Lentiviral vector-mediated dopamine D3 receptor modulation in the rat brain impairs alcohol intake and ethanol-induced conditioned place preference

Amine Bahi, Jean-Luc Dreyer

Materials and Methods

Lentiviral-mediated overexpression and siRNA knockdown of D3R

Briefly, for D3R-expressing lentiviral vectors (LV-D3R), D3R was amplified from total brain cDNA using specific primers and ligated into pTK431. D3R-specific siRNA expressing lentiviral vectors (LV-siD3R) were prepared using PCR amplification; shRNA oligos were added to the mouse U6 promoter using pSilencer 1.0-U6 (Ambion, UK) as a template and the PCR product was digested with *BamHI* and *XhoI* and cloned into similar sites in pTK431. After cloning and sequencing, all plasmids were $CsCl_2$-purified and lentiviruses were produced by the transient calcium phosphate co-transfection of HEK293T cells with pTKs vectors together with pMDG-VSV-G and pΔNRF as described previously (Bahi and Dreyer, 2012a; Bahi and Dreyer, 2012b; Bahi and Dreyer, 2013; Bahi et al., 2008b). The empty pTK431 vector (LV-Mock) was used as negative control.

Stereotaxic microinjection of lentiviral vectors

Rats (n=8) were anesthetized with Ketamine/xylazine (100 mg/kg, 10 mg/kg, i.p.) and placed in a stereotaxic apparatus. Using a precision Hamilton micro-syringe with a 26 G needle, and following the rat brain in stereotaxic coordinates (Paxinos and Watson, 1998), lentiviral vectors were injected bilaterally into the nucleus accumbens using following coordinates: 1st injection (+1.7 mm antero-posterior, ±1.0 mm lateral from the bregma, and 7.0 mm ventral from the skull); 2nd injection (+1.0 mm antero-posterior, ±1.3 mm lateral from the bregma, and 7.4 mm

ventral from the skull). For each injection 0.5 µL of viral particles were used. Viruses were infused at a rate of 0.2µL / min (final volume 1µL / hemisphere). Following vector administration, the Hamilton micro-syringe was left *in situ* for an additional 5 min to permit time for the vector to diffuse from the needle tip and minimize upward flow of viral solution after raising the needle before being slowly retracted from the brain. The Hamilton micro-syringe was rinsed with saline before being refilled for the next injection. After the surgical intervention, animals were injected subcutaneously with 5 ml pre warmed isotonic saline. Rats were singly housed with access to two-bottles of water and left to recover 7 days before behavioral experiments started.

Two-bottle choice drinking paradigm

Rats (n=8) were single housed and all experiments were conducted in the home cage. After 1 week of acclimatization, the animals were given ethanol in a two-bottle free-choice paradigm with the use of a continuous access schedule. Rats were tested for ethanol consumption and preference by offering them free choice between tap water and increasing concentrations of ethanol solution s (*v/v*) (2.5, 5, 10 and 20 % in tap water). The alcohol concentration was increased every 5 days. To avoid development of a positional bias, the positions of the drinking bottles were reversed each day. Water and ethanol intake was measured each 24 h period by weighing the bottles. Daily ethanol and water intakes were calculated from the consumed volumes of water and ethanol solution and expressed as g/kg to reduce the influence of differences in body weight. Ethanol preference was calculated as the percent ratio between ethanol consumption and total fluid (ethanol plus water) intake (i.e., (ethanol intake / total fluid intake) x 100).

In order to determine whether the difference in ethanol drinking observed following pharmacological and viral-mediated modulation of D3R was specific for ethanol or may be due to a preference for new liquids, an additional experiment was performed seven days later with the same animals with saccharin or quinine solutions, in addition to water as drinking fluids. Animals were given a choice between saccharin (at concentrations increasing from 0.04 to 0.08%) and tap water, for 5 days. 7 days later rats were given a choice between quinine (at concentrations increasing from 30 to 60 µM) and tap water, for 5 days. Solutions with different concentrations were used because a single concentration may be insufficient to detect differences between the treatments, mainly in the case of quinine solution. The 24 h consumption of water, ethanol, saccharin and quinine was measured daily around 16h00 and body weight was recorded every 5^{th} day.

Two control bottles (one with water and the other with the corresponding alcohol (or tastant) solution) were used to estimate evaporation and spillage, and the consumption amounts obtained for experimental animals were adjusted accordingly.

Conditioned Place Preference (CPP)

The CPP apparatus consisted of two-wooden chambers (30 × 30 × 30 cm) separated by a retractable guillotine door. Different visual and tactile cues distinguished the two chambers: black walls and large grid floor for one chamber, and white walls and fine grid floor for the other chamber. The floors of both chambers were raised 3 cm to reduce the accumulation of feces and urine. As for the exclusion criteria during baseline (Pre-CPP), rats exhibiting an unconditioned preference (more than 600 sec) or an aversion (less than 300 sec) for any chamber were discarded from the conditioning session (n = 5). During the Pre- and Post-CPP sessions, the room was kept in semi-darkness with only a single 60 W source bulb reflecting light off one wall

of the room. The place conditioning procedure consisted of 3 phases: Pre-CPP, conditioning and CPP-test.

At day 1 (Pre-CPP), rats were allowed to move freely in the two chambers for 15 min and the time spent in each chamber was manually scored. Most rats spent approximately 50% of the time in each chamber ($p > 0.05$). Thereafter, conditioning was performed using an unbiased protocol. At days 9 - 13 (Conditioning), rats were confined to one chamber for 30 min immediately after they had received 1 ml/kg saline i.p. injection. Four hours later, they received an i.p. injection of 0.5 g/kg ethanol prepared from 20% ethanol solution in isotonic saline (*v/v*) and were confined to the other side chamber for 30 min. The ethanol- and saline-paired conditioning chambers and the time of the ethanol or saline conditioning session (morning or afternoon) were counterbalanced across all groups. At day 14 (Post), rats were placed between the two-chambers and allowed to move freely for 15 min and time spent in ethanol-paired chamber was manually scored. EtOH-CPP was determined by plotting the difference between the time spent in the ethanol-paired chamber during the pre- and post-conditioning.

D3R mRNA quantification following LV-siD3R or LV-D3R injections in the NAcc

After completion of the behavioral experiments, rats were killed by rapid decapitation and NAcc were immediately dissected out. Total RNA was extracted by Trizol (Invitrogen) and precipitated with isopropanol. Total RNA was then quantified and used for cDNA preparation using a standard SuperScript III reverse transcriptase procedure. RNA expression was analyzed by qRT-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 sec, annealing and extension at 64°C for 45 sec. PCR for the control gene, GAPDH, was run with the

same cycling parameters. Template (2 µl) was amplified by PCR in 20 µl total reaction volume containing 0.5 µmol of each specific PCR primer.

Results

The D3R antagonist SB-277011-A did not affect saccharin and quinine consumption

Using the SB-277011-A (30 mg/kg), rats have been tested for taste neophobia using non-ethanol tastants. The one-way ANOVA repeated measure analysis revealed that SB-277011-A had no effect on saccharin intake [main effect of treatment: ($F_{(1,14)}$ = 0.470, p = 0.504). In contrast, an overall increase was found in saccharin consumption with concentration, as indicated by a significant effect of concentration on consumption ($F_{(1,14)}$ = 255.891, p < 0.0001). However, the main effect of treatment x saccharin interaction was not significant ($F_{(1,14)}$ = 0.413, p = 0.531)] (**Suppl. Fig. 1A**). In addition, saccharin preference was not affected upon SB-277011-A injection [main effect of treatment: ($F_{(1,14)}$ = 0.0001, p = 0.998), main effect of saccharin concentration: ($F_{(1,14)}$ = 31.979, p < 0.0001), main effect of treatment x saccharin interaction ($F_{(1,14)}$ = 1.359, p = 0.263)] (Data not shown). There were no significant differences in total fluid intake (g/kg/day) between vehicle and SB-277011-A (30 mg/kg) rats for saccharin [main effect of treatment: ($F_{(1,14)}$ = 1.400, p = 0.256), main effect of saccharin concentration: ($F_{(1,14)}$ = 3.596, p = 0.079), main effect of treatment x saccharin interaction ($F_{(1,14)}$ = 0.018, p = 0.896)] (**Suppl. Fig. 1B**).

The results of quinine intake are depicted in **Suppl. Fig. 1C**. One-way ANOVA for repeated measures revealed significant differences between quinine concentrations ($F_{(1,14)}$ = 32.282, p < 0.0001), but no main effect of SB-277011-A treatment ($F_{(1,14)}$ = 0.227, p = 0.641) nor quinine and treatment interaction ($F_{(1,14)}$ = 0.003, p = 0.960). For quinine preference, the one-way ANOVA revealed a main effect of quinine concentration ($F_{(1,14)}$ = 10.865, p = 0.005) but no main effect of SB-277011-A (30 mg/kg) treatment ($F_{(1,14)}$ = 0.602, p = 0.451) nor the treatment x quinine concentration interaction ($F_{(1,14)}$ = 0.134, p = 0.451) (Data not shown). As for the total

volume of fluid consumed, results are depicted in **Suppl. Fig. 1D**. The one-way ANOVA analysis revealed that the main effects of SB-277011-A treatment ($F_{(1,14)}$ = 0.132, p = 0.722), quinine concentration ($F_{(1,14)}$ = 1.735, p = 0.209) and their interaction ($F_{(1,14)}$ = 0.002, p = 0.961) were not significant.

Accumbal knockdown of D3R had no effect on saccharin and quinine intake and preference

To control for any taste neophobia following D3R knockdown in the NAcc, LV-Mock and LV-siRD3R-injected rats were tested with increasing concentrations of saccharin. As depicted in **Suppl. Fig. 2A**, the one-way ANOVA repeated measure indicated a non-significant effect of viral-injection on saccharin intake ($F_{(1,14)}$ = 0.267, p = 0.613) and a non-significant virus x concentration interaction ($F_{(1,14)}$ = 0.0001, p = 0.997). A significant concentration effect ($F_{(1,14)}$ = 43.017, p < 0.0001) reflected a general tendency of both experimental groups towards higher consumption from more concentrated saccharin solution. As expected, the one-way ANOVA (virus x concentration) indicated a non-significant effect of virus on saccharin preference ($F_{(1,14)}$ = 4.014, p = 0.065) and a non-significant virus x concentration interaction ($F_{(1,14)}$ = 0.785, p = 0.391). However, a significant concentration effect ($F_{(1,14)}$ = 67.604, p < 0.0001) reflected a general tendency of both experimental groups towards higher preference for the 0.08% saccharin solutions (Data not shown). Finally and as depicted in **Suppl. Fig. 2B**, the one-way ANOVA on total fluid intake measurement yielded no main effects of both factors: virus ($F_{(1,14)}$ = 0.284, p = 0.603); saccharin concentration ($F_{(1,14)}$ = 0.641, p = 0.437); and non-significant virus x concentration interaction ($F_{(1,14)}$ = 0.039, p = 0.846).

7 days later the same rats were tested for their intake and preference toward increasing concentrations of quinine (30 and 60 µM). The one-way ANOVA repeated measure revealed a

non-significant effect of viral-injection on quinine intake ($F_{(1,14)}$ = 1.809, p = 0.200), and a non-significant virus x concentration interaction ($F_{(1,14)}$ = 0.245, p = 0.628). However, the quinine concentration effect was significant ($F_{(1,14)}$ = 10.508, p = 0.006) (**Suppl. Fig. 2C**). The latter observation reflected the fact that quinine intake increased as its concentration increased in both experimental groups. In addition, the one-way ANOVA repeated measure showed no significant effect of viral-injection ($F_{(1,14)}$ = 1.461, p = 0.247) nor concentration ($F_{(1,14)}$ = 1.918, p = 0.188) on quinine preference. Also, a virus x concentration interaction largely missed significance ($F_{(1,14)}$ = 0.044, p = 0.837) (Data not shown). Finally and in line with the above, total fluid intake did not differ between the two experimental groups regardless of the solution available in the second bottle. In fact, the one-way ANOVA repeated measure revealed no main effect of virus ($F_{(1,14)}$ = 0.209, p = 0.654), no significant effect of concentration: ($F_{(1,14)}$ = 0.227, p = 0.641) and no virus x concentration interaction: ($F_{(1,14)}$ = 0.026, p = 0.873) as shown in **Suppl. Fig. 2D**.

The D3R agonist 7-OH-DPAT had no effect on saccharin and quinine intake and preference

For saccharin intake, data are presented in **Suppl. Fig. 3A**. The one way ANOVA analysis indicated that there was no significant effect of treatment between vehicle and 7-OH-DPAT-treated rats in terms of saccharin consumption (g/kg/24h) ($F_{(1,14)}$ = 0.470, p = 0.504). However, the ANOVA revealed a significant concentration effect ($F_{(1,14)}$ = 255.891, p < 0.0001), which reflected a general tendency towards higher intake of more concentrated saccharin solution. Interestingly, no treatment x concentration interaction was detected ($F_{(1,14)}$ = 0.413, p = 0.531). Similarly, 7-OH-DPAT also did not affect saccharin preference; [effect of treatment ($F_{(1,14)}$ = 0.0001, p = 0.998), effect of concentration ($F_{(1,14)}$ = 31.979, p < 0.0001), effect of treatment x concentration interaction ($F_{(1,14)}$ = 1.359, p = 0.263)]. Finally and depicted in **Suppl. Fig. 3B**,

there was no effect of treatment ($F_{(1,14)}$ = 1.400, p = 0.256) and no effect of saccharin concentration ($F_{(1,14)}$ = 3.596, p = 0.079) on total fluid intake.

When quinine was continuously available, there were no significant differences in mean quinine intake between vehicle and 7-OH-DPAT rats (main effect of treatment: ($F_{(1,14)}$ = 0.227, p = 0.641). Also, the interaction between treatment and concentration was not significant ($F_{(1,14)}$ = 0.003, p = 0.960). However, the main effect of concentration was significant ($F_{(1,14)}$ = 32.282, p < 0.0001) reflecting a general tendency towards higher intake of more concentrated quinine solution as depicted in **Suppl. Fig. 3C**. Mean preference for the quinine solution across the two-experimental groups was not affected neither by treatment ($F_{(1,14)}$ = 0.602, p = 0.451) nor by the interaction between treatment and concentration ($F_{(1,14)}$ = 0.134, p = 0.720). However, the main effect of concentration was significant ($F_{(1,14)}$ = 10.865, p = 0.005) (Data not shown). Analysis of total fluid intake revealed no significant effect of treatment ($F_{(1,14)}$ = 0.132, p = 0.722) or concentration ($F_{(1,14)}$ = 1.735, p = 0.209) in both groups. In addition, and as shown in **Suppl. Fig. 3D**, the interaction between treatment and concentration was not significant ($F_{(1,14)}$ = 0.002, p = 0.961).

Accumbal D3R overexpression had no effect on saccharin and quinine intake and preference

To determine if any difference existed in general taste preferences, drinking studies using saccharin and quinine were performed after the ethanol drinking study and results are depicted in **Suppl. Fig. 4**. For saccharin consumption, there was a significant effect of concentration ($F_{(1,14)}$ = 92.471, p < 0.0001). However, viral-injection had no effect on saccharin intake ($F_{(1,14)}$ = 0.184, p = 0.675). More importantly, no interaction was found between virus and saccharin concentration ($F_{(1,14)}$ = 0.003, p = 0.957). In fact and compared to the 0.04% solution, both LV-

Mock and LV-D3R-groups of rats consumed more saccharin from the 0.08% solution (**Suppl. Fig. 4A**). Similarly, saccharin preference was not affected following LV-D3R [main effect of viral-injection: ($F_{(1,14)}$ = 0.046, p = 0.834); interaction between viral-injection and concentration: ($F_{(1,14)}$ = 7.236, p = 0.018)]. However, both groups of rats preferred the 0.08% as evidenced by a main effect of concentration ($F_{(1,14)}$ = 121.252, p < 0.0001) (Data not shown). Finally and as shown in **Suppl. Fig. 4B**, total fluid intake was similar when the saccharin solutions were presented. The one-way ANOVA revealed no main effect of viral-injection ($F_{(1,14)}$ = 0.210, p = 0.654), no main effect of saccharin concentration ($F_{(1,14)}$ = 1.485, p = 0.243) and no interaction between viral-injection and saccharin concentration ($F_{(1,14)}$ = 0.067, p = 0.799).

A week later rats had access to increasing concentrations of quinine solutions. As shown in **Suppl. Fig. 4C**, the one-way ANOVA revealed that lentiviral-injection had no effect on quinine intake ($F_{(1,14)}$ = 0.325, p = 0.578). However, there was a main effect of quinine concentration ($F_{(1,14)}$ = 21.765, p < 0.0001) but no interaction between virus and quinine concentration ($F_{(1,14)}$ = 0.001, p = 0.974). Quinine preference was also similar in LV-Mock and LV-D3R-injected rats [main effect of virus: ($F_{(1,14)}$ = 0.235, p = 0.635); main effect of quinine concentration: ($F_{(1,14)}$ = 0.0001, p = 0.985); virus x quinine concentration interaction: ($F_{(1,14)}$ = 0.252, p = 0.623)] (Data not shown). Finally and as depicted in **Suppl. Fig. 4D**, the total fluid intake during quinine experiments was similar in both LV-Mock and LV-D3R groups [main effect of virus: ($F_{(1,14)}$ = 0.006, p = 0.938); main effect of quinine concentration: ($F_{(1,14)}$ = 0.398, p = 0.538); interaction between viral-injection and quinine concentration: ($F_{(1,14)}$ = 0.070, p = 0.795)].

Locomotor activity during the CPP-test following lentiviral injection

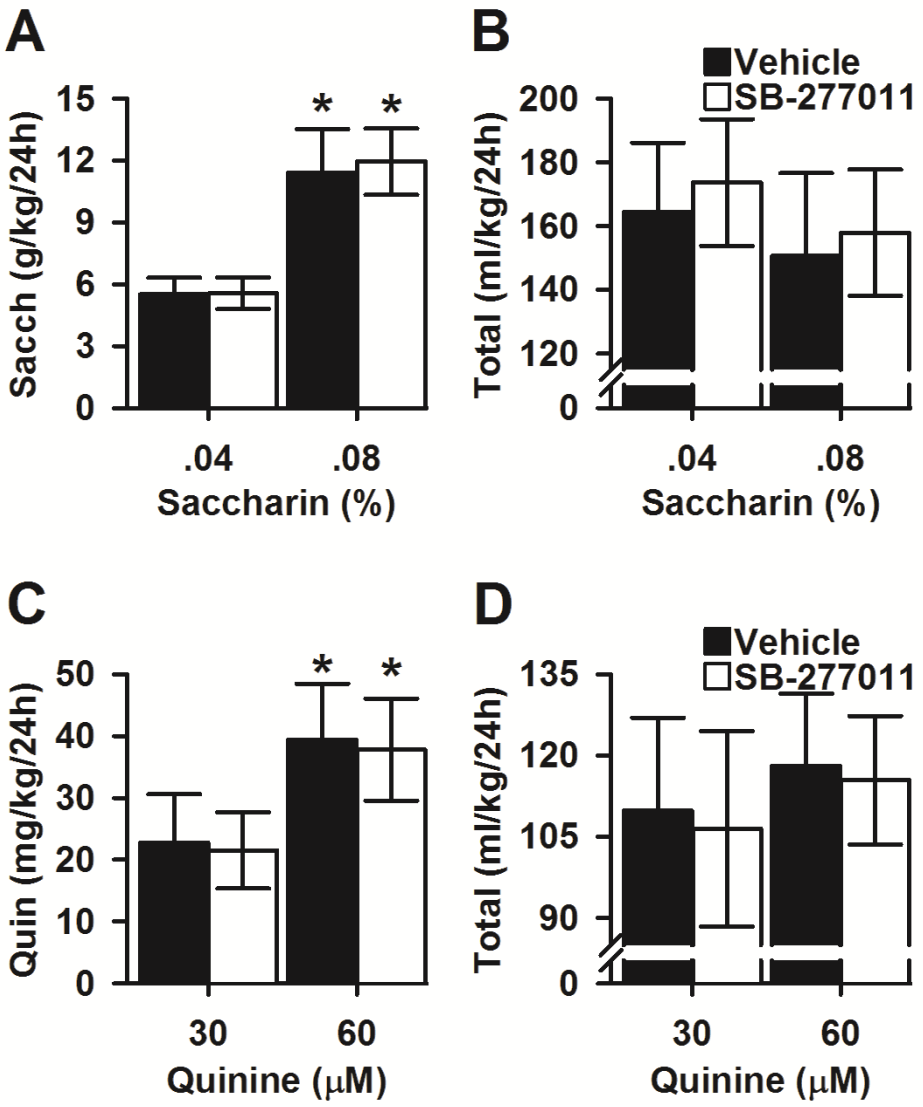
In order to rule out the possibility that the alterations in CPP were a byproduct of lentiviral vector effects on activity, we assessed locomotors activity during the CPP-test and results are

depicted in **Suppl. Fig. 5.** A one way ANOVA revealed that during the CPP-test, viral injection has no effect on spontaneous locomotr activity ($F_{(2,21)} = 0.188$, $p = 0.829$).

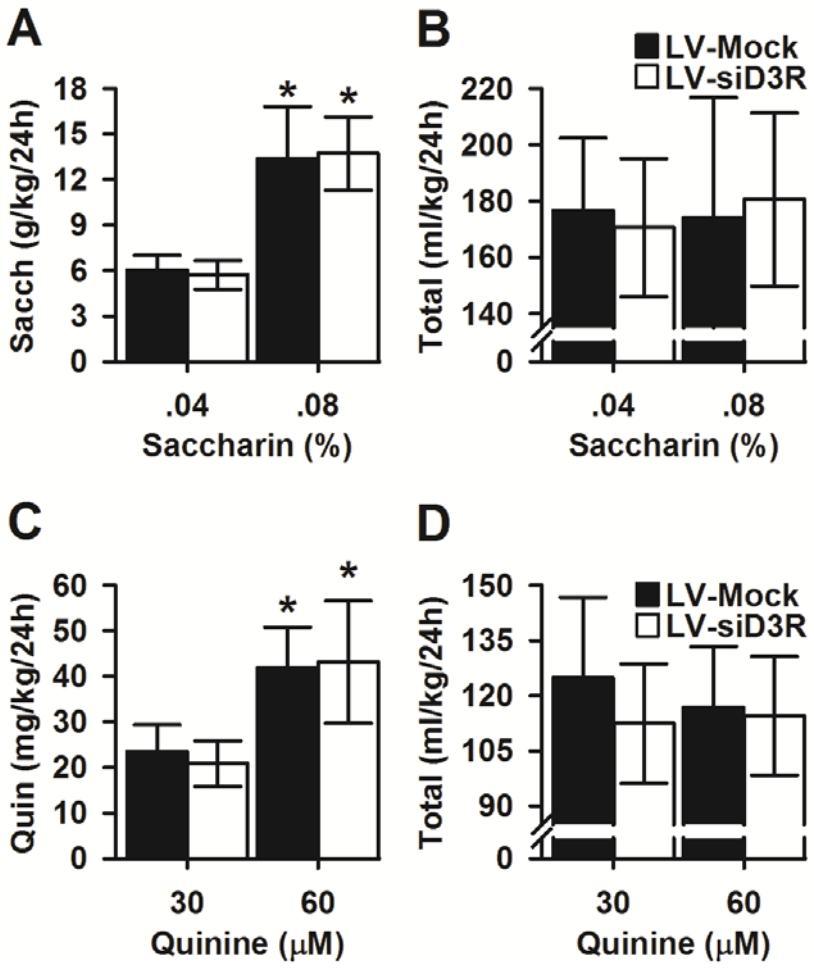
D3R quantification following lentiviral injections

At the end of the experiment, animals were killed by rapid decapitation, brains were dissected out and NAcc regions were used to prepare total RNA. cDNAs were then quantified by RT-PCR using specific sets of primers. D2R and D4R were used as control genes as they belong to the same family as D3R. Results (**Suppl. Fig. 6A**) revealed that LV-D3R injection increased D3R mRNA expression by approximately 2 folds ($F_{(1,14)} = 7.106$, $p = 0.018$). However, LV-D3R overexpression had no effect on either D2R ($F_{(1,14)} = 0.566$, $p = 0.464$) nor D4R ($F_{(1,14)} = 0.200$, $p = 0.662$). As for LV-siD3R expressing animals, results have shown that, compared to LV-Mock, siRNA knockdown only affected D3R mRNA expression ($F_{(1,14)} = 10.088$, $p = 0.007$). However, LV-siD3R had no effect on D2R ($F_{(1,14)} = 0.712$, $p = 0.413$) nor on D4R ($F_{(1,14)} = 0.567$, $p = 0.464$) mRNA expression (**Suppl. Fig. 6B**).

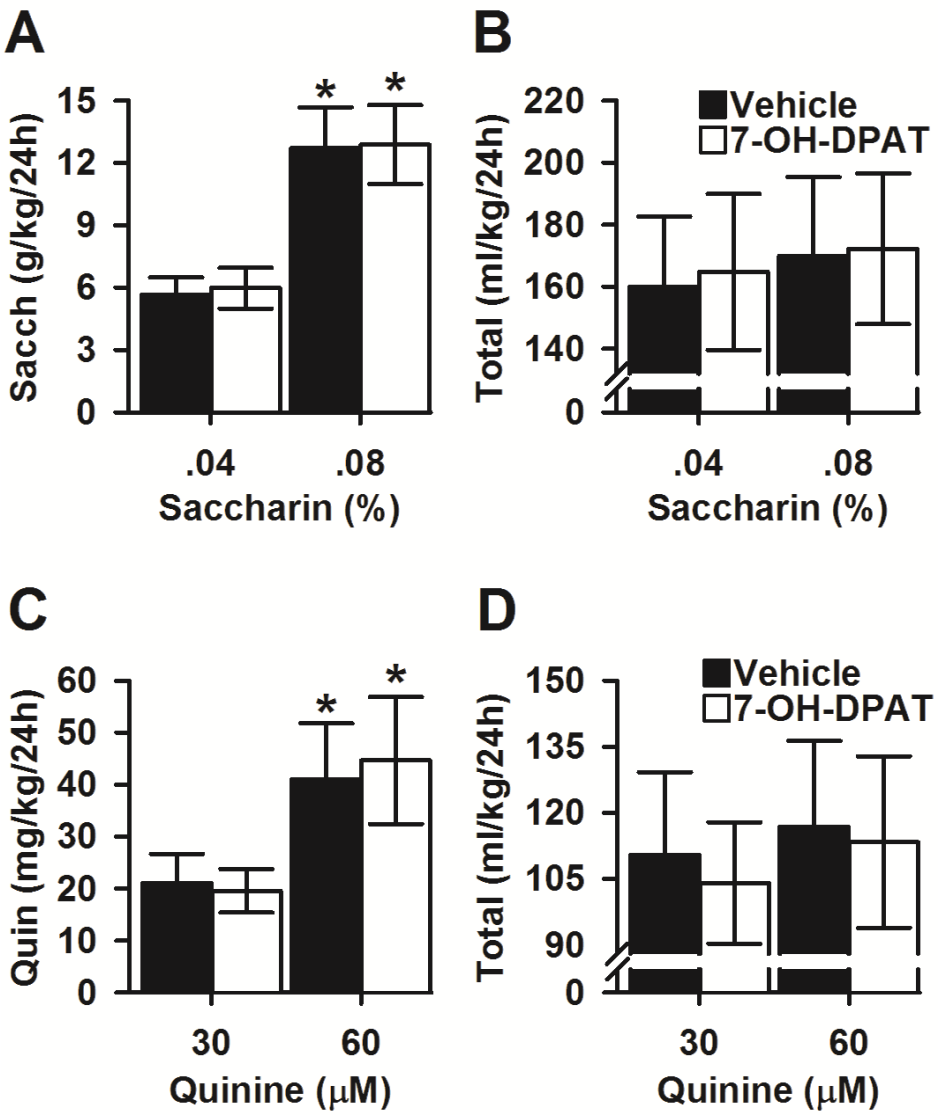
It should be emphasized that the stereotaxic coordinates we aimed for were NAcc-shell specific. The RT-PCR mRNA quantification results presented above are obtained from NAcc-shell punched tissue samples. In one hand, we do not rule out that some negligible viral particles may have infected the NAcc-core subdivision. In the other hand we didn't want to be overambitious with sub-region difference and take the risk of missing mRNA overexpression/knockdown differences. This is our 1st attempt to provide a link between D3R expression and ethanol intake in rodents and further studies will be performed to investigate whether discrete sub-regions of the NAcc may have similar / different behavioral readouts.



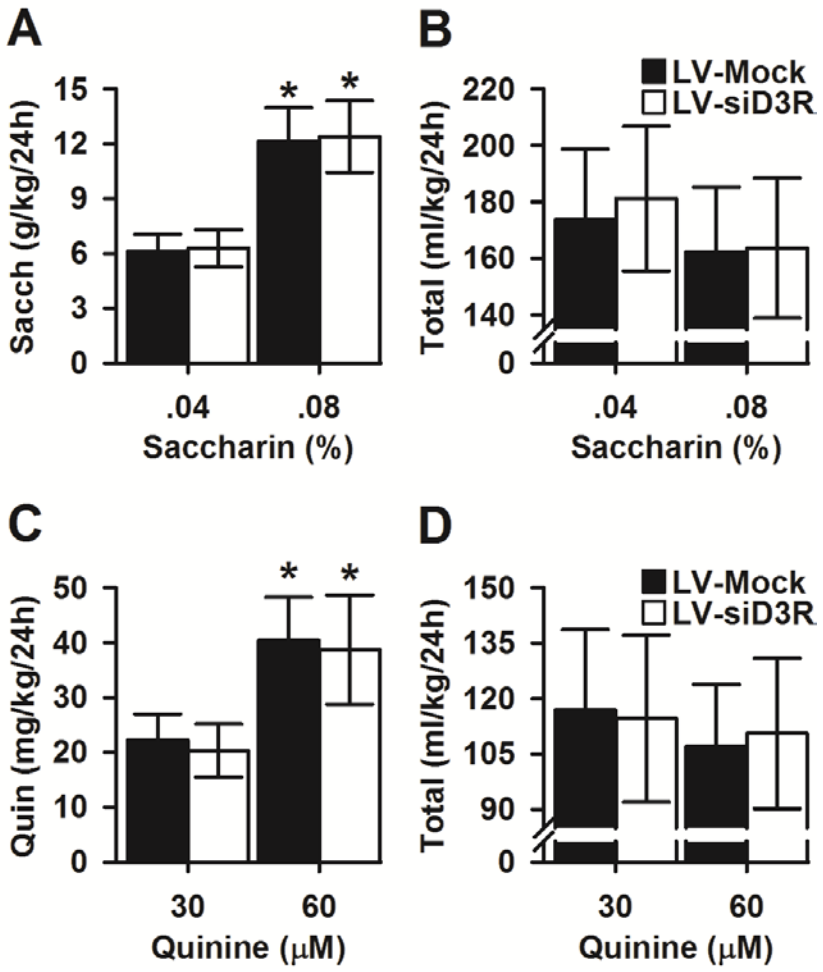
Suppl. Fig. 1 Voluntary saccharin and quinine consumption and total fluid intake following SB-277011-A injection. **A)** Saccharin consumption expressed as a gram of saccharin solution consumed per kg of body weight per day. **B)** Total fluid intake expressed as the sum of the volume of the saccharin solution and water consumed per kg of body weight per day. **C)** Quinine consumption expressed as a milligram of quinine solution consumed per kg of body weight per day. **D)** Total fluid intake expressed as the sum of the volume of the quinine solution and water consumed per kg of body weight per day. The data represent mean ± SEM. The number of animals per group was n = 8. * $p < 0.05$.



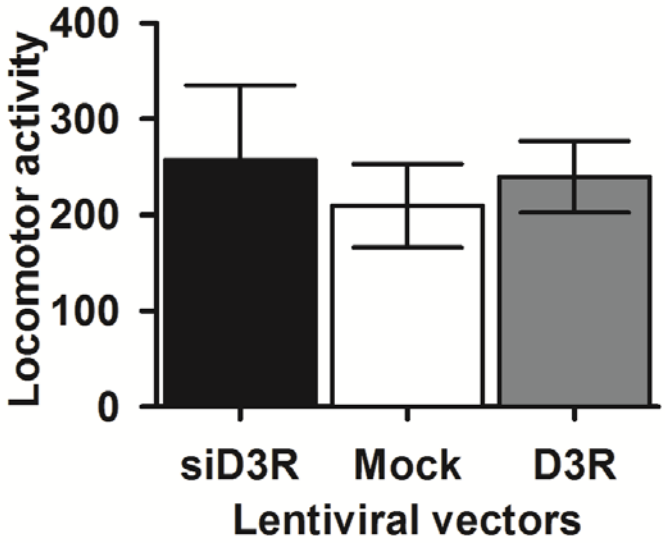
Suppl. Fig. 2 Saccharin and quinine consumption and total fluid intake following accumbal knockdown of D3R. Wistar rats were stereotaxically injected with LV-Mock (n = 8) and LV-siD3R (n = 8) into the NAcc and given access to increasing concentrations of non-alcohol tastants and water in a two-bottle choice drinking paradigm for 5 days. **A)** Saccharin consumption expressed as a gram of saccharin solution consumed per kg of body weight per day. **B)** Total fluid intake expressed as the sum of the volume of the saccharin solution and water consumed per kg of body weight per day. **C)** Quinine consumption expressed as a milligram of quinine solution consumed per kg of body weight per day. **D)** Total fluid intake expressed as the sum of the volume of the quinine solution and water consumed per kg of body weight per day. The data represent mean ± SEM. * $p < 0.05$.



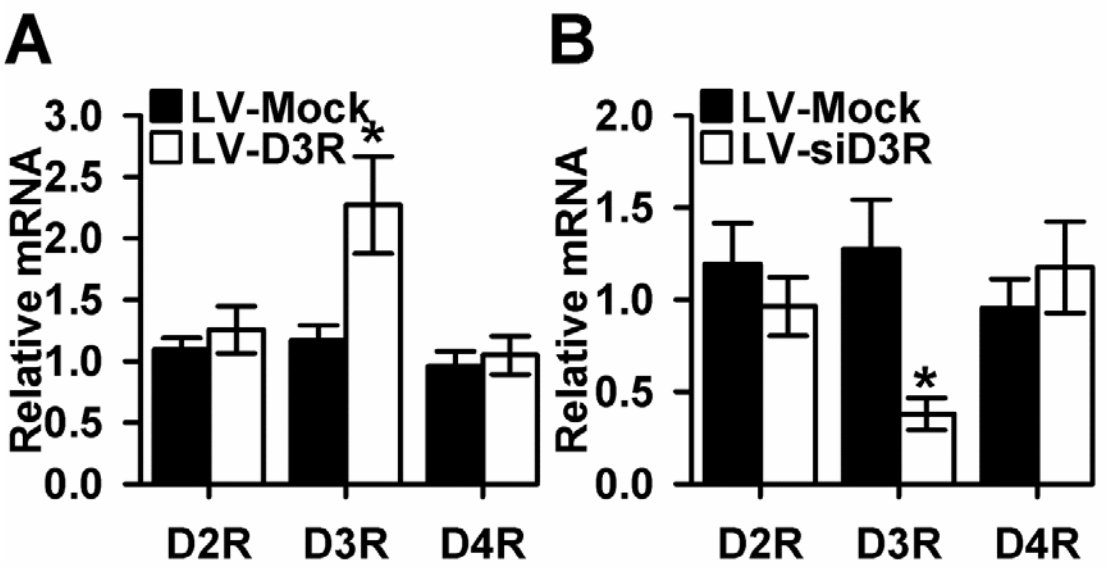
Suppl. Fig. 3 Voluntary saccharin and quinine consumption and total fluid intake following 7-OH-DPAT injection. **A)** Saccharin consumption expressed as a gram of saccharin solution consumed per kg of body weight per day. **B)** Total fluid intake expressed as the sum of the volume of the saccharin solution and water consumed per kg of body weight per day. **C)** Quinine consumption expressed as a milligram of quinine solution consumed per kg of body weight per day. **D)** Total fluid intake expressed as the sum of the volume of the quinine solution and water consumed per kg of body weight per day. The data represent mean ± SEM. The number of animals per group was n = 8. * $p < 0.05$.



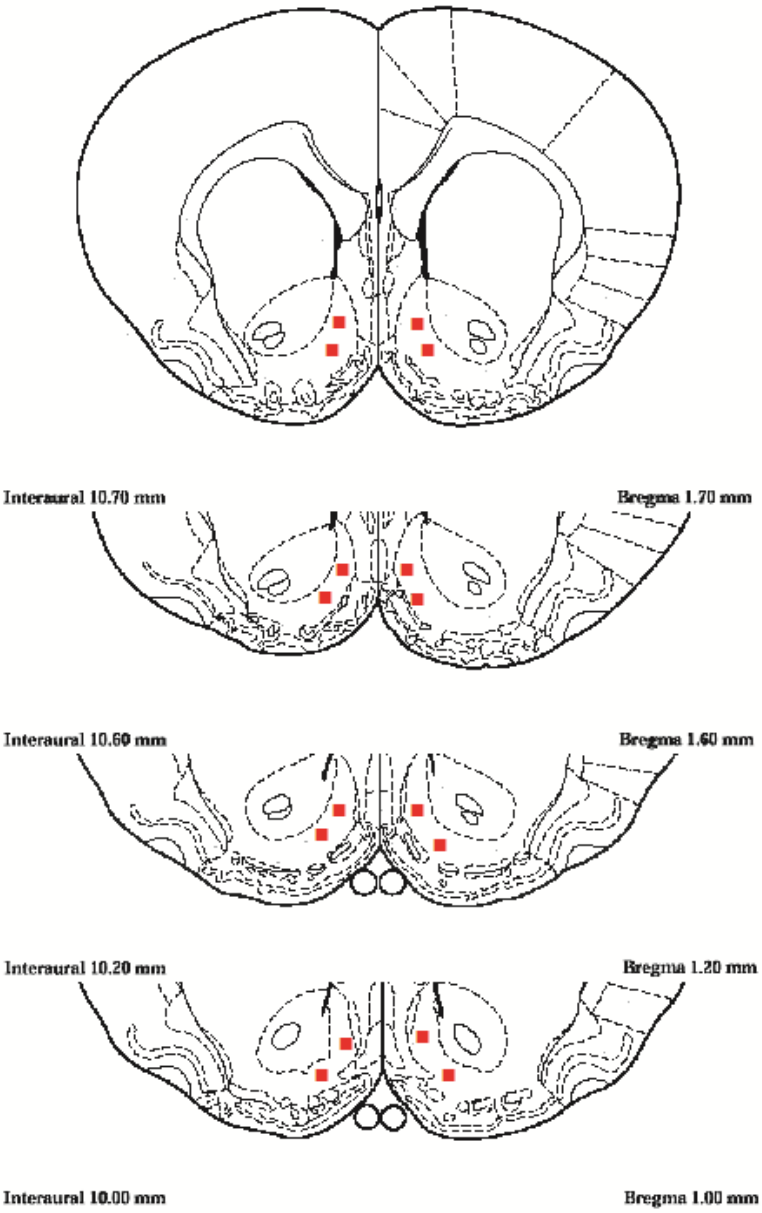
Suppl. Fig. 4 Saccharin and quinine consumption and total fluid intake following accumbal overexpression of D3R. Wistar rats were stereotaxically injected with LV-Mock (n = 8) and LV-D3R (n = 8) into the NAcc and given access to increasing concentrations of non-alcohol tastants and water in a two-bottle choice drinking paradigm for 5 days. **A)** Saccharin consumption expressed as a gram of saccharin solution consumed per kg of body weight per day. **B)** Total fluid intake expressed as the sum of the volume of the saccharin solution and water consumed per kg of body weight per day. **C)** Quinine consumption expressed as a milligram of quinine solution consumed per kg of body weight per day. **D)** Total fluid intake expressed as the sum of the volume of the quinine solution and water consumed per kg of body weight per day. The data represent mean ± SEM. * $p < 0.05$.



Suppl. Fig. 5 Effects of D3R overexpression and knockdown on locomotors activity during the CPP-test. When animals were tested for their CPP-preference test, lentiviral-mediated overexpression or knock down of the D3R did not change spontaneous locomotor activity. Data are means ± SEM. N = 8.



Suppl. Fig. 6 Effects of D3R overexpression and knockdown on mRNA expression. (A) Lentiviral-mediated overexpression increased D3R mRNA expression in the NAcc but not for D2R neither for D4R. **(B)** siD3R-expression in the NAcc decreased D3R, but not D2R nor D4R, mRNA levels. Data are means ± SEM. *$p \leq 0.05$ *vs.* Mock.



Suppl. Fig. 7 Histological representation of injection placements in the NAcc-Shell. The distance anterior to bregma (in millimeters) is indicated.