receptor blockade on ethanol-induced psychomotor sensitization

Psychomotor stimulation, resulting in increased locomotor activity, is an indirect measure of mesolimbic dopaminergic system activation (for example, Badiani et al. 1995; Di Chiara and Imperato 1985, 1988; Guan et al. 1985; Kalivas and Stewart 1991; Wise 1987, 1988; Wise and Hoffman 1992). Most drugs of abuse, including ethanol, can induce locomotor activity in experimental animals (Cott et al. 1976; Erickson and Kochhar 1985; Frye and Breese 1981; Larsson and Engel 2004; Matchett and Erickson 1977; Phillips and Shen 1996). In line with the findings of reduced voluntary ethanol drinking, LV-siD1R-injected mice were insensitive to the acute stimulant effects of ethanol (2 g/kg, i.p.) (Fig. 4a). When locomotor activity was measured after a single injection of ethanol, LV-Mock displayed a significant increase in locomotion subsequent to ethanol injection. In contrast, ethanol lacks stimulant properties in LV-siD1R-injected mice. A two-way ANOVA for virus and ethanol treatment revealed a significant interaction [$F_{(2,\ 37)}=8.674$, $p<0.01$]. In addition, as shown in Fig. 4b, viral-mediated injection of D1R siRNA prior to ethanol treatment reduced the acquisition of ethanol-induced locomotor sensitization. A three-way ANOVA with repeated measures (EtOH dose×virus×test day) for data from the LV-siD1R treatment/EtOH sensitization phase (days 3–15) revealed a significant three-way interaction [$F_{(3,\ 127)}=6.512$; $p<0.05$]. Further analyses revealed significant ethanol dose×virus interactions on each of the treatment phase days (3, 7, 11, and 15). Mean comparisons showed that the LV-Mock-Ethanol group had significantly greater activity levels compared to the LV-siD1R-

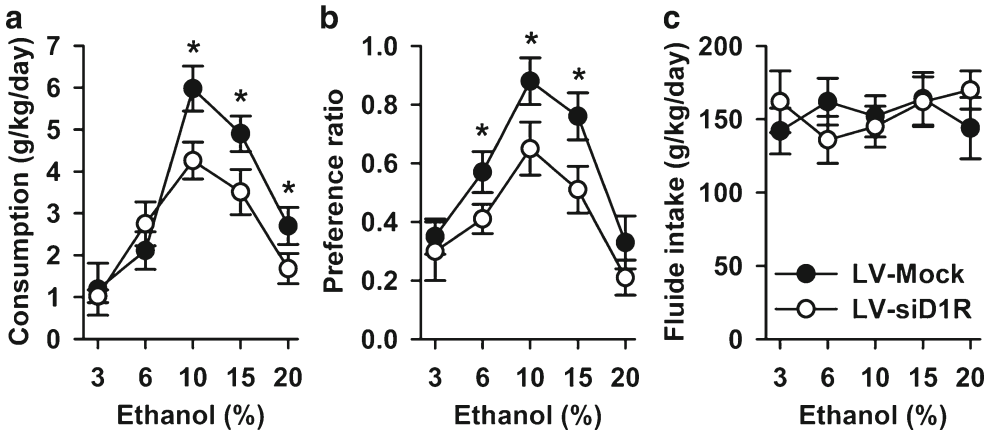

Fig. 2 Decreased ethanol consumption and preference in LV-siD1R mice. **a** Ethanol consumption was significantly lower in mutant LV-siD1R compared to LV-Mock mice. **b** Preference for ethanol was greater in LV-siD1R compared to LV-Mock mice. **c** The total amount of fluid (water+ethanol) intake was stable across ethanol concentrations for both groups of mice. Data are mean±SEM, n=10–11 for both groups

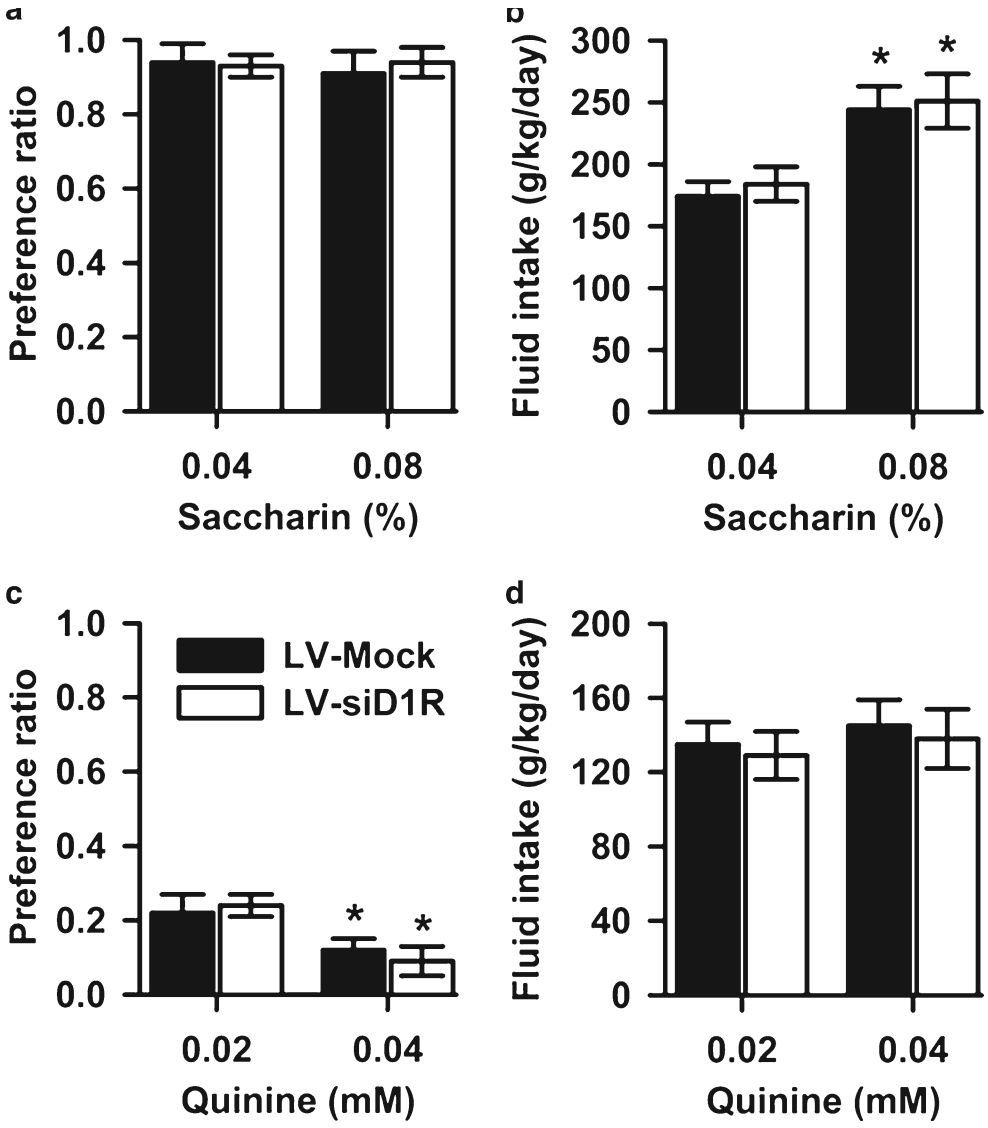

Fig. 3 No difference in preference for sweet (saccharin) or bitter (quinine) solutions between LV-siD1R compared to LV-Mock mice. **a** Preference for saccharin solutions was similar in LV-siD1R and LV-Mock mice. **b** Total fluid (saccharin+water) intake was similar in LV-siD1R and LV-Mock mice. **c** Preference for quinine was similar in LV-siD1R and LV-Mock mice. **d** Total fluid (quinine+water) intake was similar in LV-siD1R and LV-Mock mice. Data are mean±SEM, n=10–11 for both groups

Ethanol group across the entire treatment phase. No significant effect of LV-siD1R pretreatment was found in the LV-siD1R-Saline compared to the LV-Mock-Saline group. The blocking effect of LV-siD1R on ethanol-induced locomotor sensitization was also present in the challenge test (day 20), again indicating that LV-siD1R blocked the acquisition of sensitization to ethanol. A two-way ANOVA (treatment dose × virus) identified a significant interaction effect [$F_{(1,\ 44)}$=3.699; p<0.05], and pairwise comparisons confirmed significant sensitization in the LV-Mock-Ethanol, compared to the other three treatment groups. No differences among groups were found on saline test day 21.

Effects of dopamine D1 receptor blockade on ethanol-induced place preference

Effect of SCH-39166 on the acquisition of ethanol-induced CPP The effects of ethanol-induced CPP and of pretreatment with SCH-39166 (12.5 mg/kg) are shown in Fig. 5a. Mice receiving ethanol (2 g/kg) during conditioning spent more time in the drug-paired compartment during the post-conditioning test (785.62±66.32 s) with respect to the pre-conditioning test (452.31±61.29 s, p<0.001) and with respect to the post-conditioning test of the saline group (451.82±62.57 s, p<0.001). Repeated-measures three-way ANOVA of the effects of SCH-39166 on alcohol-induced CPP, with time of conditioning phases as within-subjects (dependent factors) and pretreatment and treatment as between-subjects (dependent factors), revealed a significant effect of conditioning [$F_{(1,\ 27)}$=6.945, p<0.01] but not a significant conditioning × pretreatment × treatment interaction [$F_{(1,\ 27)}$=1.032, p>0.05]. Bonferroni post hoc evaluation revealed that ethanol elicited a significant CPP and that pretreatment with SCH-39166 (12.5 μg/kg) did not affect it (p>0.05). The effects of ethanol-induced CPP and of pretreatment with SCH-39166 (50 μg/kg) are shown in Fig. 5b.

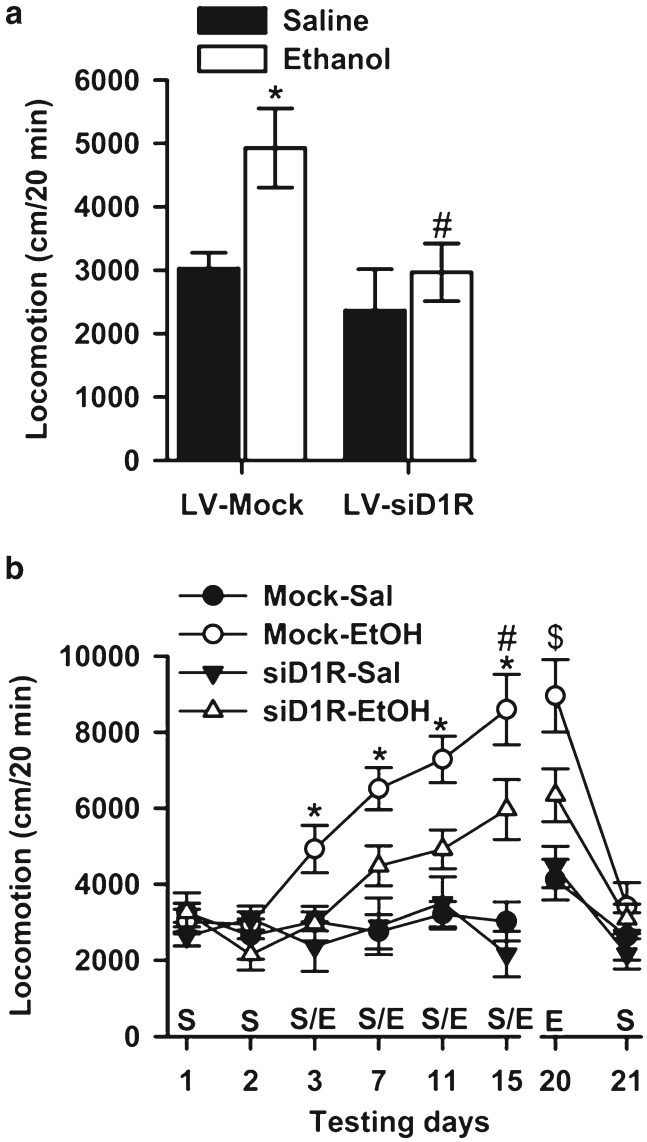

Fig. 4 Dopamine D1-siRNA effects on ethanol-induced psychomotor stimulation. Dopamine D1 receptor blockade blocks the **a** acute locomotor stimulant response, as well as **b** acquisition of locomotor sensitization to ethanol. Distance traveled (centimeter) after saline (*S*) or 2 g/kg ethanol (*E*) is shown following pre-injection in the NAc with LV-Mock or LV-siD1R prior to activity tests on days 1–15 (n=12–14 per group). *p< 0.005 for the comparison of LV-Mock-Ethanol vs. LV-siD1R-Ethanol on days 3, 7, 11, and 15. #p<0.005 for the comparison of LV-Mock-Ethanol day 12 vs. day 3. \$$p$<0.005 for the comparison of LV-Mock-Ethanol vs. the rest of the groups on day 20

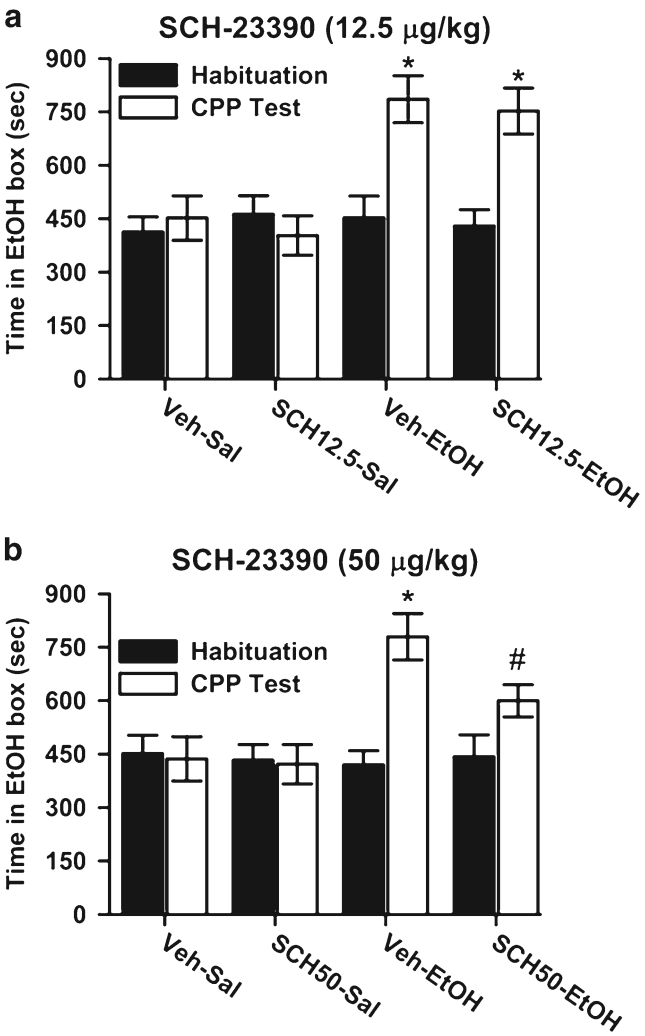

Fig. 5 Effect of SCH-39166 pretreatment on ethanol-induced acquisition of conditioned place preference. Before being conditioned with ethanol (2 g/kg), mice were pre-injected with **a** SCH-39166 (12.5 μg/kg) or **b** SCH-39166 (50 μg/kg). The number of mice was for Veh-Sal n=7 and 7; SCH-Sal n=8 and 9; Veh-EtOH n=7 and 8; SCH-EtOH n=9 and 8. Data are presented as time spent (seconds)±SEM in the ethanol-paired compartment. *p<0.01 significant differences between the time spent during post-conditioning test when compared with Veh-Sal group; #p< 0.05 significant differences between the time spent during post-conditioning test when compared with Veh-EtOH. *SCH* SCH-39166, *EtOH* ethanol, *Sal* saline, *Veh* vehicle

Mice receiving ethanol (2 g/kg) during conditioning spent more time in the drug-paired compartment during the post-conditioning test (779.35±65.21 s) with respect to the pre-conditioning test (419.88±39.82 s, p<0.001) and with respect to the post-conditioning test of the saline group (436.42±62.37 s, p<0.0186). Repeated-measures three-way ANOVA of the effects of SCH-39166 (50 μg/kg) on ethanol-induced CPP, with time of conditioning phases as within-subjects (dependent factors) and pretreatment and treatment as between-subjects (dependent factors), revealed a significant effect of conditioning [$F_{(1,\ 28)}$=10.362, p< 0.01] and a significant conditioning×pretreatment×treatment interaction [$F_{(1,\ 28)}$=4.644, p<0.05]. Bonferroni post hoc analysis revealed that ethanol elicited a significant CPP and that pretreatment with SCH-39166 reduced it (p<0.05).

Effect of LV-siD1R on the acquisition of ethanol-induced CPP The effects of D1R knock-down on ethanol-induced CPP acquisition are depicted in Fig. 6a. With respect to the pre-conditioning test, mice receiving ethanol (2 g/kg) during

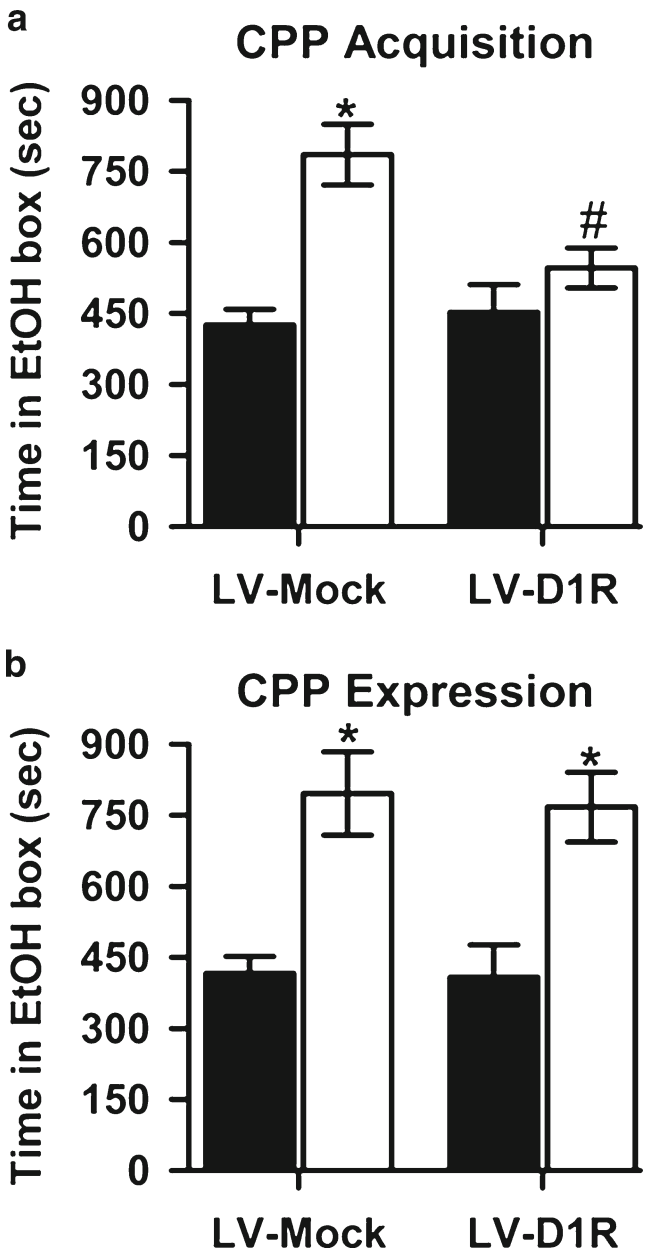

Fig. 6 Effect of D1R knock-down on ethanol-induced acquisition and expression of conditioned place preference. **a** LV-D1R disrupted the acquisition of ethanol reward memory. A significant difference was found in post-conditioning CPP scores between the LV-Mock group and LV-siD1R group ($n=10$ per group). *$p<0.01$, compared with pre-conditioning within the same group; #$p<0.01$, compared with post-conditioning in the LV-Mock group. **b** LV-D1R had no effect on ethanol-induced CPP expression. No significant difference was found in post-conditioning CPP scores between the LV-Mock group and LV-siD1R group ($n=10$–11 per group). *$p<0.01$, compared with pre-conditioning within the same group. All data are presented as time spent (seconds)±SEM in the ethanol-paired compartment, *black bars* habituation; *white bars* CPP test

conditioning spent more time in the drug-paired compartment during the post-conditioning test ($p<0.001$). Repeated-measures three-way ANOVA of the effects of LV-siD1R on ethanol-induced CPP, with time of conditioning phases as within-subjects (dependent factors) and virus and treatment as between-subjects (dependent factors), revealed a significant effect of conditioning [$F_{(1,\ 18)}=15.661$, $p<0.01$] and a significant conditioning×virus×treatment interaction [$F_{(1,\ 18)}=8.928$, $p<0.01$]. Bonferroni post hoc analysis revealed that ethanol elicited a significant CPP and that injection with LV-siD1R reduced it ($p<0.05$).

Effect of LV-siD1R on the expression of ethanol-induced CPP The effects of D1R knock-down on ethanol-induced CPP expression are depicted in Fig. 6b. Repeated-measures three-way ANOVA of the effects of LV-siD1R on the expression of ethanol-induced CPP, with time of conditioning phases as within-subjects (dependent factors) and virus and treatment as between-subjects (dependent factors), revealed a significant effect of conditioning [$F_{(1,\ 19)}=18.314$, $p<0.005$] but not a significant conditioning×pre-treatment×treatment interaction [$F_{(1,\ 19)}=0.311$, $p>0.05$]. Bonferroni post hoc evaluation revealed that ethanol elicited a significant CPP and that injection of LV-D1R did not affect it ($p>0.05$).

Discussion

While dopamine D1 receptor-dependent signaling has been widely implicated in cocaine reward, including conditioned place preference and self-administration, considerably less is known about the role of this receptor in ethanol-induced reward, although it is generally agreed that acute ethanol increases glutamate release via activation of dopamine D1 receptors (Xiao et al. 2009). Co-operative activation of D1-like and D2-like dopamine receptors in the nucleus accumbens shell is necessary to reinstate cocaine seeking in rats (Schmidt and Pierce 2006). For ethanol, studies found that ethanol increases extracellular glutamate levels in the VTA in midbrain slices and in vivo in rats and the increased glutamatergic transmission in turn modulates dopaminergic cell activity in the reward pathway and thus plays a significant role in the processes involved in alcohol addiction (Deng et al. 2009). Ethanol also modulates GABAergic synaptic transmission in the VTA (Xiao et al. 2008a, b). Alcohol dependence has been mainly associated to $GABA_A\alpha1$, $\alpha3$, and $\alpha6$ allelic polymorphisms (Sander et al. 1999; Thomas et al. 1998). Also during the formation of drug–stimulus associations, that are critical for cue-induced reinstatement, D1 receptors display unique contributions in mediating dopamine inputs within the basolateral amygdala complex (Andrzejewski et al. 2005; Berglind et al. 2006). However, specific brain regions and mechanisms are still being unknown.

In the current study, we provide the first evidence that the dopamine D1 receptor is required for ethanol intake in mice. We show that viral-mediated knockdown of D1R mRNA in the nucleus accumbens via RNAi-expressing lentiviral vectors impairs ethanol intake. There were no differences in the total fluid consumed and also no differences in the amount of ethanol consumed between groups prior to surgery. However, after surgery, the LV-siD1R group consumed less ethanol than the control group. Further, we demonstrate that ethanol-induced behavioral sensitization and conditioned place preference acquisition were also affected when D1R mRNA was inhibited. Down-regulation of endogenous D1R using viral-mediated siRNA in the NAc, significantly decreased ethanol-induced behavioral sensitization as well as acquisition, but not expression, of ethanol-induced CPP. This clearly indicates that decreased D1R expression into the NAc leads to reduced ethanol rewarding properties and to lower voluntary alcohol consumption, demonstrating that the D1 receptor pathway within the NAc controls ethanol reward and intake.

Dopamine D1 receptor and voluntary ethanol consumption

We show in the current study that D1R blockade in the NAc reduces ethanol consumption and preference in mice. There is compelling support for the contribution of the D1R in voluntary ethanol intake. In fact, chronic ethanol consumption in rats induced a significant increase in the number of dopamine D1 receptor binding sites in the caudate putamen (Lograno et al. 1993). Also, dopaminergic neurotransmission in the NAc and caudate putamen was highly affected upon ethanol exposure (Nestby et al. 1999), suggesting that these changes in dopamine receptors may be critical for ethanol response. Using genetic mapping in rhesus macaques, D1R polymorphism was associated with ethanol consumption in non-human primates in the context of early environmental stress induced by maternal separation (Newman et al. 2009). In rodents, our findings are in agreement with the documented decrease in ethanol consumption and preference in D1R-deficient mice ($D1R^{-/-}$) as compared to their heterozygous ($D1R^{+/-}$) and wild-type ($D1R^{+/+}$) littermates (El-Ghundi et al. 1998). In the same study, D1 receptor blockade with SCH-23390 reduced alcohol consumption in $D1R^{+/+}$ and $D1R^{+/-}$ mice to the level seen in untreated $D1R^{-/-}$ mice (El-Ghundi et al. 1998), suggesting that D1R signaling is highly involved in ethanol-seeking behavior in mice. In the same line, using limited access to 10% ethanol solution (4 h/day), D1R antagonist SCH-23390 dose-dependently decreased ethanol intake during the first hour of access (Dyr et al. 1993). However, pretreatment of C57BL/6 J inbred mice with D1R agonist (+/−)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride "SKF-38393" reduced voluntary ethanol intake approx. 76% in comparison to untreated controls (Ng and George 1994). Similarly, using the two-bottle free choice drinking paradigm, both ethanol consumption and preference were reduced by SKF-38393 in rats (Silvestre et al. 1996). Using operant responding behavior, systemic as well as microinjection of SCH-23390, but not SKF-38393, into the NAc reduced alcohol self-administration without affecting response rate (Chaudhri et al. 2009; Eiler et al. 2003; Hodge et al. 1997; Liu and Weiss 2002). Importantly, SKF-38393 pretreatment also decreased responding for saccharin (Cohen et al. 1999) and increased intravenous ethanol self-administration (D'Souza et al. 2003). In the light of these conflicting findings, it is difficult to tease apart the exact role of dopamine D1 receptor but clearly the partial agonist SKF-38393 may have an off target effect acting through D2R (Ruskin et al. 1998). As we used specific knock-down of D1R using shRNA-expressing lentiviral vectors, we propose that antagonizing D1R would be more beneficial to tackle alcohol abuse and alcoholism but further studies are needed to elucidate these conflicting observations.

Dopamine D1 receptor and ethanol-induced conditioned reward and locomotor stimulation

The findings of the current study demonstrate that D1R blockade inhibited ethanol-induced conditioned place preference and behavioral sensitization: D1 receptor-specific shRNA expression in the NAc consistently induced lower activity levels in sensitized animals. When DBA/2 J mice were injected systemically, the D1R partial agonist SKF-82958 showed no effect on sensitization to the locomotor-stimulating effects of ethanol (Broadbent et al. 2005). Although the i.p. administration of SKF-38393 did not affect the locomotor activity, the intra-NAc administration of SKF-38393 significantly increased the locomotor activity in sensitized mice. In contrast, both i.p. and intra-NAc administration of the D1R antagonist SCH-23390 blocked the expression of ethanol sensitization (Abrahao et al. 2011; Camarini et al. 2011). Taken together, these findings suggest that activation of D1 receptor in the NAc is necessary for the expression of ethanol sensitization.

When tested in ethanol-induced conditioned reward, we have shown that D1R blockade in the NAc attenuated ethanol-induced place preference. This effect was mostly studied in response to cocaine. In fact, large pieces of evidence suggest that dopamine D1R blockade inhibits cocaine-induced place preference (Akins et al. 2004; Baker et al. 1998; Hnasko et al. 2007; Liao et al. 1998; Sershen et al. 2010; Shippenberg and Heidbreder 1995). Our finding is in full agreement with other studies which reported that the D1 antagonist SCH-23390 significantly attenuated the ethanol-induced place preference (Matsuzawa et al. 1999). This effect may require an intact amygdala as the infusion of

the D1/D2/D3 receptor antagonist (flupenthixol) blocked ethanol CPP but not when it was injected into the NAc (Gremel and Cunningham 2009).

In summary, the present studies demonstrate that prior exposure to ethanol enhances the conditioning of cocaine-induced reward and behavioral sensitization. Viral-mediated shRNA expression studies further suggest the potential involvement of the dopamine Dl receptor in the development of this phenomenon. Given the role of reinforcement processes in the initiation of drug addiction, it is hypothesized that the sensitization that develops to the rewarding effects of alcohol may play an important role in drug craving and the reinstatement of compulsive drug-seeking behavior. Consequently, targeting D1R using SCH-23390-like compounds may be useful to approach ethanol addiction and alcoholism in humans.

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