

Dopamine D3 Receptor Is Necessary for Ethanol Consumption: An Approach with Buspirone

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Mesolimbic dopamine (DA) controls drug- and alcohol-seeking behavior, but the role of specific DA receptor subtypes is unclear. We tested the hypothesis that D₃R gene deletion or the D₃R pharmacological blockade inhibits ethanol preference in mice. D₃R-deficient mice (D₃R^{-/-}) and their wild-type (WT) littermates, treated or not with the D₃R antagonists SB277011A and U99194A, were tested in a long-term free choice ethanol-drinking (two-bottle choice) and in a binge-like ethanol-drinking paradigm (drinking in the dark, DID). The selectivity of the D₃R antagonists was further assessed by molecular modeling. Ethanol intake was negligible in D₃R^{-/-} and robust in WT both in the two-bottle choice and DID paradigms. Treatment with D₃R antagonists inhibited ethanol intake in WT but was ineffective in D₃R^{-/-} mice. Ethanol intake increased the expression of RACK1 and brain-derived neurotrophic factor (BDNF) in both WT and D₃R^{-/-}; in WT there was also a robust overexpression of D₃R. Thus, increased expression of D₃R associated with activation of RACK1/BDNF seems to operate as a reinforcing mechanism in voluntary ethanol intake. Indeed, blockade of the BDNF pathway by the TrkB selective antagonist ANA-12 reversed chronic stable ethanol intake and strongly decreased the striatal expression of D₃R. Finally, we evaluated buspirone, an approved drug for anxiety disorders endowed with D₃R antagonist activity (confirmed by molecular modeling analysis), that resulted effective in inhibiting ethanol intake. Thus, DA signaling via D₃R is essential for ethanol-related reward and consumption and may represent a novel therapeutic target for weaning.

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INTRODUCTION

The mesolimbic dopamine (DA) pathway mediates the rewarding effects of drugs of abuse (Bowers *et al*, 2010; Ikemoto and Bonci, 2013; Koob, 1992; Robbins and Everitt, 1996; Wise and Bozarth, 1987), including ethanol and opiates (Pierce and Kumaresan, 2006; Wise and Bozarth, 1987). Both oral self-administration (Weiss *et al*, 1992) and systemic administration of ethanol increase the firing rate of mesolimbic dopaminergic neurons (Gessa *et al*, 1985; Mereu *et al*, 1984) and stimulate extracellular DA release in the striatum and in the nucleus accumbens (Imperato and Di Chiara, 1986; Yoshimoto *et al*, 1992). In a recent meta-analysis on published data sets of *in vivo* microdialysis in

rat brain, the acute administrations of ethanol appear to increase the level of monoamines, including DA, globally and independent of the brain sites up to 270% of the basal concentrations (Brand *et al*, 2013). DA exerts its action through five receptor subtypes (D₁₋₅R); the D₃ receptor (D₃R) subtype has an important role in the modulation of the mesolimbic DA pathway and in the control of drug-seeking behavior (Heidbreder *et al*, 2005; Joyce and Millan, 2005). The D₃R is located both at pre- and post-synapses, in the ventral striatum (nucleus accumbens and island of Calleja (Bouthenet *et al*, 1991; Murray *et al*, 1994)); in these structures, stimulation of presynaptic D₃R may modulate DA synthesis and release (Levant, 1997). Several studies have explored the involvement of D₃R in ethanol-drinking paradigms (Cohen *et al*, 1998; Harrison and Nobrega, 2009; Heidbreder *et al*, 2007; Rice *et al*, 2012; Silvestre *et al*, 1996; Thanos *et al*, 2005), but their precise role remains unclear. Indeed, pharmacological studies generally report that D₃R blockade decreases ethanol consumption (Heidbreder *et al*, 2007; Rice *et al*, 2012; Silvestre *et al*, 1996; Vengeliene *et al*, 2006); in contrast, genetic manipulation studies did not find

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a change in ethanol intake following D₃R gene deletion (McQuade *et al*, 2003).

In the present study, we tested the hypothesis that D₃R gene deletion or the D₃R pharmacological blockade inhibits the ethanol preference and the voluntary intake in mice. Mice D₃R^{-/-} and their wild-type (WT) littermates, treated or not with D₃R selective antagonists, were tested in a long-term free choice ethanol-drinking paradigm (two-bottle choice) (McQuade *et al*, 2003; Wise, 1973) and in a binge-like ethanol-drinking paradigm (drinking in the dark, DID). Activation of the RACK1/BDNF (brain-derived neurotrophic factor)/D₃R pathway (Jeanblanc *et al*, 2006) and activation of DA transmission were assessed at the end of behavioral experiments. The RACK1/BDNF/D₃R pathway was here considered because D₃R expression is related to BDNF (Guillin *et al*, 2001; Le Foll *et al*, 2005b) and ethanol exposure is able to increase RACK1 translocation into the nucleus of neurons, which increases expression of BDNF (Jeanblanc *et al*, 2006; McGough *et al*, 2004). Finally, the effect of buspirone was evaluated in the drinking paradigms. Because buspirone is an already approved drug for anxiety disorders, endowed with D₃R antagonist activity, it may be easier to translate to the clinic practice.

MATERIALS AND METHODS

Animals

Mice D₃R null (D₃R^{-/-}) and WT littermates (males, 8–12 weeks old) were individually housed, with free access to chow and water (except in the ethanol-drinking procedures), in an air-conditioned room, with a 12-h light-dark cycle. Mice D₃R^{-/-} were 10th–12th generation of congenic C57BL/6J mice, generated by a back-crossing strategy (Accili *et al*, 1996). All experiments were carried out according to the Directive 2010/63/EU and to the Institutional Animal Care and Use Committee of the Catania University.

Two-Bottle Choice Paradigm

Mice D₃R^{-/-} ($n = 30$) and WT ($n = 30$) received 24 h free access to tap water and 10% ethanol solution (v/v), contained in 100 ml graduated tubes with stainless steel drinking spouts; the position of tubes was interchanged (left/right) every 24 h, to prevent acquisition of position bias. Ethanol and water intake was measured as daily consumption in grams. The experiments lasted 59 days. For the first 15 days, (habituation period) animals received 24 h free access to two tubes containing only tap water (time 0 in Figure 1a). After the habituation period (from 15 to 59 days), 10% ethanol solution was available in one of the bottles.

In the forced alcohol-drinking procedure, D₃R^{-/-} ($n = 12$) and WT ($n = 18$) received for the first 15 days (habituation period) tap water only (time 0), followed (from 15 to 59 days) by 10% ethanol only.

DID Paradigm

The 4 h version of the behavioral paradigm was used, as described by Rhodes *et al* (2005). The procedure started 3 h after lights off in the animal room. Water bottles were

replaced with graduated tubes with stainless steel drinking spouts containing 20% (v/v) ethanol in tap water. This was done in home cages where animals were singly housed (Rhodes *et al*, 2005). The ethanol tubes remained in place for 2 h. After the 2-h period, intakes were recorded, and the ethanol tubes were replaced with water tubes. This procedure was repeated on days 2 and 3. On day 4, the procedure was again repeated except that the ethanol tubes were left in place for 4 h, and intakes were recorded after 4 h.

Drugs and Treatments

Ethanol, U99194A maleate, SB277011A hydrochloride, buspirone hydrochloride, 8-OH-DPAT and ANA-12 were from Sigma (St Louis, MO). All drugs were dissolved in saline and intraperitoneally (i.p.) injected (in a volume of 10 ml/kg), except ANA-12 that was dissolved in 10% dimethyl sulfoxide. U99194A was used at 10 mg/kg (Harrison and Nobrega, 2009), SB277011A was used at 10 mg/kg (Song *et al*, 2012), buspirone was used in the range 0.1–10 mg/kg (Martin *et al*, 1992), 8-OH-DPAT was used at 1 mg/kg (Martin *et al*, 1992), and ANA-12 was used at 0.5 mg/kg (Cazorla *et al*, 2011).

In the two-bottle choice paradigm, after 30 days of voluntary alcohol-drinking procedure, D₃R^{-/-} and WT were randomly allocated to the eight experimental groups ($n = 6/10$ per group): WT/vehicle, WT/U99194A, WT/SB277011A, WT/buspirone, D₃R^{-/-}/vehicle, D₃R^{-/-}/U99194A, D₃R^{-/-}/SB277011A, and D₃R^{-/-}/buspirone. Animals were i.p. injected once a day, for 14 consecutive days. On day 14, animals were sacrificed 1 h after the last administration and brain tissues were taken. In another set of experiments, after 30 days of voluntary alcohol-drinking procedure, mice were randomly allocated to five experimental groups ($n = 5/7$ per group): WT naïve, WT/vehicle, WT/ANA-12, D₃R^{-/-}/vehicle, and D₃R^{-/-}/ANA-12. Animals were i.p. injected once a day, for 4 consecutive days with the selective Trkb antagonist ANA-12 at 0.5 mg/kg (Cazorla *et al*, 2011; Vassoler *et al*, 2013). On day 4, animals were sacrificed 1 h after the last administration and brain tissues were taken.

In the DID paradigm, mice were allocated to 10 experimental groups ($n = 5/6$ per group): WT naïve, D₃R^{-/-} naïve, WT/vehicle, D₃R^{-/-}/vehicle, WT/SB277011A, D₃R^{-/-}/SB277011A, WT/buspirone 0.1 mg/kg, WT/buspirone 1 mg/kg, WT/buspirone 3 mg/kg, and WT/buspirone 10 mg/kg. In another set of experiments, mice were allocated to four experimental groups ($n = 5/6$ per group): WT/vehicle, WT/8-OH-DPAT, D₃R^{-/-}/vehicle, and D₃R^{-/-}/8-OH-DPAT, and they were tested in the DID paradigm. Animals were i.p. injected 1 h before the behavioral procedure.

8-OH-DPAT-Induced Hypothermia

Body temperature was measured intrarectally using a lubricated probe inserted ~2 cm and a digital thermometer (CEM advanced thermometer; DT-610B). Mice were moved to the behavioral room and two baseline temperature measurements were taken. After 10 min, animals received an i.p. injection of vehicle or 1 mg/kg 8-OH-DPAT or 3 mg/kg buspirone. The body temperature was recorded every 15 min for a total of 45 min.

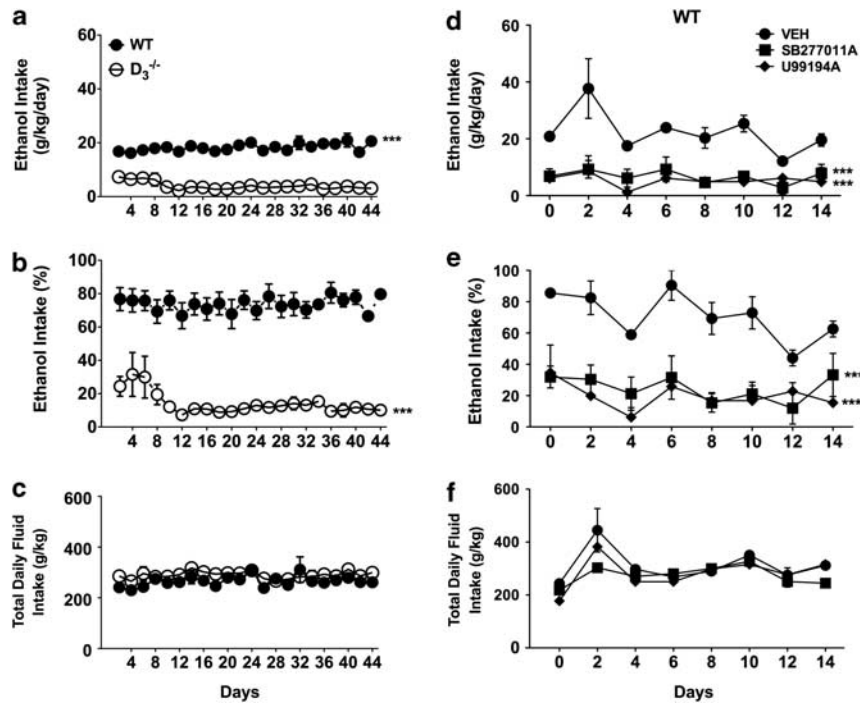


Figure 1 In the two-bottle choice paradigm, D₃^{-/-} mice show a lower voluntary ethanol intake as compared with wild-type (WT). D₃ pharmacological antagonism inhibits ethanol intake in WT mice. (a, b) Voluntary ethanol intake was measured every 24 h, for 44 days, in WT ($n = 30$) and D₃^{-/-} ($n = 30$) mice that had free access to water and ethanol solution (10%). (c) Shows total fluid intake that was not different in the two groups. (d, e), Voluntary ethanol intake was measured as in a, but in mice that had received the day before and kept receiving daily i.p. injection of either saline (vehicle, VEH, $n = 10$), U99194A ($n = 10$) or SB277011A ($n = 10$), either drug at 10 mg/kg. (f) Total fluid intake in either group that was not affected by drug treatment. *** $p < 0.001$ vs water or vehicle (VEH). One-way ANOVA and Newman-Keuls *post hoc* test.

Analysis of mRNA Expression by Real-Time Quantitative RT-PCR

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA). Single-stranded cDNA was synthesized with SuperScript III (Invitrogen), by priming with oligo-(dT)₂₀. Aliquots of cDNA were amplified in parallel reactions with external standards at known amounts, using specific primer pairs for D₃R, RACK1, BDNF, and S18 ribosomal RNA (reference gene). Each PCR reaction (20 μ l final volume) contained 0.5 μ M primers, 1.6 mM Mg²⁺, and 1 \times Light Cycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN). Amplifications were carried out in a Light Cycler 1.5 instrument (Roche Diagnostics). Quantification was obtained by the Δ C_t comparative method.

Western Blot Analysis

Protein extracts from striatum and cerebellum were run in SDS-PAGE, blotted, and probed for non-phosphorylated and phosphorylated forms of DARPP-32, GSK-3 β , and Trkb, with primary antibodies (Cell Signalling Technology, Beverly, MA), diluted at 1:1000, and secondary antibody (goat anti-rabbit IRDye; Li-Cor Biosciences, Lincoln, NE). Blots were scanned with an Odyssey Infrared Imaging System (Li-Cor Biosciences) and analyzed with ImageJ software (NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij/index.html>).

Statistical Analysis

Data were analyzed using one- or two-way analysis of variance (ANOVA). The *post hoc* Newman-Keuls test was used for multiple comparisons; p -values < 0.05 were considered as significant.

RESULTS

D₃R^{-/-} Mice Exhibited Lower Ethanol Intake

As shown in Figure 1a and b, WT mice exhibited a high intake of ethanol-containing solution. In contrast, D₃R^{-/-} mice showed a low ethanol intake (Figure 1a and b). During the entire period of observation (44 days), WT mice maintained their preferential intake of ethanol, whereas D₃R^{-/-} mice maintained a preferential intake of water ($F_{(1,307)} = 1170.08$, $p < 0.001$). There was no difference between WT and D₃R^{-/-} in terms of total amount of fluid intake (ethanol + water) (Figure 1c). In the DID paradigm, D₃R^{-/-} mice also showed a lower ethanol intake compared with their WT counterparts ($F_{(3,97)} = 13.90$, $p < 0.01$, 2nd day; $F_{(3,97)} = 21.04$, $p < 0.001$, 3rd day; Figure 2a).

Blockade of D₃R Inhibited Ethanol Intake

In the two-bottle choice paradigm, after 30 days of stable ethanol/water intake, mice were treated with D₃R antagonists (U99194A or SB277011A). As shown in Figure 1d and

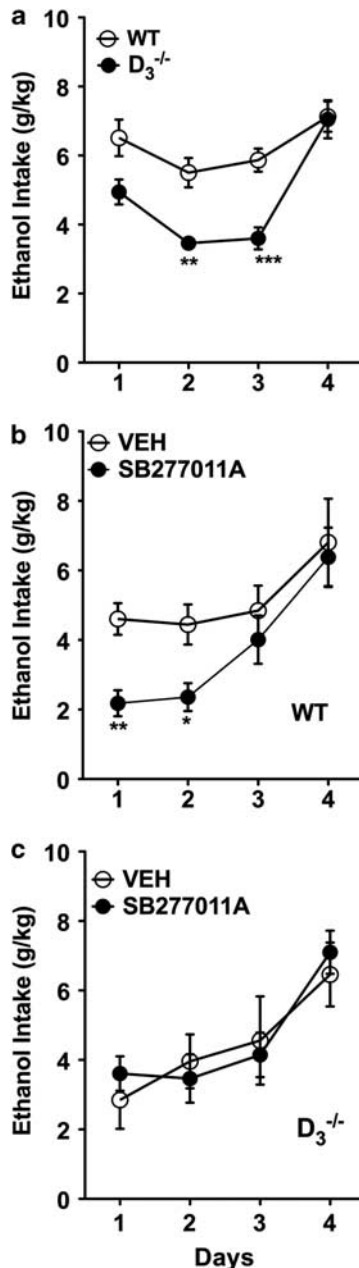


Figure 2 In the drinking in the dark (DID) paradigm, D₃^{-/-} mice show a lower ethanol intake as compared with their wild-type (WT) littermates. The D₃ antagonist SB277011A inhibits ethanol intake of WT but not in D₃^{-/-} mice. (a) DID was measured, for 4 days, in WT ($n = 12$) and D₃^{-/-} ($n = 12$) mice that had limited access (2 h/day for 3 days and 4 h the 4th day) to ethanol solution (20%). (b, c) Voluntary ethanol intake was measured as in a, but in mice that had received the day before and kept receiving daily i.p. injection of either saline (vehicle, VEH, $n = 10$), or SB277011A ($n = 10$), at 10 mg/kg. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs wild-type (WT) or vehicle (VEH). One-way ANOVA and Newman-Keuls *post hoc* test.

e, treatment of WT with each D₃R antagonist decreased voluntary ethanol intake ($F_{(2,56)} = 55.23$, $p < 0.01$, for both U99194A and SB277011A). Treatment of D₃R^{-/-} with U99194A and SB277011A did not change ethanol intake (data not shown). Neither in WT nor in D₃R^{-/-} total fluid intake was affected by treatments (Figure 1f and data not

shown). SB277011A also significantly decreased ethanol intake in WT mice tested in the DID ($F_{(3,48)} = 8.67$, $p < 0.01$, 1st day; $p < 0.05$ 2nd day; Figure 2b), while it did not change ethanol intake of D₃R^{-/-} in the DID paradigm (Figure 2c).

RACK1, BDNF, and DA D₃R Expression were Increased in the Striatum of WT Mice Following Chronic Ethanol Intake

BDNF induces D₃ receptor expression in the ventral striatum, both during development and in adulthood (Guillin *et al*, 2001). RACK1, a mediator of chromatin remodeling, regulates in an exon-specific manner the expression of the BDNF gene (He *et al*, 2010) and the RACK1/BDNF pathway is activated upon exposure to ethanol (McGough *et al*, 2004). We therefore assessed D₃R, BDNF, and RACK1 mRNA expression in striatum of WT that had free access to either water only or to both water and ethanol. Figure 3a shows that chronic ethanol intake increased D₃R mRNA expression in striatum ($F_{(3,23)} = 170.4$, $p < 0.05$). Long-term access to ethanol also increased BDNF (Figure 3b, $F_{(7,47)} = 48.05$, $p < 0.01$) and RACK1 (Figure 3c, $F_{(7,47)} = 21.14$, $p < 0.01$) mRNA in striatum of WT mice.

Long-term ethanol exposure appeared to be associated with BDNF/RACK1 overexpression, but interpretation of these data was made difficult by the different ethanol intake in the two genetic groups, as it was very high in WT and very low in D₃R^{-/-}. To address this issue, some WT and D₃R^{-/-} mice were subjected to forced ethanol intake, that is, they had access to ethanol 10% solution only. As shown in Figure 3d and e, forced ethanol intake induced a significant overexpression of BDNF ($F_{(7,47)} = 48.05$, $p < 0.05$, $p < 0.01$) and RACK1 ($F_{(7,47)} = 21.14$, $p < 0.05$, $p < 0.05$) mRNAs in striatum of both WT and D₃R^{-/-} mice. We also tested the effects of the D₃R antagonists SB277011A and buspirone (see also below) on mRNA expression of D₃R, BDNF, and RACK1. None of these values were changed by a 14-day treatment with SB277011A or buspirone (Figure 3f-h).

Blockade of the BDNF Receptor TrkB Inhibited Ethanol Intake and Decreased D₃R Expression

TrkB is the high affinity receptor for BDNF, belonging to the family of tyrosine kinase receptors, that undergo autophosphorylation upon agonist binding (Soppet *et al*, 1991). In order to assess the role of BDNF pathway in ethanol intake, we used the recently available TrkB selective antagonist ANA-12 (Cazorla *et al*, 2011). After 30 days of stable ethanol/water intake, mice received daily i.p. injections of either vehicle or ANA-12 (Figure 4a and b). ANA-12 reversed the stable ethanol intake of WT mice ($F_{(7,42)} = 30.53$, $p < 0.001$) but did not change the voluntary and the forced ethanol intake of D₃R^{-/-} (data not shown). Neither in WT nor in D₃R^{-/-} total fluid intake was affected by treatment with ANA-12 (Figure 4c and data not shown). Also in the DID paradigm ANA-12 was effective in reducing ethanol intake in WT mice ($F_{(3,55)} = 6.64$, $P < 0.05$, Figure 4d), whereas it did not change ethanol intake in D₃R^{-/-} (Figure 4e).

To assess the selective blockade of the BDNF receptor in striatum by ANA-12, we determined, by immunoblot, the

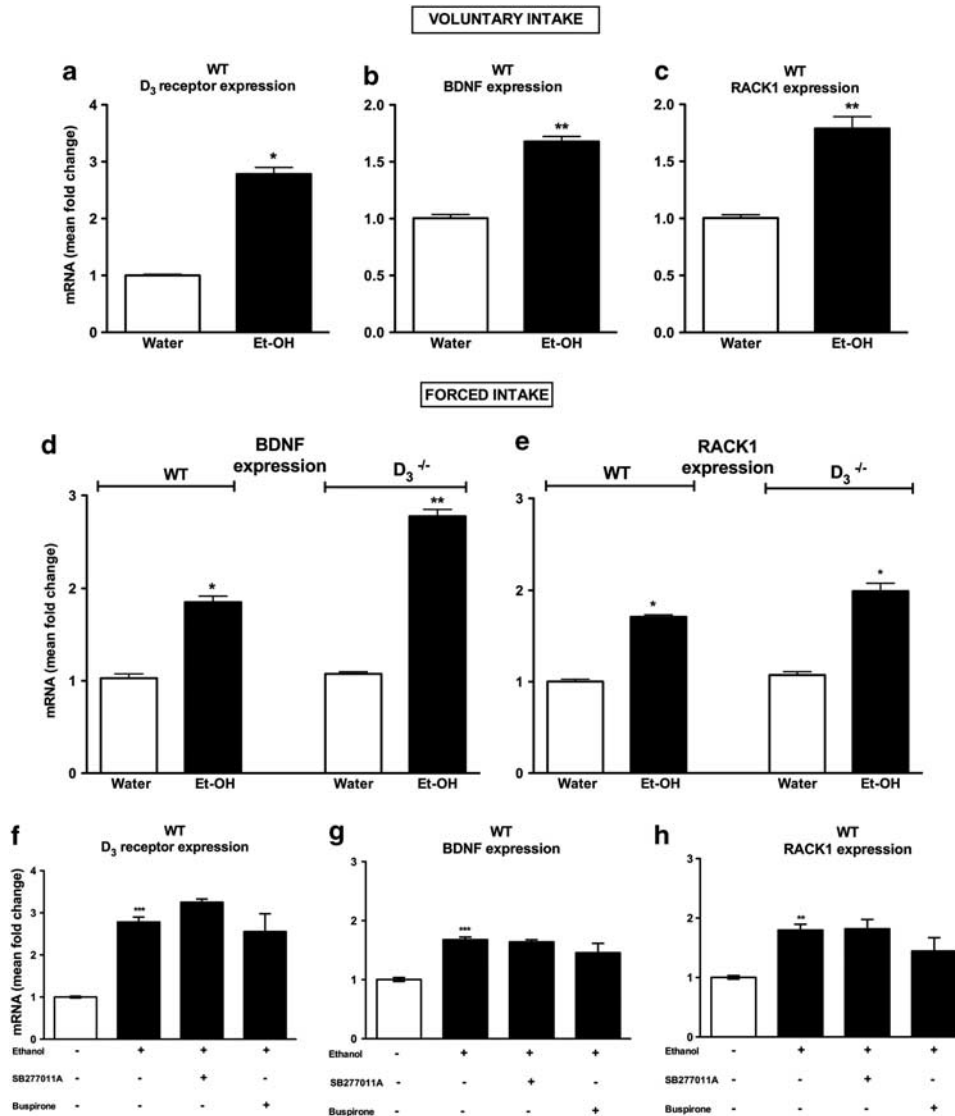


Figure 3 Chronic ethanol intake induces D₃ upregulation, associated with the activation of BDNF/RACK1 pathway. Abundance of transcripts in striatum was assessed by quantitative RT-PCR after 44 days of free access to water only (white columns), or to both water and ethanol (black columns, upper panels) or forced ethanol intake (black columns lower panels). In the forced alcohol-drinking procedure (d–h), D₃R^{-/-} and WT received 10% ethanol only, with or without SB277011A or buspirone for 14 days. (a, f) D₃ Expression profile in WT; (b, d, g) brain-derived neurotrophic factor (BDNF) expression profile in WT and D₃^{-/-}; (c, e, h) RACK1 expression profile in WT and D₃^{-/-}. Mean fold changes are expressed relative to transcript levels in controls (WT having access to water only). Each column is the mean (± SEM) from five different samples. **p* < 0.05, ***p* < 0.01 vs water. One-way ANOVA and Newman–Keuls *post hoc* test.

abundance of phosphorylated TrkB. As shown in Figure 4f, treatment of WT with ANA-12 significantly decreased phosphorylation of TrkB ($F_{(3,35)} = 184.5$, $p < 0.01$). Finally and more interestingly, ANA-12 strongly decreased D₃R mRNA expression in the striatum of WT mice exposed to voluntary ethanol intake (Figure 4f, $F_{(3,35)} = 184.5$, $P < 0.001$).

Buspirone Inhibited Ethanol Intake

In the two-bottle choice paradigm, after 30 days of stable ethanol/water intake, mice were treated with buspirone (1 mg/kg/day). As shown in Figure 5a and b, treatment of WT with buspirone significantly decreased voluntary

ethanol intake ($F_{(1,28)} = 20.88$, $p < 0.05$). Treatment of D₃R^{-/-} with buspirone did not change ethanol intake (data not shown). Neither in WT nor in D₃R^{-/-} total fluid intake was affected by treatment (Figure 5c and data not shown). The treatment with buspirone also significantly decreased ethanol intake in WT mice when tested in the DID. Dose ranging of buspirone (0.1, 1, 3, and 10 mg/kg) showed that treatment of WT with buspirone at the doses of 3 and 10 mg/kg significantly decreased ethanol intake both in the 1st day ($F_{(4,75)} = 31.24$, $p < 0.05$) and in the 2nd day ($F_{(4,75)} = 31.24$, $p < 0.01$ 3 mg/kg; $p < 0.05$ 10 mg/kg) of the behavioral paradigm (Figure 5d). Buspirone did not change ethanol intake of WT in the 3rd and 4th days of DID (Figure 5d). Furthermore, in the DID paradigm, 3 mg/kg

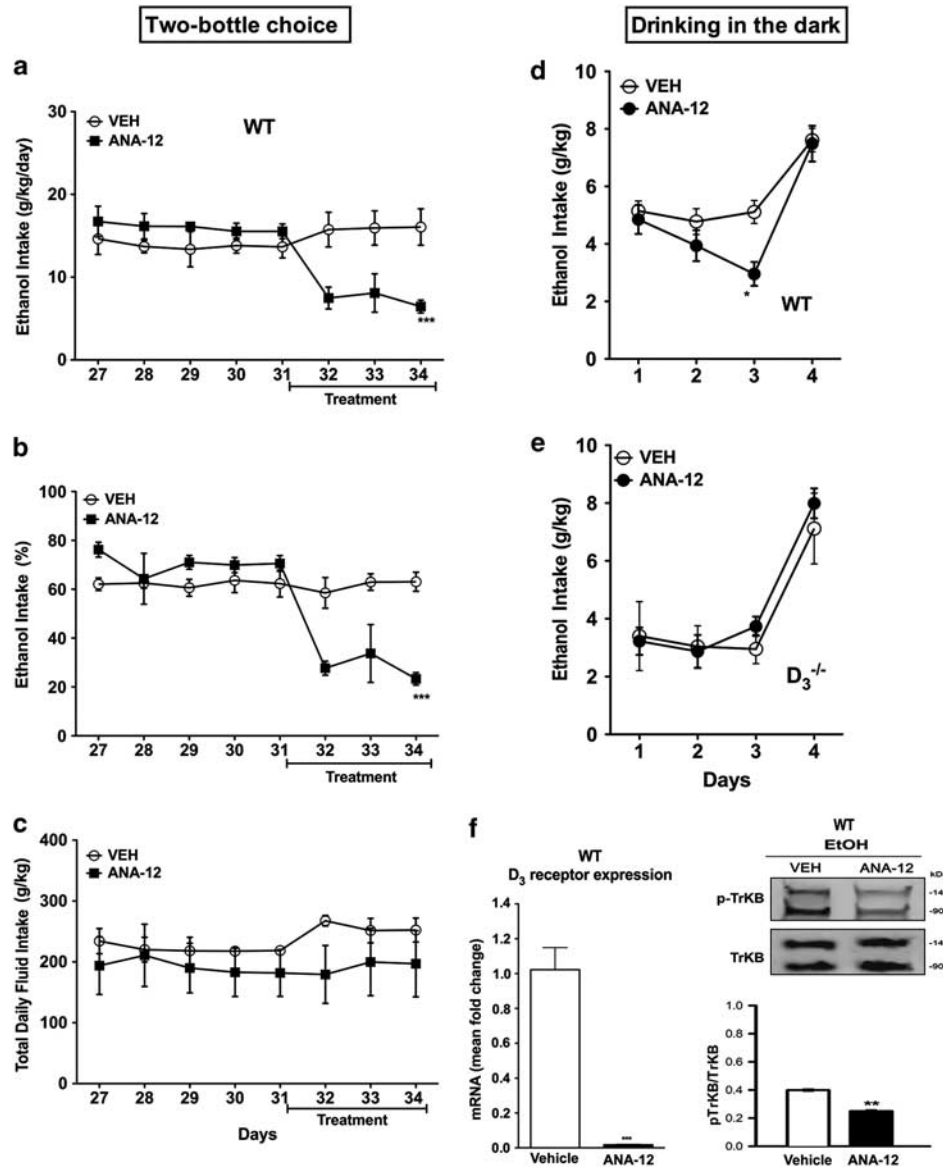


Figure 4 The selective TrkB antagonist, ANA-12 reverses ethanol intake of WT mice and induces D₃ receptor downregulation but does not change ethanol intake of D₃^{-/-} mice. (a, b and c) Voluntary ethanol intake was measured every 24 h, for 34 days, in WT ($n = 30$) and D₃^{-/-} ($n = 20$) mice that had free access to water and ethanol solution (10%). At day 31, mice received daily i.p. injection of either vehicle (VEH), or ANA-12 at 0.5 mg/kg. (d, e) Drinking in the dark (DID) was measured, for 4 days, in WT ($n = 9$) and D₃^{-/-} ($n = 9$) mice that had limited access (2 h/day for 3 days and 4 h the 4th day) to ethanol solution (20%), daily injected with vehicle or ANA-12 1 h before the test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs VEH, one-way ANOVA and Newman–Keuls *post hoc* test. (f) The abundance of transcripts of D₃ receptor in striatum was assessed by quantitative RT-PCR in WT mice exposed to chronic voluntary ethanol intake. Mean fold changes are expressed relative to transcript levels in controls. The abundance of phosphorylated TrkB was assessed by immunoblot, in the striatum WT treated with ANA-12 and exposed to the voluntary ethanol intake. Bars show mean (\pm SEM). ** $p < 0.01$, *** $p < 0.001$ vs vehicle. One-way ANOVA and Newman–Keuls *post hoc* test.

buspirone did not change ethanol intake in D₃R^{-/-} (data not shown). Because buspirone is also known as a 5-HT_{1A} agonist, the D₃R specific effect of buspirone in decreasing ethanol intake was confirmed by using the selective 5-HT_{1A} agonist, 8-OH-DPAT. As shown in Figure 5e, treatment with 8-OH-DPAT (1 mg/kg, i.p.) in WT and D₃R^{-/-} mice did not affect ethanol intake (Figure 5e and data not shown). As expected, the 5-HT_{1A} selective agonist 8-OH-DPAT decreased the body temperature of WT mice ($F_{(2,39)} = 14.99$, $p < 0.001$) (Figure 5f). Buspirone (3 mg/kg) decreased the body temperature of WT mice only transiently (Figure 5f).

DA Receptor Signaling in Striatum of WT and D₃R^{-/-} Mice Exposed to Ethanol

Activation of D₁ receptor results in activation of adenylyl cyclase/cAMP/protein kinase A (PKA) signaling; a major substrate for PKA in the striatum is DARPP-32. D₂-like receptors regulate the activity of the protein kinases Akt and GSK3 β ; stimulation of either D₂ or D₃ receptors results in phosphorylation of Akt and GSK3 β (Mannoury la Cour *et al*, 2011). In order to assess activation of dopaminergic transmission in striatum, we determined, by immunoblot,

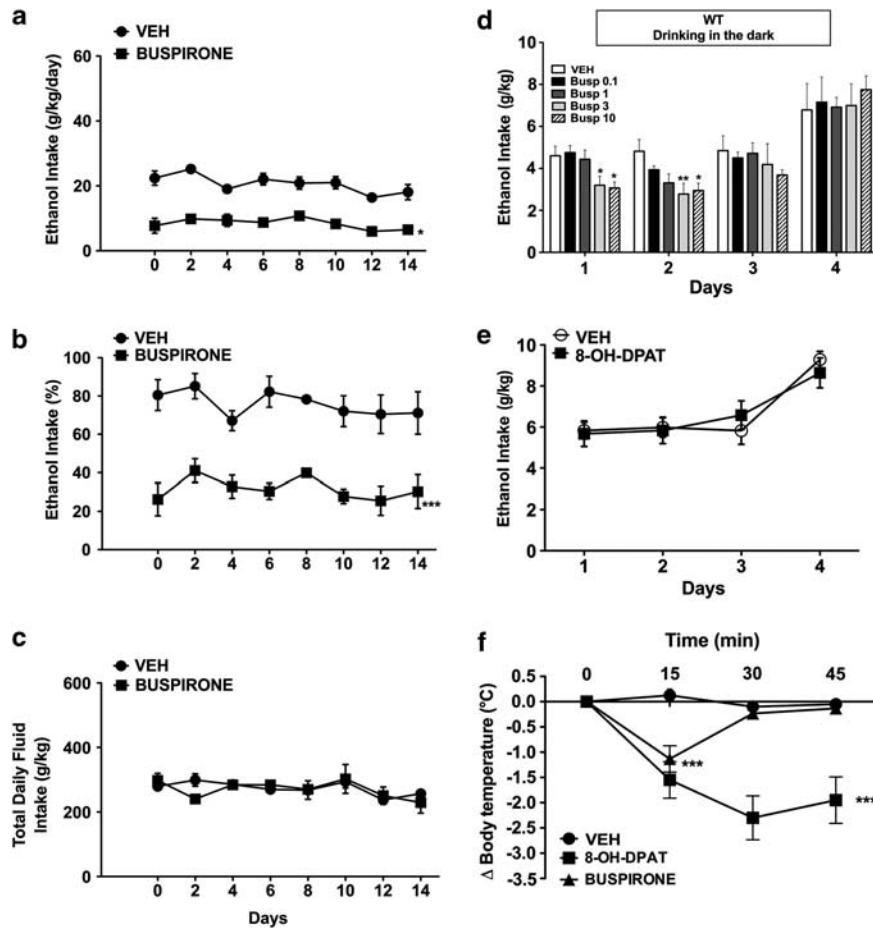


Figure 5 Buspirone inhibits ethanol intake in WT mice both in the two bottle choice and DID paradigm. (a, b) Voluntary ethanol intake was measured every 24 h, for 44 days, in WT ($n = 20$) and $D_3^{-/-}$ ($n = 20$) mice that had free access to water and ethanol solution (10%). Mice received for 14 days, from day 31, daily i.p. injection of either vehicle (VEH) or buspirone at 1 mg/kg. (c) Total fluid intake that was not changed by buspirone. $*p < 0.05$, $***p < 0.01$ vs VEH. One-way ANOVA and Newman–Keuls *post hoc* test. (d) The dose ranging of buspirone (0.1, 1, 3, and 10 mg/kg) in WT mice exposed to the drinking in the dark (DID) paradigm. DID was measured, for 4 days, in WT ($n = 33$) that had limited access (2 h/day for 3 days and 4 h the 4th day) to ethanol solution (20%). $*p < 0.05$, $***p < 0.01$ vs VEH. One-way ANOVA and Newman–Keuls *post hoc* test. (e) The effect of the selective 5-HT_{1A} agonist, 8-OH-DPAT in DID paradigm. 8-OH-DPAT at 1 mg/kg did not change ethanol intake. (f) The action on 5-HT_{1A} of 3 mg/kg buspirone was compared with 1 mg/kg 8-OH-DPAT by assessing the pharmacologically induced hypothermia. $***p < 0.001$ vs VEH. One-way ANOVA and Newman–Keuls *post hoc* test.

the abundance of phosphorylated DARPP-32 (Thr 34) and of phosphorylated GSK3 β (Ser 9). As shown in Figure 6, phosphoGSK3 β was more abundant in striatum of $D_3R^{-/-}$ than in WT mice, whereas phosphoDARPP-32 showed the same tendency, though it did not reach statistical significance. Treatment of WT mice with SB277011A induced phosphorylation of DARPP-32 and GSK3 β , up to the level seen in $D_3R^{-/-}$ mice. In contrast, in cerebellum, there was no difference in the level phosphoDARPP-32 and phosphoGSK3 β between WT and $D_3R^{-/-}$, nor it was influenced by SB277011A treatment in WT.

DISCUSSION

This study demonstrates that D_3R is necessary for ethanol consumption in mice, because either D_3R gene deletion or D_3R pharmacological blockade by selective D_3R experimental antagonists or the approved drug buspirone, inhibits

alcohol intake. The D_3R overexpression induced by ethanol intake associated with the activation of RACK1/BDNF may represent the basis for a reinforcing mechanism of ethanol intake. Indeed, although selective blockade of the TrkB reversed stable intake of ethanol in WT mice and decreased D_3R expression levels in their striatum, it was ineffective in $D_3^{-/-}$ mice.

It seems that D_3R , among D_2 -like receptors, is the key player in addiction, particularly in reward mechanisms. Indeed, although the D_2R is associated with mesocortical and mesohippocampal DA pathway, the D_3R is associated with the ventral mesolimbic DA system (Sokoloff *et al*, 1990). Previous studies reported low levels of D_2R both in animal models and in patients addicted to cocaine, alcohol, metamphetamines, and nicotine (Volkow *et al*, 2009). Conversely, upregulation of D_3R expression has been reported following exposure to DA elevating drugs (Boileau *et al*, 2012; Heidbreder and Newman, 2010; Le Foll *et al*, 2005b; Mash, 1997; Segal *et al*, 1997; Staley and Mash, 1996).

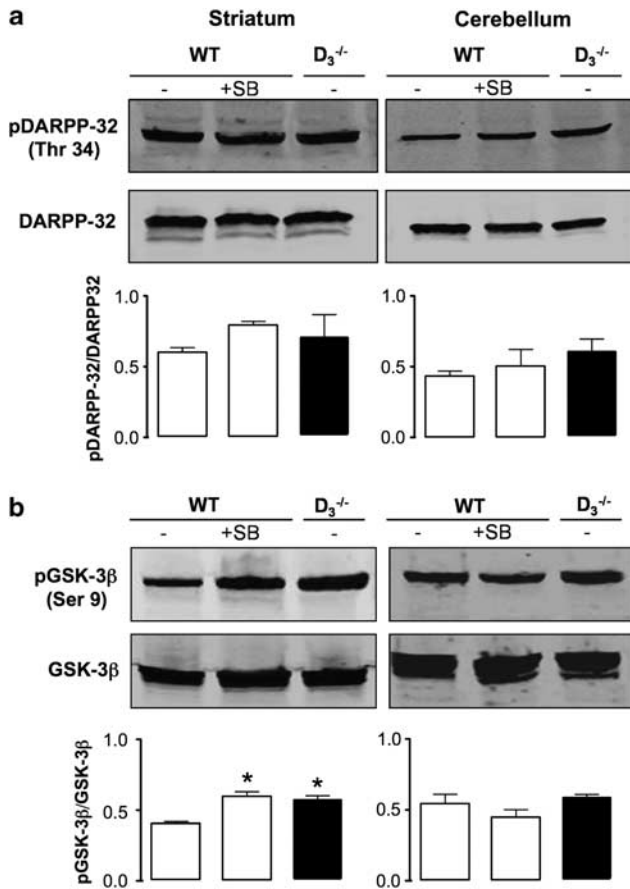


Figure 6 DA receptor signaling is enhanced in striatum of D₃R^{-/-} mice and of SB277011A-treated WT mice. The abundance of phosphorylated DARPP-32 (Thr 34) (a) and phosphorylated GSK3β (Ser 9) (b) was assessed by immunoblot, in the striatum of WT mice exposed to the long-term voluntary ethanol intake (white columns) and injected i.p. for 14 days with either vehicle or 10 mg/kg SB277011A and in D₃R^{-/-} (black columns). Brain tissues were taken 1 h after the last administration of either vehicle or SB277011A. Bar graphs show mean (± SEM) of intensities normalized against the respective non-phosphorylated protein. Each column is the mean (± SEM) from five different samples. **p* < 0.05 vs control (vehicle-injected WT). Two-way ANOVA and Newman–Keuls *post hoc* test.

An important interpretative issue is the genetic background on which the D₃R null mutation was placed. Specific behavioral phenotypes are differently expressed in different strains of mice (Nelson and Young, 1998). The D₃R^{-/-} mice we used are on the C57BL/6J background (Accili *et al*, 1996), a strain where ethanol preference and sensitivity is well documented (Crabbe *et al*, 1983). Interestingly, D₃R^{-/-} mice have extracellular DA levels twice as high as their WT littermates (Joseph *et al*, 2002; Koeltzow *et al*, 1998); this enhanced DA tone and the resulting adaptations may reflect removal of the inhibitory influence of D₃R in the control of basal extracellular DA levels (Le Foll *et al*, 2005a), giving support to an autoreceptor role for D₃R in the mesolimbic areas of the brain (Diaz *et al*, 2000). The increased DA activity in D₃R^{-/-} mice is consistent with their phenotype, including higher basal levels of grooming behavior, hyper-locomotion, and reactivity to drug-paired environmental cues (Accili *et al*, 1996; Le Foll *et al*, 2005a; Le Foll *et al*, 2002).

Here we found that D₃R^{-/-} mice chronically exposed to the voluntary ethanol intake paradigm, drink very low quantities of ethanol in comparison with their WT littermates. This observation cannot be attributed to differences in metabolism (McQuade *et al*, 2003), locomotor activity (Harrison and Nobrega, 2009), or taste reactivity (McQuade *et al*, 2003) between WT and D₃R^{-/-} mice. The lower ethanol intake of D₃R^{-/-} in comparison with their WT control mice seems apparently in contrast with the only two previous studies testing D₃R^{-/-} mice in the ethanol voluntary intake paradigm (Boyce-Rustay and Risinger, 2003; McQuade *et al*, 2003). This may be due, at least in part, to some important differences in experimental procedures used. Indeed, McQuade *et al* (2003), that have shown no difference between D₃R^{-/-} and WT in the 24-h access paradigm, used a different experimental procedure in the two-bottle choice paradigm. First, they used just 4 days of adaptation period before ethanol exposure. Second, they tested both D₃R^{-/-} and WT animals with increasing concentrations of ethanol in subsequent 7-day steps. In the first step, 3% ethanol, in the second step 6%, in the third step 10%, in the 4th 15%, and finally, in the 5th 20% ethanol. Thus, the behavioral paradigm used by McQuade and co-workers is quite different from our paradigm. From our experience, for these mice it is to have a long period of habituation in the two-bottle paradigm (15 days) before to start with the ethanol access procedure. It is likely that the progressive increase of the ethanol concentration every 7 days, may induce an adaptation to the ethanol that damps the difference between D₃R^{-/-} and WT mice. Furthermore, in the McQuade's study, the relative positions of the ethanol and water bottle were determined randomly each day, whereas in our experiments the position of tubes was interchanged (left/right) every 24 h, to prevent acquisition of position bias. The random change of bottles may expose a given animal to access the same solution (either ethanol or water) in the same position for two/three days consecutively, which may interfere with the results of the experiment during a short period of observation (7 days).

In the study by Boyce-Rustay and Risinger (2003), C57 animals were used as control of D₃R^{-/-} mice. These experiments are not comparable to our experiments using WT littermates as controls. Moreover, again, in this study increasing concentrations of ethanol were used in 8-day steps (3 and 10%). Thus, (i) the behavioral procedure is different; (ii) an adaptation to ethanol may occur and damp the difference between genotypes.

To obtain pharmacological evidence for a functional role of D₃R in the control of voluntary ethanol intake, we tested two D₃R antagonists, U99194A and SB277011A at doses reported to selectively target the D₃R (Carr *et al*, 2002; Reavill *et al*, 2000). Before administering these drugs, we performed a molecular modeling study to gain information on the interaction of U99194A and SB277011A with D₃R. As illustrated in Supplementary Information, *in silico* analysis showed that the two D₃R antagonists were (i) highly selective for the D₃R subtype and (ii) displayed a distinct interaction (different binding energy, different interaction patterns) with D₃R, consistent with their distinct chemical structure. We found that both U99194A and SB277011A induced a significant decrease in voluntary ethanol intake in

WT but not in D₃R^{-/-}. This pharmacological evidence reinforces the view that the D₃R is necessary for ethanol consumption in mice and is consistent with rat data showing that D₃R antagonism reduces relapse-like drinking and cue-induced ethanol-seeking behavior (Vengeliene *et al*, 2006).

We confirmed the primary role of D₃R in the control of ethanol-drinking behavior in a binge-like ethanol-drinking paradigm (Crabbe *et al*, 2011; Rhodes *et al*, 2005; Rhodes *et al*, 2007). Here, again, D₃R^{-/-} mice exposed to DID drank lower quantities of ethanol in comparison with their WT littermates, and D₃R blockade by SB277011A decreased ethanol intake in WT but not in D₃R^{-/-}. No differences were recorded in the DID at day 4. Indeed, there was neither a genotype effect between WT and D₃R^{-/-} nor a treatment effect with the SB277011A in WT mice. In general, the binge-like behavior is captured by the 2 h time window that detects differences between treatments/genotypes better than the 4 h window, because the cumulative intake over 4 h makes smaller the proportion of differences (Rhodes *et al*, 2005). Thus, it is likely that, the lack of differences on day 4 is due to the longer lasting access to ethanol that produced overall a higher consumption, potentially masking the genotype/treatment effect on binge-like drinking behavior occurring in the first 2 h.

Enhanced D₃R expression in striatum following long-term alcohol consumption has been previously reported in both mice and rats (Jeanblanc *et al*, 2006; Vengeliene *et al*, 2006). Our data show and confirm that chronic voluntary ethanol intake upregulated D₃R mRNA expression in the striatum of WT mice. Interestingly, D₃R expression is increased by exposure to other addictive drugs, such as nicotine and cocaine, in caudate-putamen (Neisewander *et al*, 2004) and in nucleus accumbens of rats (Le Foll *et al*, 2003, 2005b) and humans (Staley and Mash, 1996). Expression of D₃R therefore appears to be a potential basis for a reinforcing mechanism in reward-related behavior associated with voluntary intake of addictive drugs and ethanol.

A number of studies have linked D₃R expression in the nucleus accumbens to BDNF derived from cortical sources (Guillin *et al*, 2001; Le Foll *et al*, 2005b); furthermore, ethanol exposure increases both BDNF and D₃R within the striatum itself (Jeanblanc *et al*, 2006; McGough *et al*, 2004). The scaffolding protein RACK1 is a key regulator of BDNF expression; RACK1 translocates to the nucleus after exposure of neurons to ethanol and increases expression of BDNF (McGough *et al*, 2004). Jeanblanc *et al* (2006) proposed that the RACK1/BDNF/D₃R pathway is involved in the control of ethanol consumption in mice. Our hypothesis is that activation of RACK1/BDNF by ethanol may induce expression of D₃R, which in turn controls and maintains ethanol consumption. This hypothesis is supported by the data we generated showing that: (i) ethanol intake is negligible in D₃R^{-/-} and robust in WT; (ii) increase in RACK1/BDNF/D₃R is maintained during chronic ethanol intake in WT; (iii) forced ethanol intake increases RACK1/BDNF even in D₃R^{-/-}. Furthermore, chronic voluntary ethanol intake increased D₃R expression in striatum concomitant with increased expression of BDNF. It is noteworthy that, in the basal condition, D₃R^{-/-} mice exhibited higher BDNF than WT, consistent with a tendency reported in a recent study (Xing *et al*, 2012). When

subjected to forced ethanol intake, D₃R^{-/-} mice showed a robust increase in BDNF expression in the striatum. Therefore, chronic ethanol intake increases BDNF independently of D₃R receptor stimulation. The finding that chronic ethanol intake increased RACK1 in striatum of both WT and D₃R^{-/-} provides additional evidence for the role of RACK1/BDNF/D₃R pathway in ethanol intake; chronic ethanol intake stimulates RACK1/BDNF pathway leading to D₃R overexpression and addictive behavior in WT, but not in D₃R^{-/-}, because this latter lacks D₃R.

To provide additional evidence, we blocked the BDNF pathway by using the TrkB specific antagonist, ANA-12. We found that ANA-12 reversed ethanol intake both in the two-bottle choice and DID paradigms and strongly decreased the expression of D₃R in the striatum of WT-treated mice. Recently, D₃R on VTA-SN dopaminergic neurons were found to mediate neuroplasticity effects of several addictive drugs (Collo *et al*, 2012; Collo *et al*, 2013). Therefore, our conclusion about the engagement of striatal RACK1, BDNF, and D₃R in mediating ethanol consumption may be only a part of a more complex mechanism, whose elucidation may require an assessment of the effects of ethanol intake in the VTA-SN dopaminergic neurons.

Finally, in a translational perspective, we tested buspirone, a drug marketed for anxiety disorders, endowed with D₃R antagonist (Bergman *et al*, 2013; Le Foll and Boileau, 2013; Newman *et al*, 2012) and 5-HT_{1A} partial agonist activity (Wong *et al*, 2007). Notably, buspirone shows also high affinity for other D₂-like receptors (Bergman *et al*, 2013; Kula *et al*, 1994; Tallman *et al*, 1997). D₃R antagonists may be effective for treating substance use disorders and buspirone has proven effective in several preclinical model of drug abuse (Heidbreder and Newman, 2010; Higley *et al*, 2011; Song *et al*, 2012), but no studies have, so far, investigated its D₃R antagonist action in ethanol consumption. By both radioligand binding and molecular modeling studies (see Supplementary Information), we found that buspirone: (i) shows slight higher affinity at D₃R than at D₂R (K_i, 29 vs 62 nM, respectively) and may form interactions comparable with those of SB277011A in D₃R, having the antagonist binding mode at D₃ receptor, (ii) displays a distinct interaction from the other two antagonists SB277011A and U99194A (different binding energy, different interaction patterns) with D₃R, consistent with their distinct chemical structure. Thereafter, we found that buspirone induced a significant decrease in ethanol intake in both two-bottle choice and DID paradigms. The dose of 1 mg/kg inhibited ethanol intake in both paradigms, though its effect did not reach statistical significance in DID; 3 and 10 mg/kg, however produced a significant effect in DID. We confirmed the specificity of D₃R effect by using a selective 5-HT_{1A} agonist, 8-OH-DPAT, in the DID. Treatment with 8-OH-DPAT did not impact ethanol intake, whereas, as expected, decreased the body temperature in a stable manner. In a translational perspective, an important issue is the actual availability of buspirone to bind D₃R in human CNS. Reported buspirone's affinity toward human recombinant D₃R ranges from 3.5 to 98 nM (Bergman *et al*, 2013; Newman *et al*, 2012), which partially overlaps its affinity for 5-HT_{1A} receptors; because buspirone binding to 5-HT_{1A} is considered the basis of its anxiolytic activity in humans, it is likely that anxiolytic doses are sufficient to occupy also D₃R

in human CNS. However, the D₃R-related therapeutic potential of buspirone requires more detailed information, including measurements of D₃R receptor occupancy in human PET studies, as an essential prerequisite to clinical application.

Finally, as D₃R^{-/-} mice have been shown to exhibit extracellular DA levels substantially higher than WT, as assessed by microdialysis (Koeltzow *et al*, 1998), a phenomenon related to the lack of autoreceptor function (Joseph *et al*, 2002), we hypothesized that ethanol intake effectively stimulates DA release and transmission in WT, but not in D₃R^{-/-}, presumably because this latter already displays high extracellular DA levels. To test the hypothesis that treatment with D₃R antagonists mimicked the high DA phenotype documented in D₃R^{-/-} (Koeltzow *et al*, 1998), we assessed phosphorylation of DARPP32, that is increased by different addictive drugs, including ethanol (Nuutinen *et al*, 2011; Svenningsson *et al*, 2005), and of GSK3 β , that is linked to D₂-like receptors signaling cascade (Beaulieu *et al*, 2007; Li *et al*, 2009), particularly under hyper-DAergic conditions (Li *et al*, 2009). Treatment with SB277011A increased phosphorylation of DARPP32 and of GSK3 β to a level similar to that of D₃R^{-/-}. Thus, chronic blockade of the D₃R or its genetic deletion increased DA transmission in striatum, consistent with increased extracellular DA (Joseph *et al*, 2002; Koeltzow *et al*, 1998).

In conclusion, either D₃R gene deletion or D₃R pharmacological blockade inhibit ethanol intake. Thus, pharmacological antagonism selectively targeting D₃R may provide a basis for novel weaning treatments to inhibit ethanol consumption. In this context, buspirone, a drug marketed as anxiolytic since more than 25 years and endowed with D₃R antagonist activity, exhibits, translational potential for treating alcohol addiction.

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