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ARTICLE

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Overexpression of wild type glycine alpha 1 subunit rescues ethanol sensitivity in accumbal receptors and reduces binge drinking in mice

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The nucleus accumbens (nAc) is a critical region in the brain reward system since it integrates abundant synaptic inputs contributing to the control of neuronal excitability in the circuit. The presence of inhibitory α1 glycine receptor (GlyRs) subunits, sensitive to ethanol, has been recently reported in accumbal neurons suggesting that they are protective against excessive binge consumption. In the present study, we used viral vectors (AAV) to overexpress mutant and WT α1 subunits in accumbal neurons in D1 Cre and α1 KI mice. Injection of a Cre-inducible AAV carrying an ethanol insensitive α1 subunit in D1 Cre neurons was unable to affect sensitivity to ethanol in GlyRs or affect ethanol drinking. On the other hand, using an AAV that transduced WT α1 GlyRs in GABAergic neurons in the nAc of high-ethanol consuming mice caused a reduction in ethanol intake as reflected by lowered drinking in the dark and reduced blood ethanol concentration. As expected, the AAV increased the glycine current density by 5-fold without changing the expression of $GABA_A$ receptors. Examination of the ethanol sensitivity in isolated accumbal neurons indicated that the GlyRs phenotype changed from an ethanol resistant to an ethanol sensitive type. These results support the conclusion that increased inhibition in the nAc can control excessive ethanol consumption and that selective targeting of GlyRs by pharmacotherapy might provide a mechanistic procedure to reduce ethanol binge.

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INTRODUCTION

The transition from recreational alcohol use to compulsive drinking or alcohol use disorders (AUD) involves an array of neurobiological adaptation at the pre-and post-synaptic regions of mesocorticolimbic circuits. These changes are particularly relevant in the metabotropic signaling associated with the neurotransmitter dopamine, excitatory ionotropic receptors, namely AMPA and NMDA, and inhibitory neurotransmissions, both $GABA_A$ and glycine receptors (GlyRs) [1–4].

The nucleus accumbens (nAc) is one of the most critical regions in the brain reward system since it receives abundant synaptic inputs that contribute to the control of its excitability. For example, it receives dopaminergic innervation from the ventral tegmental area (VTA) and strong excitatory glutamatergic inputs from the prefrontal cortex (PFC), amygdala, and hippocampus [5, 6]. On the other hand, the main inhibitory control in the nAc is provided by GABAergic and glycinergic inputs [7], two of the most abundant fast acting inhibitory neurotransmitters believed to have a role in AUD [8, 9]. In the nAc, the principal inhibitory neurons found are the medium spiny neurons (MSNs) that release GABA at their projecting terminals. These MSNs can be classified as D1-MSNs since they primarily express D1-type dopamine receptors and form part of the direct projection pathway of the nAc, and D2-MSNs that primarily express the D2-type dopamine receptor and contribute to the indirect pathway [5]. These pathways are believed to have distinctive roles, i.e., the direct pathway is mainly associated with reward and the indirect pathway with the processing of aversion [10, 11].

The reward circuit is involved with various gratifying stimuli, both natural and those produced by drugs of abuse that also activate this circuit [12]. Independent of the associated cellular and molecular mechanisms, drugs of abuse, including ethanol, cause an increase in the level of extracellular dopamine released from the VTA into the nAc [13], and this has been associated with wide addictive behaviors [14].

GlyRs are inhibitory Cl^- channels widely expressed in the spinal cord and brain stem, where they control pain processing, respiratory rhythms, and motor coordination [15]. More recently, several GlyRs subunits (α1-3 and β) have also been reported in upper brain regions [16, 17]. The glycine-activated current from α1 GlyRs can be potentiated by ethanol through a mechanism where ethanol increase free Gβγ that interacts with basic residues (lysine 385 and 386) in the intracellular loop responsible for producing the reversible enhancement of Cl^- conductance [18]. In vivo studies using microdialysis reported that activation of GlyRs in the nAc, either by application of the agonist or a glycine transporter 1 (GlyT1) inhibitor (Org24598), increased dopamine release to nAc and lowered ethanol consumption. On the other hand, the

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administration of strychnine (STN), a highly selective GlyRs antagonist, led to a decrease in the dopamine level and increased ethanol consumption [19–21]. These results suggest that activation of GlyRs affects dopamine release, modulates reward, and perhaps ethanol intake. Supporting this notion, we recently reported that mice with a Knock-In (KI) mutation in the large intracellular loop of α1 (KK385-386AA α1 GlyR), that converts the GlyR to an ethanol insensitive receptor, showed an enhanced drinking pattern compared with WT littermates. In addition, an examination of ethanol sensitivity in nAc neurons showed a reduced ethanol-induced potentiation in the KI mice. Therefore, in the present study using a viral vector, we overexpressed the WT α1 subunit in the nAc as an attempt to rescue the neurophysiological and drinking phenotypes of these KI mice. The results show that when the α1 WT subunit is overexpressed in KI MSNs, it can recover ethanol sensitivity in the glycine-induced current and reduce ethanol intake to WT levels.

METHODS

Animals

Animal care and experimental protocols for this study were approved by the Institutional Animal Care and Use Committee at the University of Concepción. They followed the guidelines for ethical protocols and care of experimental animals established by NIH (National Institutes of Health, Maryland, USA). Male and female D1-CRE mice (Tg(Drd1-Cre)EY217Gsat, RRID: MMRRC_030778-UCD), commercially available from the Mutant Mouse Resource & Research Center, and GlyRα1-point-mutated (α1 KI) mice [22, 23] between 45 and 60 postnatal days old were used for the electrophysiological experiments. No differences were found between males and females, therefore, the data was combined. For behavioral experiments, only male mice were included, as previously reported [23]. These genetically modified mice, having a mutation in the α1 GlyRs, drank more than WT mice at three months [23]. Animals were individually housed in groups of 2–4 mice on a 12 h light/dark cycle and given food and water ad libitum. When possible, tissues from each animal were used for multiple experiments. All mice were genotyped at weaning using PCR with their respective primers.

Plasmid generation

Chemically competent XL1 Blue cells were transformed with the plasmid pAAV- mDlx-GFP (Green Fluorescent Protein) purchased from Addgene (#83900). The insert sequence was designed to achieve the correct subcloning of the GlyRα1 WT sequence flanked by the restriction sites for BglII and BstXI restriction enzymes. Both plasmids were digested and loaded on a 0.8% agarose gel, and after electrophoresis, the fragments of interest were purified and ligated with a T4 enzyme (Merck) at 16 °C for 16 h. All the positive tested candidates were digested to verify the correct insertion, and ultimately one was chosen to purify and assess its properties in PC-12 cells through electrophysiological techniques. Since the DIO (Double-Floxed Inverted Open reading frame) AAVs are Cre-inducible AAVs, those were used to exclusively express the virus in D1-Cre MSNs. For the AAV-DIO-GlyRα1KI-mCitrine plasmid, we performed site-directed mutagenesis to change the basic lysine residues 385–386 to alanines from the original plasmid AAV-DIO-GlyRα1WT-mCitrine, which has 7495 pair bases and a penicillin resistance site. The expression cassette has a synapsin promoter followed by a loxP site for the recognition of the Cre recombinase, the sequence for the reporter protein mCitrine, a sequence for internal ribosome entry site (IRES), followed by the sequence of the glycine receptor α1 subunit, (1349 pb, ref seq NM_000171.3) and finally an inverted loxP site. The AAVs packaging was done in the laboratory of Dr. Mary Kay Lobo. The viral title was 2.6×10^{13} viral particles/mL for AAV-mDlx-GFP, 1.7×10^{13} viral particles/mL for AAV-GlyRα1WT-GFP, 1.0×10^{13} viral particles/mL for AAV-DIO-GlyRα1KI-mCitrine and 1.5×10^{13} viral particles/mL for AAV-DIO-mCitrine.

Drinking in the dark (DID) protocol

This limited access drinking test produces significant levels of ethanol in the blood [24]. Mice were transferred to individual cages and allowed to acclimate for at least 1 week. Two hours after the lights were turned off, water bottles were replaced with bottles containing 15% v/v of ethanol solution for either 2 h during the first 3 days or 4 h the fourth day. The ethanol bottles were weighed before placement and after removal from the cages every day. The amount of ethanol consumed was calculated as g/kg body weight per 2 or 4 h accordingly.

Blood ethanol concentration (BEC)

Blood samples from the facial vein of the mice were collected after 10 min on the fourth day of drinking in the dark. Blood samples were centrifuged (10,000 rpm x 10 min) and ethanol concentration was determined in the serum using an Analox AM1 Analyzer (Lunenburg, Massachusetts).

Immunohistochemistry (IHC)

Brain slices were obtained as described below. Coronal slices (100 µm) containing the nAc were fixed for 50 min with 4% PFA (Bioworld, USA). After 3 washes with 1X PBS, the brain slices were blocked and permeabilized with normal horse serum (10%) and 0.3% Triton x-100 (Sigma) for 40 min with stirring. Slices were incubated with constant rocking (overnight) and a combination of primary antibodies: Pan α GlyR (1:200, guinea pig; Cat No 146 308; Synaptic Systems, Germany), MAP-2 (1:200, mouse; Cat No. 188 011, Synaptic System, Germany), Cre (1:200 rabbit; Cat No 257 003; Synaptic Systems, Germany Germany), and GFP (1:200 chicken; Cat No 132 006; Synaptic Systems, Germany). After the brain slices were washed with 1X PBS, they were incubated with a secondary antibody (anti-guinea pig Alexa Fluor 647 Cat. No. 706-605-148, anti-chicken Alexa Fluor 488 Cat. No. 703-545-155, Cy3 anti-rabbit Cat. No. 711-166-152 or Cy3 anti-mouse Cat No. 715-165-150, Jackson ImmunoResearch, USA) diluted 1:200 for 2 h with constant rocking. After 5 washes with 1X PBS, the preparations were mounted with Dako (DakoCytomation, USA) mounting solution. Confocal images of a single optical section were acquired with a 40X /1.3 n.a objective in a LSM700 laser scanning microscope and ZEN software suit (Zeiss, Oberkochen, Germany) in the CMA core facility at the University of Concepcion. Accumbal neurons in coronal slices were chosen randomly from view-fields presenting multiple cells exhibiting different levels of fluorescence. A 3D rendered image was generated from a z-stack of 16 optical sections (7.5 µm total optical thickness). Triple color immunofluorescent images were captured, processed, deconvoluted, rendered, stored, and analyzed using the ZEN (Zeiss) ImageJ program (NIH).

Western blot

Tissue homogenates from nAc after lysis treatment (10 mM Tris-HCl pH 7.4, 0.25 M sucrose, 10 mM NEM, protease inhibitor cocktail 1×) were subjected to electrophoresis on 10% SDS-PAGE gels. Proteins were blotted onto a nitrocellulose membrane (Bio-Rad) and blocked with 5% skimmed milk in 1× TBS-0.1% Tween 20 for 1 h with stirring. Subsequently, the membranes were incubated with the following primary antibodies: anti-GlyR pan α (1:500; rabbit monoclonal IgG; Cat No 146008; Synaptic Systems, Germany), and anti-Gβ (1:600; rabbit polyclonal IgG; Cat No Sc-378; Santa Cruz Biotechnology) for 1–2 h. After washes with 1× TBS and 0.1% Tween 20, membranes were incubated for 1 h with anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (1:3000; goat polyclonal anti-rabbit IgG-HRP, Cat# sc-2004, Santa Cruz Biotechnology). The immunoreactivity of the proteins was detected using an ECL Plus Western Blotting Detection System (PerkinElmer, MA, USA). Levels of Gβ were used as loading controls. Western blot was quantified using the Image J (NIH) program. The data were expressed in relative units (RU) of the normalization of the signal between GlyR Pan α divided by Gβ signal (GlyR Pan α /Gβ (RU)).

Stereotaxic viral injection

Mice were placed in a stereotaxic frame under anesthesia and bilaterally infused with 200 nL on each hemisphere with a Cre-inducible viral vector AAV-DIO-GlyRα1KI-mCitrine or AAV-DIO-mCitrine used as an empty control for the D1-CRE mice, and the AAV-mDlx-GlyRα1WT-GFP or AAV-mDlx-GFP (Empty) adenovirus for the KIα1 mice. After the viral intracerebral injections, the needle was kept for 2 min following the infusion to promote the diffusion and prevent backflow. Stereotaxic coordinates for the injections for nAc were (AP: + 1.3 mm, ML: ± 1.1 mm and DV: −4 mm from Bregma). The experiments were performed 2 weeks post-surgery, and positive viral expression in the nAc, shell and core, was confirmed by fluorescence inspection and electrophysiology. Only animals that were positively confirmed to be injected in the nAc were used in this study.

Open field assay

Mice were tested for basal locomotor activity in a modified open field assay after the stereotaxic injection to demonstrate that there were no alterations in motor activity of these mice. Cre D1 and α1KI mice were placed into the 50 × 50 cm test area and were allowed to freely explore the chamber for 20 min. The distance traveled every 5 min was recorded and analyzed using a video tracking system (ANY-maze, Stoelting Co.).

Preparation of brain slices

Cre D1 and α1KI mice (PND 21-40) were decapitated as previously described [25]. The brain was quickly excised, placed in cutting solution containing (in mM): sucrose 194, NaCl 30, KCl 4.5, $MgCl_2$ 1, $NaHCO_3$ 26, NaH_2PO_4 1.2, Glucose 10 (pH 7.4) saturated with 95% O_2 and 5% CO_2, glued to the chilled stage of a vibratome (Leica VT1200S, Germany), and sliced to a thickness of 300 µm. Slices were transferred to the aCSF solution containing (in mM): NaCl 124, KCl 4.5, $MgCl_2$ 1, $NaHCO_3$ 26, NaH_2PO_4 1.2, Glucose 10, $CaCl_2$ 2 (pH 7.4 and 310-320 mOsm) saturated with O_2 at 30 °C for 1 hr at 32 °C before the enzymatic treatment for dissociation.

Enzymatic dissociation of accumbal neurons

For enzymatic dissociation, brain slices that contained the nAc were incubated for 30 min in normal aCSF (saturated with 95% O_2/5% CO_2) in the presence of 0.5 mg/mL pronase (Calbiochem /EDM Bioscience, Darmstadt, Germany) at 37 °C. The nAc was dissected from the slices and the tissue was triturated through a series of pipette tips of decreasing diameter size in a 35 mm-culture dish in trituration buffer (in mM: NaCl 20, N-methyl-D-glucamine (NMG) 130, KCl 2.5, $MgCl_2$ 1, Hepes 10, glucose 10, adjusted to pH 7.4 and 340 mOsm). After 20 min, isolated neurons were attached to the bottom of the culture dish (Nunc ThermoFisher Scientific) and were ready for electrophysiological experiments.

Electrophysiology

Whole-cell current recordings of GFP labeled accumbal neurons were performed using the voltage-clamp technique. Patch pipettes were prepared from filament-containing borosilicate micropipettes (World Precision Instruments) using a P-1000 micropipette puller (Sutter Instruments, Novato, CA) having a 6–8 MΩ resistance used for whole-cell recording. We used an internal solution containing (in mM): 120 KCl, 4.0 $MgCl_2$, 10 BAPTA, 0.5 Na_2-GTP and 2.0 Na_2-ATP (pH 7.4, 290-310 mOsmol) and an external solution containing (in mM) 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4, 315–320 mOsm). Neurons were perfused with increasing concentrations of glycine (1–1000 µM) to obtain a concentration-response curve. For ethanol potentiation, the $EC_{10\text{-}20}$ of glycine was used to evoke the current in the presence or absence of 10, 50, and 100 mM ethanol. Recordings were done using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) at a holding potential of −60 mV. Currents were displayed and stored on a personal computer using a 1322 A Digidata (Axon Instruments, Union City, CA) and analyzed with Clampfit 10.1 (Axon Instruments, Union City, CA).

Reagents

Ethanol was purchased from Merck Millipore (USA).

Sample size

The target number of samples in each group for biochemistry and electrophysiological experiments was determined based on numbers reported in published studies [22, 26, 27].

Replication

All sample sizes indicated in the figures for electrophysiological experiments represent biological replicates. The biochemistry experiments (western blot and immunocytochemistry) were repeated at least three times.

Data analyses

Statistical analyses were performed for studies where each group size was at least $n = 3$ using the two-tailed paired or unpaired Student's *t*-tests, and for non-normally distributed data, Mann-Whitney U-test was used. Data with more than two groups or factors were analyzed by one-way or two-way ANOVA test using Origin 8 (Microcal, Inc., Massachusetts, USA) or GraphPad Prism 6 Software. After ANOVA, Bonferroni *post hoc* test was run only if F achieved the necessary level of statistical significance ($p < 0.05$) and there was no significant variance in homogeneity. Data are shown as mean ± SEM for normally distributed populations and as median and interquartile ranges (IQR) for non-normally distributed populations. The group size in this study represents independent values, and the statistical analysis was done using these independent values. As in previous studies [22, 23], in order to obtain statistical power above 95% (α = 0.05, power = 0.95) to determine the existence of statistically significant differences ($p < 0.05$), we used a sample size of 6–8 measurements for experimental group. n.s. denotes no significant difference, and asterisks denote significant difference when *p* value is lower than 0.05 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [28]. The outliers were excluded from the statistical analysis. To identify outliers, we performed the ROUT method and the Q was set to 1%.

RESULTS

Overexpression of GlyRα1KI in D1 MSNs of the nAc

GABAergic projecting MSNs predominate in the nAc and they can be distinguished as D1 and D2 MSNs [11]. These neurons represent more than 90% of the existing neurons in the nAc [29]. In a previous study [27], it was reported that ethanol potentiated GlyRs in D1 and D2 MSNs. Also, electrophysiological recordings in the nAc of KI α1 mice showed a marked attenuation in the potentiation of GlyRs in most MSNs tested, together with an increase in ethanol intake [23]. However, because this mouse model is a global mutant, further neuronal dissection was necessary. Therefore, we examined if the selective expression of α1 KI GlyRs in D1 MSNs would cause the same effect on drinking as reported in the KI mice. For this, we generated an AAV to transduce the KI α1 subunit in the nAc of Cre-D1 mice. The construct was first examined in PC-12 cells. Figure S1A is a schematic showing the co-transfection in PC-12 cells with the pCMV-Cre plasmid along with pmCitrine as a control vector (in red), pGlyRα1WT-mCitrine for the wild type GlyRα1 (green), and pGlyRα1KI-mCitrine for the Knock-In GlyRα1 (blue). The data in Fig. S2A show membrane current responses obtained from PC-12 cells co-transfected with a plasmid that expresses Cre with either the control (mCitrine), the α1 WT, or the α1 KI subunit. Analysis of the current density (pA/pF) showed that, as expected, the control mCitrine vector did not elicit any current response when we applied a concentration of 1000 µM glycine. The same concentration produced large Cl^- current responses in cells transfected with the WT and KI subunits (Fig. S2B). For example, rapid application of glycine produced a current density of 80 ± 17 pA/pF in WT and 45 ± 19 pA/pF in KI GlyRs, demonstrating that the constructs are functional (Fig. S2B, One-Way ANOVA, F (2,13) = 16.90, $p = 0.0002$; Bonferroni *post hoc* test: ***$p < 0.001$). Next, to evaluate the sensitivity of WT and KI α1 subunits to ethanol, GlyRs expressed in PC-12 cells were stimulated with ethanol, a positive allosteric modulator of the receptor [15]. The data support a functional receptor and show that only the WT α1, but not the KI α1, was potentiated by increasing ethanol concentrations (10–100 mM) (Fig. S2C, D, Two-Way ANOVA, F (1,28)=19.84, $p = 0.0001$; Bonferroni *post hoc* test: *$p < 0.05$, **$p < 0.01$). For instance, with 50 mM ethanol, the glycine-activated current was potentiated by 34 ± 6% of control ($n = 5$) in WT, but it was not affected in KI (8 ± 5, $n = 8$, $p < 0.05$). These data show that the receptors are expressed with the expected properties, including sensitivity to ethanol in WT, and resistance to the allosteric modulator in KI.

After generating the AAV with the functional α1 KI GlyR-mCitrine cDNA, we proceeded to bilaterally inject the nAc in D1 Cre mice to test the drinking behavior of the mice. The control condition was the injection of the AAV for mCitrine alone. Two weeks post-injection, the viral construct was significantly expressed along the shell and core of the nAc (Fig. 1B). The overexpression of KI GlyRs was also confirmed by western blot analysis (Fig. 1C, unpaired Student *t*-test $t_{(4)} = 4.256$, $p = 0.0131$, *$p < 0.05$). This glycine receptor overexpression allowed to record large glycine activated Cl^- currents in dissociated neurons after

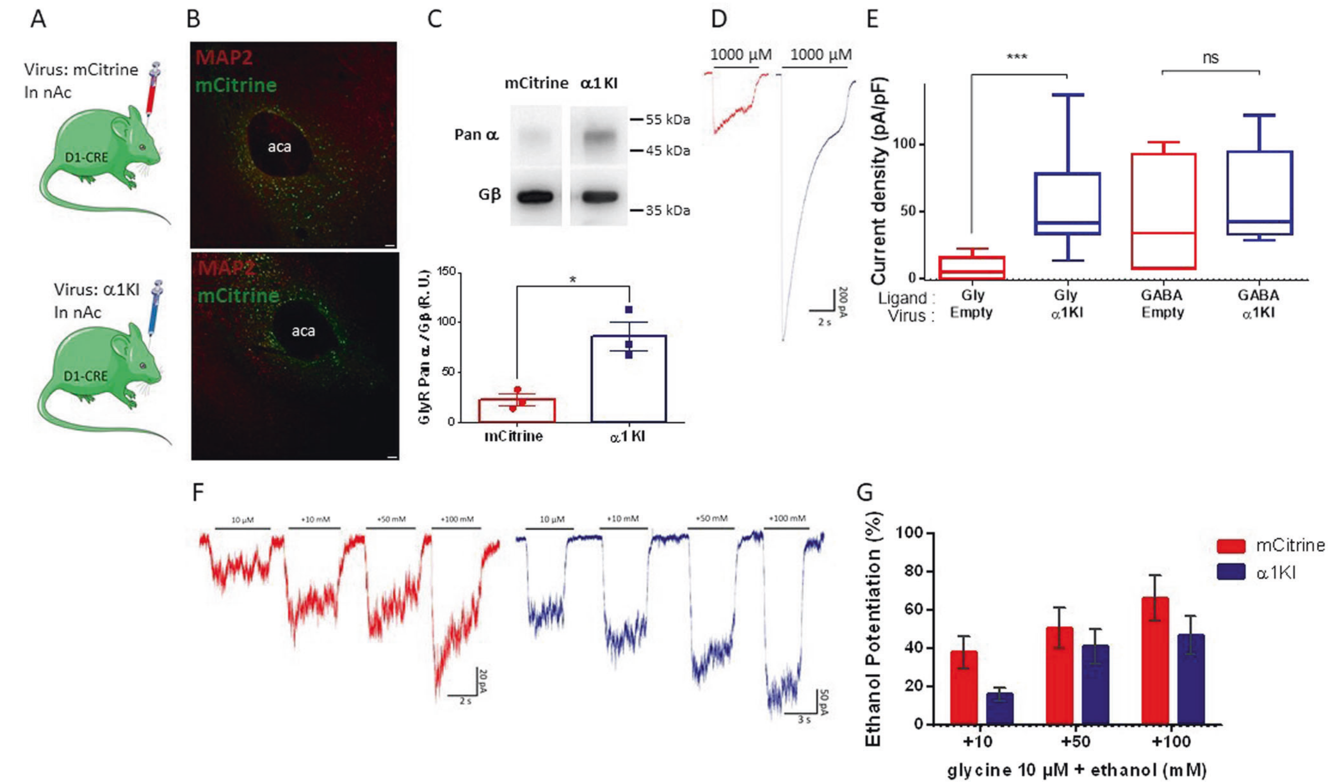

Fig. 1 Effects of overexpressing GlyRα1KI in D1-Cre mice in accumbal neurons. **A** Schematic representation of D1 mice models stereotaxically injected in the nAc with the Cre-inducible adenoassociated virus AAV-DIO-mCitrine (red, Empty) and AAV-DIO-GlyRα1KI-mCitrine (blue). **B** Immunohistochemistry of coronal brain slices showing mCitrine positive D1 MSNs in the nAc (10x) 2 weeks after injection, neurons were labeled with MAP2 in red and mCitrine in green. aca stands for anterior commissure, and the calibration bar is 50 μm. **C** Representative image of the Western blot using the antibodies pan α glycine receptor (upper panel) and Gβ (lower panel) as loading control in both conditions. Western blot quantification normalizing the pan α signal against the Gβ loading control in relative units (R.U.). **D** Representative current traces recorded in dissociated neurons infected with the Empty (red) and α1 KI (blue) containing AAVs. The neurons were identified by their green fluorescence. **E** The graph shows the values of the current density (pA/pF) for glycine- and GABA-activated currents that were recorded in dissociated D1 neurons. Note that the current density associated with $GABA_AR$ was not affected by overexpression of GlyRs. **F** Representative traces of ethanol potentiation with 10 μM glycine and 10 mM, 50 mM and 100 mM ethanol concentration in dissociated neurons from the injected mice with α1KI or the empty AAV. **G** The graph shows the ethanol potentiation of glycine receptor mediated currents. Unpaired Student's *t*-test. Data represent mean ± SEM. $n = 3$ for each Western blot condition. $^*p < 0.05$ for (**C**). Unpaired Mann Whitney U test for (**E**). Boxes indicate interquartile range (IQR); center lines, median; whiskers, 1.5×IQR (interquartile range). $n = 6$ (empty) and $n = 10$ (α1KI) for glycine current density; $n = 6$ for GABA current density with each AAV injection. Two-Way ANOVA for (**G**). Data represent mean ± SEM. $n = 10$ and $n = 8$ for each ethanol concentration in neurons from mice injected with mCitrine and α1KI AAV, respectively. $^{***}p < 0.001$.

injection of the α1- mCitrine (Fig. 1D, blue trace). These results also showed that the increase in current density was selective for GlyRs and the overexpression of KI α1 did not affect the current activated by GABA in the same MSNs (Fig. 1E, unpaired Mann–Whitney U = 1, $p = 0.0005$ for glycine; unpaired Mann Whitney U = 14, $p = 0.5714$ for GABA, $^{***}p < 0.001$). More significantly, recordings of GlyRs-activated current in α1-mCitrine positive neurons showed that the Cl^- current was still potentiated by ethanol, indicating the presence of WT GlyRs in D1 Cre neurons (red currents). On the other hand, neurons transduced with α1 KI GlyRs also showed potentiation of the glycine-activated currents by ethanol (Fig. 1F, G, Two-Way ANOVA, F (1,48)=4.596, $p = 0.0371$; Bonferroni *post hoc* test: n.s. for each concentration, blue traces and bars). Thus, transduction of GlyRα1KI was unable to overcome the WT phenotype normally expressed in these D1 MSNs.

Ethanol drinking in mice overexpressing GlyRα1KI in D1 MSNs of the nAc

The behavioral data examining drinking in the dark (DID) showed that both mice, injected with either the AAV containing α1 KI (blue) or control (mCitrine, red) (Fig. 2A), displayed similar drinking behaviors (Fig. 2B, Two-Way RM ANOVA, F (1,16) = 2.843, $p = 0.1112$; Bonferroni *post hoc* test: n.s. for both experimental groups). This result was confirmed by measuring the ethanol concentration in the blood on day 4 of the experiment which showed similar levels in both groups of animals (Fig. 2C, unpaired Student *t*-test $t_{(16)} = 1.570$, $p = 0.1359$). We also evaluated the locomotor activity of all mice injected stereotaxically. For this, we performed an open field assay to rule out an alteration in motor control after the surgical procedure. The traveled distance every 5 min was graphed in Fig. S4A. The mean total distance traveled for the KI mice was 6.691 ± 333 cm for the mCitrine control group and 7479 ± 676 cm for the α1 KI group (Fig. S4C). No differences were found among the analyzed groups in terms of the distance traveled in this assay. (Fig. S4A One-Way RM ANOVA, F(1,14) =1.258, $p = 0.2010$ Bonferroni *post hoc* test: n.s; Fig. S4C unpaired Student's *t*-test $t_{(14)} = 1.121$, $p = 0.2810$, n.s.).

Global overexpression of GlyRα1WT in accumbal neurons of KIα1 mice

Having concluded that overexpression of the α1 KI subunit in D1 MSNs did not alter the phenotype of mice regarding ethanol drinking, we proceeded to use a broader and more aggressive

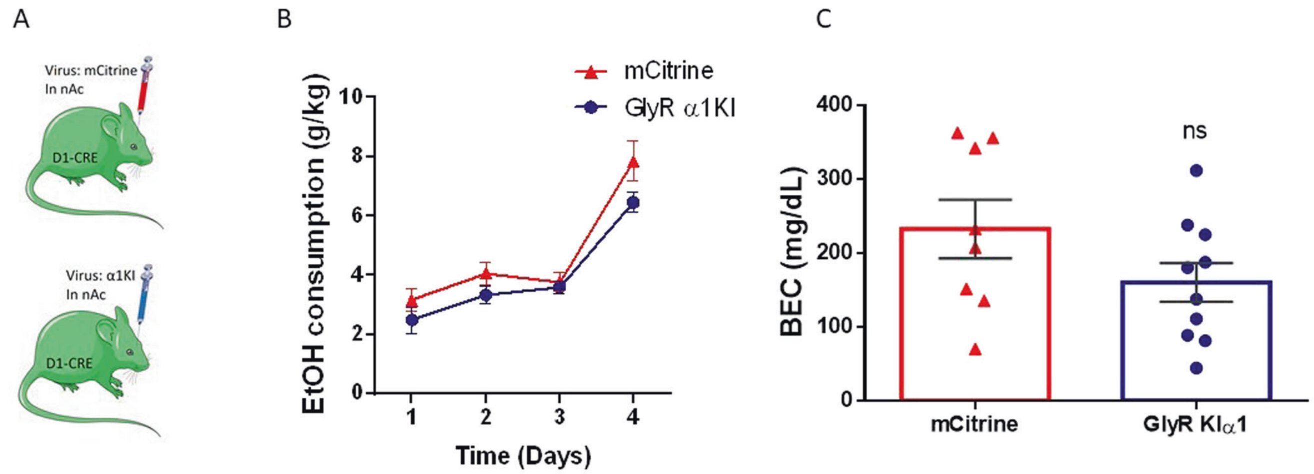

Fig. 2 Ethanol consumption behavior in D1-Cre mice that overexpressed the GlyRα1KI in the nAc. **A** The scheme depicts the two experimental models used: Cre-inducible control virus AAV-DIO-mCitrine (red) or AAV-DIO-GlyRα1KI-mCitrine (blue) stereotaxically injected in D1-Cre mice nAc. **B** Data represents ethanol intake during the drinking in the dark (DID) assay. The consumption was measured in grams of ethanol per kilogram of weight (g/kg). **C** Blood ethanol concentration (BEC) after ethanol consumption on the fourth day of the test. No statistical differences were found between these two animal models. Data represent mean ± SEM, Two-way ANOVA and Bonferroni *post hoc* test for (**B**) and unpaired Student's *t* test for (**C**). $n = 8$ (mCitrine) and $n = 10$ (α1KI) for the DID assay and BEC. ns not significant.

approach to overexpress α1 in nAc MSNs based on the fact that most of accumbal neurons have a GABAergic phenotype [30]. In addition, we also decided to use KI α1 mice, where all accumbal neurons express KI α1, in an attempt to rescue its phenotype regarding the loss of GlyRs positive allosteric modulation and high ethanol drinking [22]. Therefore, we generated a viral vector that expresses the α1 WT subunit using a recombinant AAV with the enhancer mDlx that restricts gene expression to GABAergic neurons, also expressing GAD-65 [31] (Fig. S3A). Patch-clamp recordings with these constructs showed that, unlike the empty control plasmid, the WT α1 clone was functional producing a large Cl^- current when stimulated with glycine (Fig. S3B, C).

At the onset of this series of experiments, we predicted that overexpression of α1 WT in KI mice might revert the GlyRs phenotype from an ethanol-resistant to an ethanol-sensitive receptor. The viral expression in the nAc was robust 2 weeks post-injection allowing for morphological conformation and electrophysiological characterization (Fig. 3). For example, confocal microscopy showed abundant GFP positive neurons that allowed for electrophysiological studies in the identified neurons [20], which agrees with other studies using this viral vector in various brain regions [21, 32]. Western blot analysis confirmed the overexpression of GlyRs in the nAc (Fig. 3C, unpaired Student's t-test $t_{(13)} = 2.672$, $p = 0.0192$, $^*p < 0.05$).

Before examining the ethanol sensitivity of these GlyRs, we wanted to confirm the properties of the overexpressed GlyRs in accumbal neurons by recording glycine-activated currents in dissociated neurons (GFP positive) that were previously infected with the empty or WT α1 containing AAV. The data show that the properties of GlyRs, native KI and overexpressed WT, were quite similar with EC_{50} values of 45 ± 4 µM ($n = 13$) in the KI injected with empty AAV and 58 ± 10 µM ($n = 11$) in the KI injected with WT α1 GlyR AAV (Fig. 3D, E unpaired Student's *t*-test $t_{(22)}=1.281$, $p = 0.2135$), values that are in close agreement to those reported in a previous study [23]. Interestingly, the properties of the recorded current, i.e., apparent affinity and decay time course, are similar to the responses recorded in identified D1-MSNs [7, 27], suggesting a similar neuronal nature. Further analysis showed that the current density was also increased significantly by the overexpression of α1 WT subunits compared to neurons infected with the empty AAV (Fig. 3F). For example, the values for current density were 8 ± 3 pA/pF for the empty AAV ($n = 13$) and 38 ± 6 pA/pF for the α1 WT ($n = 11$), representing an increase of 5-fold in receptor current density. On the other hand, the data showed that the current density for the GABA-activated Cl^- current was not affected (Fig. 3F, unpaired Student's *t*-test $t_{(17)} = 5.071$, $p = 0.0001$, $^{****}p < 0.0001$ for glycine; unpaired Student *t*-test $t_{(18)} = 0.1574$, $p = 0.8767$, n.s. for GABA).

Overexpression of the WT α1 in KI accumbal neurons rescued sensitivity to ethanol

We then examined the sensitivity of GlyRs expressed in accumbal neurons to ethanol (Fig. 4). The traces representing currents recorded in MSNs that were injected with an AAV containing either the empty or α1 WT subunit (Fig. 4A) show that they behaved as KI and WT GlyRs, respectively, in the sense that only those overexpressing α1 WT were potentiated by ethanol (Fig. 4B, C, green traces and bar). For example, with 50 mM ethanol, the current was potentiated by 44 ± 14% above control in the WT and only 10 ± 2% in the empty, respectively (Fig. 4C, Two-Way ANOVA, F (1,72) = 17.92, $p = 0.0001$; Bonferroni *post hoc* test: $^*p < 0.05$ for 50 mM ethanol; $^{***}p < 0.001$ for 100 mM ethanol concentration).

Overexpression of the WT α1 in KI accumbal neurons reduced binge drinking

For the locomotor activity evaluation of KI mice injected stereotaxically, we performed an open field assay to rule out an alteration in motor control after the surgical procedure. The traveled distance every 5 min was graphed in Fig. S4D. The mean total distance traveled for the KI mice was 6.477 ± 461 cm for the Empty control group, 7166 ± 508 cm for the α1 WT group, and 7196 ± 376 cm for the α1 KI group (Fig. S4F). No differences were found among the analyzed groups in terms of the distance traveled in this assay (Fig. S4C, One-Way RM ANOVA, F (2,25) =2.474, $p = 0.1046$ Bonferroni *post hoc* test: n.s. for all conditions for α1KI mice; for S4F One Way RM ANOVA F(2,25) = 0.7419, $p = 0.4864$ Bonferroni post hoc test: n.s.).

We then examined if overexpression of GlyRα1WT in the nAc of KI mice affected ethanol drinking using the DID protocol. The data in Fig. 5 show the drinking behavior of KI mice injected with the empty (control) and WT α1 subunit AAVs. The results show that ethanol consumption was lower in KI mice injected with the AAV containing the WT α1 (Fig. 5A, green squares and filled circles, Two-Way RM ANOVA, F (1,17) = 11.22, $p = 0.0038$; Bonferroni *post hoc* test: $^*p < 0.05$ for third and fourth day; $^{**}p < 0.01$ for the first day). The data show that mice carrying the KI mutation and D1 Cre consumed similar levels of ethanol in the DID study (Fig. 5). Comparison

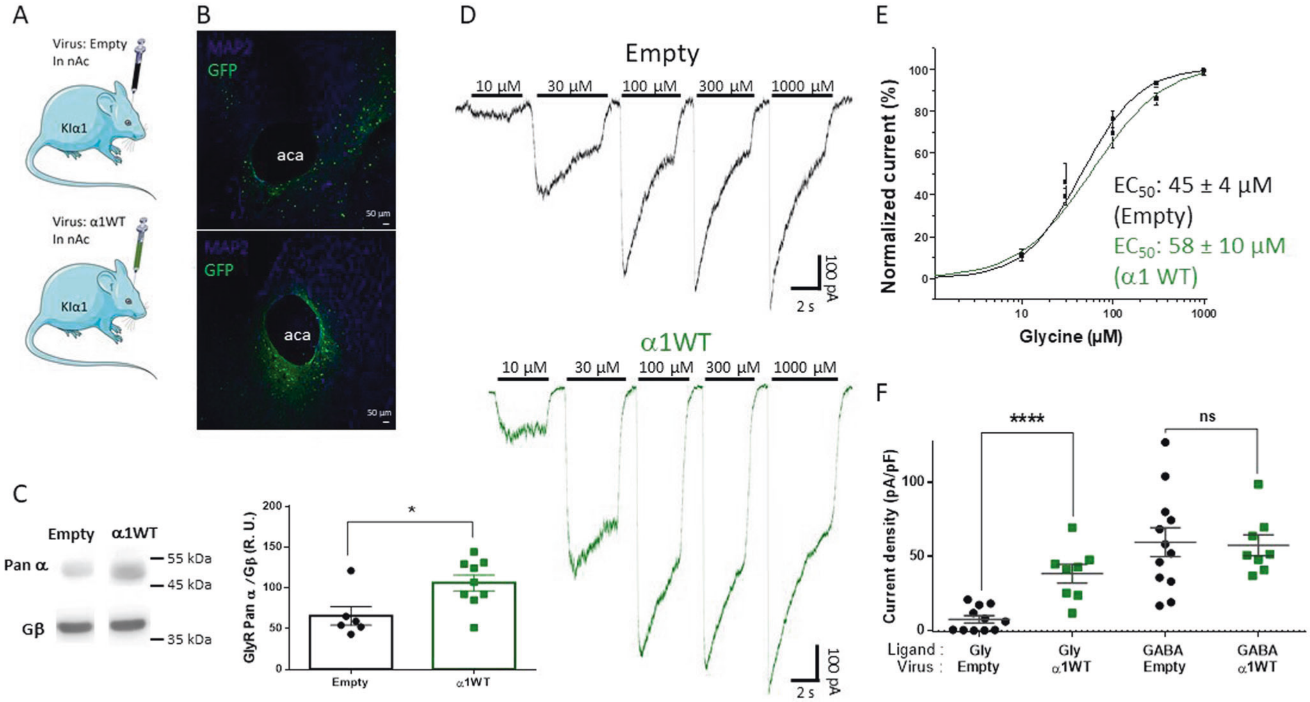

Fig. 3 Overexpression of α1 GlyRs in accumbal neurons of KIα1 mice. **A** Scheme of KIα1 mice that were injected in the nAc with Empty (black) or α1WT virus (green). **B**, **C** Confocal microscopy and western blots showing the localization of the positive neurons and the protein levels of GlyRs. **D** Representative current traces recorded at different glycine concentrations (10, 30, 100, 300 and 1000 µM) in dissociated neurons under both conditions. **E** The graph shows the glycine concentration-response curve for neurons transduced with Empty and α1 WT virus. Data represent normalized currents and shows the value for the EC_{50}. The dissociated neurons were recorded after the positive detection of the GFP reporter. **F** The graph shows the values of the current density (pA/pF) for glycine- and GABA-activated currents that were recorded in dissociated KIα1 nAc MSNs. Note that the current density associated with $GABA_AR$ was not affected by overexpression of GlyRs. Data represents mean ± SEM. Unpaired Student's *t*-test for (**C**, **E**, **F**). $n = 6$ for empty and $n = 10$ for α1WT. *$p < 0.05$ for (**C**). $n = 13$ for empty and $n = 11$ for α1WT virus for (**E**). For glycine current density (**F**) $n = 11$ (empty) and $n = 8$ (α1WT); for GABA current density $n = 12$ (empty) and $n = 8$ (α1WT). ****$p < 0.0001$, ns not significant.

between mice strains is not easy because previous studies showed that ethanol ingestion varies in different mice strains [33, 34]. Also, the KI mice used in this study were older than those used in our previous study (2 vs 3.5 months) and older animals showed a reduced intake level because of enhanced sedation [35]. As an additional control of the overexpression of inhibitory GlyRs in the nAc of KI mice, we injected an AAV that encodes for GlyR KI α1 subunit finding that overexpression of an ethanol-insensitive GlyR subunit did not change the ethanol consumption (Fig. 5B, red triangles and filled circles, Two-Way RM ANOVA, F (1,17 = 0.02502, $p = 0.8762$; Bonferroni *post hoc* test: n.s. for each day); highlighting the importance of ethanol-sensitive GlyRs in the nAc. Interestingly, the BEC measurement at day 4 in the mice injected with the WT α1 (green squares) was significantly lower than control mice: 77 ± 17 mg/dL (17 ± 4 mM) for WT α1 vs 195 ± 14 mg/dL (42 ± 3 mM) for empty (Fig. 5C, One-Way ANOVA, F (2,25) = 13.58, $p = 0.0001$; Bonferroni *post hoc* test: ***$p < 0.01$ for empty vs WT α1). None of these expressions affected water consumption (Fig. 5D, One-Way ANOVA, F (2,25) = 2.474, $p = 0.1046$ Bonferroni *post hoc* test: n.s. for all conditions), nor locomotor activity.

DISCUSSION

Presence of α and β subunits in accumbal neurons

Previous studies showed that mice nAc MSNs expressed synaptic and non-synaptic GlyRs with different ethanol sensitivities. The data also showed that concentrations of ethanol, between 10 and 100 mM, potentiated the glycine activated current in a scaled concentration-dependent manner in most neurons [23]. Further studies using D1 and D2-Cre-RiboTag mice showed that both cell types expressed mRNAs for α1 and α2 subunits. In addition, D1 and D2 neurons expressed β subunits indicating the presence of αxβ heteropentameric complexes. Thus, GlyRs in D1 and D2 MSNs are sensitive to different ethanol concentrations, reflecting a mixture of α1, α2, and β subunit expression. Recordings in neurons isolated from GFP-D1 mice indicated that D1 MSNs were more sensitive to lower concentrations of ethanol [7].

The experiments involving the overexpression of KI α1 only in D1 Cre MSNs, although causing a decrease in ethanol potentiation in D1 MSNs, did not result in changes in the drinking pattern of the injected mice. Our interpretation for this result is two-fold: 1) overexpression of KI α1 in a single type of MSNs was unable to affect native GlyRs in other neuronal populations sensitive to ethanol that can still exert inhibitory effects, and 2) this experimental approach does not affect D2 MSNs that also express α1 subunits [27]. In the second experiment, the global replacement of KI α1 with the WT α1 GlyRs in accumbal neurons, primarily D1 and D2 MSNs, was indeed more efficacious supporting the involvement of both cell types in high ethanol intake.

Higher drinking behavior of mice with mutant GlyRs

Studies in recombinant α1 and α2 GlyRs subunits showed that their positive allosteric modulation by ethanol depended primarily on the presence of two basic residues in the intracellular domain near the four transmembrane domain (TM4). Subsequent studies allowed the generation of two KI mice bearing the mutations KK-AA and KR-AA for α1 and α2, respectively [32, 36]. Electrophysiological studies on glycine-activated Cl^{-} currents in the nAc showed that most MSNs in KI mice were insensitive to ethanol, supporting the conclusion that α1 and α2 are important molecular

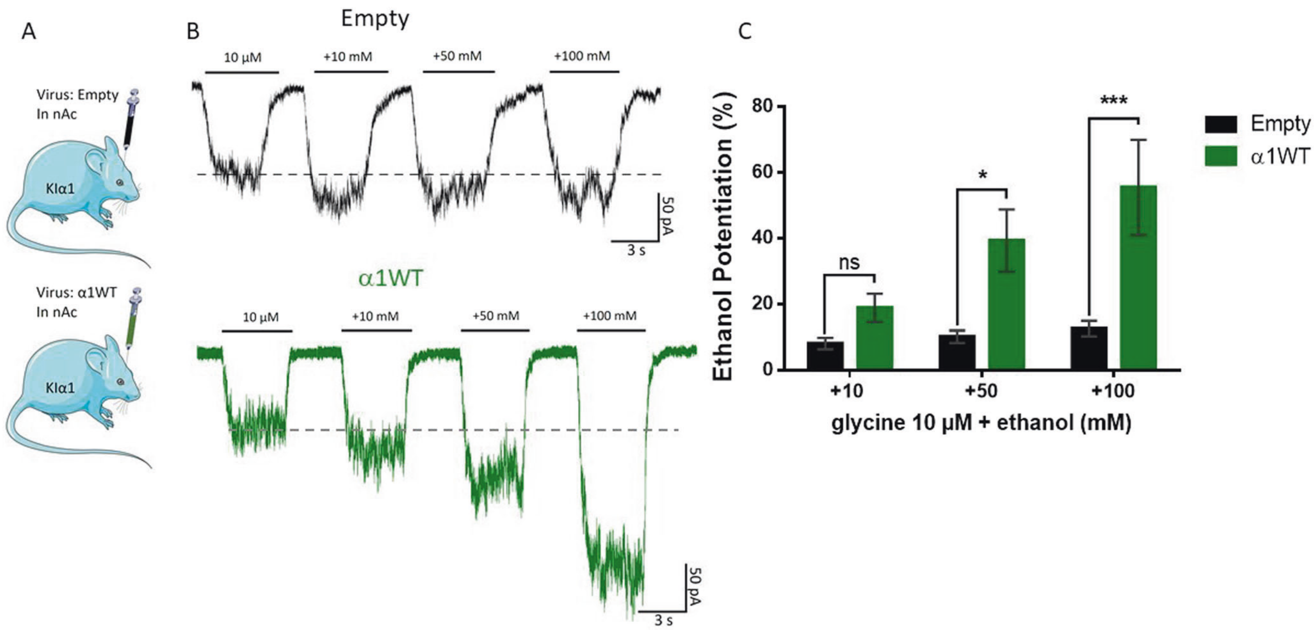

Fig. 4 Overexpression of α1 WT GlyRs in KIα1 mice recovered the sensitivity to ethanol in dissociated accumbal neurons. A The nAc in KIα1 mice was injected with Empty (black) or α1WT (green) virus. **B** Representative traces for responses in the presence of several ethanol concentrations that were recorded in neurons infected with the Empty (upper) and α1 WT (lower) AAVs. Potentiation glycine response using glycine 10 μM and ethanol 10, 50 and 100 mM. **C** The graph shows ethanol potentiation recorded in GFP-positive dissociated neurons from both conditions. Data represents mean ± SEM. Two-Way ANOVA and Bonferroni *post hoc* test. $n = 12$ for empty and $n = 14$ for α1WT virus. *$p < 0.05$, ***$p < 0.001$, ns. not significant.

targets for low ethanol concentrations. Interestingly, these mice showed a higher level of ethanol drinking, concluding that WT GlyRs have a protective role on consumption. This role was related to an enhanced inhibition produced by ethanol in the firing of action potentials in the nAc [36], an effect that was blocked by strychnine, a selective antagonist for GlyRs.

Expression of WT α1 reduced drinking in the KI mice

The present study showed that a viral vector to overexpress WT α1 subunits in the nAc of KI mice reduced their ethanol intake. Although previous data showed that accumbal MSNs express functional GlyRs [27], we cannot disregard the contribution of GlyRs in GABAergic interneurons, a smaller neuronal type [37], explaining the reduced ethanol intake found after overexpression of WT α1 GlyRs. Dlx1 is a lineage-specific transcription factor required for the differentiation of diverse types of GABAergic neurons, including projecting neurons and interneurons [38–40]. The significant increase, about 60%, in the protein level of WT α1 detected with Western blot support the idea of a wide expression in GABAergic neurons. The electrophysiological data showed that the current density associated with GlyRs activation in the nAc increased by 5-fold after the overexpression of the WT subunit in the nAc. The effect was selective for the response mediated by GlyRs since the current activated by $GABA_A$ receptors was not altered by the overexpression of GlyRs in MSNs, suggesting that the approach did not cause any noticeable compensation, at least in inhibitory neurotransmission. One possibility to explain the effect of reduced drinking is that the shift between ethanol insensitive to sensitive GlyRs increases the inhibition of MSNs, reducing the activation (see scheme in Fig. S5), which agrees with the notion that GlyRs are protective against excessive drinking [7]. Since the overexpression of WT GlyRs in the nAc did not affect water consumption, it appears that its main effect on DID was related to the positive allosteric modulation of GlyRs produced by ethanol (Fig. 4). Interestingly, the recording of GlyRs-activated currents showed that the effect of ethanol was already detected at 10 mM and became significant at 50 mM, a concentration that was detected in the measured BEC and that can produce a significant intoxication in humans [41].

The present results agree with other previous studies using a dopamine D2 vector producing 50% overexpression [42] and causing a significant reduction in ethanol intake and preference. However, more recent studies in mice did not report reduced intake of ethanol during continuous or intermittent access after the D2R upregulation [32]. Still, questions concerning the animal models used and the prior alcohol exposure were possible explanations for the reported differences.

Neuronal activity within the nAc appears to control ethanol binge drinking

The level of neuronal activation in the nAc appears to be essential for rewarding behaviors. For example, increased excitatory neurotransmission in the nAc affected ethanol drinking [43]. Homer2, known to affect the localization and function of NMDA receptors [44], increased ethanol intake and preference after its overexpression in the nAc [43]. Expression of Homer2 did not affect the sensitivity of the NMDA receptor to ethanol, but only its expression as a functional ion channel in the membrane [44]. Ethanol intake can also be affected by changing the firing activity of accumbal neurons. Using DREADDs in D1 Cre mice (Designer Receptors Exclusively Activated by Designer Drugs), it was reported that the activator CNO (clozapine-n-oxide) increased ethanol intake after the activation of D1 MSNs [45]. Thus, the global level of neuronal activity in MSNs appears to affect the rewarding properties of ethanol, with activation of D1 MSNs tending to increase the intake and preference. The overall data suggest that the enhanced inhibitory action of WT GlyRs, but not KI ethanol insensitive GlyRs, in the nAc [23, 36, 46] would tend to reduce excitation and the rewarding effect of the drug (Fig. S5).

Relationship between ethanol, brain reward, and GlyRs

How can GlyRs in the nAc control drinking behavior? The nAc is a critical hub center that integrates and controls the motivational circuit by receiving several synaptic inputs that control its

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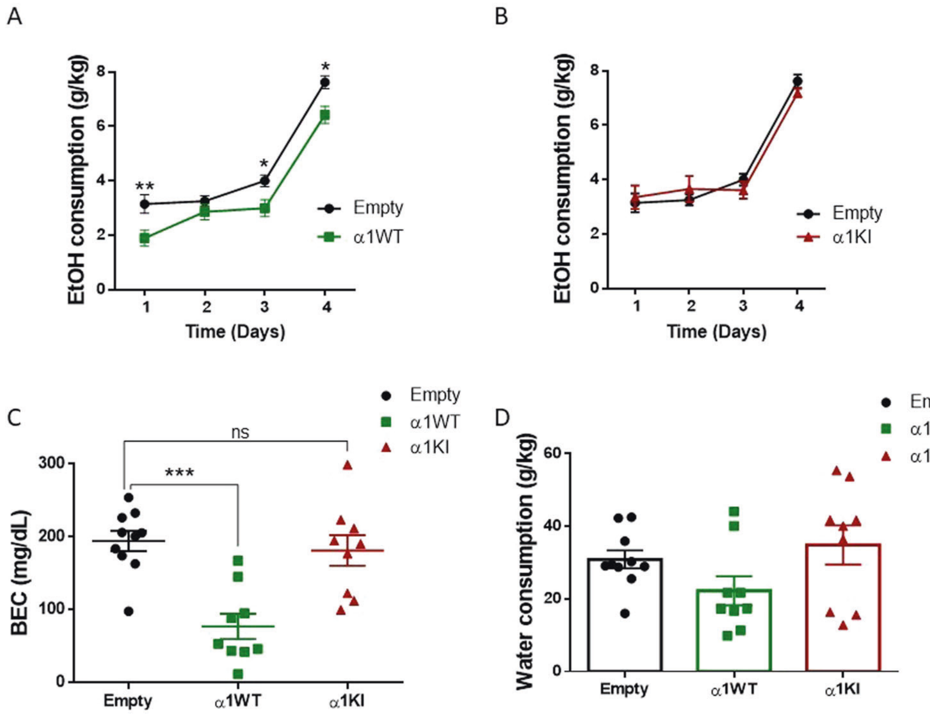

Fig. 5 Effect of GlyRs α1WT overexpression in the nAc of KIα1 mice on ethanol consumption behavior. A Graph shows ethanol consumption in the Drinking in the Dark (DID) assay for KIα1 mice injected with Empty (black circles), and α1WT (green squares) virus in the nAc. Mice injected with α1WT virus lowered their consumption on days 1, 3 and 4 vs KIα1 mice injected with the Empty virus. **B** Graph shows the consumption after the overexpression of the insensitive α1 GlyRs subunit (Dark red triangles) in the nAc of KIα1 mice. No differences were found between the KIα1 injected with the empty and α1KI virus. **C** The graph shows the blood ethanol concentration on the fourth day of the DID assay in mg/dL. Only the BEC from KIα1 mice injected with α1WT were significantly lower than the BEC from KIα1 mice injected with empty or α1KI virus. **D** Graph shows the water intake from the three mice groups. No differences were found between each group. Data represent mean±S.E.M. Two-Way ANOVA and Bonferroni *post hoc* test for (**A**, **B**), One-Way ANOVA and bonferroni *post hoc* test for (**C**, **D**). For DID, BEC and water consumption tests, $n = 10$ for empty, $n = 9$ for α1WT and $n = 9$ for α1KI virus. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

neuronal activity and project to other brain regions regulating their excitability. It receives dopaminergic innervation from the VTA and strong excitatory glutamatergic inputs from the PFC and hippocampus, among others [5, 6]. The inhibitory control of the accumbal MSNs is provided by GABAergic and glycinergic inputs [7], which exert a fast inhibitory control. These synaptic inputs and their Cl^- permeable ion channels appear to be involved in alcohol addiction [8, 9]. Because MSNs synthesize and release GABA within the nAc and at their projecting terminals, the most important function is to provide an inhibitory influence on the rewarding network. As a result of the neuronal interplay induced by intrinsic excitability and synaptic inputs, such as activation of GlyRs, the decoding between action potential firing and behavior is ultimately associated with the number of action potentials generated in these neurons. Thus, upon activation of GlyRs, neuronal firing is reduced [46]. Therefore, in the presence of ethanol, a positive allosteric modulator of GlyRs, the MSNs will be more inhibited.

Several studies have allowed us to strongly imply an interaction between ethanol, accumbal dopamine release, and GlyRs. Like other drugs of abuse, ethanol can increase the release of dopamine in the nAc, an effect that is blocked by strychnine [47]. On the other hand, the increase in GlyRs activation by glycine and ORG-25935, a GlyT1 transporter inhibitor, have the opposite effect (i.e., increasing dopamine) [21]. However, it appears that the increase in GlyRs function leads to a reduction in intake and preference for ethanol [48]. Mechanistically, the effect of GlyRs on the release of dopamine is not well understood. Still, it is possibly linked to the fact that ethanol depresses neuronal excitability in the MSNs, a GABAergic projecting inhibitory neuron, thus causing an increase in VTA dopaminergic function [23]. Subsequently, nAc activation might produce neuronal disinhibition in the cortex and thalamus, facilitating the generation of actions (Go-noGo) to promote drinking. Future studies should evaluate what aspects of AUD, reward and/or addiction, are affected by GlyRs and if pharmacotherapy guided to the glycinergic function can be of use for its management.

In conclusion, our study sheds light on the role of ethanol-sensitive α1 GlyRs present in the nAc and how their activity affects ethanol consumption, as shown by the rescue of the ethanol sensitivity function of α1 GlyRs in nAc from alcohol insensitive mice.

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AUTHOR CONTRIBUTIONS

AA and LA participated in the research design. AA, MR, AM and RC designed and generated AAVs. AA, SG, and AM performed the experiments and analyzed the data. AA, SG, MKL and LA wrote or contributed to the writing of the manuscript. All authors reviewed the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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