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Dopaminergic-GABAergic interplay and alcohol binge drinking

Gian Marco Leggio[a,1], Roberta Di Marco[a,1], Walter Gulisano[a], Marcello D'Ascenzo[b,d], Sebastiano Alfio Torrisi[a], Federica Geraci[a], Gianluca Lavanco[a], Kristiina Dahl[c], Giovanni Giurdanella[a], Alessandro Castorina[a,2], Teemu Aitta-aho[c], Giuseppe Aceto[b], Claudio Bucolo[a], Daniela Puzzo[a], Claudio Grassi[b,d], Esa R. Korpi[c], Filippo Drago[a], Salvatore Salomone[a,*]

[a] Department of Biomedical and Biotechnological Sciences, School of Medicine, University of Catania, Catania, Italy
[b] Institute of Human Physiology, Medical School, Università Cattolica, Rome, Italy
[c] Department of Pharmacology, Faculty of Medicine, University of Helsinki, Helsinki, Finland
[d] Fondazione Policlinico Universitario A. Gemelli IRCCS, Roma, Italia

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ABSTRACT

The dopamine D_3 receptor (D_3R), in the nucleus accumbens (NAc), plays an important role in alcohol reward mechanisms. The major neuronal type within the NAc is the GABAergic medium spiny neuron (MSN), whose activity is regulated by dopaminergic inputs. We previously reported that genetic deletion or pharmacological blockade of D_3R increases $GABA_A$ α6 subunit in the ventral striatum. Here we tested the hypothesis that D_3R-dependent changes in $GABA_A$ α6 subunit in the NAc affect voluntary alcohol intake, by influencing the inhibitory transmission of MSNs.

We performed *in vivo* and *ex vivo* experiments in D_3R knockout ($D_3R^{-/-}$) mice and wild type littermates ($D_3R^{+/+}$). Ro 15-4513, a high affinity α6-$GABA_A$ ligand was used to study α6 activity.

At baseline, NAc α6 expression was negligible in $D_3R^{+/+}$, whereas it was robust in $D_3R^{-/-}$; other relevant $GABA_A$ subunits were not changed. In situ hybridization and qPCR confirmed α6 subunit mRNA expression especially in the NAc. In the drinking-in-the-dark paradigm, systemic administration of Ro 15-4513 inhibited alcohol intake in $D_3R^{+/+}$, but increased it in $D_3R^{-/-}$; this was confirmed by intra-NAc administration of Ro 15-4513 and furosemide, a selective α6-$GABA_A$ antagonist. Whole-cell patch-clamp showed peak amplitudes of miniature inhibitory postsynaptic currents in NAc medium spiny neurons higher in $D_3R^{-/-}$ compared to $D_3R^{+/+}$; Ro 15-4513 reduced the peak amplitude in the NAc of $D_3R^{-/-}$, but not in $D_3R^{+/+}$.

We conclude that D_3R-dependent enhanced expression of α6 $GABA_A$ subunit inhibits voluntary alcohol intake by increasing GABA inhibition in the NAc.

1. Introduction

Alcohol is the most widely used and abused of all psychoactive drugs. Despite its mechanism of action being still elusive, general consensus recognizes its major impact on the brain reward system. Repeated intake of ethanol induces alterations in the nucleus accumbens (NAc), a main component of the mesolimbic reward circuit [1], as several other drugs of abuse [2]. In this brain region more than 95% of the cells are GABAergic Medium Spiny Neurons (MSNs), whose activity is regulated by dopaminergic and glutamatergic inputs [3]. MSNs comprise three distinct cell subpopulations; one expressing dopamine D_1-like receptors (D_1R and D_5R), a second one expressing dopamine D_2-like receptors (D_2R, D_3R, D_4R), and a small third one expressing both D_1-like and D_2-like receptors [4,5]. $GABA_A$ receptors ($GABA_ARs$) in the NAc have been considered as a primary target for alcohol, and may be involved in voluntary alcohol consumption [6];

Abbreviations: DID, drinking in the dark paradigm; DR, dopamine receptor; $D_{1\text{-}5}R$, dopamine $D_{1\text{-}5}$ receptor; GABA, *gamma*-aminobutyric acid; $GABA_ARs$, $GABA_A$ receptors; ISH, in situ hybridization; mIPSCs, miniature inhibitory postsynaptic currents; MSN, medium spiny neuron; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area

* Corresponding author at: Department of Biomedical and Biotechnological Sciences, University of Catania, Via Santa Sofia 97, 95123 Catania, Italy.
E-mail address: salomone@unict.it (S. Salomone).
[1] Equally contribution author.
[2] Present address University of Technology Sydney, Sydney, Australia.

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moreover, chronic alcohol intake alters GABAergic function in the NAc, which sustains behavioral addictive patterns [1,6]. $GABA_AR$ is an heteromeric pentamer chloride channel assembled from a variety of subunits from the 19 known up to now, α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3 [7]. This lead to the formation of multiple isoforms that are likely to differ in their alcohol sensitivity [8]. This ionotropic receptor represents a major pharmacological target for many drugs, including benzodiazepines, barbiturates and ethanol. While GABA binds to an orthosteric site, these exogenous compounds (and some endogenous modulators) bind to allosteric sites, affecting the gating of the channel and/or the response to GABA [7]. Previous findings reported that $GABA_AR$ containing α6 subunit is particularly sensitive to alcohol; indeed, rats expressing the naturally occurring R100Q allelic variation of α6 exhibit a higher sensitivity to motor incoordination induced by moderate doses of ethanol [9] and avoid alcohol consumption [10]. This mutation was originally found in a selectively bred, alcohol-sensitive rat line [11], which also shows reduced voluntary acceptance of alcohol solutions [12]. Furthermore, the hypersensitivity to ethanol was also seen in tonic inhibitory currents mediated by the α6βδ-type $GABA_ARs$ in cerebellar slices [13]. GABAergic MSNs receive dopaminergic inputs from the ventral tegmental area (VTA) [14]; activation of this circuitry, the dopaminergic mesolimbic pathway, is classically considered as responsible for the reward response to physiological (e.g. food intake, sexual activity) or pathological (drug of abuse) stimuli. Activation of D_3R, highly expressed in the NAc, is involved in the control of alcohol consumption [15–17]. Indeed, either D_3R gene deletion or D_3R pharmacological blockade inhibit alcohol intake [15]. Because DRs and $GABA_ARs$ are co-localized in MSNs, both contributing to the control of NAc output [18], we hypothesized that some cross-talk may exist between D_3R and $GABA_ARs$ in the regulation of reward system. In this respect, we have already shown that genetic deletion or pharmacological blockade of D_3R, by using the selective D_3R antagonist SB 277011 A, increases $GABA_A$ α6 subunit expression in the ventral striatum [19]. Thus, this behavioral effect on alcohol intake might be linked with changed $GABA_A$ α6 subunit expression levels in the NAc, due to the D_3R gene deletion or D_3R pharmacological blockade by SB 277011 A. Here, we tested the hypothesis that D_3R-dependent changes in $GABA_A$ α6 subunit expression in the NAc affect the alcohol intake behavior, and, at the cell level, the electrical activity of MSNs, thereby influencing the inhibitory synaptic transmission in the NAc. To do so, we attempted to directly reveal $GABA_A$ α6 activity, by using Ro 15-4513, an imidazobenzodiazepine $GABA_A$ ligand exerting differential effects depending on the α subunit present in the $GABA_AR$ isoform, showing negative allosteric agonism with α1,2,3 and 5, but positive agonism with α4 and α6 [20,21]. Interestingly, based on molecular docking analysis and ligand binding interactions, Ro 15-4513 has been proposed to compete with ethanol within a binding pocket involving α6 [22,23]. More importantly, Ro 15-4513 has shown efficacy in reducing alcohol drinking in rodents [24,25], but the detailed mechanisms of action have remained unknown. However, Ro 15-4513 may be considered a high affinity α6-$GABA_AR$ ligand, since its binding is obvious in a α6 rich brain structure, such as the cerebellum, while it is hardly detectable in the very same structure in α6 null mice [26].

2. Materials and methods

2.1. Animals

Mice $D_3R^{-/-}$, $D_3R^{+/-}$ and $D_3R^{+/+}$ 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 $D_3R^{-/-}$ and $D_3R^{+/-}$ were congenic after 10th–12th generation of back crossing into C57BL/6 J mouse line [27]. All experiments were carried out according to the Directive 2010/63/EU and to the Institutional Animal Care and Use Committee of the University of Catania.

2.2. Analysis of mRNA expression by real-time quantitative RT-PCR

NAc was freshly dissected out for real-time quantitative RT-PCR by using punches (bilateral) of 14-gauge on ice, held in ice-cold PBS solution and frozen on dry ice according to Koo et al. [18]. Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) from the brain tissues. Single-stranded cDNA was synthesized with Super-Script III (Invitrogen), by random priming. Aliquots of cDNA were amplified in parallel reactions with external standards at known amounts, using specific primer pairs for α6-$GABA_A$ subunit, D_3R and GAPDH (reference gene). GAPDH levels did not differ among different groups and were not changed by alcohol exposure in the DID paradigm. Each PCR reaction (20 µl final volume), contained 0.5 mM primers, 1.6 mM Mg^{2+}, and 1 X Light Cycler-Fast Start DNA Master SYBR Green I (Roche Diagnostics, IN). Amplifications were carried out in a Light Cycler 1.5 instrument (Roche Diagnostics). Quantification was obtained by the ΔCt comparative method.

2.3. Drinking in the dark paradigm (DID)

The 4-hour version of the behavioral paradigm was used, as described by Rhodes et al. [28]. 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 (Sigma, St Louis, MO) in tap water; this was done in home cages where animals were singly housed [28]; 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.

2.4. In situ hybridization and [^{3}H]Ro 15-4513 autoradiography

The in situ hybridization (ISH) and [^{3}H]Ro 15-4513 autoradiography were carried out as described earlier [29,30]. The detailed protocols are reported in Supplemental Information section.

2.5. Systemic administrations

Ro 15-4513 and SB 277011 A hydrochloride were from Tocris (Ellisville, MO). Drugs were intraperitoneally (i.p.) injected. Ro 15-4513 (5 mg/kg) [31] was dissolved in 10% dimethyl sulfoxide whereas SB 277011 A hydrochloride (10 mg/kg) [15,19] was dissolved in saline. All drugs and their respective vehicles were injected in a volume of 10 ml/kg. In the DID paradigm, we first tested $D_3R^{+/+}$, $D_3R^{+/-}$ and $D_3R^{-/-}$ naïve (n = 8/10 per group). For pharmacological experiments with Ro 15-4513, we allocated $D_3R^{+/+}$ and $D_3R^{-/-}$ mice to 4 experimental groups: $D_3R^{+/+}$ treated with vehicle, $D_3R^{+/+}$ treated with Ro 15-4513, $D_3R^{-/-}$ treated with vehicle and $D_3R^{-/-}$ treated with Ro 15-4513 (n = 8/10 per group).

In another set of experiments, $D_3R^{+/+}$ and $D_3R^{-/-}$ were randomly allocated to 3 experimental groups (n = 8/13 per group): $D_3R^{+/+}$ treated with SB277011 A for 7 days before SB 277011 A plus Ro 15-4513 during the DID procedure; $D_3R^{+/+}$ treated with Vehicle for 7 days before Vehicle plus Ro 15-4513 during the DID procedure and $D_3R^{+/+}$ treated with Vehicle for 7 days before Vehicle plus Vehicle during the DID procedure. SB 277011 A and Ro 15-4513 were i.p. injected, respectively 1 h and 15 min before DID. On day 4, animals were sacrificed 1 h after ethanol-drinking procedure and the brain tissues were taken.

2.6. Intra-accumbens administrations

Ro 15-4513 and furosemide (Tocris) were dissolved in 10% dimethyl sulfoxide and 90% synthetic cerebrospinal fluid (CSF) [15,19]. Cannulas were implanted as previously described (11). After anesthesia

with tiletamine + zolazepam (60 mg/kg) and medetomidine (40 μg/kg), mice were implanted with a 26-gauge guide cannula into the NAc (coordinates from *Bregma*: anterior-posterior = + 1.42 mm, latero-lateral ± 0.75 mm to a depth of 4.1 mm). The cannulas were fixed to the skull with acrylic dental cement (RelyX™ Unicem). After 6–8 days recovery, drugs (10 nmol/mouse) were bilaterally injected in a final volume of 1 μl over 1 min through infusion cannulas connected to a Hamilton microsyringe by a polyethylene tube. Ro 15-4513 was injected 15 min before the DID, whereas furosemide was injected 5 min before Ro 15-4513. Animals were handled gently to minimize stress during infusion. After the infusion procedure, the needle was left in place for another minute to allow diffusion. In the DID paradigm, mice were allocated to three experimental groups (n = 8/10 per group): $D_3R^{-/-}$ / vehicle, $D_3R^{-/-}$ / Ro 15-4513, $D_3R^{-/-}$ / furosemide + Ro 15-4513. After behavioral testing, a solution of 4% methylene blue was infused for histological localization of infusion cannulas.

2.7. Electrophysiology

For the preparation of brain slices, we followed the protocol described by Scala et al. [32], with minor modifications. The detailed protocol is reported in Supplemental Information section. The electrophysiological recordings were analyzed using the Clampfit 10.7 software (Molecular Devices). A template was constructed using the "Event detection/create template" function, as described in [33], then, miniature inhibitory postsynaptic currents (mIPSCs) were detected using the "Event detection/template search" function. All the waveforms detected during a single recording using template analysis were averaged and amplitude, rise time and decay time calculated.

2.8. Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical significance was assessed with the Student's t test (when used, paired-t test has been indicated in the text), one- or two-way analysis of variance (ANOVA) and post hoc Newman-Keuls. The level of significance was set at 0.05.

3. Results

3.1. Alcohol intake and $GABA_A$ α6 subunit expression

We previously reported that $D_3R^{-/-}$ mice have low ethanol intake [15] and exhibit higher basal expression of $GABA_A$ α6 in the ventral striatum [19]. Here, we assessed whether a link exists between alcohol consumption and $GABA_A$ α6 subunit expression in the NAc. $D_3R^{-/-}$ exhibited about 5-fold higher basal mRNA expression of α6 subunit as compared with $D_3R^{+/+}$in the NAc [main effect of genotype F (2, 14) = 9.447, P < 0.01; *post hoc:* P < 0.01], but not in the prefrontal cortex (PFC), while other relevant $GABA_A$ subunits were not changed (Fig. 1A–B). Based on these data, we compared $D_3R^{+/+}$, heterozygous $D_3R^{+/-}$ and homozygous $D_3R^{-/-}$ in the drinking-in-the-dark (DID) paradigm. As shown in Fig. 1C, $D_3R^{+/+}$exhibited obvious ethanol preference in DID paradigm on day 1, 2 and 3, whereas $D_3R^{-/-}$ had significantly lower ethanol intake [main effect of day: F (3, 60) = 40.58, P < 0.01; main effect of genotype F (2, 20) = 7.812, P < 0.01; *post hoc:* P < 0.01 and P < 0.05]. $D_3R^{+/-}$ showed alcohol intake similar to $D_3R^{+/+}$and, consistently, a low α6 expression in the NAc (Fig. 1D). The lack of difference in ethanol intake on day 4 might be linked to the 4 h-time window used instead of a 2 h-time window (see Methods). Overall, these data suggest that there is a link between α6 mRNA expression and alcohol intake such that the high level of $GABA_A$ α6 subunit expression in the NAc is associated to reduced alcohol consumption. To precisely assess the spatial expression of α6 subunit in the brain of $D_3R^{+/+}$and $D_3R^{-/-}$, we carried out in situ hybridization (ISH) experiments and analyzed the results in a blinded manner. These experiments confirmed that, while heavily enriched in the cerebellar granule cell layer, significant α6 expression in the forebrain of $D_3R^{-/-}$ occurred specifically in the NAc [P < 0.05], being very low in the other examined brain areas (Fig. 2A–D, Tab. S1-S2). Furthermore, the expression of other relevant $GABA_A$ subunits was not changed in $D_3R^{-/-}$ (Tab. S1–S2). Data obtained by ISH confirmed the qPCR data (Fig. 1A–B). Autoradiography following incubation with a high 15 nM concentration of [^{3}H]Ro 15-4513 showed a statistically significant increase of [^{3}H]Ro 15-4513 binding in the NAc [P < 0.05] (Fig. 2E–F). Ro 15-4513 binds at α6/4β3δ-type $GABA_A$ receptors with high affinity (K_D ≈ 10 nM) [21,34], consistent with an increased expression of α6/4β3δ-type $GABA_A$ receptors in the NAc.

3.2. Alcohol antagonist Ro 15-4513 increased ethanol consumption in mice expressing $GABA_A$ α6 in NAc

Ro 15-4513 was earlier named "alcohol antagonist" [35], because, in some studies, it inhibited alcohol intoxication, preference and self-administration in wild type rodents [31,36]. Therefore, based on ISH and [^{3}H]Ro 15-4513 binding data, we tested the hypothesis that Ro 15-4513 differently affects ethanol intake in mice expressing different levels of α6 in the NAc. As shown in Fig. 3A, systemic administration of Ro 15-4513 decreased voluntary ethanol intake in $D_3R^{+/+}$ [main effect of day F (3, 63) = 55.62, P < 0.01; main effect of treatment F (1, 21) = 7.198, P < 0.05; post hoc: P < 0.05], but increased voluntary ethanol intake in $D_3R^{-/-}$ (Fig. 3B) [main effect of day F (3, 39) = 34.87, P < 0.01; main effect of treatment F (1, 13) = 9.384, P < 0.01; post hoc: P < 0.05]. Worthy of note, $D_3R^{-/-}$, which normally show low preference for alcohol [15], following Ro 15-4513 –treatment reached a level of ethanol consumption similar to that of $D_3R^{+/+}$. To gain stronger evidence of the specific role of D_3R-dependent expression of α6 $GABA_A$ subunit in the NAc, we tested $D_3R^{-/-}$ mice in the DID after intra-NAc administration of Ro 15-4513, with or without furosemide, an α6-$GABA_A$ receptor antagonist [37]. As shown in Fig. 3C, intra-NAc administration of Ro 15-4513 increased voluntary ethanol intake in $D_3R^{-/-}$ [main effect of treatment F (2, 13) = 22.31, P < 0.001; main effect of days X treatment interaction F (6, 39) = 3.297 P < 0.05, post hoc: P < 0.05, P < 0.01 and P < 0.001 vs vehicle]; the effect of Ro 15-4513 injected in this brain area was blocked by pretreatment with furosemide [main effect of treatment F (2, 13) = 22.31, P < 0.001; main effect of days X treatment interaction F (6, 39) = 3.297, post hoc: P < 0.001 vs furosemide + Ro 15-4513] (Fig. 3D). This result confirms that the increased expression of α6-$GABA_A$R in the NAc has a key role in modulating the paradoxical effect of Ro 15-4513 in $D_3R^{-/-}$ mice, ruling out potential off target and/or non-specific effects of Ro 15-4513 (on other brain areas, because of intraNAc injection, and on other $GABA_A$ receptor isoforms, because of furosemide antagonism). Thus, the paradoxical response to Ro 15-4513 seen in $D_3R^{-/-}$ is related to increased expression of α6-$GABA_A$R in the NAc, which also accounts for the low ethanol consumption observed in these mice, as mentioned above.

Changes of $GABA_A$R function induced by alterations in dopaminergic transmission may have clinical relevance, because a number of DR ligands are currently used to treat different neuropsychiatric disorders [38]. In this respect, consistent with data obtained in $D_3R^{-/-}$ mice, we previously reported that chronic treatment with the selective D_3R antagonist SB 277011 A increases α6 expression in the ventral striatum and accelerates the appearance of tolerance to the anxiolytic effect of diazepam [19]. Here, to assess the functional relevance of the D_3R/α6-$GABA_A$R cross-talk, we treated $D_3R^{+/+}$with SB 277011 A for 7 days, (10 mg/kg i.p. as done in [19]) before testing in the DID paradigm. As shown in Fig. 3D, pretreatment of $D_3R^{+/+}$ with SB 277011 A for 7 days, which increased the expression of α6-$GABA_A$R in the NAc (Fig. S1), induced a paradoxical effect of Ro 15-4513 on alcohol intake, similar to $D_3R^{-/-}$ [main effect of days F (3, 108) = 31.59, P < 0.001; main effect of treatment F (2, 36) = 19.34, post hoc: P < 0.05,

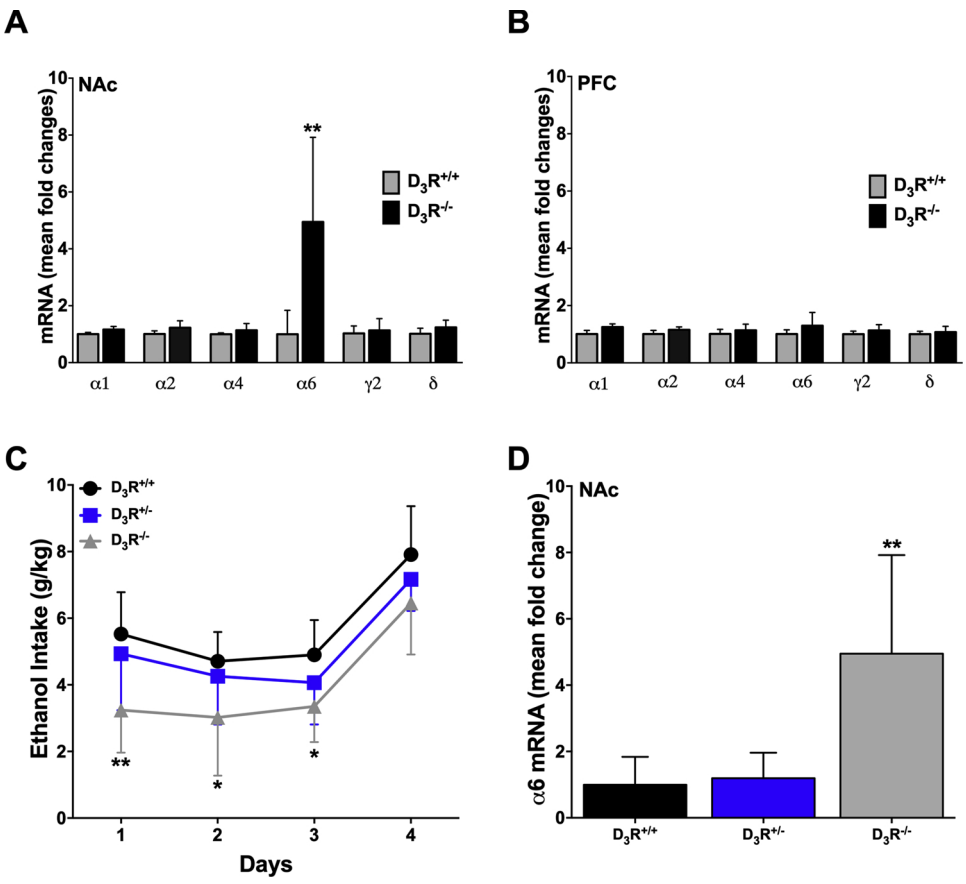

Fig. 1. Alcohol intake and D_3R-dependent $GABA_A$ α6 subunit mRNA expression in the NAc. A and B, $GABA_AR$ α1, α2, α4, α6, γ2 and δ subunits mRNA expression in the nucleus accumbens (NAc) and in the prefrontal cortex (PFC) of wild type ($D_3R^{+/+}$) and D_3R null mice ($D_3R^{-/-}$). Abundance of transcripts was assessed by qPCR (primer sequences are reported in Tab. S3). C and D, ethanol intake (in the drinking in the dark paradigm, DID) and α6 expression in wild type ($D_3R^{+/+}$) heterozygous ($D_3R^{+/-}$) and null mice ($D_3R^{-/-}$). DID was measured for 4 days, in mice with limited access (2 h/day for 3 days and 4 h the 4th day) to ethanol solution (20%). Abundance of transcripts in the NAc was assessed by qPCR after DID; expression level is given as mean fold changes relative to controls. *P < 0.05, **P < 0.01 *vs.* the corresponding control ($D_3R^{+/+}$,); one- or two-way ANOVA and Newman–Keuls post hoc test. Each experimental group included 8–10 mice.

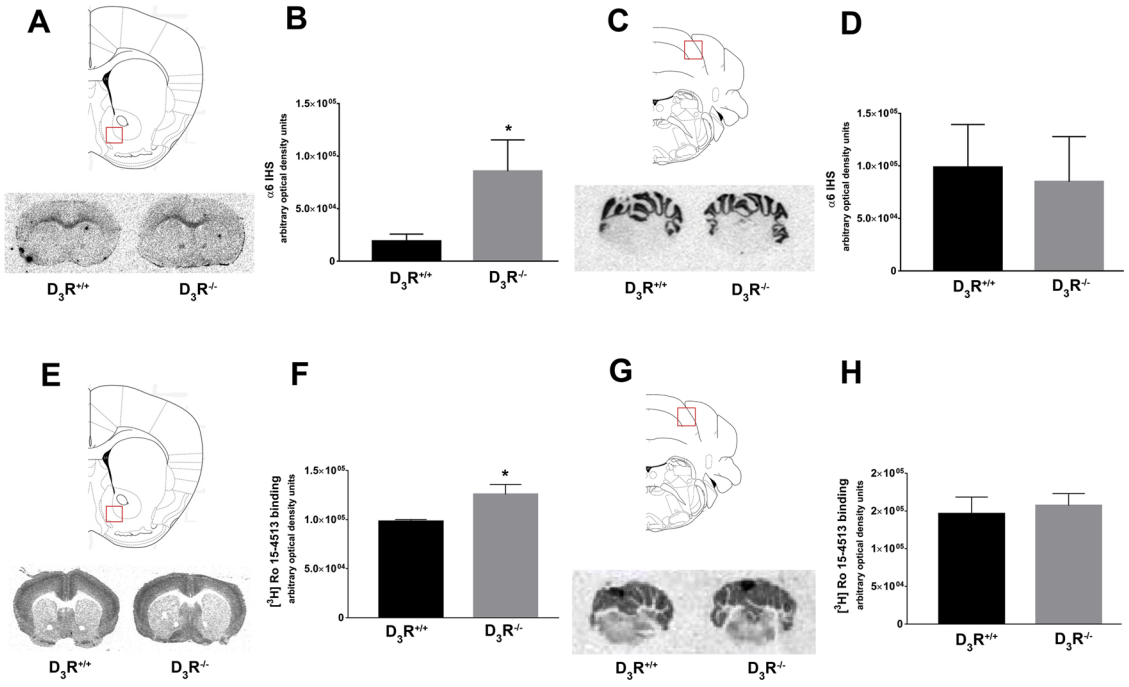

Fig. 2. Expression of α6 $GABA_A$ subunit mRNA and [^{3}H]-Ro 15-4513 binding in the NAc and Cerebellum of $D_3R^{+/+}$ and $D_3R^{-/-}$ mice. A, B, C and D *in situ* hybridization (ISH) detection of α6; E, F, G and H, [^{3}H]-Ro 15-4513 autoradiography. A, C, E and G show representative images. B, D, F and H show average optical density, (expressed in arbitrary units); n = 6–8 per group. *P < 0.05 *vs.* $D_3R^{+/+}$, unpaired *t* test.

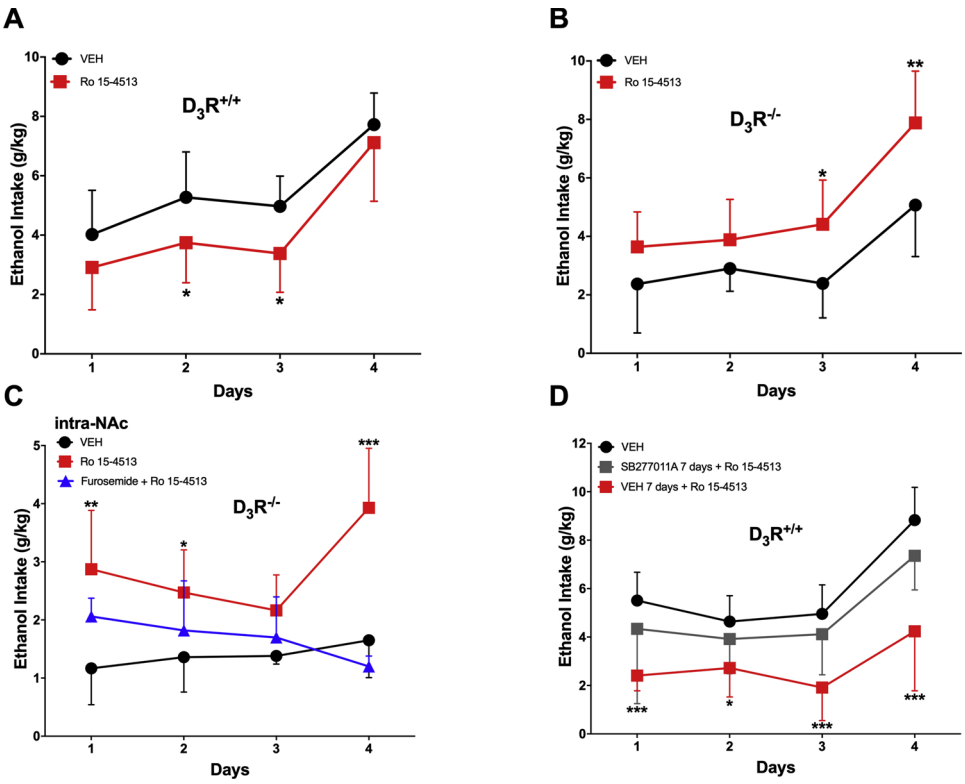

Fig. 3. Opposite effect of RO 15-4513 on alcohol intake, in $D_3R^{+/+}$ and $D_3R^{-/-}$ (drink in the dark paradigm, DID). A and B, ethanol intake in $D_3R^{+/+}$ and $D_3R^{-/-}$ intraperitoneally (i.p.) treated with vehicle (VEH) or Ro 15-4513 (5 mg/kg); C, ethanol intake in $D_3R^{-/-}$ locally injected into the NAc with VEH, Ro 15-4513 (10 nmol/mouse) or furosemide (10 nmol/mouse) plus Ro 15-4513 ; D, ethanol intake in $D_3R^{+/+}$ pretreated with VEH or the selective D_3R antagonist, SB 277011 A for 7 days (10 mg/kg, i.p.) plus Ro 15-4513 (5 mg/kg, i.p.) over DID paradigm.
Each experimental group included 8–13 mice. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle (VEH). One- or two- way ANOVA and Newman–Keuls post hoc test.

P < 0.001 vs vehicle]. These data indicate that treatment with a D_3R antagonist, sufficient to change the expression of α6-$GABA_AR$ in the NAc, determines changes in ethanol intake.

3.3. $D_3R^{-/-}$ mice exhibited Ro 15-4513 -driven decrease of mIPSC amplitude in medium spiny neurons

To test the hypothesis that α6 subunit expression in the NAc shell, as seen in $D_3R^{-/-}$ mice, modifies inhibitory transmission, we performed whole-cell patch-clamp recordings on GABAergic MSNs, which represent > 95% of the cell population in this brain region, and recorded miniature inhibitory postsynaptic currents (mIPSCs). Analysis of the peak amplitudes of mIPSCs revealed a significant increase in $D_3R^{-/-}$ compared to $D_3R^{+/+}$(Fig. 4A–D; 38.58 ± 3.35 pA, n = 19 versus 29.51 ± 2.96 pA, n = 16; P < 0.05). In contrast, there was no significant difference in mIPSC frequency ($D_3R^{-/-}$: 1.98 ± 0.30 Hz, $D_3R^{+/+}$: 1.77 ± 0.26 ms) and mIPSC kinetics (Fig. 4G, H; rise time, $D_3R^{-/-}$: 0.72 ± 0.06 ms; $D_3R^{+/+}$: 0.72 ± 0.06 ms; decay time, $D_3R^{-/-}$: 16.96 ± 1.10 ms; $D_3R^{+/+}$: 16.14 ± 1.31 ms). Next, we tested the effects of Ro 15–4513 on mIPSCs in MSNs from $D_3R^{+/+}$and $D_3R^{-/-}$. Based on ISH and qPCR data, indicating that α6-$GABA_ARs$ in the NAc are scarce in naïve $D_3R^{+/+}$mice and given the opposite effect of Ro 15-4513 treatment on ethanol intake observed in $D_3R^{-/-}$ mice, we expected that Ro 15-4513 would have differential effects on mIPSCs. For this *in vitro* experiment we selected the 0.3 μM Ro 15-4513 concentration, because it completely antagonizes ethanol enhancement of α4β3δ-type $GABA_AR$ current [21]. As shown in Fig. 4, bath application of 0.3 μM Ro 15-4513 did not significantly alter the frequency, rise time, decay time and amplitude of mIPSCs in $D_3R^{+/+}$ (n = 16; paired t test), but induced a significant reduction of amplitude in the NAc of $D_3R^{-/-}$ (Fig. 3B–F; 38.58 ± 3.35 pA, versus 31.93 ± 3.03 pA, n = 19 P < 0.05; paired t test) while frequency, rise time and decay time were not affected. These results suggest that the activity of α6-$GABA_AR$ in $D_3R^{-/-}$ influences inhibitory synaptic transmission of MSN within NAc shell, possibly because α6 expression, higher than in $D_3R^{+/+}$, is sufficient to generate a population of heteromeric $GABA_ARs$ containing α1 and α6 [39].

4. Discussion

We found that increased expression of α6 $GABA_A$ subunit, induced by D_3R deletion or pharmacological blockade, is associated to reduced alcohol intake and increased GABA inhibition in the NAc. We revealed $GABA_A$ α6 activity by using Ro 15-4513, both in terms of behavior (ethanol intake) as well as of neuronal excitability (electrophysiology). Ro 15-4513 is considered a high affinity α6-$GABA_AR$ ligand, since its binding is obvious in a α6 rich brain structure, such as cerebellum, while it is hardly detectable in the very same structure in α6 null mice [26].

We previously reported that alcohol sensitization is linked to increased D_3R expression induced by ethanol intake and is associated with the activation of RACK1/BDNF pathway. In fact, selective blockade of the TrkB, the receptor for BDNF, reverses stable intake of ethanol in WT mice and decreases D_3R expression levels in their striatum, while it results ineffective in $D_3R^{-/-}$ mice [15].

The α6 subunit came to the attention of the alcohol addiction studies following the identification of the R100Q mutation in the Sardinian non-ethanol-preferring rat line, suggesting a possible involvement of the $GABA_AR$ containing α6 subunit in the genetic predisposition to alcohol preference [10]. This mutation is associated with hypersensitivity to motor-impairing effects of ethanol and tonic inhibitory currents mediated by α6βδ-type $GABA_AR$ in cerebellar granule cells [8,13]. Worthy of note, this mutation strongly increases diazepam effect on GABA-evoked currents [11]. Consistently, a model where the amino acidic residue at position 100 affects ethanol sensitivity in the $GABA_ARs$ is part of the benzodiazepine ligand-binding pocket on the α6-subunit [19,40]. Other studies have also described α6 polymorphisms that correlate to alcohol dependence in humans [41,42]. Our observation that genetic deletion or pharmacological blockade of D_3R increased $GABA_A$ α6 subunit expression in the ventral striatum [16], a brain structure involved in voluntary ethanol intake, provides a tool to study how the increased expression of α6 subunit-containing receptors may

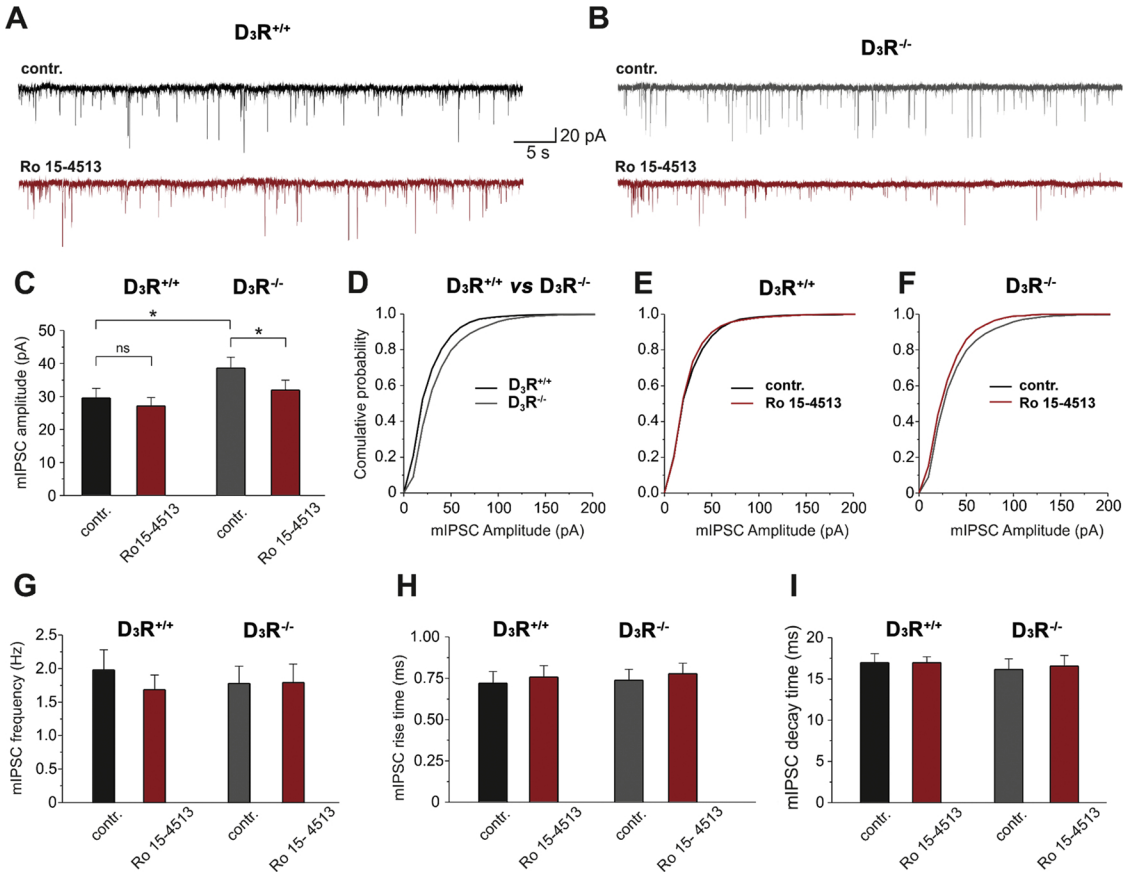

Fig. 4. NAc medium spiny neurons from $D_3R^{-/-}$ mice exhibited increased $GABA_A$ inhibitory currents sensitive to Ro 15-4513. A and B, representative traces showing mIPSC recordings in slice from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice before and after treatment with Ro 15-4513 (0.3 μM; in red). C, analysis of the peak amplitudes of mIPSCs; notice an increase in $D_3R^{-/-}$ compared to $D_3R^{+/+}$ and a decrease following Ro 15-4513 application in $D_3R^{-/-}$ only. D–F, cumulative frequency distributions for mIPSC amplitude in the experimental conditions shown in A and B. G–I, analysis of mIPSC frequency, rise time and decay time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
*P < 0.05, unpaired ($D_3R^{-/-}$ *vs.* $D_3R^{+/+}$) or paired (pre- *vs.* post- Ro 15-4513) *t* test ($D_3R^{-/-}$, n = 19; $D_3R^{+/+}$, n = 16).

affect alcohol intake. Indeed, some studies have evaluated the contribution of other $GABA_AR$ subunits, such as α4 and δ, but no data are available on the role of NAc $GABA_A$ α6 subunit in alcohol intake; this latter has only been studied for its involvement in the motor incoordination associated to alcohol, given its abundant localization in cerebellum granule cells.

Several studies, in the last two decades, have tried to elucidate how the subunit composition of different $GABA_ARs$ determines their electrophysiological and pharmacological features (inhibitory currents, ligand binding), or, at the organism level, the animal behavior (anxiety, addiction, response to anxiolytics). While most studies have dealt with recombinant systems, such as *Xenopus laevis* oocytes injected either with cRNA coding for the different subunits [9,21] or with cRNA coding for concatenated subunits [43], no studies had the opportunity to examine native systems, i.e. animals spontaneously and stably expressing specific subunits in defined CNS structures. Polymorphisms of α6 subunit have been found to be associated both to anxiety-related traits [44] and to benzodiazepine sensitivity in humans [45]. It is not yet known whether increased expression of α6 subunit containing $GABA_AR$ isoforms in brain areas that normally express negligible amounts of α6 produces different responses to GABA (i.e. different inhibitory currents) and/or to exogenous modulators. This might be due to the lack of *in vivo* systems with significant changes in α6 expression. Early studies with α6 subunit knockout mice [26,46] remained inconclusive as it was later discovered that the knockout construct affected the expression of neighboring subunits in the $GABA_A$ gene cluster [47].

We took advantage of Ro 15-4513, because it has been proposed to compete with ethanol within a binding pocket involving α6 [23]. We expected a different effect of Ro 15-4513 in $D_3R^{+/+}$, which poorly express α6 in the NAc, versus $D_3R^{-/-}$, which robustly express α6. Indeed, we found an opposite effect of Ro 15-4513 in the two groups; in $D_3R^{+/+}$, the systemic administration of Ro 15-4513 reduced ethanol intake, presumably as a result of its action as a negative allosteric modulator in multiple $GABA_ARs$ [21], where it would behave as an "ethanol antagonist" [23,48]. Conversely, in $D_3R^{-/-}$, Ro 15-4513 paradoxically increased ethanol intake, a surprising finding that might be explained in terms of differential modulation of the $GABA_AR$ containing α6 subunit by Ro 15-4513. These data were confirmed and validated by intra-NAc injection experiments, where the local administration of furosemide, a selective α6-$GABA_A$ receptor antagonist [37], completely blocked the effect of Ro 15-4513.

The antagonism between Ro 15-4513 and ethanol might be more at the functional level, rather than at the binding level. While the reported affinity of Ro 15-4513 for α4 and α6 containing $GABA_AR$ is quite similar in the nanomolar range [9,21,23], the effect on the GABA-dependent currents in cells expressing exclusively α4 or α6 subunits is not clear and might be quite different. This is consistent with the paradoxical activation of neurons by gaboxadol in a transgenic Thy1α6 mouse line, ectopically expressing the $GABA_AR$ α6 subunit gene under the Thy-1.2 promoter [20]. We directly address this issue by measuring MSN mIPSCs in the NAc and their sensitivity to Ro 15-4513. Based on the above premises, we hypothesized that a change in $GABA_A$ α6 subunit expression would increase spontaneous mIPSCs and that Ro 15-4513 would inhibit mIPSCs in MSN from $D_3R^{-/-}$, robustly expressing

α6, whereas it would be ineffective in α6-deficient MSNs from $D_3R^{+/+}$. The electrophysiological analysis of MSNs revealed a significant increase in mIPSC amplitude in $D_3R^{-/-}$, which expressed $GABA_AR$ containing α6 subunit in NAc, compared to $D_3R^{+/+}$. Accordingly perfusion with Ro 15-4513 induced a significant reduction of amplitude in the NAc of $D_3R^{-/-}$, but was ineffective in $D_3R^{+/+}$. This latter observation clearly indicates that the modulation of the $GABA_AR$ channel by Ro 15-4513 depends on the presence of α6 subunit and is consistent with the observation of opposite effects of this drug on ethanol intake in $D_3R^{+/+}$ and $D_3R^{-/-}$. To precisely assess the spatial expression of α6 subunit in the brain of $D_3R^{+/+}$ and $D_3R^{-/-}$, we carried out in situ hybridization (ISH) experiments. The systematic assessment of α6 expression in the CNS by ISH confirmed qPCR results, indicating that α6 expression in $D_3R^{-/-}$ was restricted to a limited brain area, corresponding to NAc. These results were reinforced also by autoradiography data obtained with [^{3}H]Ro 15-4513. The fact that genetic or pharmacological manipulation of D_3R induced changes in the $GABA_AR$ α6 subunit expression specifically in NAc is consistent with the restricted expression of D_3R in this brain region [49]. To the best of our knowledge, it is not known in detail how D_3R controls $GABA_AR$ subunit mRNA expression; however, other studies have shown dynamic D_3R-dependent down-regulation of GABAergic control over lateral/basolateral amygdala neurons [50], NAc [51] and hippocampus [52]. A direct dynamic interplay between metabotropic DA receptors and other ionotropic receptors in plasma membrane has been documented by single-molecule detection imaging and electrophysiology in live hippocampal neurons [53]. Furthermore, cell signaling downstream of D_3R affects $GABA_ARs$ in NAc [51], but numerous other complex mechanisms may impact $GABA_ARs$ trafficking [54] and deserve further studies to be elucidated. Finally, because these changes in $GABA_AR$ function can be related to dopaminergic transmission, they may assume further relevance in clinical situations, such as schizophrenia and Parkinson's disease, where D_3R are chronically blocked or stimulated by drug-treatments [38].

In conclusion, these data indicate that α6-containing $GABA_ARs$ in NAc play an important role in controlling alcohol intake by increasing GABAergic-inhibition in the MSNs. Because changes in α6-containing $GABA_ARs$ are specifically induced in NAc by D_3R-blockade, the interplay between DAergic and GABAergic transmission may present a novel relevant mechanisms in reinforcing properties of alcohol and other addictive drugs.

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Competing interests

None of the authors have competing financial interests in relation to the work described.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2019.01.022.

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