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RESEARCH PAPER

Ethanol consumption and sedation are altered in mice lacking the glycine receptor α2 subunit

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Background and Purpose: The precise mechanism/s of action of ethanol, although studied for many years, are not well understood. Like other drugs of abuse, ethanol affects dopamine levels in the nucleus accumbens (nAc), an important region of the mesolimbic system, causing a reinforcing effect. It has been shown that glycine receptors (GlyRs) present in the nAc are potentiated by clinically relevant concentrations of ethanol, where α1 and α2 are the predominant subunits expressed.

Experimental Approach: Using a combination of electrophysiology and behavioural assays, we studied the involvement of GlyR α2 subunits on the effects of low and high doses of ethanol, as well as on consumption using mice lacking the GlyR α2 subunit (male *Glra2*$^{-/Y}$ and female *Glra2*$^{-/-}$).

Key Results: GlyR α2 subunits exist in accumbal neurons, since the glycine-evoked currents and glycinergic miniature inhibitory postsynaptic currents (mIPSCs) in *Glra2*$^{-/Y}$ mice were drastically decreased. In behavioural studies, differences in ethanol consumption and sedation were observed between wild-type (WT) and *Glra2* knockout (KO) mice. Using the drinking in the dark (DID) paradigm, we found that *Glra2*$^{-/Y}$ mice presented a binge-like drinking behaviour immediately when exposed to ethanol rather than the gradual consumption seen in WT animals. Interestingly, the effect of knocking out *Glra2* in female (*Glra2*$^{-/-}$) mice was less evident, since WT female mice already showed higher DID.

Conclusion and Implications: The differences in ethanol consumption between WT and KO mice provide additional evidence supporting the conclusion that GlyRs are biologically relevant targets for the sedative and rewarding properties of ethanol.

1 | INTRODUCTION

The nucleus accumbens (nAc) is a major input structure of the basal ganglia and a pivotal region in the mesolimbic dopaminergic system mediating goal-directed behaviour, addiction-related behaviour, and motivational processes (Di Chiara & Imperato, 1988; Russo & Nestler, 2013). The nucleus accumbens receives glutamatergic innervations from the medial prefrontal cortex (PFC), amygdala and hippocampus and also receives GABAergic and cholinergic projections from the lateral septum. In addition, it sends and receives GABAergic projections to and from the ventral tegmental area (VTA) and receives an important dopaminergic input from the ventral tegmental area

Abbreviations: ASD, autism spectrum disorder; AUD, alcohol use disorder; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPP, conditioned place preference; DID, drinking in the dark; GlyR, glycine receptor; mIPSCs, miniature inhibitory postsynaptic currents; nAc, nucleus accumbens; LORR, loss of the righting reflex; PFC, prefrontal cortex; STN, strychnine; TTX, tetrodotoxin; VTA, ventral tegmental area.

(Koob & Nestler, 1997; Russo & Nestler, 2013). It is well known that ethanol and other drugs of abuse activate the mesolimbic dopaminergic reward system and increase dopamine levels in the nucleus accumbens (Di Chiara, 1997; Jonsson, Adermark, Ericson, & Soderpalm, 2014; Soderpalm, Lido, & Ericson, 2017). Microdialysis studies have indicated that **glycine receptors** (GlyRs) play an important role in the increase of dopamine levels in the nucleus accumbens produced by **ethanol** (Molander & Soderpalm, 2005b; Soderpalm et al., 2017).

Glycine receptors are expressed throughout the CNS in mammals and are the major inhibitory receptors present in spinal cord and brainstem (Legendre, 2001). Recent studies have shown that glycine receptors are also expressed in supraspinal regions such as prefrontal cortex (Lu & Ye, 2011; Salling & Harrison, 2014), nucleus accumbens (Martin & Siggins, 2002; Molander & Soderpalm, 2005b; Muñoz, Yevenes, Forstera, Lovinger, & Aguayo, 2018), dorsal striatum (Comhair et al., 2018; Molchanova et al., 2018), raphe nuclei (Maguire et al., 2014), and ventral tegmental area (Li et al., 2012; Ye et al., 2001). The expression of glycine receptors in mesolimbic areas is of special interest because their presence might be relevant for the rewarding properties of ethanol since it has been demonstrated that activation of glycine receptors regulate **dopamine** release and ethanol consumption in rodents (Lido, Ericson, Marston, & Soderpalm, 2011; Molander, Lof, Stomberg, Ericson, & Soderpalm, 2005; Molander & Soderpalm, 2005a).

Previous studies have demonstrated that ethanol potentiates the function of glycine receptors (Yevenes et al., 2010; Yevenes, Moraga-Cid, Peoples, Schmalzing, & Aguayo, 2008). However, the behavioural role of brain glycine receptors in ethanol-induced effects has not been thoroughly examined. Recent data from several laboratories have shown that glycine receptors are important in the brain reward system and that α1 and α2 are the predominant subunits expressed in the nucleus accumbens (Forstera et al., 2017; Jonsson et al., 2012). Our laboratory recently characterized the properties of glycine receptors in the nucleus accumbens (Forstera et al., 2017) and determined that accumbal medium spiny neurons express functional, ethanol-sensitive glycine receptors, primarily α1 and α2 subunits. A genetically modified knock-in (KI) mouse for the glycine receptor α1 subunit (K385/386A) that has an impaired interaction with G proteins demonstrated reduced ethanol sensitivity in the nucleus accumbens, a higher ethanol intake and increased conditioned place preference (CPP) to ethanol (Aguayo et al., 2014; Muñoz et al., 2019). Since **glycine receptor α2 subunits** are also known to be regulated by ethanol (Yevenes et al., 2010) and they are expressed in nucleus accumbens (Forstera et al., 2017; Jonsson et al., 2012), glycine receptor α2 subunits might also play an important role in regulating the mesolimbic dopaminergic reward circuitry and addictive behaviours. Previous studies have shown that α2 subunits modulate neuronal migration and cortical development (Avila et al., 2013), while several mutations and a microdeletion in *GLRA2* have been implicated in autism spectrum disorder (ASD) (Pilorge et al., 2016; Zhang, Ho, Harvey, Lynch, & Keramidas, 2017).

What is already known

- Ethanol activates the mesolimbic dopaminergic system and increases dopamine levels in the nucleus accumbens (nAc).
- Clinically relevant concentrations of ethanol potentiate the function of accumbal glycine receptors (GlyRs).

Wha this study adds

- *Glra2* knockout mice displayed a significant deficit in the expression of GlyRs in the nAc.
- Mice lacking the GlyR α2 subunit showed increased ethanol consumption and reduced sedation.

What is the clinical significance

- *Glra2* knockout mice are a useful model for studying the rewarding properties of ethanol.
- Pharmacological modification of α2 subunits might represent a novel therapy for alcohol abuse disorders.

The aim of this study was to evaluate the contribution of α2-containing glycine receptors in the nucleus accumbens and to characterize the effects of ethanol on relevant behaviours in mice lacking the glycine receptor α2 subunit. Since previous studies have reported sex differences in alcohol-related behaviours using rodents (Becker & Koob, 2016; Caruso et al., 2018; DeFries, Wilson, Erwin, & Petersen, 1989; Rhodes et al., 2007), we used male and female mice for behavioural tests. The knockout (KO) mice displayed a large deficit in the expression of glycine receptors in the nucleus accumbens as demonstrated by experiments on **glycine**-evoked currents and glycinergic neurotransmission. Interestingly, we found that male *Glra2*$^{-/Y}$ mice exhibited an increase in ethanol consumption and decreased sedation, suggesting that *Glra2* KO mice might be a useful model for the study of alcohol use disorders (AUDs). Nevertheless, the precise mechanisms underlying these results have to be treated with caution because of potential compensations in other brain neurotransmitter systems.

2 | METHODS

2.1 | Animals

C57BL/6J (wild-type [WT]) and *Glra2* KO mice (male: *Glra2*$^{-/Y}$ and female: *Glra2*$^{-/-}$) ages 7–12 weeks were used in this study. *Glra2* KO

mice were initially generated in the laboratories of Harvey and Dear by deletion of the exon 7 in the *Glra2* gene (Avila et al., 2013). Breeding pairs were transferred from Dr. Rigo's lab in Belgium to Chile where they were bred and maintained in a 12-h light/dark cycle. *Glra2* KO mice were backcrossed to C57BL/6J (IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664) and genotyped as described previously (Avila et al., 2013). All the animals used in this study were generated from crosses between hemizygous males ($Glra2^{-/Y}$) and heterozygous females ($Glra2^{-/+}$). Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Concepción and conducted according to the ethical protocols established by the National Institutes of Health (NIH, Bethesda, Maryland). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*.

2.2 | Experimental protocol

All the studies were designed to generate groups of equal size and randomly assigned. Operator and data analyses were blinded.

2.3 | Preparation of brain slices

WT and $Glra2^{-/Y}$ mice were decapitated as previously described (Forstera et al., 2017). Coronal slices were prepared immediately after excision and placement of the brain in ice-cold cutting solution (in mM: sucrose 194, NaCl 30, KCl 4.5, $MgCl_2$ 1, $NaHCO_3$ 26, NaH_2PO_4 1.2, and glucose 10, saturated with 95% O_2/5% CO_2 and adjusted to pH 7.4), glued to the chilled stage of a vibratome (Leica VT1200S, Leica Biosystems, Germany), and sliced to a thickness of 300 μm. Slices were transferred to an artificial CSF (aCSF) solution (in mM: NaCl 124, KCl 4.5, $MgCl_2$ 1, $NaHCO_2$ 26, NaH_2PO_4 1.2, glucose 10, and $CaCl_2$ 2, pH 7.4, 315–320 mOsm) saturated with 95% O_2/5% CO_2 at 30°C for at least 1 h.

2.4 | Enzymatic dissociation of accumbal neurons

For enzymatic dissociation, brain slices that contained the nucleus accumbens were incubated for 30 min in normal aCSF (saturated with 95% O_2/5% CO_2) in the presence of 0.5 $mg{\cdot}ml^{-1}$ of pronase (Calbiochem/EDM Bioscience, Darmstadt, Germany) at 37°C. The nucleus accumbens 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, and glucose 10, adjusted to pH 7.4 and 340 mOsm). After 20 min, isolated neurons were attached to the bottom of the culture dish and were ready for electrophysiological experiments.

2.5 | Electrophysiology

Recordings were done using an Axopatch 200B amplifier (Axon Instrument, Union City, California) at a holding potential of −60 mV. Currents were displayed and stored on a personal computer using a 1322A Digidata (Axon Instruments) and analysed with Clampfit 10.1 (Axon Instruments) and MiniAnalysis 6.0 (Synaptosoft, Inc.). Patch pipettes with a resistance of 4–6 MΩ were prepared from filament-containing borosilicate micropipettes (World Precision Instruments, Sarasota, FL, USA) using a P-87 micropipette puller (Sutter Instrument, Novato, CA, USA) and filled with an internal solution (in mM: KCl 120, $MgCl_2$ 4.0, BAPTA 10, Na_2-GTP 0.5, and Na_2-ATP 2.0, pH 7.4 and 290–310 mOsm).

2.5.1 | Dissociated accumbal neurons

Glycine-activated currents were studied in dissociated accumbal neurons using whole-cell recordings and an external solution containing (in mM) NaCl 150, KCl 5.4, $CaCl_2$ 2.0, $MgCl_2$ 1.0, glucose 10, and HEPES 10 (pH 7.4, 315–320 mOsm). The amplitude of the glycine current was measured by using a short pulse (1–2 s) of different concentrations of glycine. We used an array of external tubes (internal diameter, 200 μm) placed within 50 μm of the neuron and solutions containing the ligands flowed continuously from the tubes by gravity.

2.5.2 | Brain slice recording

Coronal brain slices (300 μm) containing the nucleus accumbens region were used for electrophysiology recordings. Glycinergic miniature inhibitory postsynaptic currents (mIPSCs) were pharmacologically isolated via bath application using 6-cyano-7-nitroquinoxaline-2,3-dione (**CNQX**, 10 μM) as an AMPA receptor antagonist; **bicuculline** (10 μM) as a $GABA_A$ antagonist; **mecamylamine** (10 μM) as a nicotine ACh receptor antagonist and **tetrodotoxin** (TTX, 500 nM). Analysis of frequency (Hz), decay constant (ms) and amplitude (pA) was used to compare between WT and $Glra2^{-/Y}$ mice. The decay phase was fitted with a single exponential curve and between 10% and 90% of its amplitude.

2.6 | Real-time quantitative PCR

For analysis of relative gene expression, WT mice and $Glra2^{-/Y}$ mice of 9–16 weeks were anaesthetized with isoflurane and killed by decapitation. When possible, tissues from each animal were used for multiple experiments. Nucleus accumbens tissue from WT mice was collected and preserved for 5–14 days at −80°C in TRIzol (Ambion, Life Technologies, UK) before further processing. Total RNA was isolated from samples using the TRIzol Reagent and chloroform (Darmstadt, Germany) followed by digestion with DNase I

(Thermo Scientific, Waltham, MA, USA) for 30 min. cDNA was prepared from 0.5 μg of total RNA with the Affinity Script qPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA, USA), including preparation of RNA sample-free negative controls (H_2O). PCRs were run in duplicate using 1-μl cDNA for 40 cycles with an annealing temperature of 57°C in a Strategene Mx3005P cycler (Agilent Technologies) for real-time quantitative PCR (qPCR) (n = 5–6 in WT; n = 6 in *Glra2*$^{-/Y}$) using the Brilliant II SYBR QPCR Master Mix (Agilent Technologies), including melting curves to control qPCR specificity. qPCR data were collected and analysed with MxPro (Agilent Technologies), and the δ–δ Ct method was used. 18S was used as a reference gene, and threshold cycles of glycine receptor expression were normalized to 18S. The primers used for real-time qPCR are shown in Table S1. The primers used for detection of α2 subunit mRNA hybridize to exon 7, which corresponded to the deleted region of the *Glra2* gene in the KO mice line.

2.7 | Western blots

Tissue homogenates from nucleus accumbens (50 μg) after lysis treatment (10-mM Tris–HCl, pH 7.4, 0.25-M sucrose, 10-mM *N*-ethylmaleimide [NEM], and protease inhibitor cocktail 1X) were loaded in a 10% SDS-PAGE and placed in an electrophoresis chamber. Subsequently, proteins were blotted onto nitrocellulose membranes, blocked with 5% milk in Tris-buffered saline (TBS) with 0.1% Tween 20 for 1 h, and incubated with primary anti-pan α glycine receptor antibody (1:500, rabbit monoclonal IgG; Cat# 146008, Synaptic Systems, RRID:AB_2636914), anti-glycine receptor β antibody (1:200, rabbit polyclonal IgG; Cat# AGR-014, Alomone, RRID:AB_2340973), and anti-Gβ antibody (1:1,000, rabbit polyclonal IgG; Cat# sc-378, Santa Cruz Biotechnology, RRID:AB_631542) overnight at 4°C. After washing steps, the membranes were incubated with anti-rabbit secondary antibodies conjugated to HRP (1:5,000, goat polyclonal anti-rabbit IgG-HRP, Cat# sc-2004, Santa Cruz Biotechnology, RRID:AB_631746). The immunoreactivity of the proteins was detected using an ECL Plus Western Blotting Detection System (PerkinElmer, Boston, Massachusetts). The relative expression of protein was normalized using the expression of the Gβ subunit. The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.8 | Behavioural studies

Both male (*Glra2*$^{-/Y}$) and female (*Glra2*$^{-/-}$) mice were used in this study, unless otherwise indicated. Mice were allowed to acclimate to the experimental room for at least 1 h prior to behavioural assays. Ethanol was diluted in 0.9% saline (20% v/v) and administered via intraperitoneal injections in doses adjusted by injected volumes.

2.8.1 | Open-field assay

Mice were tested for basal locomotor activity in a novel open field using a video tracking system (ANY-maze, Stoelting Co.). Mice were allowed to freely explore the 50 × 50-cm test area for 25 min. Total distance travelled and time in the centre were analysed.

2.8.2 | Accelerating rotarod

Basal motor skill performance was tested using an accelerating rotarod assay. Mice were placed on a non-rotating rod (IITC, Life Science). The test started when the rod began accelerating from 4 to 40 rpm in 120 s, and the latency to fall was recorded. Each mouse was tested 5 times on day 1 and 10 times on day 2.

2.8.3 | Fixed speed rotarod

Mice were tested for sensitivity and tolerance to the motor ataxic effects of ethanol using a fixed speed rotarod assay (IITC, Life Science). Mice were trained (rod speed 5 rpm) for 3 days, and training was considered complete when mice were able to stay on the rotarod for 120 s. Mice were injected with ethanol (2.0 g·kg^{-1} i.p.), and every 15 min after injection, mice were placed back on the rotarod, and latency to fall was measured until mice were able to remain on the rotarod for the full 120 s.

2.8.4 | Loss of the righting reflex

Mice were tested for sedative effects of ethanol using 3.5 g·kg^{-1} i.p. Mice were injected with ethanol, and when mice became ataxic, they were placed in the supine position in V-shaped plastic troughs until they were able to right themselves three times within 30 s. Loss of the righting reflex (LORR) was defined as the time from being placed in the supine position until the righting reflex was regained. These experiments were performed in Chile and Belgium. Although we found similar results in the duration of loss of the righting reflex between the two laboratories, the onsets of loss of the righting reflex were distinct. We attributed these differences to the speed of intraperitoneal injection, mice diet, and/or background noise at both sites. Nevertheless, the similarity in results on the duration of loss of the righting reflex is highly significant because it is the most widely used measure for ethanol sedation/intoxication in our and other laboratories (Aguayo et al., 2014; Blednov et al., 2015; Blednov, Benavidez, Homanics, & Harris, 2012).

2.8.5 | Drinking in the dark (DID)

This limited access drinking test produces significant levels of ethanol in the blood (Rhodes, Best, Belknap, Finn, & Crabbe, 2005). 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 $g \cdot kg^{-1}$ body weight per 2 or 4 h accordingly.

2.8.6 | Blood ethanol concentration

Blood samples from the facial vein from WT and *Glra2*$^{-/Y}$ (or *Glra2*$^{-/-}$) were collected after 10 min on day 1 and day 4 of drinking in the dark. Blood samples were centrifuged (10,000 rpm × 10 min), and ethanol concentration was determined in the serum using an Analox AM1 Analyzer (Lunenburg, Massachusetts).

2.8.7 | Conditioned place preference

The conditioned place preference was performed as previously described (Muñoz et al., 2019). This paradigm is based on a Pavlovian conditioning behaviour. Six identical acrylic boxes (33 × 27 × 20 cm) were separately enclosed in ventilated, light- and sound- attenuating chambers. Briefly, the place-conditioning study involved a pre-conditioning session, eight conditioning sessions, and a post-conditioning session. For the pre-conditioning session, mice received an injection of saline immediately before being placed in the box with both compartments available for 15 min. There were eight conditioning sessions, and mice received an injection of either ethanol 2.0 $g \cdot kg^{-1}$ (Cs+) or saline (Cs−) on alternating days, and their movement was recorded for 10 min. The compartments had different floor grid sizes: 0.6-cm grid for the positive stimulus (Cs+) and 0.1-cm grid for the negative stimulus (Cs−). Finally, for the post-conditioning, mice received an injection of saline before being placed in the centre of the box without separations to test activity in a 30-min preference session. Preference recording video was analysed using a video tracking system (ANY-maze software, Stoelting Co.).

2.9 | Reagents

Bicuculline and CNQX were purchased from Tocris (Bristol, UK). Glycine and strychnine (STN) were obtained from Sigma-Aldrich (USA). TTX was purchased from Alomone labs (Jerusalem, Israel). Ethanol was purchased from Merck Millipore (USA).

2.10 | Data analyses

Results are expressed as the mean ± SE, and statistical analyses were performed using unpaired Student's *t*-test or two-way repeated measures ANOVA for studies where each group size was at least $n = 5$. The group size in this study represents independent values. Differences with $P < 0.05$ were considered statistically significant. 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 inhomogeneity. As in previous studies (Aguayo et al., 2014; Muñoz et al., 2019), in order to obtain statistical power above 95% ($\alpha = 0.05$, power = 0.95) to determine existence of statistically significant differences ($P < 0.05$), we used a sample size of 6–8 measurements for experimental group. For behaviour studies, we considered at least 10–12 animals per group; however, there were some small variations in group size due to unreliable intraperitoneal injections or problems with the bottles. OriginPro 9.0 (Microcal Origin, RRID:SCR_002815, Northampton, MA, USA) software was used for all statistical analyses. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Presence of α2-containing glycine receptors in the nucleus accumbens

Previous studies demonstrated that nucleus accumbens expresses glycine receptor α and β subunits (Jonsson et al., 2012; Muñoz et al., 2019). Analysis of glycine receptor subunit relative expression in the nucleus accumbens using real-time PCR revealed high levels of α2 and β subunits in WT mice, while in the *Glra2*$^{-/Y}$ mice, the expression of α2 was completely abolished, as expected (Figure 1a). Interestingly, the expression levels of α1 and α3 were increased but were only significant for α3 in the *Glra2*$^{-/Y}$ mice compared to WT, while β expression levels, on the other hand, had a surprising significantly decrease in mice lacking the α2 subunit. In order to examine protein expression levels, we performed Western blot experiments in tissue lysates from nucleus accumbens dissected from brain slices using pan α and β subunit antibodies. Preliminary results revealed that the α2 subunit constitutes an important component of glycine receptors expressed in accumbal neurons in WT mice because the expression of total α subunits in mice lacking α2 subunits was significantly diminished as reflected by the densitometry analysis (Figure 1b). The presence of the β subunit, a protein important for the anchoring of glycine receptors to synaptic sites (Grudzinska et al., 2005), was also analysed in the *Glra2*$^{-/Y}$ mice using Western blot. Surprisingly, the results

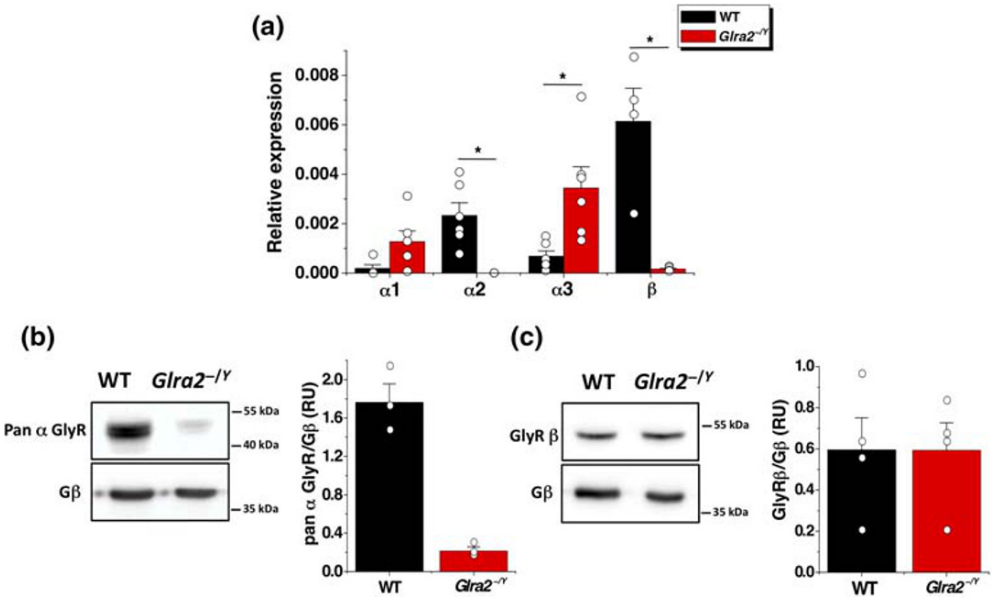

FIGURE 1 Presence of glycine receptor (GlyR) α and β subunits in the nucleus accumbens (nAc) of WT and *Glra2*$^{-/Y}$ mice. (a) Quantitative real-time PCR of GlyR subunits in nAc of wild-type (WT) and *Glra2*$^{-/Y}$ mice. (b,c) Western blot of the nAc from WT and *Glra2*$^{-/Y}$ mice for GlyR α and β subunits. The graphs show low levels of α subunits in *Glra2*$^{-/Y}$ mice compared to WT mice (n = 3 WT and n = 3 *Glra2*$^{-/Y}$) and similar levels of the β subunit in both mice (n = 4 WT and n = 4 *Glra2*$^{-/Y}$). Unpaired Student's t-test. Data represent mean ± SEM. *P < 0.05

revealed no significant changes in the expression of β subunits in *Glra2*$^{-/Y}$ mice compared to WT mice (Figure 1c). Although this result is contradictory to the qPCR analysis of the β subunit transcript, this could be due to differences in mRNA versus protein stability.

3.2 | Loss of glycine-activated currents in accumbal neurons in *Glra2*$^{-/Y}$ mice

Recent findings from our laboratory concluded that there are functional ethanol-sensitive glycine receptors present in accumbal neurons (Forstera et al., 2017) and that α1 and α2 are the predominant subunits expressed in this region (Forstera et al., 2017; Jonsson et al., 2012). Using electrophysiological recordings in dissociated neurons (up to 8 weeks old), we found that only 17% of the *Glra2*$^{-/Y}$ neurons (4 out of 24 accumbal neurons) presented amplitudes of more than 30 pA using a saturating concentration of glycine (Figure 2b). These neurons exhibited very small glycine-evoked currents (∼100 pA at 1 mM of glycine in these four cells) (Figure 2d) compared to WT mice (∼500 pA at 1 mM of glycine) (Figure 2a). Analysis of current densities revealed that *Glra2*$^{-/Y}$ mice had significantly smaller values (1.7 ± 0.7 pF·pA^{-1}) compared to WT mice (39.5 ± 6.3 pF·pA^{-1}; Figure 2c). Interestingly, the current density of GABA responses was also significantly decreased in *Glra2*$^{-/Y}$ mice (49.2 ± 5.1 pF·pA^{-1} vs. 73.3 ± 7.7 pF·pA^{-1} for WT, Figure 2c). The glycine concentration–response curve for the four neurons from *Glra2*$^{-/Y}$ mice showed an EC_{50} of 55 ± 8 μM, while WT mice exhibited an EC_{50} of 64 ± 5 μM (Figure 2d). In order to quantify ethanol potentiation, a concentration of glycine that activates 10%–15% of the maximal current (EC_{10}) was used (Yevenes et al., 2008). Due to the small amplitude of the glycine-evoked current obtained in these *Glra2*$^{-/Y}$ neurons, it was not possible to evaluate ethanol sensitivity, concluding that accumbal glycine receptors are basically absent in the KO mice. This was at the least surprising because we had predicted only a reduction in the glycine-activated current because of the presence of α1 and α3 subunits (see Section 4).

3.3 | The glycine receptor α2 subunit is important for glycinergic synaptic currents

A recently published article showed the presence of glycinergic synaptic currents in accumbal neurons (Muñoz et al., 2018). This was supported by Western blot and immunohistochemistry analyses that showed the presence of α and β subunits and GlyT2, a presynaptic glycine transporter (Bradaia, Schlichter, & Trouslard, 2004). In the present study, we wanted to evaluate the contribution of glycine receptor α2 subunits to the glycinergic inputs of the nucleus accumbens. Using a cocktail of inhibitors (TTX, bicuculline, CNQX and mecamylamine), we isolated glycinergic synaptic currents in slices that contained the nucleus accumbens. We found that 64% of the total neurons in WT mice had glycinergic activity, whereas only 38% of the total neurons in *Glra2*$^{-/Y}$ mice had glycinergic synaptic currents (Figure 3a). Figure 3b shows representative traces of total miniature postsynaptic currents (mPSCs) recorded in the nucleus accumbens of *Glra2*$^{-/Y}$ mice in the absence of inhibitors. After pharmacological isolation of miniature glycinergic postsynaptic currents (glycinergic mIPSCs), we detected a small number of synaptic events. The total blockade of these miniature events in presence of strychnine was indicative of glycinergic synaptic currents (Figure 3b). The average traces of these events are shown in Figure 3c. Analysis of synaptic parameters showed a significant decrease in frequency of total mPSCs in *Glra2*$^{-/Y}$ mice (0.81 ± 0.24 Hz) compared to WT (2.50 ± 0.28 Hz) and a significant diminished frequency of pharmacologically isolated glycinergic mIPSC in the *Glra2*$^{-/Y}$ mice (0.68 ± 0.09 Hz in WT mice vs. 0.17 ± 0.06 Hz in *Glra2*$^{-/Y}$ mice, Figure 3d). When analysing the amplitude, no changes were found between genotypes for total mPSCs (15.9 ± 1.3 pA in WT mice vs. 15.9 ± 1.7 pA in *Glra2*$^{-/Y}$ mice)

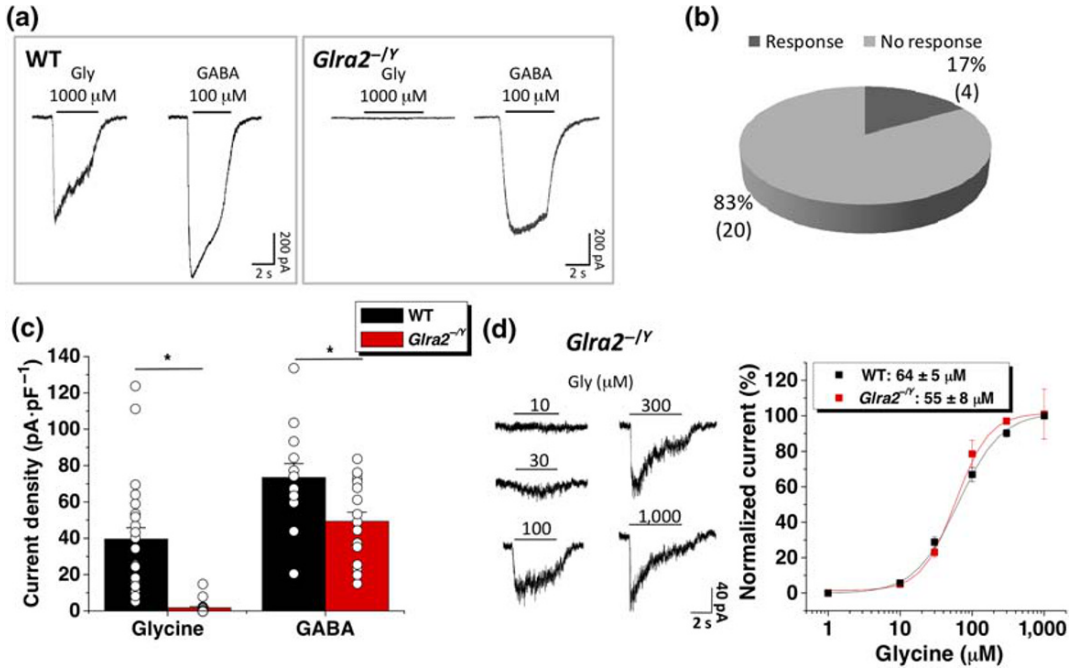

FIGURE 2 Glycine response of accumbal neurons from *Glra2*$^{-/Y}$ mice. (a) Representative current traces induced by a saturating concentration of agonist in dissociated neurons from the nucleus accumbens (nAc) in WT and *Glra2*$^{-/Y}$ mice. (b) Only 4 out of 24 accumbal neurons (17%) from *Glra2*$^{-/Y}$ mice had a glycine-evoked current greater than 30 pA. (c) The graph shows the current density of accumbal neurons from WT and *Glra2*$^{-/Y}$ mice. (d) Representative traces of glycine-evoked currents from responding neurons (1–1,000 μM) in *Glra2*$^{-/Y}$ mice. The graph shows the glycine concentration–response curve in accumbal neurons from WT (black squares) and *Glra2*$^{-/Y}$ (red squares) mice. Unpaired Student's *t*-test. Data are mean ± SEM. *$P < 0.05$

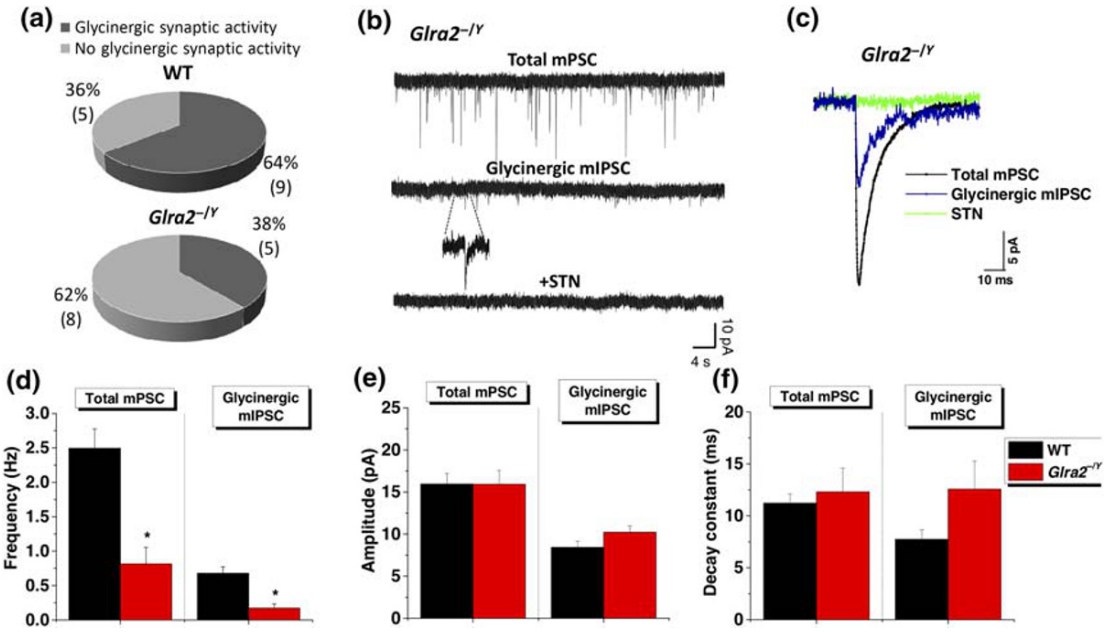

FIGURE 3 Synaptic activity in accumbal neurons from WT and *Glra2*$^{-/Y}$ mice. (a) Percentage of neurons that have glycinergic synaptic currents from accumbal neurons of WT and *Glra2*$^{-/Y}$ mice. (b) Representative synaptic current traces from *Glra2*$^{-/Y}$ mice for total miniature postsynaptic current (mPSC), pharmacologically isolated glycinergic mIPSC, and glycinergic mIPSC blocked with strychnine (STN). (c) Average traces for mPSC (black), glycinergic mIPSC (blue), and STN (green). (d–f) The graphs summarize synaptic parameters: (d) frequency, (e) amplitude, and (f) decay constant from mPSCs and glycinergic mIPSCs in accumbal neurons from WT and *Glra2*$^{-/Y}$ mice ($n = 14$ WT and $n = 13$ *Glra2*$^{-/Y}$ mice). Unpaired Student's *t*-test. Data are mean ± SEM. *$P < 0.05$

or glycinergic mIPSCs (8.4 ± 0.7 pA in WT mice vs. 10.2 ± 0.8 pA in *Glra2*$^{-/Y}$ mice, Figure 3e). Finally, analysis of the decay constant showed that total mPSCs revealed similar values for both genotypes (11.2 ± 0.9 ms in WT mice vs. 12.3 ± 2.3 ms in *Glra2*$^{-/Y}$ mic). However, there was an increase, although not significant, in the decay constant in glycinergic mIPSCs in *Glra2*$^{-/Y}$ mice of 12.5 ± 2.7 ms compared to 7.7 ± 0.9 ms found in WT mice (Figure 3f). Since the accumbal glycinergic currents in WT mice were insensitive to ethanol (Muñoz et al., 2018), we did not study ethanol effects on accumbal glycinergic currents in *Glra2*$^{-/Y}$ mice.

3.4 | *Glra2* KO mice displayed normal locomotor activity but impaired motor skill learning

Because *Glra2* is located on the X chromosome, all adult males used in the behavioural study were hemizygous (*Glra2*$^{-/Y}$), while females were homozygous (*Glra2*$^{-/-}$) for the exon 7 deletion. Similar to previous studies (Pilorge et al., 2016), this genetically modified mouse did not display foot clasping behaviour when the mouse was lifted by the tail (Figure S1A), indicating the absence of alterations in muscle tone and motor reflexes. Furthermore, using a qualitative grip test, *Glra2*$^{-/Y}$ mice did not show gross alterations in muscle strength (Figure S1B). The *Glra2*$^{-/Y}$ mice exhibited normal brain and total weight compared to WT mice (Figure S1C,D).

Basal locomotor activity was studied using the open-field test in both WT and *Glra2* KO mice. Initial analysis of sex demonstrated that there were no significant differences of total distance travelled between males and females in WT mice and in *Glra2* KO mice. Therefore, we combined the data of both sexes in this assay. Analysis of total distance travelled showed no differences between WT and *Glra2* KO mice (Figure 4a,b). There were also no differences between the genotypes when we analysed the time spent in the centre, an indicator of anxiety-like behaviour (Figure 4c).

When we performed the accelerating rotarod assay, we found that the latency to fall was significantly increased in female WT compared to male WT mice (i.e., for trial 15: 70 ± 4 vs. 57 ± 4 s). Therefore, we decided to split the data by sex. Interestingly, in this experiment, the latency to fall was significantly increased in male *Glra2*$^{-/Y}$ mice compared to male WT mice (Figure 5a). Additionally, there was a significant effect of trial. Similarly, analysis of latency to fall in female mice also demonstrated that *Glra2*$^{-/-}$ mice performed significantly better than WT (Figure S2A). A significant effect of trial was also observed, indicating a difference in motor skill performance between sexes and genotypes.

3.5 | Reduced ethanol-induced sedation in mice lacking the glycine receptor α2 subunit

Mice were also tested using high doses of ethanol to study motor ataxic effects and sedation. Using a fixed speed rotarod assay, we tested motor performance every 15 min after an intraperitoneal injection of 2.0 g·kg^{-1} of ethanol. Since we found that female WT recovered significantly faster than male WT mice (i.e. minute 60: 118 ± 2 vs. 80 ± 8 s; minute 75: 118 ± 2 vs. 102 ± 6 s), the data were also split by sex. After ethanol injection, we found no differences in recovery from ataxia between genotypes in male (Figure 5b) and female mice (Figure S2B). It is important to note that both genotypes were able to perform during the training trials before injection.

Interestingly, when we assayed male *Glra2*$^{-/Y}$ mice for loss of the righting reflex in presence of a sedative ethanol dose (3.5 g·kg^{-1} of ethanol i.p.), we found a significant reduction in the onset of loss

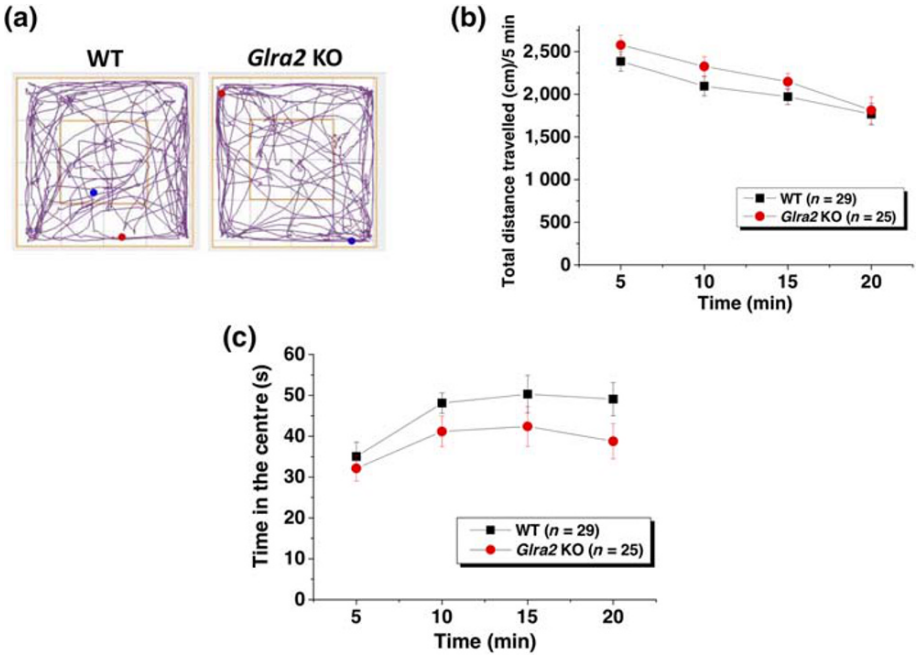

FIGURE 4 Basal motor activity of *Glra2* knockout mice (*Glra2* KO). (a) The distance travelled in an open-field environment to test locomotor activity was examined. Representative trajectories of a WT and a *Glra2* knockout mouse for the first 5 min in the open-field test. (b) The graph shows the total distance travelled during 20 min quantified every 5 min. (c) No differences were found in the time that the mice spent in the centre. Both males and females were used in this test (n = 29 WT and n = 25 *Glra2* KO mice). Two-way repeated measures ANOVA. Data are mean ± SEM

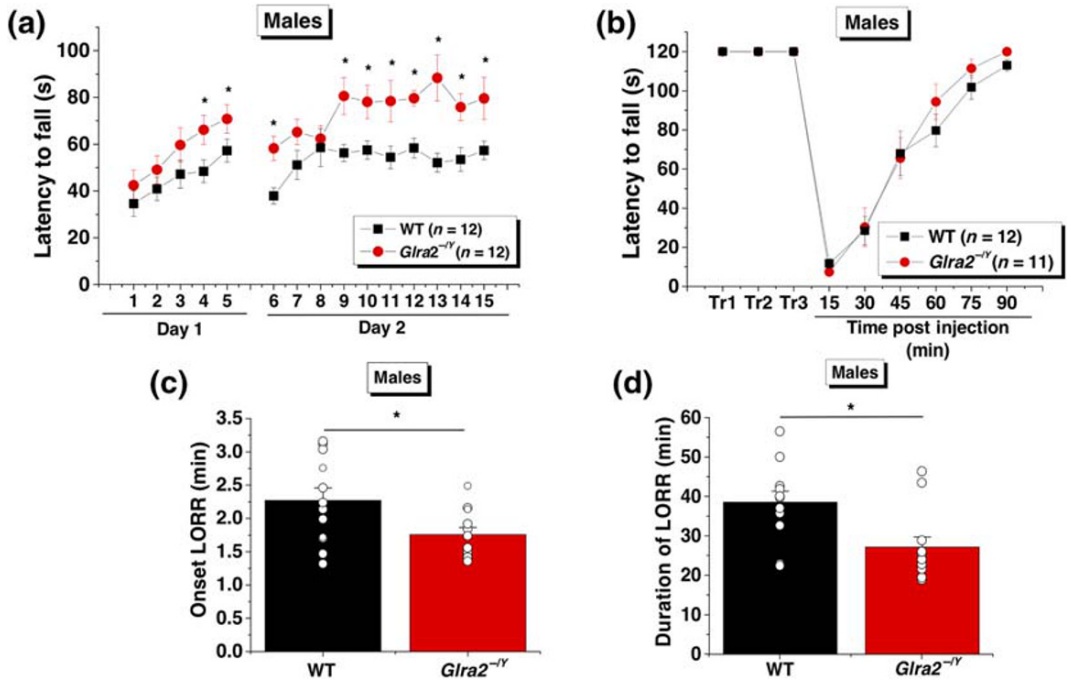

FIGURE 5 Effect of high doses of ethanol in male *Glra2* knockout mice (*Glra2*$^{-/Y}$). (a) Mice were tested for motor skill performance using an accelerating rotarod that consisted of multiple trials over a 2-day period. The graph revealed a significant difference in performance, where the *Glra2*$^{-/Y}$ mice had increased latency to fall compared to WT mice (n = 12 WT and n = 12 *Glra2*$^{-/Y}$ mice). (b) Ataxic effect of 2.0 g·kg^{-1} of ethanol on the fixed speed rotarod test in male WT and *Glra2*$^{-/Y}$ mice. Mice were trained for 3 days to a maximum time of 120 s at 8 rpm. On day 4, mice received an intraperitoneal injection of ethanol and were tested every 15 min post injection. No significant differences were found between genotypes (n = 12 WT and n = 11 *Glra2*$^{-/Y}$). Two-way repeated measures ANOVA and Bonferroni post hoc test. (c) Loss of the righting reflex (LORR) assays were used as an index of CNS depression. Mice received an intraperitoneal injection of 3.5 g·kg^{-1} of ethanol, and the latency to LORR was measured. *Glra2*$^{-/Y}$ mice lost their reflex ability faster than WT mice. (d) The ability to recover the righting reflex was measured as duration of LORR, where *Glra2*$^{-/Y}$ mice recovered faster than WT mice (n = 12 WT and n = 10 *Glra2*$^{-/Y}$). Unpaired Student's t-est. Data are mean ± SEM. *P < 0.05

of the righting reflex (2.3 ± 0.2 min for WT; 1.7 ± 0.1 min for *Glra2*$^{-/Y}$) and the duration of loss of the righting reflex (39 ± 3 min for WT; 27 ± 3 min for *Glra2*$^{-/Y}$) (Figure 5c,d). The same assay was performed in our collaborator's laboratory in Belgium also finding that the duration of loss of the righting reflex was reduced in *Glra2*$^{-/Y}$ mice compared to WT (Figure S3B). However, the onset to loss of the righting reflex was increased in *Glra2*$^{-/Y}$ mice compared to WT (Figure S3A), which could likely be associated to a smaller speed of intraperitoneal injection, mice diet and/or background noise. The analysis in female mice indicated that loss of the righting reflex was not affected in *Glra2*$^{-/-}$ mice (Figure S2C,D). It is interesting to note that the duration of loss of the righting reflex significantly differed between sexes in WT mice (39 ± 3 min for males vs. 28 ± 2 min for females). To confirm that these differences were not due to ethanol pharmacokinetics, blood ethanol concentrations were measured 30 min after intraperitoneal injection of ethanol (3.5 g·kg^{-1}). Blood ethanol concentrations in male mice were 394 ± 14 and 429 ± 15 mg·dl^{-1} for WT and *Glra2*$^{-/Y}$ mice, respectively, while in female mice, the values were 419 ± 24 and 435 ± 8 mg·dl^{-1} for WT and *Glra2*$^{-/-}$ mice, respectively. Thus, females had a shorter loss of the righting reflex than males with similar blood ethanol concentration values.

3.6 | *Glra2*$^{-/Y}$ mice had a high ethanol consumption on their first exposure compared to WT mice

Several reports have demonstrated that glycine receptors present in the reward system are important for ethanol consumption and preference (Lido et al., 2011; Molander et al., 2005). Similarly, the increase in dopamine levels produced by ethanol in the nucleus accumbens is affected by glycine receptor activation (Molander & Soderpalm, 2005a). We used the drinking in the dark paradigm as previously described (Rhodes et al., 2005) to evaluate behaviours associated with consumption. Initial analysis demonstrated that there was a significant difference in ethanol consumption between males and females in WT mice. Therefore, we split the data by sex for further analysis. Our results showed that male WT started to binge gradually over the 4 days of the assay. *Glra2*$^{-/Y}$ mice, on the other hand, had significantly greater ethanol consumption (binge levels) compared to male WT mice (P < 0.05) already at days 1 and 2 (day 1: 2.3 ± 0.2 g·kg^{-1} in WT vs. 3.0 ± 0.2 g·kg^{-1} in *Glra2*$^{-/Y}$; day 2: 2.4 ± 0.2 g·kg^{-1} in WT mice vs. 3.5 ± 0.3 in *Glra2*$^{-/Y}$, Figure 6a). Analysis of blood ethanol concentration showed that *Glra2*$^{-/Y}$ mice had significantly higher levels of ethanol in the blood on the first day of consumption compared to WT mice (62 ± 10 mg·dl^{-1} in WT vs. 106 ± 18 mg·dl^{-1} in

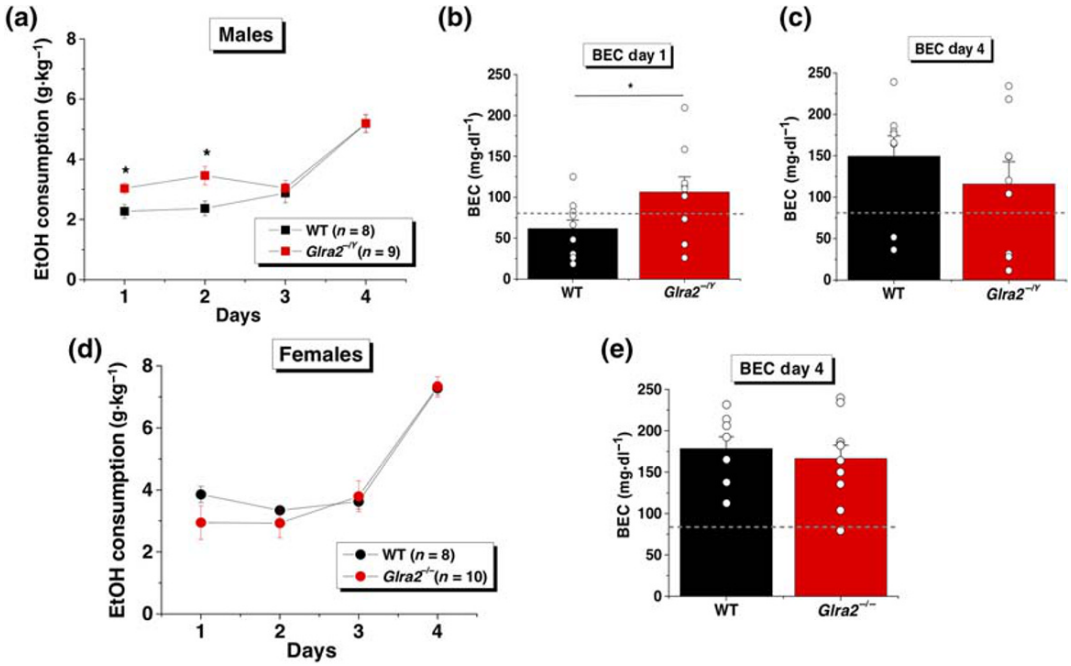

FIGURE 6 Male *Glra2* knockout mice consume higher amounts of ethanol in the drinking in the dark test. (a) Graph summarizes the drinking in the dark (DID) test in male WT and *Glra2*$^{-/Y}$ mice. During the dark phase of the day, the mice were able to drink a 15% v/v ethanol solution for 2 h the first 3 days. The fourth day, mice were allowed to drink for 4 h. Male *Glra2*$^{-/Y}$ mice consumed more ethanol on days 1 and 2 compared to WT mice (measured in grams of ethanol per kilogram of weight [g·kg^{-1}]; n = 8 WT and n = 9 *Glra2*$^{-/Y}$). Two-way repeated measures ANOVA and Bonferroni post hoc test. (b) The graph shows that after the first day of consumption, *Glra2*$^{-/Y}$ mice had higher blood ethanol concentrations (BECs) than WT mice. (c) BEC on day 4 showed similar levels for ethanol. Unpaired Student's t-test. (d) The graph summarizes the DID test in female WT and *Glra2*$^{-/-}$ mice (n = 8 WT and n = 10 *Glra2*$^{-/-}$). (e) Analysis of BEC indicates no differences in ethanol levels between genotypes. Unpaired Student's t-test. Data are mean ± SEM. n.s. $P > 0.05$, $^*P < 0.05$

Glra2$^{-/Y}$, Figure 6b). Interestingly, the levels in *Glra2*$^{-/Y}$ mice were above the limit of binge drinking defined by the National Institute of Alcohol Abuse and Alcoholism. The analysis of blood ethanol concentration on day 4, after 4 h of consumption, revealed no significant differences between genotypes (149 ± 24 mg·dl^{-1} for WT vs. 116 ± 26 mg·dl^{-1} for *Glra2*$^{-/Y}$, Figure 6c). When the experiment was performed in females, we found a similar pattern of consumption in female *Glra2*$^{-/-}$ as compared to male *Glra2*$^{-/Y}$ mice (Figure 6d), but differences were not statistically significant when we compared female WT and *Glra2*$^{-/-}$ mice. As previously reported (Rhodes et al., 2007), the female WT mice had higher ethanol consumption than male WT mice with values reaching 3.8 ± 0.3 g·kg^{-1} for females and 2.3 ± 0.2 g·kg^{-1} for males the first day of consumption (Figure 6d). Analysis of blood ethanol concentration at day 4 indicated that female mice showed no differences between genotypes (178 ± 14 mg·dl^{-1} for WT vs. 166 ± 16 mg·dl^{-1} for *Glra2*$^{-/-}$, Figure 6e).

3.7 | Decreased ethanol-conditioned place preference in the *Glra2*$^{-/Y}$ mice

Finally, we performed a conditioned place preference test to evaluate the ethanol preference of WT and *Glra2*$^{-/Y}$ mice. This test is a form of Pavlovian conditioning used to measure the rewarding and motivational effects of abused drugs (Cunningham, Gremel, & Groblewski, 2006). It was previously reported that KI mice for the α1 glycine receptor subunit showed increased preference for the ethanol side compared to WT mice, demonstrating the relevance of glycine receptors in the regulation of behaviours related with alcohol seeking (Muñoz et al., 2019). The WT and *Glra2*$^{-/Y}$ mice used in the conditioned place preference test did not show preference for either side of the cage during the 15-min pre-conditioning session (Figure 7a,c). After eight conditioning sessions, WT mice had a significant preference for the ethanol injection side (Cs+) in the 30-min post-conditioning session (35 ± 2 vs. 25 ± 2 s·min^{-1}, Figure 7b,d). Interestingly, no ethanol preference was detected in *Glra2*$^{-/Y}$ mice after eight conditioning sessions (31 ± 3 vs. 29 ± 3 s·min^{-1}, Figure 7b,d). These results indicate that *Glra2*$^{-/Y}$ mice lost ethanol preference compared to WT.

4 | DISCUSSION

The function of glycine receptors in supraspinal regions is achieving ever increasing attention with regard to behaviours related to reward-based learning and ethanol consumption (Burgos, Munoz, Guzman, & Aguayo, 2015; Soderpalm et al., 2017). In part, this is due to the current existence of genetically modified animal models that allow to examine behaviours in the absence of significant neurological

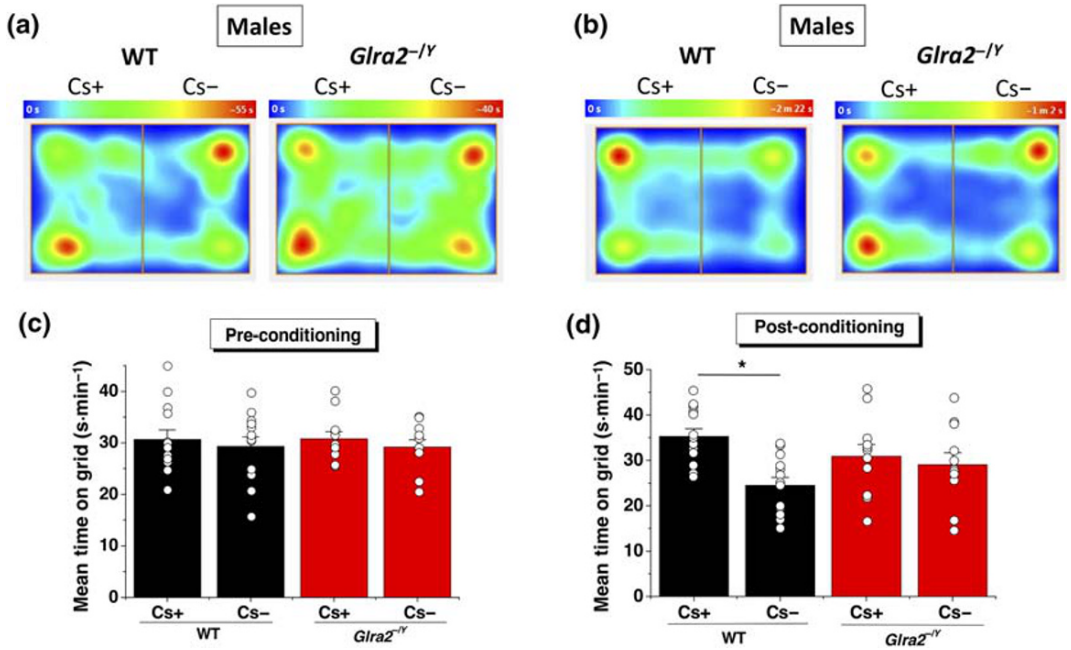

FIGURE 7 No changes in ethanol-conditioned place preference in male *Glra2*$^{-/Y}$ mice. (a) Representative traces of the trajectory obtained in the 15-min pre-conditioning session obtained from WT (left) and *Glra2*$^{-/Y}$ (right) mice. (b) Representative traces of the trajectory in the 30-min post-conditioning session obtained from WT (left) and *Glra2*$^{-/Y}$ (right) mice. (c) The graph summarizes the pre-conditioning place preference obtained in WT and *Glra2*$^{-/Y}$ mice and showed no differences in the time spent on the ethanol side (Cs+) compared to the vehicle side (Cs−). (d) The graph summarizes the post-conditioning place preference after eight conditionings in WT and *Glra2*$^{-/Y}$ mice. The results demonstrated that WT mice spent more time on the ethanol side (Cs+) than on the vehicle side (Cs−), while *Glra2*$^{-/Y}$ mice showed no preference for either side (n = 12 WT and n = 12 *Glra2*$^{-/Y}$). Two-way repeated measures ANOVA and Bonferroni post hoc test. Data are mean ± SEM. *P < 0.05

alterations that might occur when a critical brain membrane protein, such as a ligand-gated ion channels, is modified (Aguayo et al., 2014; Blednov et al., 2003; Blednov et al., 2012). Previous studies suggested that these receptors were not present in the striatum (Malosio, Marqueze-Pouey, Kuhse, & Betz, 1991). However, recent studies established that they present widespread expression in most brain areas (Forstera et al., 2017; Jonsson et al., 2012). Despite this, the particular glycine receptor subtypes expressed in these regions remain largely unknown. Previous studies using electrophysiology, qPCR, and immunohistochemistry have shown the presence of α1 and α2 glycine receptor subunits in the nucleus accumbens (Forstera et al., 2017; Muñoz et al., 2018; Muñoz et al., 2019). Studies in knock in (KI) mice showed the importance of the α1 subunit in glycine receptor potentiation with clinically relevant concentrations of ethanol, as well as in ethanol consumption and preference (Aguayo et al., 2014; Muñoz et al., 2019). However, the possible contribution of glycine receptor α2 subunits to ethanol-related effects was largely unknown. In the present study, we used a *Glra2* KO mouse that lacks the glycine receptor α2 subunit to evaluate the contribution of this subunit in a region important for reward-based learning. Using this mouse model, it was previously concluded that the gene is critical for brain development and its deletion caused deficits in object recognition memory and impaired LTP in the prefrontal cortex (Pilorge et al., 2016). Alteration in glutamatergic transmission, cortical synaptic plasticity (Morelli et al., 2017) and impaired motor memory consolidation were also detected in this mouse (Molchanova et al., 2018) suggesting the presence of compensatory mechanisms. On the other hand, using another model of *Glra2* KO mice, it was shown that this mouse displayed impaired adult hippocampal neurogenesis and deficits in spatial memory (Lin et al., 2017).

4.1 | Role of glycine receptors containing the α2 subunit in nucleus accumbens neurons

Previous studies have demonstrated the expression of glycine receptor α2 subunits in the nucleus accumbens (Jonsson et al., 2012; Jonsson, Kerekes, Hyytia, Ericson, & Soderpalm, 2009) and our results are in agreement with these findings. For example, Western blot analysis of *Glra2*$^{-/Y}$ mice showed lower protein levels of glycine receptor α subunits in the nucleus accumbens, while qPCR analysis demonstrated that the α2 subunit was completely abolished. Interestingly, results obtained with qPCR showed a higher relative expression of glycine receptor α1 and α3 subunits and a lower relative expression of glycine receptor β subunits in *Glra2*$^{-/Y}$ mice. Nevertheless, electrophysiological recordings in accumbal neurons showed that functional receptors were mostly absent in most *Glra2*$^{-/Y}$ mice neurons, which correlates well with the Western blot results. Glycine concentration–response curves for those neurons that responded to glycine revealed an EC_{50} value that suggested the presence of glycine receptor α1

subunits, since this value is comparable to the EC_{50} of 41 ± 1 μM for glycine receptor α1 subunit obtained in HEK293 cells (Yevenes et al., 2010). A possible explanation for such a small current in the presence of even high levels of other glycine receptor α subunit mRNAs and β subunit proteins might be based on the stability of the resulting mRNA or potential dominant-negative effects of the *Glra2* KO mice. A similar effect was observed in KO mice for the **$GABA_A$ receptor α6 subunit**, where removal of the α6 subunit altered δ subunit levels in cerebellar granular cells (Jones et al., 1997). Our results, nevertheless, are in agreement with previous findings that showed an absence of glycine-activated currents in the striatum in *Glra2* KO mice (McCracken et al., 2017; Molchanova et al., 2018), a region where α2 is the predominant subunit in WT mice (Comhair et al., 2018). The deficit, however, was not only in glycinergic function since we found that the amplitude of the GABA-evoked currents was also reduced in the *Glra2*$^{-/Y}$ mice.

Previous reports have shown the presence of glycinergic neurotransmission in the mesolimbic system that was insensitive to ethanol (Muñoz et al., 2018). In the present study, we found a reduction in the number of neurons with glycinergic synaptic currents in *Glra2*$^{-/Y}$ mice when compared to WT mice. Interestingly, neurons that displayed glycinergic mIPSCs also showed a tendency towards a decrease in the frequency of total mPSCs, suggesting a role of glycine receptor α2 subunits in the establishment of GABAergic and glutamatergic inputs in the nucleus accumbens. Additionally, we detected a small lengthening in the value for the decay time constant in neurons having glycinergic IPSC in *Glra2*$^{-/Y}$ mice suggesting changes in the conformation of synaptic glycine receptors, where both α1 and α3 subunits might be the predominant forms, according to qPCR analysis. Despite the reduced glycinergic synaptic neurotransmission in the nucleus accumbens of *Glra2*$^{-/Y}$ mice, there were no changes in **glycine receptor β** subunit levels, as revealed by Western blot analysis. Since the β subunit is necessary for the clustering of glycine receptors at postsynaptic sites (Meyer, Kirsch, Betz, & Langosch, 1995) and does not form functional homomeric channels (Grudzinska et al., 2005), this subunit in the *Glra2*$^{-/Y}$ mouse probably remains in a non-functional state explaining the reduced input.

Taken together, these results demonstrate an important contribution of glycine receptor α2 subunits to the glycinergic component in accumbal neurons, since the glycinergic currents in *Glra2*$^{-/Y}$ mice were markedly reduced. On the other hand, these findings also suggest that α2 glycine receptor subunits might be important for the correct expression and function of α1 and α3 glycine receptor subunits and **$GABA_A$ receptors** in the nucleus accumbens.

4.2 | Behavioural effects of high ethanol doses in *Glra2* KO mice

The nucleus accumbens is an important input structure of the basal ganglia that integrates information from cortical and limbic structures to mediate goal-directed behaviours (Scofield et al., 2016). Thus, although several studies have reported that mouse pups lacking the glycine receptor α2 subunit demonstrated microcephaly postnatally (Avila et al., 2014) and that α2 glycine receptor subunits promote cortical interneuron migration (Avila et al., 2013), the adult *Glra2*$^{-/Y}$ mice used in this study did not have differences in brain weight or total weight and they did not display an increase in muscle tone. This is consistent with previous reports that showed that this reduced brain weight is gradually attenuated with age (Morelli et al., 2017) and eliminated in adulthood (Pilorge et al., 2016). Despite that this animal model is reasonably normal and it may enable us to learn about alcohol consumption-related mechanisms, the wide-ranging impacts on brain development and associated compensatory adaptations to multiple other neurotransmitter receptors somewhat prevent ascribing more specific behavioural outcomes to the normal role of the α2 subunit, or actions of alcohol on the α2 subunit specifically.

Open-field assays did not show differences between genotypes in total distance travelled or time spent in the centre, which is in agreement with previously published data (Blednov et al., 2015; Lin et al., 2017;Molchanova et al., 2018 ; Pilorge et al., 2016). However, we detected changes in motor learning skills using the accelerating rotarod that showed an increased latency to fall in both male and female *Glra2* KO mice. Although this result was not found in previous studies (Molchanova et al., 2018; Pilorge et al., 2016), this might be due to differences in protocol since we tested the mice for two consecutive days with five trials on day 1 and 10 trials on day 2, while Molchanova et al. tested the mice for five consecutive days with four trials for each day. The increased latency to fall found in the *Glra2* KO mice might be explained by neuronal compensation in the basal ganglia, an important region for motor skill learning.

Acute consumption of intoxicating doses of ethanol produces rapid changes in brain functions from lack of coordination, motor ataxia and sedation, to respiratory depression, coma and death at higher doses of ethanol. On the other hand, its chronic use is associated to alcohol-seeking behaviour, binge drinking, tolerance and dependence (Spanagel, 2009). In order to begin understanding the role of glycine receptor α2 subunits in behaviour associated with intoxicating doses of ethanol, we evaluated ataxia and sedation induced by ethanol. While the recovery time from ataxia using the fixed rotarod assay did not differ between genotypes in both male and female, we found differences for the onset and duration of loss of the righting reflex in males. In addition, the similar blood ethanol concentration found in both genotypes showed that this difference in sedation was not due to ethanol pharmacokinetics. The reduced duration of loss of the righting reflex in male *Glra2*$^{-/Y}$ mice revealed the importance of the α2 subunit in regulating awakening, sedation, and loss of consciousness. It is important to note that the reduced duration of loss of the righting reflex found in *Glra2*$^{-/Y}$ mice was observed in two independent laboratories, supporting the results using this KO line. In addition, these findings are in agreement with unpublished results in a KI mouse model for the glycine receptor α2 subunit in which a lysine and arginine residue were replaced with alanines at amino acid positions in the large intracellular loop corresponding to KK385–386 in the glycine receptor α1 subunit (manuscript in preparation). In addition, the reduced duration of loss of the righting reflex is

consistent with the idea that ethanol potentiates glycine receptor function leading to effects on sedation (Burgos et al., 2015). Surprisingly, we did not find differences for onset or duration of loss of the righting reflex between genotypes in female mice. Nevertheless, it is important to note that female WT mice showed a reduced duration of loss of the righting reflex compared to male WT mice, similar to previous reports (Blednov et al., 2003). We attributed this difference to sex-associated mechanisms in females. For example, previous studies have reported sex differences in sleep time induced by ethanol (DeFries et al., 1989) and implied the action of neurosteroids (King, 2008; Morrow, VanDoren, Fleming, & Penland, 2001). These findings highlight that although the glycine receptor α2 subunit is an important contributor to some ethanol effects, such as loss of the righting reflex, this behaviour is more complex and likely involves multiple targets.

4.3 | Changes in ethanol intake and preference

Alcohol use disorder is a relapsing brain disease with serious social consequences whose exact underlying mechanism is still unknown. There are only three drugs approved by the U.S. Food and Drug Administration to treat alcohol use disorders (**disulfiram**, **naltrexone** and **acamprosate**) (Mann, 2004; Spanagel, 2009). Binge drinking is a form of abusive alcohol drinking defined by the National Institute of Alcohol Abuse and Alcoholism as drinking to obtain a blood ethanol concentration of at least 80 $mg{\cdot}dl^{-1}$. This typically occurs after four drinks for women and five drinks for men, in about 2 h. While binge drinking is not the same as alcohol use disorder, it significantly increases the risk of developing an alcohol abuse problem (Gowin, Sloan, Stangl, Vatsalya, & Ramchandani, 2017). Repeated cycles of binge drinking and abstinence are key components in the development of dependence. We used a drinking in the dark paradigm to evaluate if there was a difference in the consumption pattern between WT mice and *Glra2* KO mice. An important feature of this procedure is that mice self-administer alcohol and reach blood ethanol concentrations to levels of binge-like drinking behaviour (Rhodes et al., 2005; Rhodes et al., 2007). The drinking in the dark test showed higher consumption on days 1 and 2 in male $Glra2^{-/Y}$ mice as compared to WT mice, revealing blood ethanol concentration levels above binge drinking. Interestingly, there were no differences in consumption at day 4 between both genotypes. We believe that this ceiling effect on ethanol consumption represents a type of homeostatic regulation in the intake, where the previous intoxication limits the successive consumption. For the KO mice, they reach a level of binge drinking during the first exposure, whereas the WT reach binge level on the fourth day (Figure 6b). Previous studies showed a similar pattern of consumption in a glycine receptor α1 subunit KI mouse (Muñoz et al., 2019), and recent unpublished results show the same drinking behaviour in a glycine receptor α2 subunit KI mouse model (manuscript in preparation), supporting the conclusion that glycine receptors play a critical role in ethanol consumption. Surprisingly, we found that the high ethanol consumption in female WT mice was not different from the $Glra2^{-/-}$ mice on day 1, but then their intake became similar over time. However, it is important to note that female WT mice consumed more ethanol than male WT mice, a behaviour that might be explained by the fact that females need more blood ethanol concentration to reach the same ethanol effect as reflected by loss of the righting reflex assays. This result supports the idea of the homeostatic control reaching a ceiling on ethanol consumption, leading to intoxication.

Previous studies have reported sex differences in alcohol intake using rodents in several ethanol consumption tests (Becker & Koob, 2016; Caruso et al., 2018; Rhodes et al., 2007) and have demonstrated that ovarian physiology contributes to increased ethanol intake by females (Satta, Hilderbrand, & Lasek, 2018). In addition, it was demonstrated that **17-β-estradiol** inhibits glycine receptors (Jiang et al., 2009), suggesting a sex-differential modulation of glycine receptors that might explain the differences found in behaviour between male and female mice. On the other hand, when we examined conditioned place preference, we found that $Glra2^{-/Y}$ mice did not condition to ethanol after eight conditioning sessions, possibly because in the KO mice ethanol is less rewarding to produce a conditioning compared to WT mice (Cunningham et al., 2006). Hence, they need to consume more ethanol in the drinking in the dark assay to produce a rewarding effect. Another explanation is that although male $Glra2^{-/Y}$ mice lost ethanol preference, they will consume high amounts of alcohol because they might be less sensitive to the aversive effect of ethanol. These results suggest that *Glra2* KO mice would need more conditioning sessions to show preference for the ethanol side compared to WT mice. Alternatively, it is possible that because the KO mice have shown memory deficits (Lin et al., 2017; Pilorge et al., 2016), they might not be able to recall the side with the positive reinforcement. The results obtained in conditioned place preference and drinking in the dark demonstrated important differences in motivation and preference for ethanol in the *Glra2* KO mice that might be explained due to the absence of α2 subunits or compensatory effects that may occur in the KO animal.

The present results suggest that the glycine receptor α2 subunit, like the glycine receptor α1 subunit, plays an important role in the reward circuitry of the mesolimbic dopaminergic system. Regarding previous studies using another GlyR α2 subunit KO line that examined the effects on ethanol behaviour (Blednov et al., 2015), those mice had a different genetic background with less backcrosses to C57BL/6J mice compared to our mouse line. In addition, the *Glra2* KO mice used in Blednov's study were generated by the deletion of exons 6 and 7 (Young-Pearse, Ivic, Kriegstein, & Cepko, 2006), a different approach compared to our KO mouse. These differences in mouse lines might explain the discrepancies found between the two studies in terms of ethanol consumption and sedation. Although our results might seem contradictory to the aforementioned study, it is important to highlight that there is consistent evidence that accumbal glycine receptors regulate alcohol intake (Lido et al., 2011; Molander et al., 2005; Molander, Lido, Lof, Ericson, & Soderpalm, 2007; Muñoz et al., 2019). Based on our data, we postulate that glycine receptors protect against alcohol consumption, since glycine receptors in the

nucleus accumbens are able to reduce ethanol intake. A plausible mechanism for this regulation is that in the neuronal circuitry involving nucleus accumbens- ventral tegmental area, activation of accumbal glycine receptors will cause the inhibition of medium spiny neurons generating an excitatory response in the feedback pathway to the ventral tegmental area, disinhibiting dopaminergic neurons. It is also important to mention that our behavioural studies were performed in mice between the ages of 8–12 weeks old, representing young adolescent animals, a stage at which there is a high susceptibility to addiction, especially in humans (Bava & Tapert, 2010; Caruso et al., 2018; Varlinskaya & Spear, 2015).

In summary, the present study using α2 KO mice represents a comprehensive, physiological, and behavioural characterization on ethanol effects of an animal model that appears to exhibit subtle compensatory effects, albeit not lethal nor neurologically noticeable. To have a more decisive conclusion on the role of this subunit, we are currently examining an α2 KI mouse model that does not exhibit noticeable compensations and analysing subunit knockdown in specific brain regions with adeno-associated virus (AAV). Finally, this study provides evidence that mice with a deletion in *Glra2* are a useful model not only in the study of autism spectrum disorder (Pilorge et al., 2016) but also for alcohol use disorders.

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AUTHOR CONTRIBUTIONS

L.S.M., S.G., and L.G.A. participated in research design. L.S.M., S.G., A.A., N.R., J.C., and G.M. performed experiments and analysed the data. R.J.H. constructed the *Glra2* KO mouse line. L.S.M., L.G.A., R.J.H., J.M.R., and B.B. wrote or contributed to the writing of the manuscript. All authors reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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