Manuscript Details

Manuscript number	YPHRS_2018_1683_R2
Title	Dopaminergic-GABAergic interplay and alcohol binge drinking
Article type	Research Paper

Abstract

The dopamine D3 receptor (D3R), 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 D3R increases GABAA α6 subunit in the ventral striatum. Here we tested the hypothesis that D3R-dependent changes in GABAA 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 D3 knockout (D3R -/-) mice and wild type littermates (D3R +/+). Ro 15-4513, a high affinity α6-GABAA ligand was used to study α6 activity. At baseline, NAc α6 expression was negligible in D3R+/+, whereas it was robust in D3R−/−; other relevant GABAA 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 D3R+/+, but increased it in D3R−/−; this was confirmed by intra-NAc administration of Ro 15-4513 and furosemide, a selective α6-GABAA antagonist. Whole-cell patch-clamp showed peak amplitudes of miniature inhibitory postsynaptic currents in NAc medium spiny neurons higher in D3R-/- compared to D3R+/+; Ro 15-4513 reduced the peak amplitude in the NAc of D3R-/-, but not in D3R+/+. We conclude that D3R-dependent enhanced expression of α6 GABAA subunit inhibits voluntary alcohol intake by increasing GABA inhibition in the NAc.

Keywords	dopamine D3 receptor; GABAA receptor; alpha6 subunit; ethanol; nucleus accumbens; Ro 15-4513

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Suggested reviewers	Robert Nisticò, Jose Nobrega

Submission Files Included in this PDF

File Name [File Type]

Leggio cover letter 10jan19.pdf [Cover Letter]

Leggio_Author checklist.pdf [Checklist]

10jan19_Responses to the Referee's comments.docx [Response to Reviewers]

Leggio 10jan19 revised highlighted copy.docx [Response to Reviewers]

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Fig1 Leggio revised.tiff [Figure]

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Università di Catania

Sezione di Farmacologia - Prof. Salvatore Salomone

Prof. Emilio Clementi,MD, PhD
Editor-in-Chief
Pharmacological Research

Catania, January 10th, 2019

Dear Prof. Clementi,

Please find, attached the second revised version of our manuscript entitled "**Dopaminergic-GABAergic interplay and alcohol binge drinking**", by Leggio et al., submitted for publication to *Pharmacological Research*.

We have revised the manuscript according to the recommendation of the reviewer.

We hope that, in this revised form, our manuscript could now be published in Pharmacological Research.

Looking forward to hear from you.

Sincerely yours

Salvatore Salomone, MD, PhD

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Table 1: ***Author Checklist for Original Articles to be submitted to Pharmacological Research***

Questions	***reply***		
	Yes	***No***	***Not applicable***
Formatting - The submission will automatically be rejected if the first six questions are not marked "yes" (questions 1-6) or "not applicable" (limited to questions 3-6)			
1. Are all tables and figures numbered and appropriately titled with descriptive legends that permit stand-alone interpretation?	X		
2. Are all data shown on figures and tables mentioned in the text of the Results section?	X		
3. Are the whole un-cropped images of the original western blots from which figures have been derived shown as supplemental figures?			X
4. In case of human studies, has specific mention been made of the study compliance with the regulations of the country(ies) in which the study was carried out ?			X
5. In case of human studies, has the study been registered on an accessible international registry/database of clinical trials (e.g. EudraCT, ClinicalTrials.gov, ChiCTR, ANZCTR, JPRN and the like)			X
6. In case of studies on animals, is there a statement indicating compliance with regulations on the ethical treatment of animals including identification of the institutional committee that approved the experiments?	X		
Introduction			
7. Is there a clear statement with background describing the hypothesis being tested by this study?	X		
8. Are the primary endpoints clearly described?	X		
Materials and Methods			
9. Are the sources of all materials clearly indicated?	X		
10. Is(are) the chemical structure(s) of any new compound(s) presented as a figure in the manuscript or referenced in the literature?	X		
11. Are the source(s) and passage number of cell lines indicated?			X
12. Are the source, catalogue number and lot for commercial antibodies indicated?			X
13. Are the species, strain, sex, weight and source of the animal subjects provided?	X		
14. Is the rationale provided for the selection of concentrations, doses, route and frequency of compound administration?	X		
15. Are quantified results (*e.g.* IC_{50} and/or EC_{50} values) of concentration- and dose-response experiments included in the report?			X
16. Is the method of anaesthesia described?			X
17. Are all group sizes approximately the same?	X		
18. Were the criteria used for excluding any data from analysis determined prospectively and clearly stated?	X		
19. Was the investigator responsible for data analysis blinded to which samples/animals represent treatments and controls?	X		
20. Is the exact sample size (n) for each experimental group/condition clearly indicated in the text and/or on the tables and figures?	X		

21. Are the reported data displayed as the mean +/- an estimate of variability (SD, SEM) of three or more independent experimental replications?	X		
22. Is the number of replications used to generate an individual data point in each of the independent experiments clearly indicated?	X		
23. Were the statistical tests employed to analyse the primary endpoints predetermined as part of the experimental design?	X		
24. Is the threshold for statistical significance (P value) clearly indicated?	X		
25. Were the data normalised?	X		
26. Were post-hoc tests used to assess the statistical significance among means?	X		
27. Was the study exploratory rather than hypothesis-driven?		X	
28. Were human tissues or fluids used in this study?		X	
Results			
29. If western blots are shown, are the following included: i) appropriate loading controls for each western blot, ii) replication data, iii) quantification, and iv) the results of a statistical analysis?			X
30. Were MIQE guidelines followed in the quantitative analysis and presentation of PCR and RT-PCR findings?		X	
31. Was a reference standard (positive or negative controls) included in the study to validate the experiment?	X		
Discussion			
32. Are all the findings considered within the context of the hypothesis presented in the Introduction?	X		
33. Are the primary conclusions and their implications clearly stated?	X		
34. Are any secondary endpoints reported and are these sufficiently powered for appropriate statistical analysis?			X
35. Are the limitations of the current study or alternative interpretations of the findings clearly stated?	X		
For Meta-analyses only			
36. Were the PRISMA reporting guidelines and checklist followed in the case of meta-analyses on randomised controlled trials?			X
37. Were the MOOSE reporting guidelines followed in the case of meta-analyses on observational studies in epidemiology?			X
38. Was the protocol submitted into the PROSPERO International prospective register of systematic reviews and a registration number obtained?			X
Conflict of Interest/Financial Support			
39. Is the conflict of interest statement is included in the manuscript?	X		
40. Are all organisations providing funding for this work listed in the Acknowledgments?	X		

Feedback/suggestions on the checklist by the author

Responses to the Referee's comments

We do thank the Editor and the Reviewer for their helpful comments. We have revised our submission accordingly, and hope it can now be considered acceptable for publication in Pharmacological Research.
Below our point-by-point rebuttal

Reviewer #2

Query: The location of the regions of interest for RT-PCR and autoradiographic measurements does not seem to be fully accounted for by the nucleus accumbens. In fact, the left and right ROIs in the image of [3H]-Ro 15-4513 binding in D3-/- mice clearly cover anatomically heterogenous brain regions (see Response to Referee's comment, page 4), and even a small shift of the ROI between a densely labeled area and the much less densely labeled nucleus accumbens would produce large differences in the measured average optical density. In this context, it would be absolutely essential to make these measurements in a blinded manner. A statement to that effect should be added in the methods.
Answer: We fully agree with the referee's view; in fact all measurements had been carried out in blind. We have now added a statement in the methods and results sections of the revised manuscript, as suggested.

Query: The labels/units in Figs 2B, D, F and H don't seem to make sense (optical density values of 10,000?).
Answer: We apologize for this misprint. Values represented in the graphs are expressed as arbitrary optical density units. We changed optical density with arbitrary optical density units in all panels of the figure 2.

Query: The film exposure times don't seem to have been added to the SI.
Answer: We apologize. We added the film exposure time (12 weeks) in the SI section.

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2. Are all data shown on figures and tables mentioned in the text of the Results section?	***X***		
3. Are the whole un-cropped images of the original western blots from which figures have been derived shown as supplemental figures?			***X***
4. In case of human studies, has specific mention been made of the study compliance with the regulations of the country(ies) in which the study was carried out ?			***X***
5. In case of human studies, has the study been registered on an accessible international registry/database of clinical trials (e.g. EudraCT, ClinicalTrials.gov, ChiCTR, ANZCTR, JPRN and the like)			***X***
6. In case of studies on animals, is there a statement indicating compliance with regulations on the ethical treatment of animals including identification of the institutional committee that approved the experiments?	***X***		
Introduction			
7. Is there a clear statement with background describing the hypothesis being tested by this study?	***X***		
8. Are the primary endpoints clearly described?	***X***		
Materials and Methods			
9. Are the sources of all materials clearly indicated?	***X***		
10. Is(are) the chemical structure(s) of any new compound(s) presented as a figure in the manuscript or referenced in the literature?	***X***		
11. Are the source(s) and passage number of cell lines indicated?			***X***
12. Are the source, catalogue number and lot for commercial antibodies indicated?			***X***
13. Are the species, strain, sex, weight and source of the animal subjects provided?	***X***		
14. Is the rationale provided for the selection of concentrations, doses, route and frequency of compound administration?	***X***		
15. Are quantified results (*e.g.* IC_{50} and/or EC_{50} values) of concentration- and dose-response experiments included in the report?			***X***
16. Is the method of anaesthesia described?			***X***
17. Are all group sizes approximately the same?	***X***		
18. Were the criteria used for excluding any data from analysis determined prospectively and clearly stated?	***X***		
19. Was the investigator responsible for data analysis blinded to which samples/animals represent treatments and controls?	***X***		
20. Is the exact sample size (n) for each experimental group/condition clearly indicated in the text and/or on the tables and figures?	***X***		
21. Are the reported data displayed as the mean +/- an estimate of variability (SD, SEM) of three or more independent experimental replications?	***X***		
22. Is the number of replications used to generate an individual data point in each of the independent experiments clearly indicated?	***X***		
23. Were the statistical tests employed to analyse the primary endpoints predetermined as part of the experimental design?	***X***		
24. Is the threshold for statistical significance (P value) clearly indicated?	***X***		
25. Were the data normalised?	***X***		
26. Were post-hoc tests used to assess the statistical significance among means?	***X***		
27. Was the study exploratory rather than hypothesis-driven?		***X***	
28. Were human tissues or fluids used in this study?		***X***	
Results			
29. If western blots are shown, are the following included: i) appropriate loading controls for each western blot, ii) replication data, iii) quantification, and iv) the results of a statistical analysis?			***X***
30. Were MIQE guidelines followed in the quantitative analysis and presentation of PCR		***X***	

and RT-PCR findings?			
31. Was a reference standard (positive or negative controls) included in the study to validate the experiment?	***X***		
Discussion			
32. Are all the findings considered within the context of the hypothesis presented in the Introduction?	***X***		
33. Are the primary conclusions and their implications clearly stated?	***X***		
34. Are any secondary endpoints reported and are these sufficiently powered for appropriate statistical analysis?			***X***
35. Are the limitations of the current study or alternative interpretations of the findings clearly stated?	***X***		
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Feedback/suggestions on the checklist by the author			

Dopaminergic-GABAergic interplay and alcohol binge drinking

Gian Marco Leggio[1*], Roberta Di Marco[1*], Walter Gulisano[1], Marcello D'Ascenzo[2], Sebastiano Alfio Torrisi[1], Federica Geraci[1,] Gianluca Lavanco[1], Kristiina Dahl[3], Giovanni Giurdanella[1], Alessandro Castorina[1,4], Teemu Aitta-aho[3], Giuseppe Aceto[2], Claudio Bucolo[1], Daniela Puzzo[1], Claudio Grassi[2], Esa R. Korpi[3], Filippo Drago[1] and Salvatore Salomone[1]

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Short/running title: D_3R-$GABA_A$ interplay

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 D3 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.

Key words: dopamine D3 receptor; $GABA_A$ receptor; alpha6 subunit; ethanol; nucleus accumbens; Ro 15-4513

Chemical compounds studied in this article Ro 15-4513 (PubChem CID: 5081); SB 277011A (PubChem CID: 75358288); Furosemide (PubChem CID: 3440)

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; $GABA_AR$s, $GABA_A$ receptors; ISH, in situ hybridization; mIPSCs, miniature inhibitory postsynaptic currents; MSN, medium spiny neuron; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

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 D1-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 ($GABA_A$Rs) in the NAc have been considered as a primary target for alcohol, and may be involved in voluntary alcohol consumption [6]; moreover, chronic alcohol intake alters GABAergic function in the NAc, which sustains behavioral addictive patterns [1, 6]. $GABA_A$R 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 $GABA_A$R 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 $GABA_A$Rs 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 $GABA_A$Rs 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 $GABA_A$Rs 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 277011A, 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 277011A. 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 $GABA_A$R 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-$GABA_A$R

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/6J 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 3h 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 277011A 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 277011A 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 SB277011A for 7 days before SB 277011A 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 277011A and Ro 15-4513 were i.p. injected, respectively 1h and 15 minutes 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 minutes 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.1 A-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 4h-time window used instead of a 2h-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. 2 A-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. 1 A-B). Autoradiography following incubation with a high 15 nM concentration of [^{3}H]Ro15-4513 showed a statistically significant increase of [^{3}H]Ro15-4513 binding in the NAc [$P<0.05$] (Fig. 2 E-F). Ro 15-4513 binds at α6/4β3δ-type $GABA_A$ receptors with high affinity ($K_D \approx 10$ 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

Ro15-4513 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]Ro15-4513 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. 3 A, 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. 3 B) [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. 3 C, 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 -$GABA_A$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 277011A 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 277011A 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 277011A for 7 days, which increased the expression of α6-$GABA_A$R in the NAc (Figure 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, P<0.001 vs vehicle]. These data indicate that treatment with a D_3R antagonist, sufficient to change the expression of α6-$GABA_A$R 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. 4; A-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. 4 G, 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-$GABA_A$Rs 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 $GABA_A$R 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 $D_3R^{-/-}$ (Figure 3; B-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-$GABA_A$R 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 $GABA_A$Rs 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) ~~a $GABA_A$ ligand that exerts α6-dependent effects~~. Ro 15-4513 is considered a high affinity α6-$GABA_A$R 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].

We previously reported that alcohol sensitization is linked to increased D3R 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 D3R expression levels in their striatum, while it results ineffective in $D3R^{-/-}$ 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 $GABA_A$R 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 $GABA_A$R 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 $GABA_A$Rs 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 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 the 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 the NAc is consistent with the ~~leaving relatively unchanged other brain areas~~ is ~~not so surprisingly~~, ~~considering that~~, ~~at variance with D_2R~~, restricted expression of D_3R in this brain region ~~t same structures where we observe increased α6 expression~~ [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 the 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 the 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 the 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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Figure Legends

Figure 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 (2h/day for 3 days and 4h 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.

Figure 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.

Figure 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 277011A 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.

Figure 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.

*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).

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Supplementary Information

Table S1. *In situ* hybridization (ISH) signals for $GABA_A$??1, ??2, ??4, ??6, **γ**2 and δ subunit mRNA in the prefrontal cortex from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice.

$GABA_A$ subunit ISH signal ($D_3R^{-/-}$ over $D_3R^{+/+}$ ratio)	
α1	0.92 ± 0.02
α2	1.10 ± 0.11
α4	0.80 ± 0.07
α6	1.15 ± 0.13
γ2	0.89 ± 0.30
δ	0.83 ± 0.17

Table S2. *In situ* hybridization (ISH) signals for $GABA_A$??1, ??2, ??4, ??6, **γ**2 and δ subunit mRNA in the hippocampus from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice.

$GABA_A$ subunit ISH signal ($D_3R^{-/-}$ over $D_3R^{+/+}$ ratio)	
α1	0.91 ± 0.09
α2	1.19 ± 0.13
α4	1.07 ± 0.03
α6	1.20 ± 0.10
γ2	0.96 ± 0.26
δ	0.85 ± 0.16

Table S3. Primers for Real-Time PCR

Target gene	**Primer sequence**
Gabra1	5’-GACCAGGTTTGGGAGAGCGTGT-3’
	3’-GCCGGAGCACTGTCATGGGTC-5’
Gabra2	5’-CCCAGTCAGGTTGGTGCTGGC-3’
	3’-ACAGGGCCAAAACTGGTCACGT-5’
Gabra4	5’-CCTGTGCCTGGCGGCTTGTTTA-3’
	3’-CCCCAAATCCAGGACGCAGCC-5’
Gabra6	5’-GGCCAGGATTTGGGGGTGCTG-3’
	3’-TCAGTCCAAGTCTGGCGGAAGA-5’
Gabrg2	5’-ACCCAGAGGCGAGAGGCGAG-3’
	3’-GCTTGTGAAGCCTGGGTAGAGCG-5’
Gabrd	5’-CCGACCAGGCATTGGAGGTGC-3’
	3’-TGCTGTCCCGCCAGCTCTGA-5’
Gapdh	5’-CAACTCACTCAAGATTGTCAGCAA-3’
	3’-GGCATGGACTGTGGTCATGA-5’

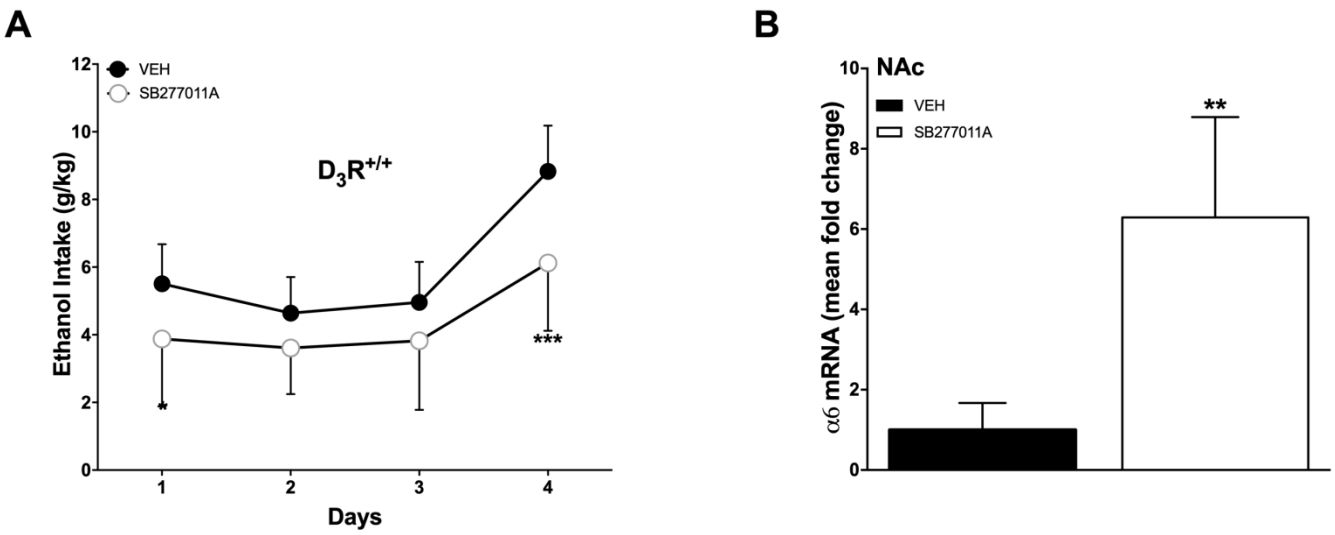

Figure S1. Pharmacological blockade of D3R counteracts alcohol intake and induced overexpression of $GABA_A$ α6 subunit in the NAc of $D_3R^{+/+}$. A and B, ethanol intake (DID) and α6 expression in $D_3R^{+/+}$ treated with vehicle (VEH) or the selective D_3R antagonist, SB 277011A (10 mg/kg, i.p.) for 7 days. Each experimental group included 8-13 mice. *P<0.05, ***P<0.001 *vs.* VEH; two-way ANOVA and Newman–Keuls post hoc test.

Material and Methods

In situ hybridization

Air-dried slides were fixed in ice-cold 4% paraformaldehyde for 5 min. The sections were washed in 1 PBS at room temperature for 5 min, dehydrated in 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until used. The antisense DNA oligonucleotide probe (Oligomer Oy, Helsinki, Finland) sequences were as follows: α6, 5'-CAG TCT CTC ATC AGT CCA AGT

CAT-3'; was complementary to the mouse $GABA_AR$ subunit mRNA sequence. Poly[^{35}S]dATP (PerkinElmer Life and Analytical Sciences, Boston, MA) tails were added to the 3'-ends of the probes by deoxynucleotidyl transferase (Promega Corporation, Madison, WI). Unincorporated nucleotides were removed by Illustra ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, UK). Labelling efficiency (360,000 cpm/µl) was determined by a scintillation counter. The labeled probe was diluted to 0.06 fmol/µl of hybridization buffer consisting of 50% formamide and 10% dextran sulfate in 4X Saline Sodium Citrate (SSC). Nonspecific controls for the antisense probes were produced by adding 100-fold excess of unlabeled probes. The hybridization occurred under glass Menzel-Gläser coverslips (Thermo Fisher Scientific, Boston, MA) overnight at 42 °C. Finally, the slides were washed in 1X SSC at room temperature for 10 min, in 1X SSC at 55°C for 30 min, and 1X SSC, 0.1X SSC, 70% EtOH and 95% EtOH at room temperature for 1 min each. The slides were then air-dried and exposed with plastic [^{14}C]-radioactivity standards (GE Healthcare) to BioMax MR films (Eastman Kodak Company, Rochester, NY). Films were scanned (Epson expression 1680 Pro). Images were imported into the *FIJI* version of the free image processing software *ImageJ*. The [^{14}C]-standards were exposed simultaneously with the brain sections as the reference. The hybridization values were converted to arbitrary optical density units. Non-specific signal was subtracted to obtain the specific signal. All measurements were analyzed in blind.

[^{3}H]Ro 15-4513 autoradiography

Slides were pre-incubated in ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl for 15 min. The final incubation for basal [^{3}H]Ro 15-4513 binding was performed in the

pre-incubation buffer containing 15 nM [^{3}H]Ro 15-4513 (23 Ci/mmol, PerkinElmer Life and Analytical Sciences) at 4 °C for 1 h. This high ligand concentration was aimed at estimating the receptor number rather than affinity. The non-specific binding was determined in the presence of 10 µM flumazenil. The sections were then washed in ice-cold pre-incubation buffer twice for 1 min, dipped in ice-cold distilled water, air-dried at room temperature and exposed with [^{3}H]-plastic standards for 12 weeks (GE Healthcare) to Biomax MR films (Eastman Kodak). The films were scanned (Epson expression 1680 Pro) and binding density was expressed as arbitrary optical density units (*FIJI IMAGE-J*). The [^{3}H]-standards were exposed simultaneously with the sections as the reference. Non-specific binding was subtracted to obtain the specific binding values. All data were analyzed in blind.

Electrophysiology

Animals were sacrificed by cervical dislocation. Brains were rapidly removed and placed in ice-cold cutting solution containing (in mM): TRIS-HCl 72, TRIZMA base 18, NaH_2PO_4 1.2, $NaHCO_3$ 30, KCl 2.5, glucose 25, HEPES 20, $MgSO_4$ 10, Na-pyruvate 3, ascorbic acid 5, $CaCl_2$ 0.5, sucrose 20. Slices (300 µm thick) were cut on a vibratome (VT1200S; Leica Microsystems, Germany) and immediately transferred to an incubation chamber held at 32°C and filled with a recovery solution containing (in mM): TRIS-HCl 72, TRIZMA base 18, NaH_2PO_4 1.2, $NaHCO_3$ 25, KCl 2.5, glucose 25, HEPES 20, $MgSO_4$ 10, Na-pyruvate 3, ascorbic acid 5, $CaCl_2$ 0.5, sucrose 20. After 30 min, slices were transferred to a second incubation chamber held at 32°C and filled with artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124, KCl 3.2, NaH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10, pH 7.4. During incubations, the chambers were continuously bubbled with 95% O_2/5% CO_2.

Slices were equilibrated at room temperature for at least 45 min. Slices were then transferred to a submerged recording chamber constantly perfused with heated aCSF (32°C) and bubbled with 95% O_2/5% CO_2. Medium spiny neurons (MSNs) within the NAc shell subregion were identified with a 40X water-immersion objective on an upright microscope equipped with differential interface contrast optics under infrared illumination (BX5IWI, Olympus, Center Valley, PA) and video observation. Electrodes were made from borosilicate glass micropipettes (Warner Instruments, Hamden, CT) prepared with a P-97 Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA). Patch pipettes had a resistance of 4-6 MΩ when filled with an internal solution containing (in mM): CsCl 135, HEPES 10, EGTA 1.1, $CaCl_2$ 0.1; Mg-ATP 2.5, Na-GTP 0.25, phosphocreatine 5, pH 7.2. After establishing a gigaseal, the patch was broken by applying negative pressure to achieve a whole-cell configuration. A series resistance lower than 15 MΩ was considered acceptable, and monitored constantly throughout the entire recording. Neurons were held at -70 mV. Tetrodotoxin (TTX, 0.5 µM, Tocris), D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 µM, Tocris) and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX, 10 µM, Tocris) were applied to the bath to block action potential-mediated neurotransmitter release, NMDA and AMPA receptors, respectively. Ro 15-4513 (0.3 µM) was applied in the bath after 5-7 min of TTX, APV and NBQX perfusion. All recordings were carried out at least 10 min after application of any drug to the bath. Recordings were performed using a Multiclamp 700B/Digidata 1550A system (Molecular Devices, Sunnyvale, CA) and digitized at a 10,000 Hz sampling frequency.

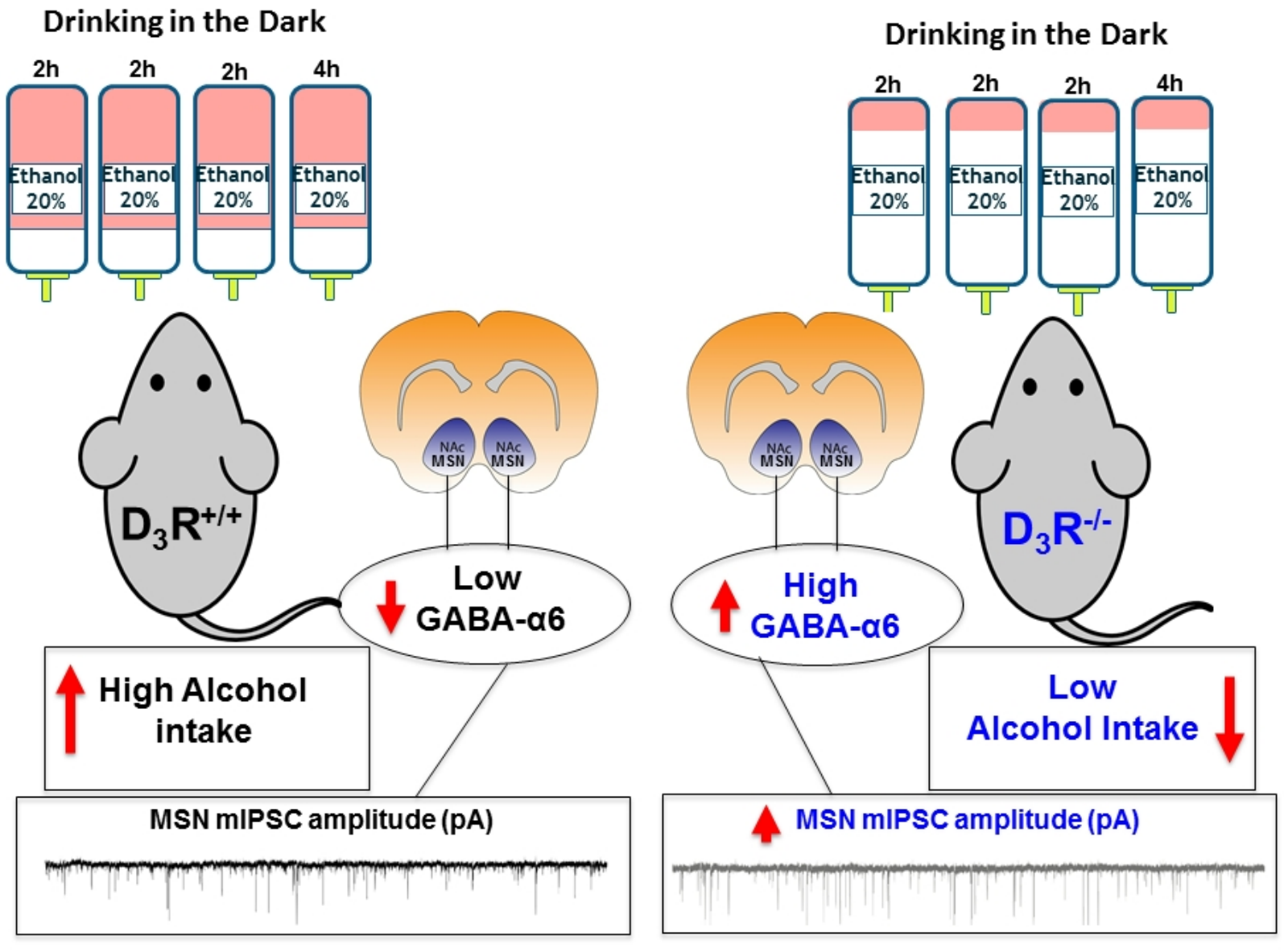
Drinking in the Dark
2h
2h
2h
4h
Ethanol 20%
Ethanol 20%
Ethanol 20%
Ethanol 20%
NAc MSN
NAc MSN
D3R+/+
Low GABA-α6
High Alcohol intake
MSN mIPSC amplitude (pA)
Drinking in the Dark
2h
2h
2h
4h
Ethanol 20%
Ethanol 20%
Ethanol 20%
Ethanol 20%
NAc MSN
NAc MSN
D3R-/-
High GABA-α6
Low Alcohol Intake
MSN mIPSC amplitude (pA)

Table 1: ***Author Checklist for Original Articles to be submitted to Pharmacological Research***

Questions	***reply***		
	Yes	***No***	***Not applicable***
Formatting - The submission will automatically be rejected if the first six questions are not marked "yes" (questions 1-6) or "not applicable" (limited to questions 3-6**)**			
1. Are all tables and figures numbered and appropriately titled with descriptive legends that permit stand-alone interpretation?	***X***		
2. Are all data shown on figures and tables mentioned in the text of the Results section?	***X***		
3. Are the whole un-cropped images of the original western blots from which figures have been derived shown as supplemental figures?			***X***
4. In case of human studies, has specific mention been made of the study compliance with the regulations of the country(ies) in which the study was carried out ?			***X***
5. In case of human studies, has the study been registered on an accessible international registry/database of clinical trials (e.g. EudraCT, ClinicalTrials.gov, ChiCTR, ANZCTR, JPRN and the like)			***X***
6. In case of studies on animals, is there a statement indicating compliance with regulations on the ethical treatment of animals including identification of the institutional committee that approved the experiments?	***X***		
Introduction			
7. Is there a clear statement with background describing the hypothesis being tested by this study?	***X***		
8. Are the primary endpoints clearly described?	***X***		
Materials and Methods			
9. Are the sources of all materials clearly indicated?	***X***		
10. Is(are) the chemical structure(s) of any new compound(s) presented as a figure in the manuscript or referenced in the literature?	***X***		
11. Are the source(s) and passage number of cell lines indicated?			***X***
12. Are the source, catalogue number and lot for commercial antibodies indicated?			***X***
13. Are the species, strain, sex, weight and source of the animal subjects provided?	***X***		
14. Is the rationale provided for the selection of concentrations, doses, route and frequency of compound administration?	***X***		
15. Are quantified results (*e.g.* IC_{50} and/or EC_{50} values) of concentration- and dose-response experiments included in the report?			***X***
16. Is the method of anaesthesia described?			***X***
17. Are all group sizes approximately the same?	***X***		
18. Were the criteria used for excluding any data from analysis determined prospectively and clearly stated?	***X***		
19. Was the investigator responsible for data analysis blinded to which samples/animals represent treatments and controls?	***X***		
20. Is the exact sample size (n) for each experimental group/condition clearly indicated in the text and/or on the tables and figures?	***X***		
21. Are the reported data displayed as the mean +/- an estimate of variability (SD, SEM) of three or more independent experimental replications?	***X***		
22. Is the number of replications used to generate an individual data point in each of the independent experiments clearly indicated?	***X***		
23. Were the statistical tests employed to analyse the primary endpoints predetermined as part of the experimental design?	***X***		
24. Is the threshold for statistical significance (P value) clearly indicated?	***X***		
25. Were the data normalised?	***X***		
26. Were post-hoc tests used to assess the statistical significance among means?	***X***		
27. Was the study exploratory rather than hypothesis-driven?		***X***	
28. Were human tissues or fluids used in this study?		***X***	
Results			
29. If western blots are shown, are the following included: i) appropriate loading controls for each western blot, ii) replication data, iii) quantification, and iv) the results of a statistical analysis?			***X***
30. Were MIQE guidelines followed in the quantitative analysis and presentation of PCR		***X***	

and RT-PCR findings?			
31. Was a reference standard (positive or negative controls) included in the study to validate the experiment?	***X***		
Discussion			
32. Are all the findings considered within the context of the hypothesis presented in the Introduction?	***X***		
33. Are the primary conclusions and their implications clearly stated?	***X***		
34. Are any secondary endpoints reported and are these sufficiently powered for appropriate statistical analysis?			***X***
35. Are the limitations of the current study or alternative interpretations of the findings clearly stated?	***X***		
For Meta-analyses only			
36. Were the PRISMA reporting guidelines and checklist followed in the case of meta-analyses on randomised controlled trials?			***X***
37. Were the MOOSE reporting guidelines followed in the case of meta-analyses on observational studies in epidemiology?			***X***
38. Was the protocol submitted into the PROSPERO International prospective register of systematic reviews and a registration number obtained?			***X***
Conflict of Interest/Financial Support			
39. Is the conflict of interest statement is included in the manuscript?	***X***		
40. Are all organisations providing funding for this work listed in the Acknowledgments?	X		
Feedback/suggestions on the checklist by the author			

Dopaminergic-GABAergic interplay and alcohol binge drinking

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Short/running title: D_3R-$GABA_A$ interplay

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 D3 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.

Key words: dopamine D3 receptor; $GABA_A$ receptor; alpha6 subunit; ethanol; nucleus accumbens; Ro 15-4513

Chemical compounds studied in this article Ro 15-4513 (PubChem CID: 5081); SB 277011A (PubChem CID: 75358288); Furosemide (PubChem CID: 3440)

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; $GABA_AR$s, $GABA_A$ receptors; ISH, in situ hybridization; mIPSCs, miniature inhibitory postsynaptic currents; MSN, medium spiny neuron; NAc, nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.

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 D1-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 ($GABA_A$Rs) in the NAc have been considered as a primary target for alcohol, and may be involved in voluntary alcohol consumption [6]; moreover, chronic alcohol intake alters GABAergic function in the NAc, which sustains behavioral addictive patterns [1, 6]. $GABA_A$R 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 $GABA_A$R 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 $GABA_A$Rs 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 $GABA_A$Rs 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 $GABA_A$Rs 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 277011A, 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 277011A. 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 $GABA_A$R 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-$GABA_A$R

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/6J 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 3h 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 277011A 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 277011A 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 SB277011A for 7 days before SB 277011A 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 277011A and Ro 15-4513 were i.p. injected, respectively 1h and 15 minutes 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 minutes 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.1 A-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 4h-time window used instead of a 2h-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. 2 A-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. 1 A-B). Autoradiography following incubation with a high 15 nM concentration of [^{3}H]Ro15-4513 showed a statistically significant increase of [^{3}H]Ro15-4513 binding in the NAc [$P<0.05$] (Fig. 2 E-F). Ro 15-4513 binds at α6/4β3δ-type $GABA_A$ receptors with high affinity ($K_D \approx 10$ 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

Ro15-4513 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]Ro15-4513 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. 3 A, 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. 3 B) [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. 3 C, 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 -$GABA_A$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 277011A 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 277011A 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 277011A for 7 days, which increased the expression of α6-$GABA_A$R in the NAc (Figure 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, P<0.001 vs vehicle]. These data indicate that treatment with a D_3R antagonist, sufficient to change the expression of α6-$GABA_A$R 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. 4; A-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. 4 G, 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-$GABA_A$Rs 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 $GABA_A$R 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 $D_3R^{-/-}$ (Figure 3; B-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-$GABA_A$R 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 $GABA_A$Rs 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) ~~a $GABA_A$ ligand that exerts α6-dependent effects~~. Ro 15-4513 is considered a high affinity α6-$GABA_A$R 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].

We previously reported that alcohol sensitization is linked to increased D3R 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 D3R expression levels in their striatum, while it results ineffective in $D3R^{-/-}$ 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 $GABA_A$R 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 $GABA_A$R 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 $GABA_A$Rs 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 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 $GABA_A$Rs [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 $GABA_A$R 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 $GABA_A$R 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 $GABA_A$R 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 $GABA_A$R 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 $GABA_A$R 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 the 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 the NAc is consistent with the ~~leaving relatively unchanged other brain areas~~ is ~~not so surprisingly~~, ~~considering that~~, ~~at variance with D_2R~~, restricted expression of D_3R in this brain region ~~t same structures where we observe increased α6 expression~~ [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 the 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 the 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 the 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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Figure Legends

Figure 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 (2h/day for 3 days and 4h 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.

Figure 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.

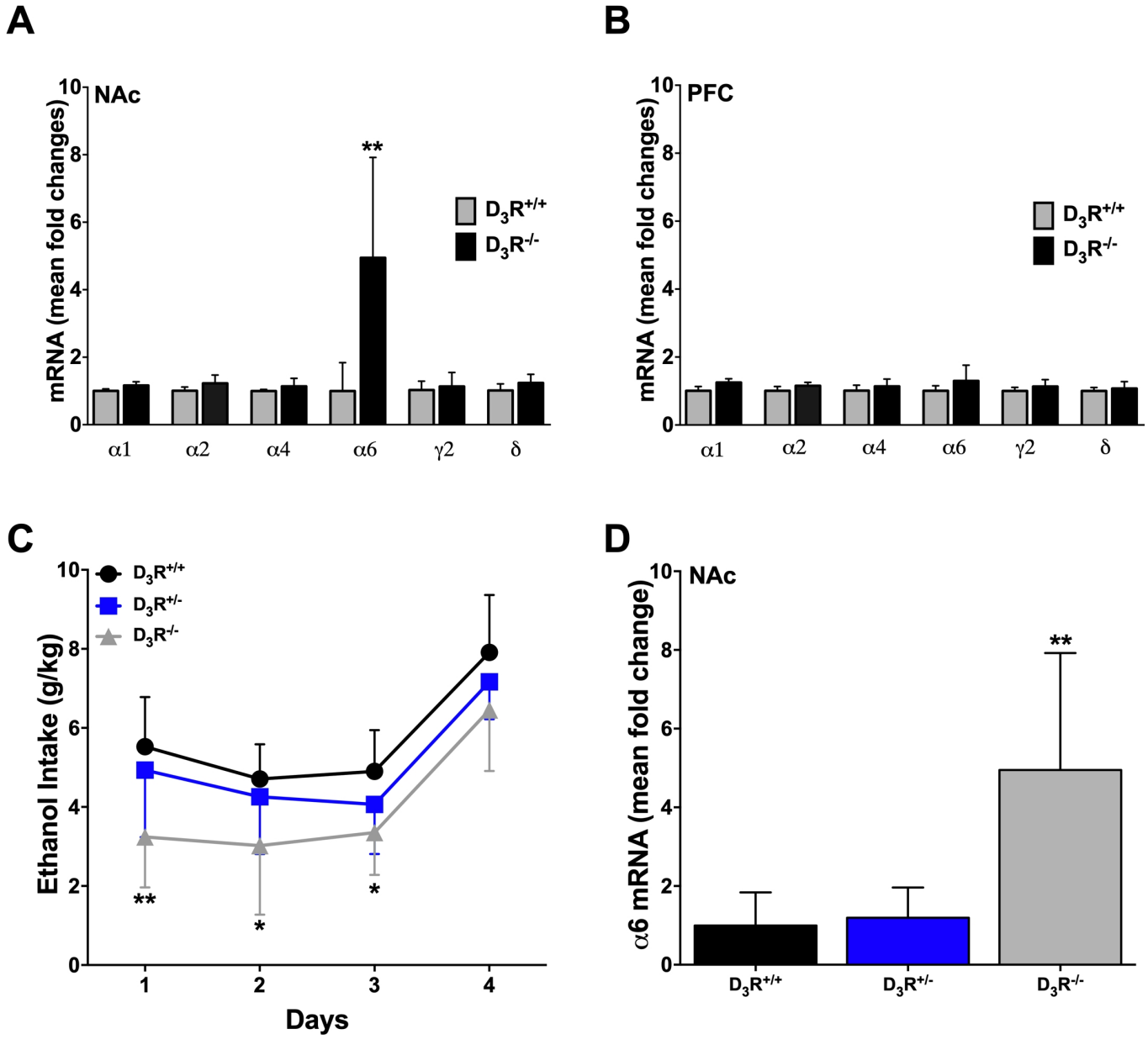
Figure 3. Opposite effect of RO 15-4513 on alcohol intake, in $D_3R^{+/+}$ and $D_3R^{-/-}$ (drink in the dark paradigm, DID). A and B**,** ethanol intake in $D_3R^{+/+}$ and $D_3R^{-/-}$ intraperitoneally (i.p.) treated with vehicle (VEH) or Ro 15-4513 (5 mg/kg); C, ethanol intake in $D_3R^{-/-}$ locally 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 277011A 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.

Figure 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.

*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).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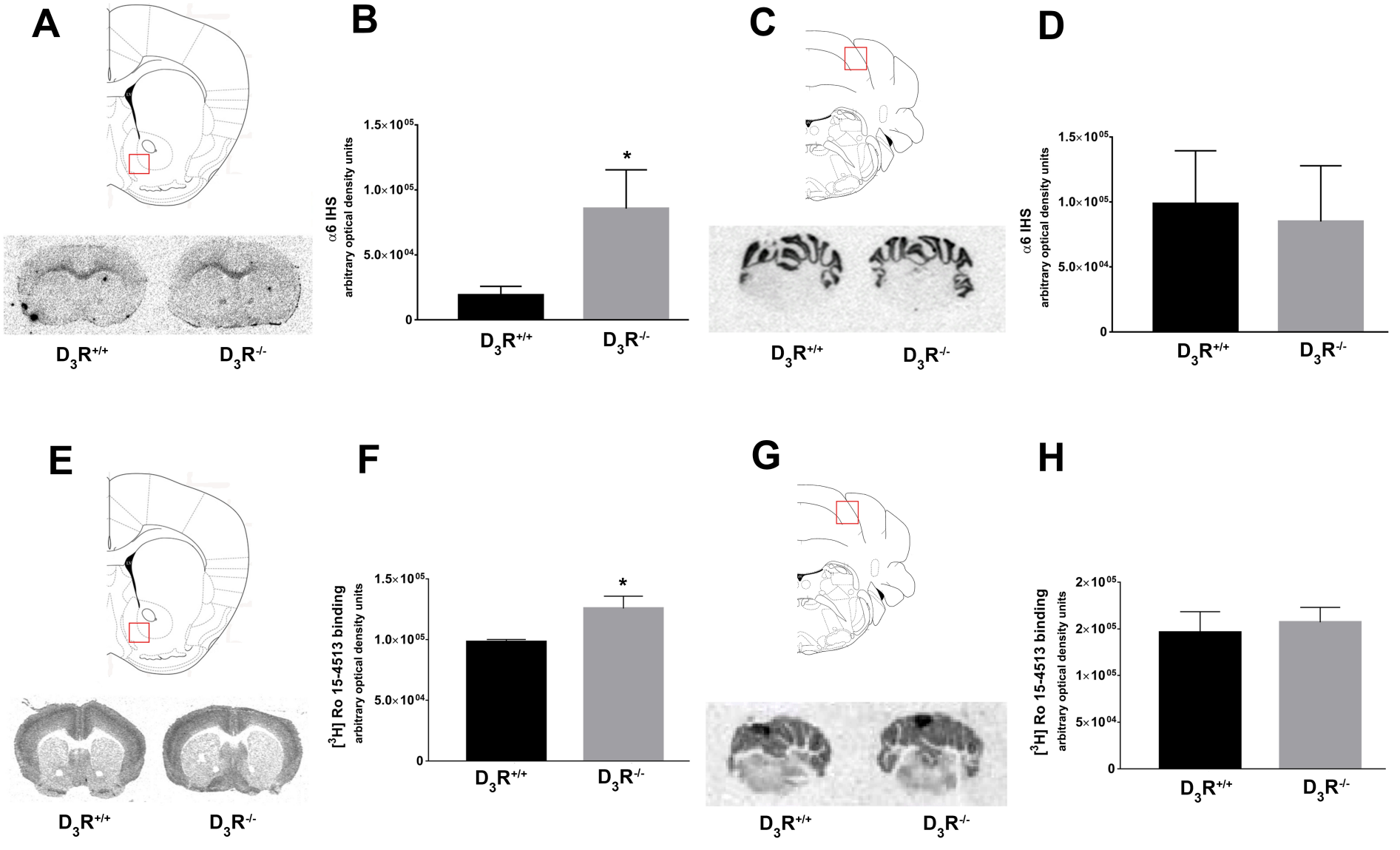
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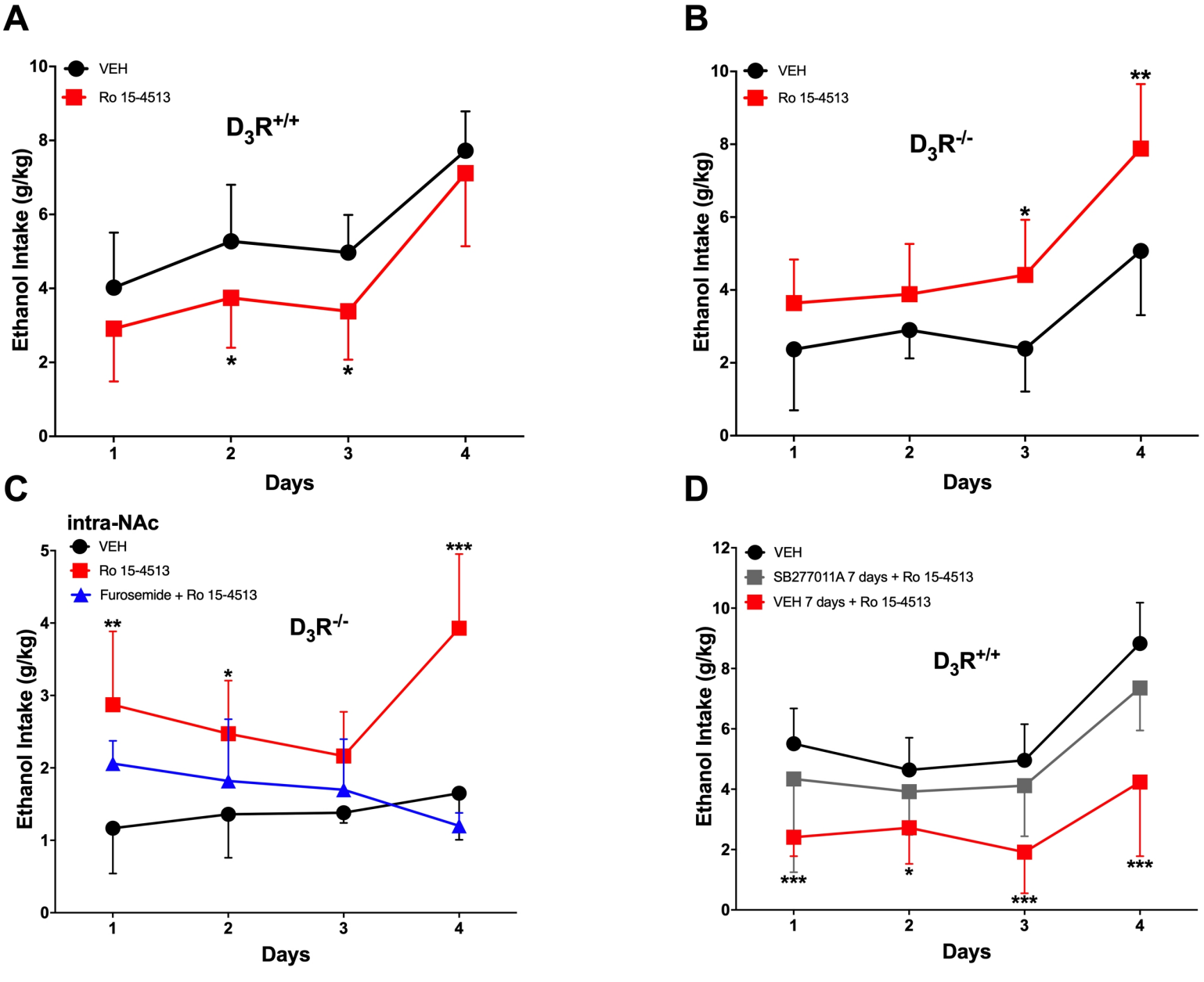
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C
intra-NAc
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Ro 15-4513
Furosemide + Ro 15-4513
D3R-/-
Ethanol Intake (g/kg)
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D3R+/+
Ethanol Intake (g/kg)
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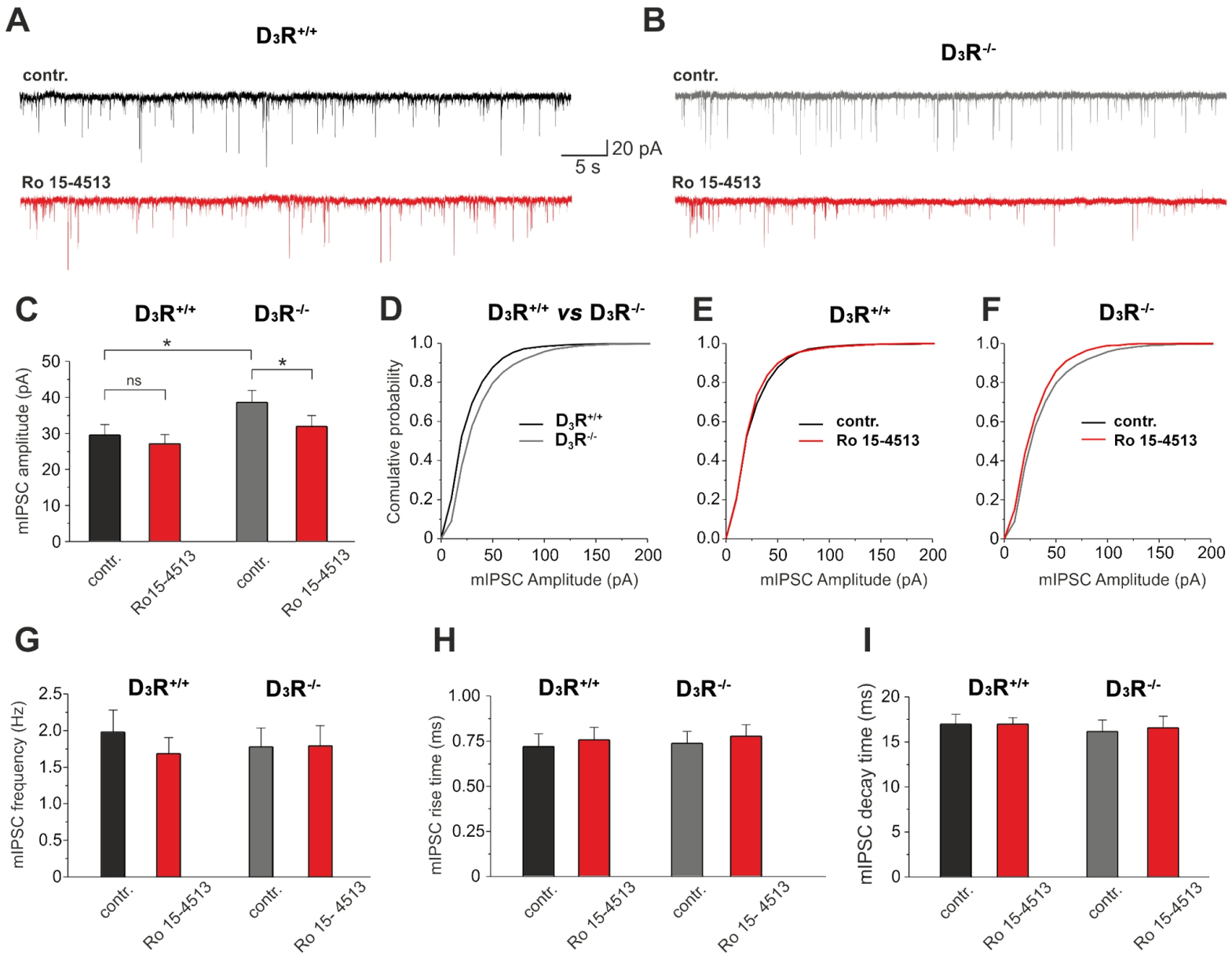
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mIPSC Amplitude (pA)
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D3R+/+
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mIPSC frequency (Hz)
contr.
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D3R-/-
mIPSC rise time (ms)
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D3R-/-
mIPSC decay time (ms)
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Biometec
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Università di Catania

Sezione di Farmacologia - Prof. Salvatore Salomone

To Prof. Emilio Clementi, MD, PhD
Editor in Chief
Pharmacological Research

This statement applies to the manuscript "**Dopaminergic-gabaergic interplay controls alcohol binge drinking",** by Leggio et al., submitted for publication to Pharmacological Research.

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Leggio et al.

Dopaminergic-GABAergic interplay and alcohol binge drinking

Supplementary Information

Table S1. *In situ* hybridization (ISH) signals for $GABA_A$??1, ??2, ??4, ??6, **γ**2 and δ subunit mRNA in the prefrontal cortex from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice.

$GABA_A$ subunit ISH signal ($D_3R^{-/-}$ over $D_3R^{+/+}$ ratio)	
α1	0.92 ± 0.02
α2	1.10 ± 0.11
α4	0.80 ± 0.07
α6	1.15 ± 0.13
γ2	0.89 ± 0.30
δ	0.83 ± 0.17

Table S2. *In situ* hybridization (ISH) signals for $GABA_A$??1, ??2, ??4, ??6, **γ**2 and δ subunit mRNA in the hippocampus from $D_3R^{+/+}$ and $D_3R^{-/-}$ mice.

$GABA_A$ subunit ISH signal ($D_3R^{-/-}$ over $D_3R^{+/+}$ ratio)	
α1	0.91 ± 0.09
α2	1.19 ± 0.13
α4	1.07 ± 0.03
α6	1.20 ± 0.10
γ2	0.96 ± 0.26
δ	0.85 ± 0.16

Table S3. Primers for Real-Time PCR

Target gene	**Primer sequence**
Gabra1	5’-GACCAGGTTTGGGAGAGCGTGT-3’
	3’-GCCGGAGCACTGTCATGGGTC-5’
Gabra2	5’-CCCAGTCAGGTTGGTGCTGGC-3’
	3’-ACAGGGCCAAAACTGGTCACGT-5’
Gabra4	5’-CCTGTGCCTGGCGGCTTGTTTA-3’
	3’-CCCCAAATCCAGGACGCAGCC-5’
Gabra6	5’-GGCCAGGATTTGGGGGTGCTG-3’
	3’-TCAGTCCAAGTCTGGCGGAAGA-5’
Gabrg2	5’-ACCCAGAGGCGAGAGGCGAG-3’
	3’-GCTTGTGAAGCCTGGGTAGAGCG-5’
Gabrd	5’-CCGACCAGGCATTGGAGGTGC-3’
	3’-TGCTGTCCCGCCAGCTCTGA-5’
Gapdh	5’-CAACTCACTCAAGATTGTCAGCAA-3’
	3’-GGCATGGACTGTGGTCATGA-5’

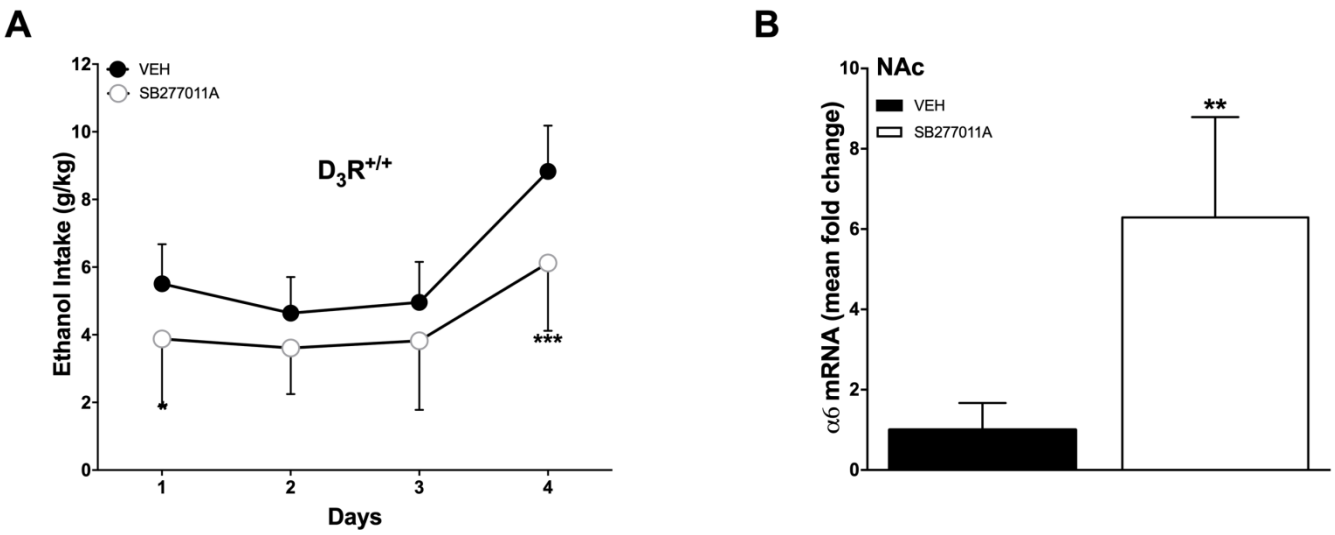

Figure S1. Pharmacological blockade of D3R counteracts alcohol intake and induced overexpression of $GABA_A$ α6 subunit in the NAc of $D_3R^{+/+}$. A and B, ethanol intake (DID) and α6 expression in $D_3R^{+/+}$ treated with vehicle (VEH) or the selective D_3R antagonist, SB 277011A (10 mg/kg, i.p.) for 7 days. Each experimental group included 8-13 mice. *P<0.05, ***P<0.001 *vs.* VEH; two-way ANOVA and Newman–Keuls post hoc test.

Material and Methods

In situ hybridization

Air-dried slides were fixed in ice-cold 4% paraformaldehyde for 5 min. The sections were washed in 1 PBS at room temperature for 5 min, dehydrated in 70% ethanol for 5 min and stored in 95% ethanol at 4 °C until used. The antisense DNA oligonucleotide probe (Oligomer Oy, Helsinki, Finland) sequences were as follows: α6, 5'-CAG TCT CTC ATC AGT CCA AGT

CAT-3'; was complementary to the mouse $GABA_AR$ subunit mRNA sequence. Poly[^{35}S]dATP (PerkinElmer Life and Analytical Sciences, Boston, MA) tails were added to the 3'-ends of the probes by deoxynucleotidyl transferase (Promega Corporation, Madison, WI). Unincorporated nucleotides were removed by Illustra ProbeQuant G-50 Micro Columns (Amersham Biosciences, Buckinghamshire, UK). Labelling efficiency (360,000 cpm/µl) was determined by a scintillation counter. The labeled probe was diluted to 0.06 fmol/µl of hybridization buffer consisting of 50% formamide and 10% dextran sulfate in 4X Saline Sodium Citrate (SSC). Nonspecific controls for the antisense probes were produced by adding 100-fold excess of unlabeled probes. The hybridization occurred under glass Menzel-Gläser coverslips (Thermo Fisher Scientific, Boston, MA) overnight at 42 °C. Finally, the slides were washed in 1X SSC at room temperature for 10 min, in 1X SSC at 55°C for 30 min, and 1X SSC, 0.1X SSC, 70% EtOH and 95% EtOH at room temperature for 1 min each. The slides were then air-dried and exposed with plastic [^{14}C]-radioactivity standards (GE Healthcare) to BioMax MR films (Eastman Kodak Company, Rochester, NY). Films were scanned (Epson expression 1680 Pro). Images were imported into the *FIJI* version of the free image processing software *ImageJ*. The [^{14}C]-standards were exposed simultaneously with the brain sections as the reference. The hybridization values were converted to arbitrary optical density units. Non-specific signal was subtracted to obtain the specific signal. All measurements were analyzed in blind.

[^{3}H]Ro 15-4513 autoradiography

Slides were pre-incubated in ice-cold 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl for 15 min. The final incubation for basal [^{3}H]Ro 15-4513 binding was performed in the

pre-incubation buffer containing 15 nM [^{3}H]Ro 15-4513 (23 Ci/mmol, PerkinElmer Life and Analytical Sciences) at 4 °C for 1 h. This high ligand concentration was aimed at estimating the receptor number rather than affinity. The non-specific binding was determined in the presence of 10 µM flumazenil. The sections were then washed in ice-cold pre-incubation buffer twice for 1 min, dipped in ice-cold distilled water, air-dried at room temperature and exposed with [^{3}H]-plastic standards for 12 weeks (GE Healthcare) to Biomax MR films (Eastman Kodak). The films were scanned (Epson expression 1680 Pro) and binding density was expressed as arbitrary optical density units (*FIJI IMAGE-J*). The [^{3}H]-standards were exposed simultaneously with the sections as the reference. Non-specific binding was subtracted to obtain the specific binding values. All data were analyzed in blind.

Electrophysiology

Animals were sacrificed by cervical dislocation. Brains were rapidly removed and placed in ice-cold cutting solution containing (in mM): TRIS-HCl 72, TRIZMA base 18, NaH_2PO_4 1.2, $NaHCO_3$ 30, KCl 2.5, glucose 25, HEPES 20, $MgSO_4$ 10, Na-pyruvate 3, ascorbic acid 5, $CaCl_2$ 0.5, sucrose 20. Slices (300 µm thick) were cut on a vibratome (VT1200S; Leica Microsystems, Germany) and immediately transferred to an incubation chamber held at 32°C and filled with a recovery solution containing (in mM): TRIS-HCl 72, TRIZMA base 18, NaH_2PO_4 1.2, $NaHCO_3$ 25, KCl 2.5, glucose 25, HEPES 20, $MgSO_4$ 10, Na-pyruvate 3, ascorbic acid 5, $CaCl_2$ 0.5, sucrose 20. After 30 min, slices were transferred to a second incubation chamber held at 32°C and filled with artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl 124, KCl 3.2, NaH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10, pH 7.4. During incubations, the chambers were continuously bubbled with 95% O_2/5% CO_2.

Slices were equilibrated at room temperature for at least 45 min. Slices were then transferred to a submerged recording chamber constantly perfused with heated aCSF (32°C) and bubbled with 95% O_2/5% CO_2. Medium spiny neurons (MSNs) within the NAc shell subregion were identified with a 40X water-immersion objective on an upright microscope equipped with differential interface contrast optics under infrared illumination (BX5IWI, Olympus, Center Valley, PA) and video observation. Electrodes were made from borosilicate glass micropipettes (Warner Instruments, Hamden, CT) prepared with a P-97 Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA). Patch pipettes had a resistance of 4-6 MΩ when filled with an internal solution containing (in mM): CsCl 135, HEPES 10, EGTA 1.1, $CaCl_2$ 0.1; Mg-ATP 2.5, Na-GTP 0.25, phosphocreatine 5, pH 7.2. After establishing a gigaseal, the patch was broken by applying negative pressure to achieve a whole-cell configuration. A series resistance lower than 15 MΩ was considered acceptable, and monitored constantly throughout the entire recording. Neurons were held at -70 mV. Tetrodotoxin (TTX, 0.5 µM, Tocris), D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5, 50 µM, Tocris) and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX, 10 µM, Tocris) were applied to the bath to block action potential-mediated neurotransmitter release, NMDA and AMPA receptors, respectively. Ro 15-4513 (0.3 µM) was applied in the bath after 5-7 min of TTX, APV and NBQX perfusion. All recordings were carried out at least 10 min after application of any drug to the bath. Recordings were performed using a Multiclamp 700B/Digidata 1550A system (Molecular Devices, Sunnyvale, CA) and digitized at a 10,000 Hz sampling frequency.