Extrasynaptic glycine receptors of rodent dorsal raphe serotonergic neurons: a sensitive target for ethanol.

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Abstract

Alcohol abuse is a significant medical and social problem. Several neurotransmitter systems are implicated in ethanol's actions, with certain receptors and ion channels emerging as putative targets. The dorsal raphe (DR) nucleus is associated with the behavioural actions of alcohol, but ethanol actions on these neurons are not well understood. Here, using immunohistochemistry and electrophysiology we characterise DR inhibitory transmission and its sensitivity to ethanol. DR neurons exhibit inhibitory "phasic" postsynaptic currents mediated primarily by synaptic $GABA_A$ receptors ($GABA_AR$) and to a lesser extent by synaptic glycine receptors (GlyR). In addition to such phasic transmission mediated by the vesicular release of neurotransmitter, the activity of certain neurons may be governed by a "tonic" conductance resulting from ambient GABA activating extrasynaptic $GABA_ARs$. However, for DR neurons extrasynaptic $GABA_ARs$ exert only a limited influence. By contrast, we report that unusually the GlyR antagonist strychnine reveals a large tonic conductance mediated by extrasynaptic GlyRs, which dominates DR inhibition. In agreement, for DR neurons strychnine increases their input resistance, induces membrane depolarization and consequently augments their excitability. Importantly, this glycinergic conductance is greatly enhanced in a strychnine-sensitive fashion, by behaviourally-relevant ethanol concentrations, by drugs used for the treatment of alcohol withdrawal and by taurine, an ingredient of certain "energy drinks" often imbibed with ethanol. These findings identify extrasynaptic GlyRs as critical regulators of DR excitability and a novel molecular target for ethanol.

Keywords

Glycine - tonic inhibition- alcohol- serotonin-dorsal raphe

Introduction

Alcoholism is a significant addictive disorder, associated with considerable medical, social and economic costs (Nutt *et al,* 2010). The behavioural effects of ethanol include euphoria, loss of motor control, sedation and anxiolysis (Harris *et al,* 2008; Mihalek *et al,* 2001). The mesolimbic dopaminergic system has been implicated in the rewarding properties of ethanol (Gonzales *et al,* 2004). However, animal and clinical studies identify an additional important role for serotonergic neurotransmission originating from the raphe nuclei (Lanteri *et al,* 2008; Lemarquand *et al,* 1994a & b; Lyness and Smith, 1992). Recombinant receptor studies reveal the function of certain $GABA_A$ receptors ($GABA_ARs$) and strychnine-sensitive glycine receptors (GlyRs) to be enhanced by behaviourally relevant concentrations (10 – 100 mM) of ethanol, suggesting these inhibitory receptors may be alcohol targets (Harris *et al,* 2008; Perkins *et al,* 2010). Although the dorsal raphe (DR) is implicated in rewarding and addictive behaviour (Kranz *et al,* 2010), the influence of ethanol on glycinergic and GABAergic inhibition in the DR neurons has not been assessed. Here, we have utilised electrophysiology and immunohistochemistry to investigate the physiological and pharmacological properties of mouse serotonergic DR $GABA_ARs$ and GlyRs. We reveal that these neurons utilise a synergistic organisation for inhibition whereby $GABA_ARs$ primarily mediate synaptic "phasic" inhibition, but act in concert with extrasynaptic GlyRs, which provide a large "tonic" inhibition that profoundly decreases neuronal excitability. Importantly, although a behaviourally relevant concentration (30 mM) of ethanol had little effect upon $GABA_AR$-mediated synaptic inhibition, it greatly and selectively enhanced the extrasynaptic glycinergic tonic and phasic conductance, thereby suppressing DR neuron firing. Furthermore, this GlyR-mediated conductance was greatly increased by clomethiazole, used clinically to treat alcohol withdrawal (Williams and McBride, 1998), sarcosine, which inhibits the GlyT1 transporter, an action reported to decrease ethanol intake and preference in rats (Molander *et al,* 2007) and by taurine, an ingredient of certain "energy drinks" often consumed with ethanol.

Collectively, our findings reveal a crucial role for DR extrasynaptic GlyRs in influencing neuronal excitability. Our pharmacological studies suggest an interaction with DR GlyRs may contribute to the behavioural actions of ethanol and the efficacy of certain treatments for alcoholism. These extrasynaptic receptors should allow a better understanding of how ethanol influences behaviour and may represent a new target to treat alcohol abuse (Li *et al,* 2012; Yevenes and Zeilhofer*,* 2011).

Materials and Methods

Husbandry Mice were group housed throughout and were given free access to food and water in a vivarium at 21 ± 2 ^{o}C and 55% humidity. The holding room lights were on between 0600 hr. and 1800 hr. daily. The $\delta^{0/0}$ mice, together with wild type (WT) control mice utilized for immunocytochemical analysis were generated on a single C57BL6 background as described previously (Mihalek *et al,* 2001). All procedures involving experimental animals were performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and under the auspices of the Project Licence PIL 60/4005.

Slice Preparation and Electrophysiology Dorsal raphe (DR) or nucleus accumbens (NAcc) slices were prepared from C57BL6 mice (P17–30) of either sex. Mice were killed by cervical dislocation. The brain was removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing, in mM, 126 NaCl, 26 $NaHCO_3$, 10 $MgSO_4$, 10 Glucose, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, pH 7.4 when bubbled with 95% O_2, 5% CO_2. Coronal sections (300 μm) were cut using a Vibratome Series 1000 microtrome (Intracel) and incubated in oxygenated aCSF at room temperature for a minimum of one hour.

Whole-cell patch-clamp recordings were made from DR or medium spiny neurons of the NAcc core (Dixon *et al,* 2010) visually identified with an Axioskop 2 FS (Zeiss) microscope, equipped with IR-DIC optics. Patch electrodes were prepared from borosilicate glass (Garner Glass Co.) with an open tip resistance of 3–5 MΩ when filled with intracellular solution (ICS) containing, in mM, 140 CsCl, 10 HEPES, 10 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 2 Mg-ATP and 5 QX-314 (pH 7.2-7.3, 305–310 mOsm).

Using an Axopatch-1D amplifier (Molecular Devices) in the whole-cell configuration of the patch-clamp recording technique, miniature inhibitory post-synaptic currents (mIPSCs) and the holding current were recorded at a holding potential (V_H) of -60 mV and 35 °C in an extracellular solution (ECS) containing in mM, 126 NaCl, 26 $NaHCO_3$, 10 glucose, 2.95 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 1.25

NaH_2PO4 (306-309 mOsm) in the presence of 2 mM kynurenic acid and 0.5 μM tetrodotoxin (TTX). $GABA_A$ and GlyR mIPSCs were isolated using selective antagonists of the GlyR, (strychnine hydrochloride), and the $GABA_AR$ (bicuculline methobromide) respectively.

Cell-attached recordings were made at 30 °C, in the absence of a glutamate receptor antagonist, in ECS containing the α_1 adrenergic receptor agonist phenylephrine, to elicit regular neuronal firing (Judge *et al,* 2004). Patch electrodes were filled with ECS. Action currents were recorded with a voltage-clamp that maintained a zero pA leak current (Perkins, 2006). Whole-cell current-clamp recordings were made at 30 °C in ECS. Patch electrodes were filled with a potassium gluconate-based ICS, composed of (in mM) 130 K gluconate, 10 HEPES, 10 EGTA, 2 Mg-ATP, 1 NaCl, 1 $MgCl_2$ and 0.5 Na-GTP (Sigma), (pH 7.2, 300-310 mOsm adjusted with D-mannitol). The liquid junction potential was corrected (Neher, 1992).

Data analysis Recordings were acquired *via* digital audiotape using a Sony PCM-R300 and a DRA-200 interface (BioLogic) and analysed offline with Strathclyde Electrophysiology Software, Electrophysiology Data Recorder / Whole cell Analysis Program (WinEDR / WinWCP; courtesy of Dr. John Dempster, University of Strathclyde, U.K.). Details of the data analysis are supplied in the Supplementary Information.

Reagents and drugs All reagents were obtained from Sigma-Aldrich-RBI, Tocris, or VWR unless otherwise stated. GABA, strychnine hydrochloride, sarcosine, clomethiazole (all from Sigma-Aldrich-UK), bicuculline methobromide (Tocris, Bristol, UK) and GES (Toronto Research Chemicals Inc., Canada) were prepared as aqueous stock solutions and then diluted to the required concentration in ECS. Cyclothiazide (Tocris, Bristol, UK) was prepared as a 1000 fold concentrated stock in DMSO and diluted to the desired concentration with ECS. The final DMSO concentration (0.1%) had no effect upon the GlyR-mediated response. All drugs were applied to the brain slice *via* the perfusion system (3-6 ml/min) and allowed to infiltrate the slice for a minimum of 10 min. before recordings were acquired. Because of the low frequency of GlyR mIPSCs, the effect of

ethanol upon GlyR mIPSCs properties was evaluated by comparing GlyR mIPSCs recorded from slices pre-treated for 10 minutes with ethanol (30 mM) with those recorded from slices incubated in ECS for the same duration.

Statistical analysis All data are presented as the arithmetic mean ± S.E.M. (standard error of the mean). The statistical significance of measurements was calculated using the Student's *t*-test (paired or unpaired), regular ANOVA, or repeated measure ANOVA (for normalised data) using Microsoft Excel and SigmaStat as appropriate. The non-parametric Kolmogorov-Smirnoff (KS) test (SPSS v15) was used to compare the inter-event interval (IEI) in control recordings and after drug application and, with the exception of the effect of ethanol upon GlyR mIPSCs (see above), to compare populations of individual events (mIPSCs) prior to and after drug application to a given cell using a stringent criterion for significance ($P < 0.01$).

Immunohistochemistry

Tissue preparation for immunohistochemistry The data presented in this study is derived from two WT and two $\delta^{0/0}$ adult male mice. Anaesthesia was induced with isoflurane and maintained with urethane (1.25 g/kg of bodyweight; i.p.). The animals were perfused transcardially with 0.9 % saline solution for 3 min, followed by a 15 min fixation with 1 % paraformaldehyde and 15 % v/v saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4. The brains were kept in the same fixative solution overnight at 4 °C. Coronal sections of the DR and NAcc, 50 µm thick, were prepared on a Vibratome and stored in 0.1 M PB containing 0.05 % sodium azide.

Immunohistochemical reactions. Visualisation of biocytin-filled cells To confirm that the recorded cells were serotonergic, biocytin was added to the intracellular electrolyte of the patch electrode. Immunohistochemistry for tryptophan hydroxylase (TPH) -to identify the DR- and visualization of biocytin (to identify the labelled cell) was performed according to our previous protocols (Swinny *et al,* 2010).

"Visualisation" of glycine receptor expression The immunohistochemical protocols are as described previously (Corteen *et al,* 2011). The following antibodies were used: 1) a mouse

monoclonal antibody mAb4a against the GlyR, (1:2000; Pfeiffer *et al,* 1984; a gift from Heinrich Betz, Max-Planck Institute for Brain Research, Frankfurt, Germany); 2) a polyclonal antibody against neuroligin2 used to visualise inhibitory synapses, (1:1000; Briatore *et al,* 2010; Synaptic Systems, catalogue number 129203); 3) a polyclonal antibody against TPH used to visualise serotonergic neurons, (1:3000) (Millipore, catalogue number AB1541); 4) a polyclonal antibody against dopamine and adenosine 3',5'-monophosphate-regulated phosphoprotein of 32 kDa (DARPP-32) used to visualise MSNs of the NAcc (1:250; Santa Cruz, catalogue number sc-8483) and; 5) a polyclonal antibody against the δ subunit of the $GABA_AR$ (a gift from Werner Sieghart). Sections were examined with a confocal laser-scanning microscope (LSM710; Zeiss) using a Plan Apochromatic 100x DIC oil objective (NA1.46). All images presented represent a single optical section.

Quantification of the density of NL2 and GlyR clusters and their relative proportion of co-localisation on TPH or DARPP-32 immunopositive profiles Tissue from two animals (4 DR sections and 4 NAcc sections, 2 sections *per* region *per* animal) was used to quantify the density (number of clusters *per* 1000 μm^2) of NL2 and GlyR clusters and the proportion of GlyR clusters which co-localise with NL2 according to previously published methods (Corteen *et al,* 2011). Three fields of view (FOV) were randomly selected within the midline of the DR, or the core of the NAcc of each tissue section and Z-stacks consisting of three optical sections were acquired for each FOV (n = 9 optical sections *per* section of tissue). Within a FOV, the number of puncta within an optical section and the number of puncta which co-localised, was manually counted using ImageJ software. The means ± SD for all fields of view within, between sections and between animals were compared for statistical differences using Kruskal–Wallis one-way analysis of variance. These values were then pooled since there were no statistical differences ($P > 0.05$) between the different FOV, between sections and between animals. The mean ± SD density data presented are thus derived from a total of 36 optical sections for the DR and 36 for the NAcc.

Results

Phasic and tonic inhibitory transmission in serotonergic dorsal raphe neurons.

All recordings were made from DR neurons situated medially throughout the rostral caudal axis. In the mouse the large majority of these neurons are serotonergic, with GABAergic neurons restricted to the lateral wings (Brown *et al,* 2008; Calizo *et al,* 2011). Confirmation that the recorded cells were serotonergic was obtained by *post-hoc* immunohistochemical analysis of biocytin-filled cells (**Figure 1a**) and by current-clamp recordings, revealing the focal application of 5-HT (100 μM) to produce a membrane hyperpolarisation of such neurons (data not shown). To characterise neuronal phasic inhibition *i.e.* mediated by the transient activation of synaptic receptors, DR neurons were voltage-clamped (-60 mV) to record miniature inhibitory postsynaptic currents (mIPSCs-**Figure 1b)**. The frequency of such events was greatly reduced by bicuculline (30 μM), confirming that they are primarily mediated by synaptic $GABA_A$Rs. However, in the presence of bicuculline and the ionotropic glutamate receptor antagonist kynurenic acid, mIPSCs were still evident, albeit at a low frequency. Such events were abolished by strychnine (0.5 μM), demonstrating that they are mediated by synaptic GlyRs (**Figure 1b**). Although both $GABA_A$R- and GlyR-mediated mIPSCs were evident for all DR neurons, their properties were distinct. In particular, the decay times of glycine-mediated mIPSCs were ~ 50% less than those mediated by GABA and, in agreement with the limited glycinergic innervation of the DR (Rampon *et al,* 1999), their frequency of occurrence was considerably lower (**Figure 1c**, **Table S1A**). Note that gender did not influence the properties of either $GABA_A$R, or GlyR-mediated mIPSCs and data were therefore pooled (**Table S1A**).

To investigate whether DR neurons exhibit a tonic inhibitory conductance *i.e.* mediated by ambient transmitter activating either $GABA_A$Rs and/or GlyRs (Farrant and Nusser, 2005), the effect on the holding current (Vh = -60 mV) of bicuculline (30 μM), or strychnine (0.5 μM) respectively, was determined. The $GABA_A$R antagonist induced an outward current (26 ± 8 pA; n = 5) in only 28% of neurons (n = 5 of 18 tested). Bicuculline-insensitive neurons may nevertheless express extrasynaptic $GABA_A$Rs that are inactive, due to low ambient GABA. However, the bath

application of 10 μM GABA, a concentration greater than that usually employed to activate extrasynaptic $GABA_A$ receptors (Scimemi *et al,* 2005; Wlodarczyk *et al,* 2013), did not induce a change in the holding current, or RMS (**Figure S1a**), suggesting that only a limited neuronal population express extrasynaptic $GABA_A$Rs. Moreover, after the application of GABA, the bath application of bicuculline (30 μM) revealed a small tonic current in only ~30% of the neurons tested (data not shown). By contrast, in the absence of exogenous glycine, 62% of neurons were sensitive to strychnine (31 of 50 tested), producing an outward current for responsive neurons (-45 ± 7 pA; n = 31), considerably greater than that produced by bicuculline (**Figure 2a, top trace and b**). Furthermore, the bath application of glycine induced an inward current in 90 % (30 μM; -64 ± 8 pA, 44 of 49 tested) and 100 % (100 μM; -273 ± 66 pA, n = 10) of neurons tested (**Figure 2a, bottom trace and b**). Gender did not influence the glycinergic tonic conductance, the amplitude of the glycine-evoked current, or the percentage of sensitive neurons and thus, data were pooled (**Table S1B**). Similarly, the focal pressure application of glycine (15 psi, 10-30 ms duration, 300 μM) induced an inward current for all neurons tested (-120 ± 19 pA, n = 5). Collectively, these findings reveal all serotonergic DR neurons to express GlyRs and, for the majority of neurons, these receptors mediate a large resident tonic current.

Confocal immunohistochemistry was used to further investigate the expression of GlyRs in the DR and to elucidate the proportion of receptors expressed within synapses as indicated by the inhibitory synaptic marker, neuroligin 2 (NL2; Varoqueaux *et al,* 2004). Labelling of GlyRs with the well-characterised mouse monoclonal pan antibody mAb4a (Pfeiffer *et al,* 1984) revealed numerous, membrane bound immunoreactive clusters on somatic and dendritic compartments of tryptophan hydroxylase (TPH)-immunopositive cells (**Figure 3a, b and c**), with the glycinergic clusters being similar to those described for other brain regions *e.g.* brain stem (Lorenzo *et al,* 2007). However, the majority of GlyR immunopositive puncta did not co-localise with NL2 puncta (**Figure 3a, b & c**). Thus, quantitative analysis of GlyR and NL2 immunopositive clusters revealed a mean cluster density of 75 ± 14 and 54 ± 12 (clusters/1000 μm^2) respectively, although only ~ 41% (31 ± 11

/1000 μm^2) of GlyR clusters co-localised with NL2 clusters. This finding contrasts with the comparative labelling density of GlyR clusters determined on medium spiny neurons (MSNs) of the nucleus accumbens (NAcc - **Figure 3d, e and f**), made under identical conditions in the same animals. The quantified density of GlyR clusters in the NAcc was 25 ± 8/1000 μm^2, which equates to only 33% of the GlyR cluster density measured in the DR. However, 61% (14 ± 6 /1000 μm^2) of GlyR puncta in these neurons co-localised with NL2, suggesting that the level of expression, as well as the trafficking of GlyRs between synaptic and extrasynaptic compartments, is cell-type specific (see below).

The influence of the resident glycinergic tonic conductance on DR neuronal excitability was investigated by utilising strychnine. In both current- and voltage-clamp recordings this GlyR antagonist (1 μM) increased the input resistance in 8 of 9 neurons tested (**Table S2**). In these 9 neurons, strychnine depolarised 7 cells by an average of 6 ± 1 mV (**Table S2**), with no change in the remaining 2 cells. To further assess the impact of GlyR activation upon the excitability of DR neurons, cell-attached voltage-clamp recordings of action currents were made (Perkins, 2006). To mimic the physiological noradrenergic input to the DR and produce regular neuronal firing, recordings were made in the presence of the α1-adrenoceptor agonist phenylephrine (Judge *et al,* 2004). Focally applied glycine (300 μM) induced a transient, cessation of DR neuronal firing, which was sensitive to block by strychnine, in all cells tested (control inter-event interval (IEI) = 0.27 ± 0.02 s; glycine IEI = 0.85 ± 0.11 s, $P < 0.01$, n = 7 - **Figure 4a and b**). Additionally, in current-clamp recordings, in 7 of 9 cells tested strychnine (1 μM) produced a leftward shift of the input-output relationship and reduced the minimum current required to elicit action potential firing i.e. the rheobase, thus increasing the cell excitability (rheobase, control = 71 ± 16 pA; + strychnine = 48 ± 14 pA, n= 7, $P < 0.05$; see Materials and Methods and **Figure 4c and d**).

Dorsal raphe glycine receptors: a relevant ethanol target?

Independently, both the DR and GlyRs have been implicated in the actions of ethanol (Aguayo and Pancetti, 1994; Eggers and Bergers, 2004; Lemarquand *et al,* 1994a & b; Mascia *et al,* 1996; Perkins *et al,* 2010; Pistis *et al,* 1997; Valenzuela *et al,* 1998). Supporting a contribution of DR GlyRs to the behavioural actions of alcohol, the bath application of a concentration of ethanol (30 mM), which results in mild intoxication, induced an inward current (ΔI = -43 ± 18 pA; n = 6; **Figure 5a,c**), which was blocked by the subsequent application of strychnine, and an associated increase in membrane current noise (ΔRMS = 4.2 ± 1.1 pA; n = 6) in 6 of 9 (67%) neurons tested (*i.e.* a similar proportion of neurons to those sensitive to strychnine). Furthermore, in the presence of pre-applied glycine (30 μM), ethanol (30 mM) induced an additional strychnine-sensitive inward current in all neurons tested (ΔI = -77 ± 12 pA; n = 7; *i.e.* an ~ 2 fold increase, $P < 0.05$ *vs.* glycine alone- **Figures 5b,c** and **6b**). Confirming ethanol specificity, a mannitol-based (30 mM) hyper-osmotic extracellular solution had no effect on the neuronal holding current, or RMS ($P > 0.05$). This facilitatory effect of ethanol was additionally investigated by the brief, focal application of glycine (300 μM). For all neurons tested, bath applied ethanol (30 mM) clearly enhanced the glycine-evoked current and this effect was quantified as the total charge transfer *i.e.* area under the curve (control = 85 ± 16 fC; ethanol = 166 ± 37 fC; n = 5, $P < 0.05$) of such glycine-evoked currents (**Figure 5d**).

We next investigated whether DR synaptic GlyRs are ethanol sensitive. Ethanol (30 mM) had no effect on the frequency, or amplitude of GlyR-mediated mIPSCs, but caused a clear prolongation of their decay time (**Figure 5e, Table 1**). Hence, both synaptic and extrasynaptic GlyRs of DR neurons are highly sensitive to ethanol. By contrast, ethanol (30 mM) had no effect upon $GABA_AR$-mediated mIPSC kinetics or amplitude, although it produced a modest, but significant increase in their frequency in 4 of 6 neurons tested ($P < 0.01$ *KS* test; 134 ± 3% of control; n = 4, $P < 0.01$- **Figure 5e, Table 1**). Moreover, in the presence of GABA (10 μM), ethanol (30 mM) did not affect the holding current, or the associated RMS (**Figure S1b**). Extrasynaptic $GABA_ARs$ containing the δ

subunit (δ-$GABA_A$Rs) are proposed targets of ethanol (Korpi *et al,* 2007; Mody *et al,* 2007). However, no δ protein immunoreactivity was detected in DR neurons compared to the dentate gyrus and thalamic relay nuclei (**Figure** S**1c**).

To further assess the impact of ethanol upon the excitability of DR neurons, cell-attached voltage-clamp recordings of action currents were performed in the presence of phenylephrine (10 μM; Judge *et al,* 2004) and bicuculline (30 μM). In the absence of added glycine, ethanol (30 mM) reduced the frequency of action currents in 1 out of 7 cells tested (Control: 3.4 Hz; + Ethanol: 2.5 Hz; $P < 0.01$ by KS test; *i.e.* a 25 % reduction), while having no significant effect in the remaining neurons possibly because glycine levels in the slices are insufficient for ethanol's action to affect phenylephrine-evoked firing. In agreement, bath-applied glycine (100 μM) reduced the frequency of action currents from 2.6 ± 0.2 Hz to 1.2 ± 0.4 Hz (*i.e.* a 54 % reduction; $P < 0.01$ *vs.* control; n = 5, see **Figure 5f** for representative plot). In these cells, the subsequent addition of ethanol (30 mM) further reduced neuronal firing to 0.4 ± 0.4 Hz ($P < 0.05$ *vs.* Gly 100 μM; n = 5, **Figure 5f**). Both effects were sensitive to strychnine (1 μM), which increased the frequency of action current firing to above that of the original control (3.6 ± 0.3 Hz *i.e.* 138 % of control, $P < 0.05$; n = 5, **Figure 5f**). Collectively, these findings identify the DR extrasynaptic GlyR as an important molecular target for ethanol.

Taurine, an agonist of GlyRs and $GABA_A$Rs (Albrecht and Schousboe, 2005), is present in certain "energy drinks" *e.g.* "Red Bull", in substantial amounts (1 g/250 ml/can http://www.canadian-seeker.com/ADHD/Taurine.htm.). Given the popular trend to consume alcohol in combination with such "energy drinks", it is conceivable that ethanol and taurine act in unison to enhance GlyR function. Unfortunately, commercially available taurine is contaminated by glycine (Lape *et al,* 2008). Therefore, we investigated the action of ethanol (30 mM) in the presence of guanidinoethanesulfonic acid (GES), a selective taurine re-uptake inhibitor (Albrecht and Schousboe, 2005; Alexander *et al,* 2011). In the presence of bicuculline (30 μM), to antagonise any $GABA_A$R-mediated contribution by taurine (Albrecht and Schousboe, 2005), GES (300 μM), on all

neurons tested, induced an inward current (-23 ± 3 pA, n = 5), which was further enhanced by 30 mM ethanol (ΔI = -30 ± 13 pA, n = 5, *i.e.* an ~ 2 fold increase, $P < 0.05$ *vs.* GES alone- Figure **6a, b** and **d**). Both the GES and the GES + ethanol-induced currents were blocked by strychnine (0.5 μM). Our observation of an interaction of ethanol and taurine on the DR GlyR tonic current suggests that the popular trend to drink ethanol together with "energy drinks" containing considerable amounts of taurine warrants caution.

We next explored whether known (clomethiazole) and putative treatments (inhibitors of glycine transporters) for alcohol abuse, in common with ethanol, influenced these extrasynaptic GlyRs. Clomethiazole enhances the function of both $GABA_A$Rs and GlyRs (Hales and Lambert, 1992) and in Europe is a common treatment for alcohol withdrawal

(Williams and McBride, 1998). In common with ethanol, the glycine (30 μM)-induced inward current (-67 ± 16 pA) was further enhanced by the subsequent application of clomethiazole (100 μM, ΔI = -55 ± 12 pA in 7 of 8 cell recorded (88%); *i.e.* an ~ 2 fold increase of the glycine (30 μM)-evoked conductance) and blocked by strychnine (0.5 μM) in all neurons tested (**Figure 6b**). However, in contrast to ethanol, clomethiazole (100 μM) additionally enhanced $GABA_A$R-mediated synaptic transmission by increasing the amplitude and prolonging the decay of $GABA_A$R-mediated mIPSCs, but with no effect on their frequency (**Table S3**). A selective GlyT1 inhibitor (ORG 25935) decreases ethanol intake and preference in rats (Molander *et al,* 2007). Here, in all neurons tested, the selective GlyT1 inhibitor, sarcosine (300 μM; Alexander *et al*, 2011) induced an inward current (-85 ± 26 pA, n =11), that was reversed by strychnine (0.5 μM - **Figure 6c** and **d**).

Glycine receptors of the nucleus accumbens are relatively insensitive to ethanol.

Previous reports have indicated the presence of GlyRs in the NAcc (Martin and Siggins, 2002), a finding consistent with our immunohistochemical analysis (**Figure 3d, e & f**). Given the established role of this brain region in addiction and reward (Koob and Volkow, 2010), we explored whether our findings for DR neurons extended to accumbal MSNs. Under identical recording conditions to those utilised for DR neurons, strychnine (0.5 μM) did not reveal any synaptic, or tonic glycinergic

conductance in MSNs. However, on all MSNs tested, the focal application of glycine (1 mM) consistently elicited an inward current (-353 ± 57 pA; n = 16), that was blocked by strychnine (0.5 μM). Similarly, bath applied glycine (100 μM) induced an inward current (-41 ± 4 pA, n = 4) on all neurons tested, albeit of a much smaller magnitude than that produced by this concentration of agonist for DR neurons (-273 ± 66 pA, n = 10; **Figure** S**2a**). However, in contrast to DR neurons, ethanol (30 mM), in the presence of glycine (100 μM), had no significant effect on the glycinergic conductance of MSNs (ΔI = -11 ± 5 pA; ΔRMS = 1.2 ± 0.7 pA; n = 6; $P > 0.05$; **Figure** S**2b**), revealing neuronal specificity for the ethanol-GlyR interaction.

Recombinant expression studies propose ethanol enhancement of GlyR function to be subunit dependent *i.e.* α1 >> α2/α3 (Mascia *et al,* 1996). To explore the subunit composition of GlyRs in the DR and accumbal MSNs, we utilised cyclothiazide, a relatively selective inhibitor of α2- compared to α1-GlyRs (Zhang *et al,* 2008). For DR neurons the inward current evoked by the focal application of glycine was unaffected by the bath application of 100 μM cyclothiazide (101 ± 2% of control, n = 5; $P > 0.05$), but in contrast was reduced (80 ± 3% of control; n = 5; $P < 0.01$) for accumbal MSNs (**Figure** S**2c**). These findings are consistent with the reported abundant and selective expression of α1-GlyR in the DR compared to the NAcc (Jonsson *et al,* 2009; http://mouse.brain-map.org/welcome.do;jsessionid=0CDE823DFD3D02505E0EB0DF5D4C95C4) and provide a parsimonious explanation for the selective modulation by ethanol of the glycinergic conductance in DR, but not accumbal MSN neurons.

Discussion

We reveal that DR serotonergic neurons utilise an unusual synergistic organisation for inhibition, whereby extrasynaptic GlyRs mediate a large "tonic" inhibition, which profoundly decreases neuronal excitability, and acts in concert with synaptic $GABA_A$Rs that primarily mediate fast "phasic" inhibition. Importantly, a behaviourally relevant concentration of ethanol selectively enhances the GlyR phasic and tonic conductance to suppress DR neuronal firing, with little effect upon $GABA_A$R-mediated synaptic or tonic inhibition.

DR neurons exhibit a large extrasynaptic glycinergic tonic conductance, which influences neuronal excitability.

Strychnine-sensitive GlyRs have traditionally been associated with the regulation of inhibition in lower centres of the CNS *e.g.* spinal cord (Yevenes and Zeilhofer, 2011). However, it is now recognised that these receptors are additionally expressed in higher centres, including the hippocampus and prefrontal cortex, where they are primarily expressed extrasynaptically (Badanich *et al,* 2013; Chattiparkon and McMahon, 2002; Keck and White, 2009; Lu and Ye, 2011; Xu and Gong, 2010). For $GABA_A$Rs, tonic inhibition mediated by extrasynaptic $GABA_A$Rs has emerged as a powerful mechanism to influence neuronal excitability of higher CNS centres and an important target for a variety of clinically relevant drugs (Belelli *et al,* 2009; Farrant and Nusser, 2005). By contrast, equivalent evidence for an analogous form of inhibition mediated by extrasynaptic GlyRs is limited. Utilising immunohistochemistry and electrophysiology we reveal that all serotonergic DR neurons express extrasynaptic GlyRs and for the majority of these neurons their receptor expression is sufficient to mediate a large tonic conductance, caused by their activation by ambient endogenous agonist. The neurophysiological significance of this conductance is revealed by the antagonist strychnine, which both depolarized and increased the excitability of these serotonergic neurons, suggesting that this GlyR-mediated tonic conductance will influence 5-HT release. Modulation of anxiety-like behaviour in rodents is associated with region-selective changes in serotonergic transmission in the terminal fields of the DR projections (Rueter and Jacobs, 1996;

Storey *et al,* 2006).Whether in vivo this glycinergic conductance influences 5HT release and consequently influences behaviours such as anxiety provides scope for future investigation.

Ethanol selectively decreases DR neuronal excitability via an action at strychnine- and ethanol-sensitive glycine receptors.

Importantly, a concentration of ethanol impairing physiological functions (30 mM) caused a large enhancement of the GlyR-mediated tonic conductance and a consequent decrease in DR excitability. Interestingly, *in vivo* acute ethanol decreased the firing rate in a proportion (67%) of rat DR serotonergic neurons similar to that affected *in vitro* in the present study (Pistis *et al,* 1997).

Potentially, ethanol could indirectly increase the glycinergic conductance by releasing taurine as a consequence of astrocyte swelling (Albrecht and Schousboe, 2005; Adermark *et al*, 2011). However, we suggest this scenario is unlikely for the following reasons. Firstly, ethanol actions are selective for DR neurons GlyRs, as accumbal GlyRs appeared insensitive, despite ethanol inducing both taurine release and astrocyte swelling in this region (Adermark *et al*, 2011). As taurine will activate GlyRs in the NAcc, these observations strongly suggest ethanol actions to be mediated by a potentiation of GlyR function. Secondly, GlyR mIPSCs are significantly prolonged by ethanol. As taurine would not prolong the synaptic response, this observation suggests that ethanol directly modulates glycine receptors expressed synaptically.

Amongst other putative ethanol candidates, $GABA_A$Rs, particularly extrasynaptic receptors incorporating the δ subunit, have been proposed as clinically relevant targets (Korpi *et al,* 2007; Mody *et al,* 2007). However, our immunohistochemistry revealed no evidence for expression of δ-$GABA_A$Rs in serotonergic DR neurons. Furthermore, the glycine-enhancing effect of ethanol was highly selective. Thus, in common with other reports (Badanich *et al*, 2013; Wietlauf *et al,* 2008), ethanol was ineffective on the few neurons that exhibited a $GABA_A$R-mediated tonic current, although for some neurons it did cause a modest increase in the frequency of $GABA_A$R-mediated mIPSCs.

Is this effect of ethanol neuron selective? Consistent with previous reports for the rat (Martin and Siggins, 2002; Molander and Soderpalm, 2005), our immunohistochemistry reveals expression of GlyRs in mouse accumbens MSNs, albeit at a significantly lower level than in the DR. However, in contrast to the DR, our electrophysiological studies reveal these GlyRs are not tonically active. Furthermore, when activated by added glycine these GlyRs are ethanol-insensitive. This differential action of ethanol may reflect the preferential expression of ethanol sensitive α1-GlyRs in the DR compared to accumbal MSNs (Jonsson *et al,* 2009), where our pharmacological analysis suggests α2-GlyRs to be the dominant isoform. This interpretation is consistent with the reported selectivity of ethanol for α1- *vs.* α2-GlyRs (Mascia *et al,* 1996; Yevenes *et al,* 2010) and the recent report selectively implicating α1-GlyRs in the potentiating actions of zinc on ethanol sensitivity of GlyRs (McCracken *et al,* 2013). Interestingly, some of the actions of ethanol in the NAcc have been proposed to be secondary to an elevation in the dopamine (DA) outflow, mediated by strychnine-sensitive GlyRs located on accumbal GABA-ergic MSNs (Chau *et al,* 2010). Our findings would appear inconsistent with this interpretation, although it remains possible that ethanol elevates DA content in the NAcc *via* a distinct strychnine-sensitive GlyR isoform (other than α2), at locations other than those on MSN neurons *e.g.* on GABAergic terminals impinging on the dopaminergic cell body within the VTA, or dopaminergic terminals within the NAcc. The former suggestion is supported by the proposal that glycine regulation of dopamine levels requires activation of dopaminergic cell bodies (Hernandes *et al,* 2007; Molander and Soderpalm, 2005). Furthermore, in support, intra-VTA injections of glycine decreases ethanol consumption and preference in rats in a strychnine-sensitive fashion (Li *et al,* 2012).

Could these extrasynaptic GlyRs be a behaviourally relevant ethanol target? Given that blockade of the tonic current by strychnine is associated with an increase in neuronal excitability, it is conceivable that ethanol enhancement of this conductance and the associated decrease of excitability may reduce 5-HT release and consequently influence behaviour. Indeed, drugs that reduce ethanol intake, interfere with the serotonergic system (Lanteri *et al,* 2008; Lemarquard *et al,*

1994a,b). The terminal fields of the DR serotonergic neurons include prefrontal cortex, hippocampus, amygdala and NAcc, all areas implicated in the mediation of anxiety, reward and alcohol action (Koob and Volkow, 2010; Kranz *et al,* 2010; Vengeline *et al,* 2008). Furthermore, a reduction in 5-HT activity in the mPFC is associated with reduced ethanol intake (Deckel *et al,* 1997) and a paradigm that decreases anxiogenic-like behaviour in rodents is associated with a reduction of 5-HT release in the mPFC (Storey *et al,* 2006). Therefore, we speculate that ethanol enhancement of DR GlyR function may influence serotonergic activity and consequently contribute to the anxiolytic properties of this alcohol. Future behavioural studies are required to explore this concept.

Summary.

Collectively, we provide the first demonstration that the activity of DR serotonergic neurons is greatly influenced by a tonic conductance mediated by extrasynaptic GlyRs. Importantly, this inhibitory conductance is selectively enhanced by ethanol. A schematic representation of the proposed role of inhibitory transmission mediated by GlyRs in the mouse DR and its influence on neuronal excitability is depicted in **Figure 7**.

Given that the DR is implicated in stress, anxiety and reward, these extrasynaptic GlyRs may be a clinically relevant target for ethanol (Baer *et al,* 2003), and may provide the impetus to develop GlyR-based therapeutics for the treatment of alcohol withdrawal. This concept is supported by the demonstration that both clomethiazole (a current treatment for alcohol withdrawal) and a GlyT1 inhibitor (active in animal models of alcohol withdrawal) greatly enhance the DR tonic conductance at clinically relevant concentrations (Harris *et al,* 2010).

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Table 1: A comparison of the effect of 30 mM ethanol (EtOH), upon the properties of GlyR- and $GABA_AR$-mediated mIPSCs.

	Control GlyR mIPSCs (n=14)	+30 mM EtOH GlyR mIPSCs (n=5)	Control $GABA_AR$ mIPSCs (n=6)	+30 mM EtOH $GABA_AR$ mIPSCs (n=6)
Peak Amplitude (pA)	-69 ± 4	-69 ± 4	-66 ± 6	-65 ± 2
Rise Time (ms)	0.6 ± 0.1	0.7 ± 0.1 *	0.6 ± 0.1	0.6 ± 0.1
τ (ms)	3.8 ± 0.2	5.8 ± 0.3 ***	5.5 ± 0.3	6.2 ± 0.4
Frequency (Hz)	0.4 ± 0.1	0.8 ± 0.6	1.5 ± 0.3 $^{+\ddagger}$	2.0 ± 0.4 $^{+\ddagger}$

* $P < 0.05$, *** $P < 0.001$, unpaired Student's *t* test, comparing control and +30 mM ethanol for GlyR mIPSCs; ‡ $P < 0.01$, paired Student's *t* test for $GABA_A$ mIPSCs $^{+}$ n = 4.

Figure legends

Figure 1 The majority of mIPSCs recorded from serotonergic DR neurons are mediated by synaptic $GABA_A$Rs. (**a**) A representative image of one of the recorded and filled DR neurons indicating that it is a serotonergic neuron. (i) Shows biocytin labelling within the recorded cell, (ii) illustrates immunoreactivity for tryptophan hydroxylase (TPH), a marker for serotonergic neurons, (iii) the overlay confirms that the recorded cell expresses TPH. Scale bar 10 μm. (**b**) Illustrated are sections (2 s duration) of whole-cell voltage-clamp recordings of mIPSCs obtained from a representative DR neuron in the presence of 2 mM kynurenic acid and 0.5 μM TTX before (top trace) and after the bath application of the $GABA_A$R antagonist bicuculline (BIC, 30 μM-middle trace) or the combined application of bicuculline (30 μM) and the glycine receptor antagonist strychnine (STRY, 0.5 μM - bottom trace). The majority of mIPSCs are blocked by bicuculline, indicating that they are mediated by $GABA_A$Rs. The remaining mIPSCs are mediated by GlyRs as demonstrated by their blockade following the application of strychnine. Note the higher frequency and slower decay of the $GABA_A$Rs compared to GlyRs mIPSCs. (**c**) Normalised (to peak amplitude) ensemble averages of exemplar $GABA_A$R (black) and GlyR (grey) mIPSCs superimposed on the same scale to illustrate the differences in their decay kinetics. Note the GlyR-mediated mIPSCs exhibit a faster decay, which is approximately half that of the $GABA_A$R-mediated mIPSCs.

Figure 2 DR neurons exhibit a large tonic inhibitory conductance mediated by extrasynaptic GlyRs. (**a**) Representative recordings (V_H = -60 mV) from DR neurons (left), illustrating the outward and inward current induced by 0.5 μM strychnine (STRY, top trace) and 30 μM glycine (GLY, bottom trace), respectively. The corresponding all-points histograms are given to the right of the traces. Note that the exemplar 5-HT neuron illustrated in the top trace exhibits a large tonic conductance as revealed by strychnine, whereas, although the neuron illustrated in the bottom trace exhibits an inward current when challenged with 30 μM glycine, the subsequent application of strychnine only returns the holding current to the pre-glycine level *i.e.* in the absence of added glycine, this particular neuron does not exhibit an endogenous tonic conductance. (**b**) A graph summarising the changes in holding current evoked by strychnine (0.5 μM, n = 31) and glycine (30 and 100 μM, n = 44 and 10 respectively) for the responsive neurons *i.e.* 62%, 90% and 100% for strychnine, glycine 30 μM and glycine 100 μM challenges, respectively. Error bars indicate S.E.M. Labels are the same for calibration bars shown in top and bottom traces.

Figure 3 Distribution of GlyR expression and inhibitory synapses in the DR and NAcc. (a) An intense punctate GlyR staining (red) is evident on the membranes of serotonergic cells stained with TPH (blue) in the DR. (**b**) The inhibitory synaptic marker protein, neuroligin2 (NL2; green) shows punctate staining mainly on the dendrites of TPH-immunopositive cells (blue). (**c**) An overlay of **a** and **b** reveals that some GlyR puncta co-localise with NL2 puncta (yellow). Note that a significant proportion of GlyR puncta, particularly on the TPH dendrites, do not co-localise with NL2, suggesting an extrasynaptic locus of expression. (**d**) In contrast to the DR, putative medium spiny neurons of the NAcc stained for DARPP-32 (blue) show much fewer GlyR immunopositive puncta (red). (**e)** NL2 puncta (green) in the NAcc are located on somata and dendritic compartments. (**f**) In contrast to the DR, the majority of the GlyR puncta co-localise with NL2 in the NAcc (yellow). For each pair of images, the bottom image is a magnified section (defined by the white rectangle) of the corresponding top image. Scale bar of top images, 5 μm; bottom images, 2 μm**.**

Figure 4 In DR neurons extrasynaptic GlyRs greatly influences neuronal excitability. (**a**) A representative cell-attached voltage-clamp recording of action currents illustrating the suppression of their discharge (in the presence of phenylephrine 10 μM) by the focal application (duration 20 ms, pressure 15 psi) of 300 μM glycine (GLY) to an individual DR neuron. (**b**) A bar chart illustrating the mean increase (for 7 neurons tested) of the inter-event interval produced by the focal application of 300 μM glycine. **(c)** Representative whole-cell current-clamp recording of action potentials elicited in response to a subset of current pulses (bottom traces; 60 – 100 pA) in control conditions (top traces) and following the bath application of 0.5 μM strychnine (STRY- lower traces). Note the increase in AP frequency following strychnine application in these exemplar traces. (**d**) A graph depicting the input-output relationship for 7 DR neurons in control conditions (●) and in the presence of strychnine (○). The response is expressed normalised to the averaged maximum number of APs elicited in response to current injection steps. Note that the input-output relationship is shifted to the left in the presence of strychnine, thus indicating an increased neuronal excitability. * $P < 0.05$; ** $P < 0.01$, *vs.* control by paired Student's *t* test. Error bars indicate S.E.M.

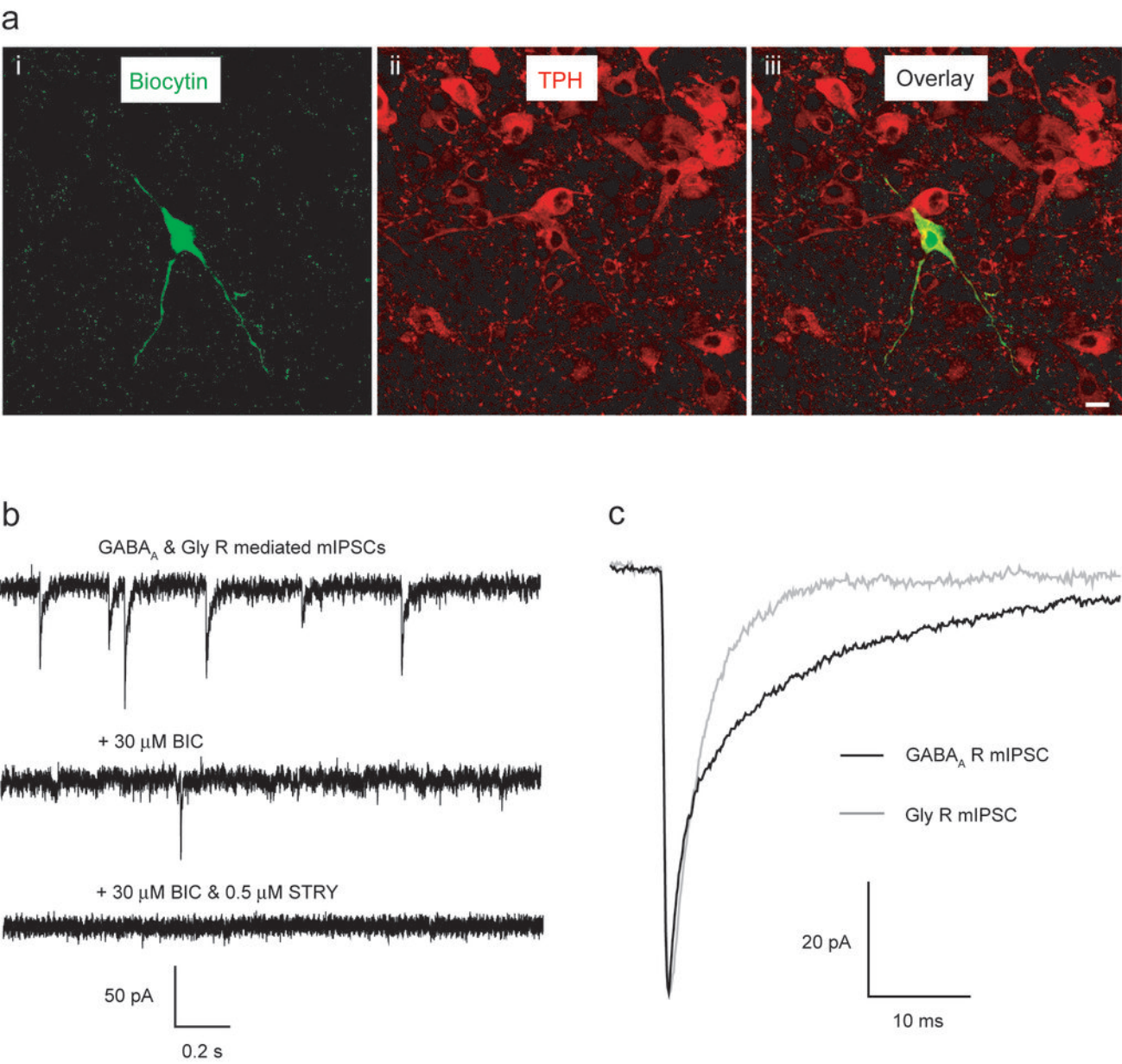
Figure 5 Ethanol enhances the function of DR GlyRs receptors and suppresses neuronal excitability. (**a**) A representative whole-cell voltage-clamp recording illustrating that ethanol (30 mM) - in the absence of added glycine- produces a large inward current, that is reversed by the subsequent application of strychnine (STRY, 0.5 μM) to beyond baseline (see corresponding all point histogram on the right of the trace). Thus, ethanol acts to enhance the endogenous glycinergic tonic conductance. **(b)** A representative whole-cell voltage-clamp recording illustrating that ethanol

(30 mM) - in the presence of 30 μM glycine (GLY)- produces a large inward current, that is reversed by strychnine (STRY, 0.5 μM) to beyond baseline (see corresponding all point histogram on the right). Thus, both the effect of glycine and ethanol are mediated by GlyRs. **(c)** A bar graph summarising the current induced by the bath application of 30 mM ethanol in the absence (white), or the presence of 30 μM glycine (grey) in 6 and 7 DR neurons respectively. Data are derived from ethanol-sensitive neurons only (6 out of 9 and 7 out of 7 in the absence and presence of 30 μM glycine respectively). Note that in the absence of added glycine, the proportion of ethanol-sensitive neurons (67 %) was similar to those sensitive to strychnine i.e. exhibiting an endogenous glycinergic conductance. **(d)** A representative whole-cell voltage-clamp recording of the current induced by the focal application (duration 20 ms, pressure 10 psi) of 300 μM glycine to a DR neuron before and after the bath application of 30 mM ethanol (EtOH). Note that ethanol increases the total charge transfer. **(e)** Representative normalised (to control peak amplitude) ensemble averages of GlyR- (top traces) and $GABA_A$R-mediated (bottom traces) mIPSCs in control conditions (black) and in the presence of 30 mM ethanol (grey). Note that ethanol prolongs the decay of GlyR- but not that of $GABA_A$R-mediated mIPSCs. Labels are the same for calibration bars shown for top and bottom traces. **(f)** A frequency plot depicting the inhibition of action current discharge by 100 μM glycine (in the presence of 10 μM phenylephrine) obtained from a representative recording of a DR neuron. The plot further demonstrates that ethanol (30 mM) enhances this inhibitory effect of glycine. Note that the subsequent application of strychnine not only reverses the inhibitory effects of glycine and ethanol, but increases the frequency of action current discharge to a level greater than control, thus suggesting the presence of a GlyR-mediated inhibitory tone.

Figure 6 The GlyR-mediated tonic current of DR neurons is enhanced by inhibition of the GlyT1 or the taurine transporter, by clomethiazole and by ethanol. (**a**) A representative whole-cell voltage-clamp recording from a DR neuron illustrating the ethanol (EtOH, 30 mM)-mediated increase of the GES (300 μM)-evoked current. Note that both effects are sensitive to block by strychnine (0.5 μM STRY). The corresponding all points histogram is given on the right of the trace. (**b**) A summary graph illustrating the fold increase for the responsive neurons of the 1) glycine (30 μM)-evoked current by: ethanol (30 mM, n = 7; left column) and clomethiazole (100 μM, n = 7; centre column) and 2) the GES (300 μM)-evoked current by ethanol (30 mM, n = 5; right column). Error bars indicate the S.E.M. (**c**) A representative whole-cell voltage-clamp recording from a DR neuron illustrating the GlyR-mediated inward current produced by the bath application of the GlyT1 inhibitor sarcosine (SARC, 300 μM) Note that the current induced by

sarcosine is sensitive to block by strychnine (STRY, 0.5 μM). (**d**) A bar graph summarising the current induced in DR neurons by sarcosine (300 μM, n = 11) and the taurine transporter blocker, GES (300 μM, n = 5). Error bars denote S.E.M.

Figure 7 A model for the role of inhibitory transmission in the mouse dorsal raphe nuclei and the relevance of modulation of the glycinergic conductance to the DR neuronal output. (a) A simplified schematic representation of the topographical organization of dorsal raphe nuclei Illustrating its principal neuronal inputs and outputs. Inhibitory inputs to 5-HT neurons (ochre) are mediated by both $GABA_A$ receptors ($GABA_A$Rs) and strychnine-sensitive glycine receptors (GlyRs). GABAergic inputs (grey) are mainly, but not exclusively of local origin, whereas the glycinergic neuronal inputs (yellow) originate out with the dorsal raphe (*e.g.* PAG and the reticular system). In addition, glial-derived taurine/β-alanine (salmon pink) and glycine can activate the inhibitory tonic conductance (see below). The glycinergic conductance exerts a powerful control over DR neuronal excitability (see traces right). Enhancement of the conductance by glycine, or by ethanol and suppression by strychnine, significantly reduces and increases respectively the action potential discharge of DR 5-HT neurons. **(b)** Our findings indicate that DR 5-HT neurons utilise a unique synergistic organisation for inhibition whereby $GABA_A$Rs (blue) primarily mediate synaptic or "phasic" inhibition, whereas extra-synaptic GlyRs (yellow) provide a large "tonic" conductance that profoundly decreases neuronal excitability (top trace right). A behaviourally relevant concentration (30 mM) of ethanol selectively enhances the extrasynaptic (bottom trace right) and synaptic (left trace) glycinergic conductance to consequently suppress DR neuronal firing [see traces in **(a)**], but has little effect upon $GABA_A$R-mediated synaptic inhibition. The large GlyR-mediated conductance is additionally greatly increased by clomethiazole, which is used clinically to treat alcohol withdrawal (Williams & McBride, 1998) and by sarcosine, which inhibits the GlyT1 transporter, an action recently reported to decrease ethanol intake and preference in rats (Molander *et. al.,* 2007). Moreover, taurine, an ingredient of certain energy drinks, often taken together with ethanol, enhances the effect of ethanol upon the tonic glycinergic conductance (see Discussion for additional details). PFC: prefrontal cortex; LC: locus coreleus; Aq: aqueduct; PAG: peri-aqueductal grey; DR: dorsal raphe; lwDR: DR lateral wing; NAcc: nucleus accumbens; GlyT1: glycine transporter 1; TauT: taurine transporter.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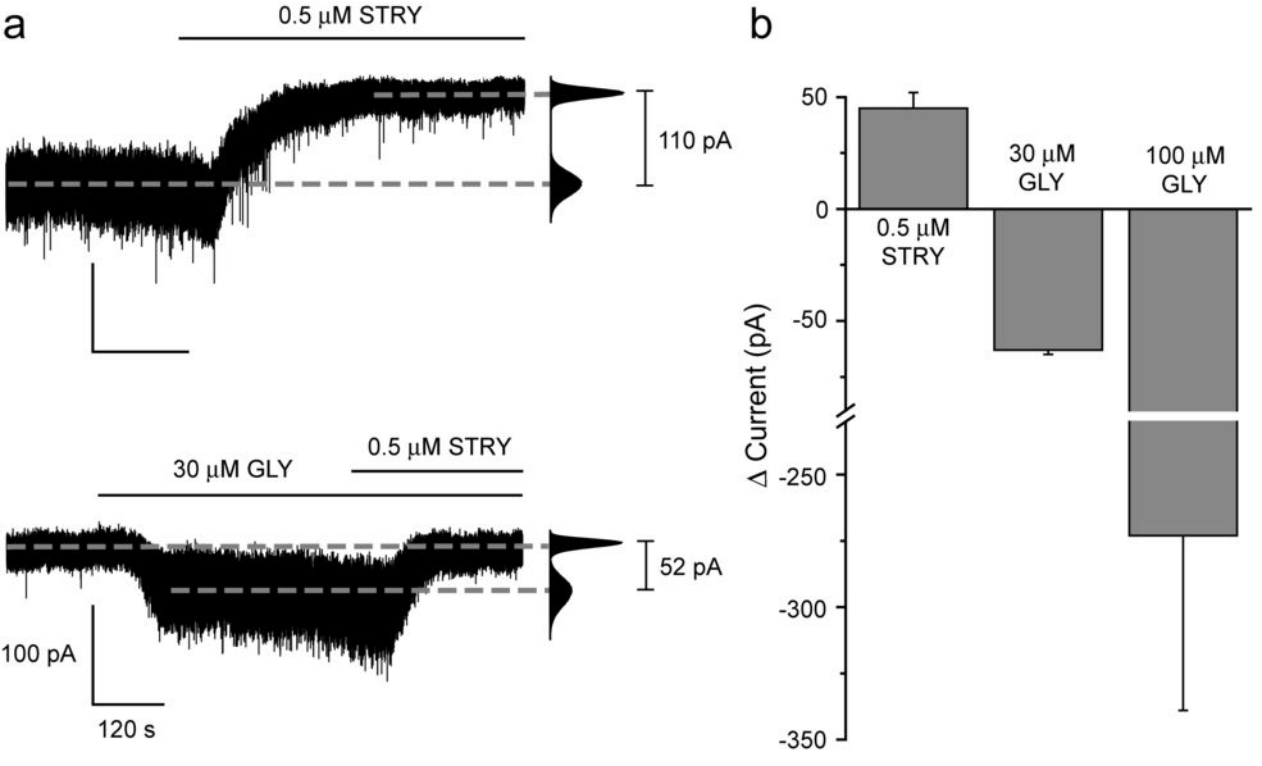
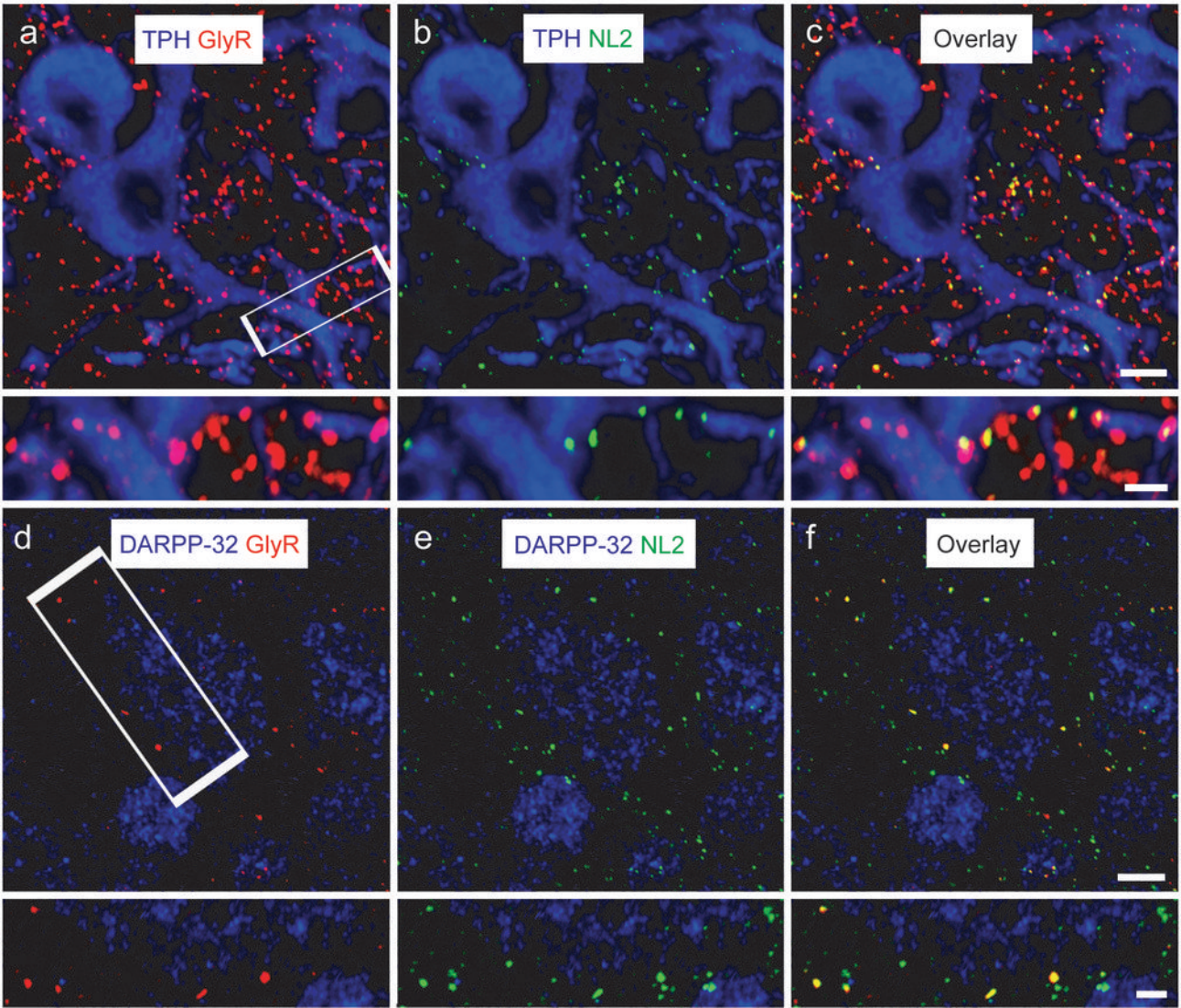
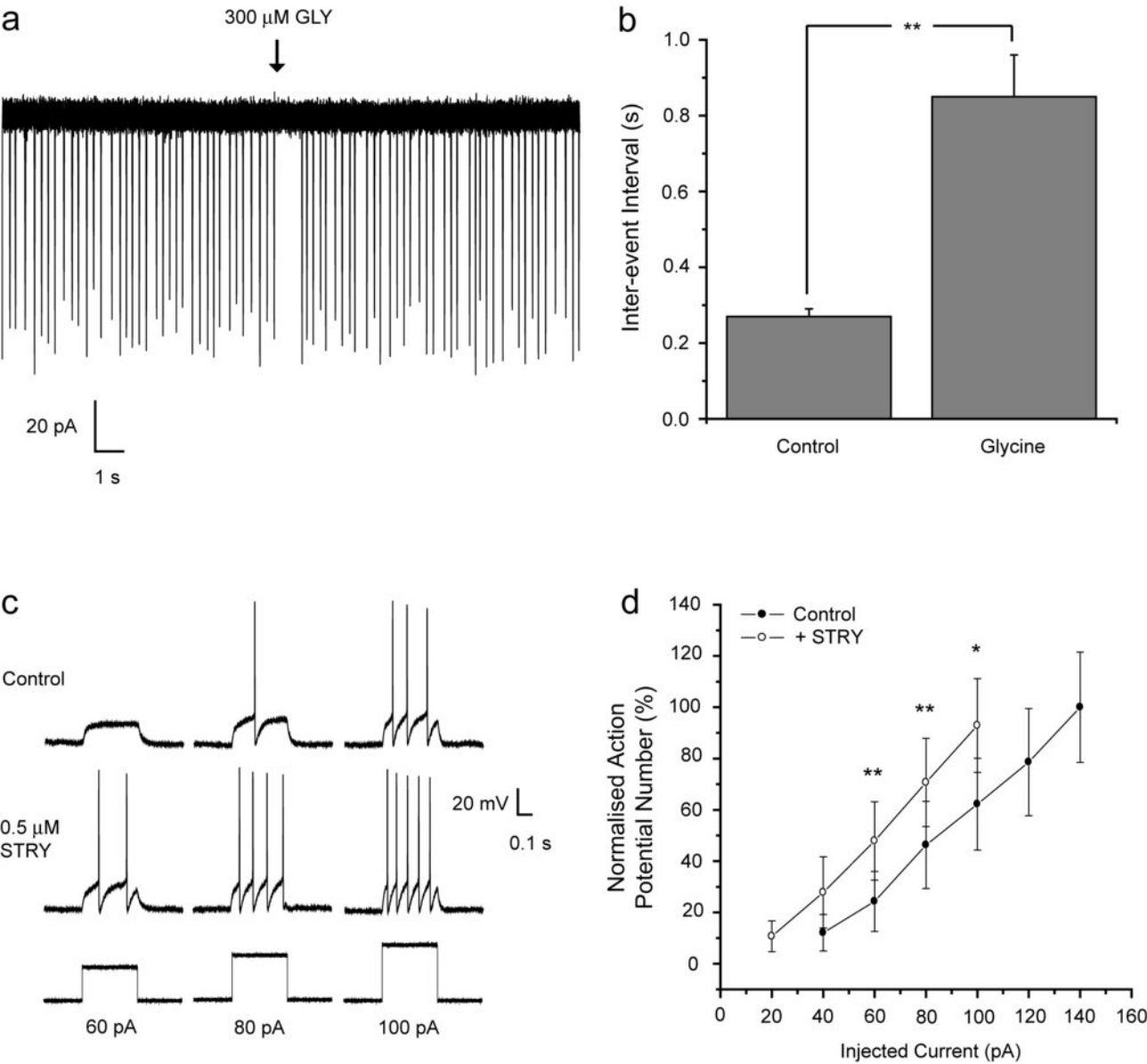
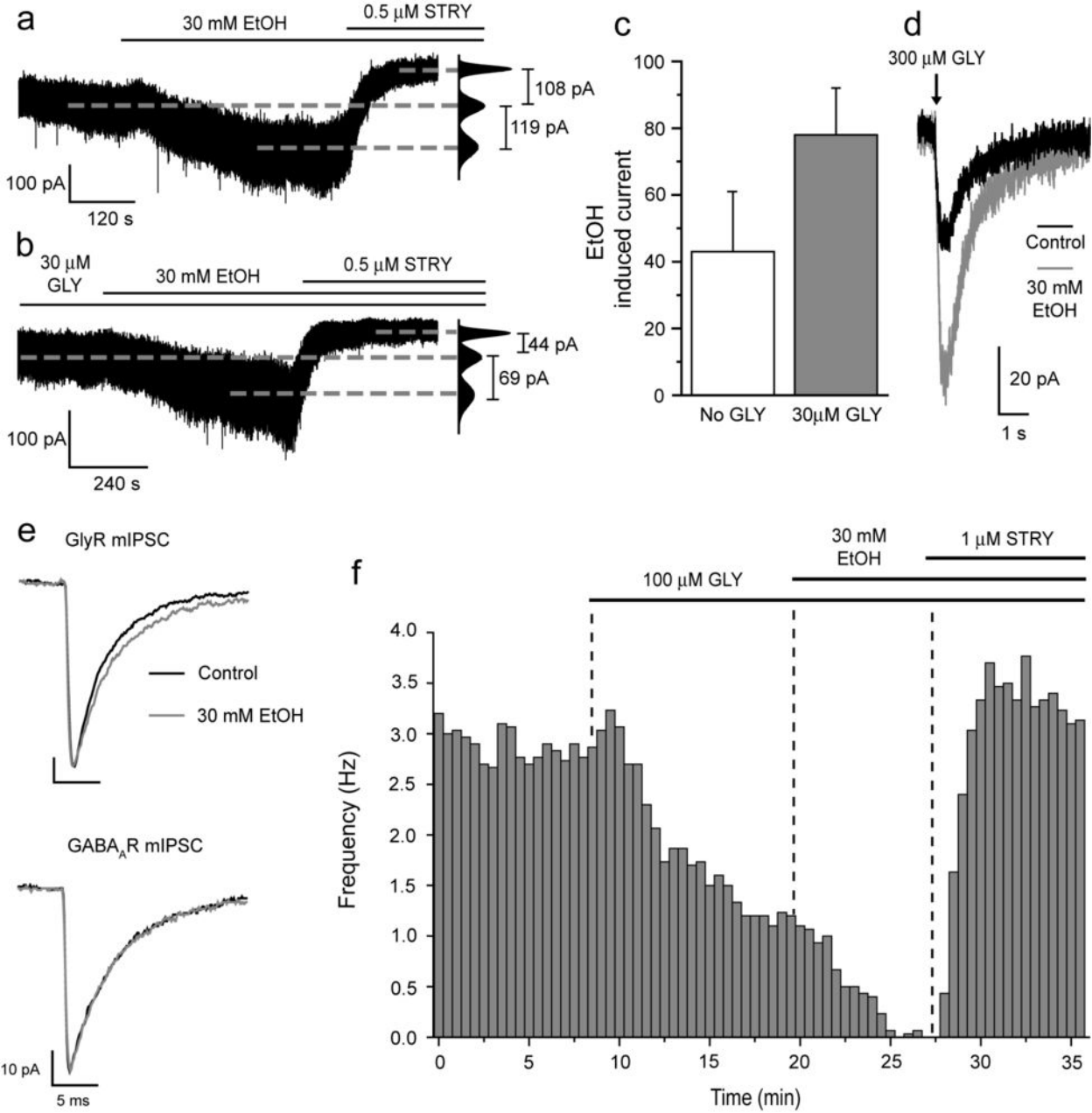
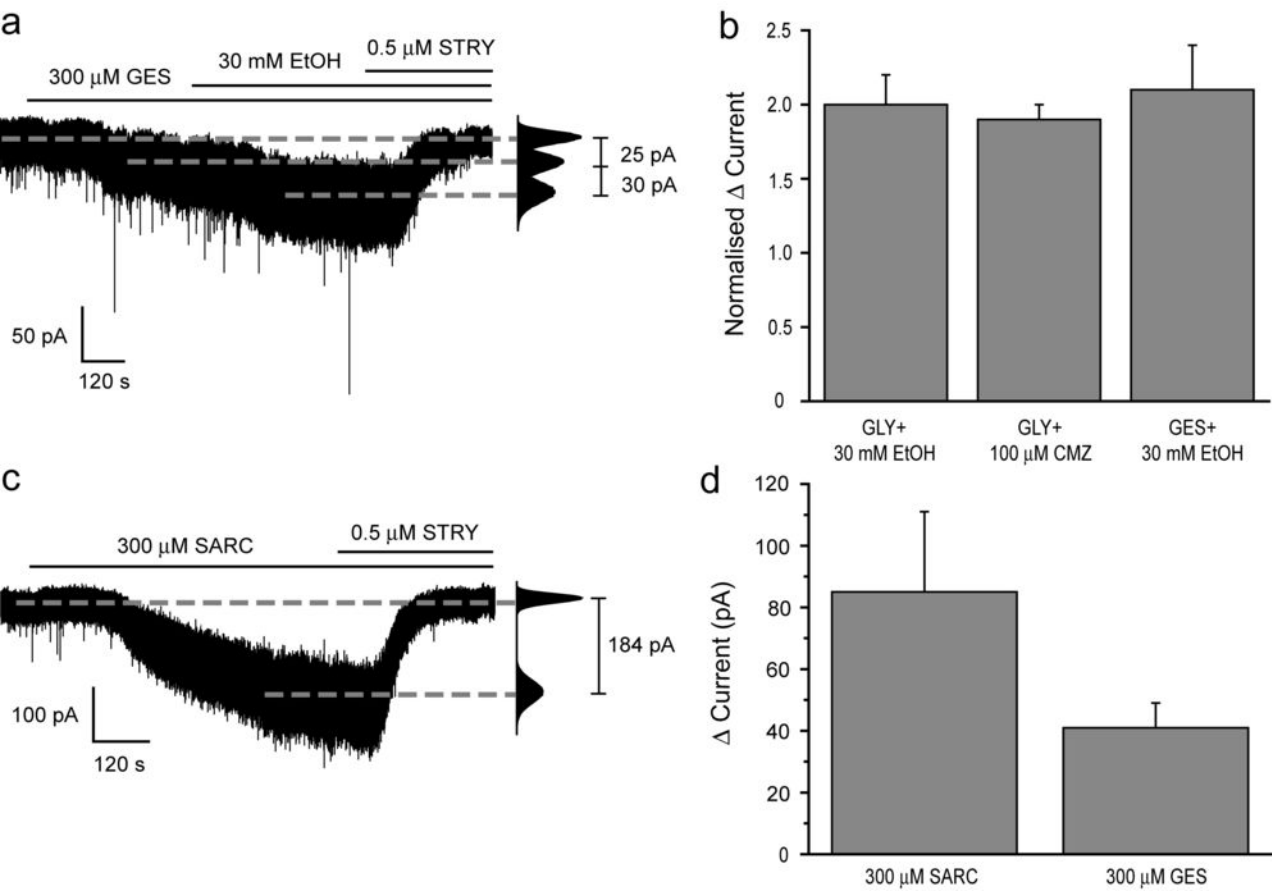
GABA$_A$ & Gly R mediated mIPSCs
+ 30 μM BIC
+ 30 μM BIC & 0.5 μM STRY
50 pA
0.2 s
c
GABA$_A$ R mIPSC
Gly R mIPSC
20 pA
10 ms

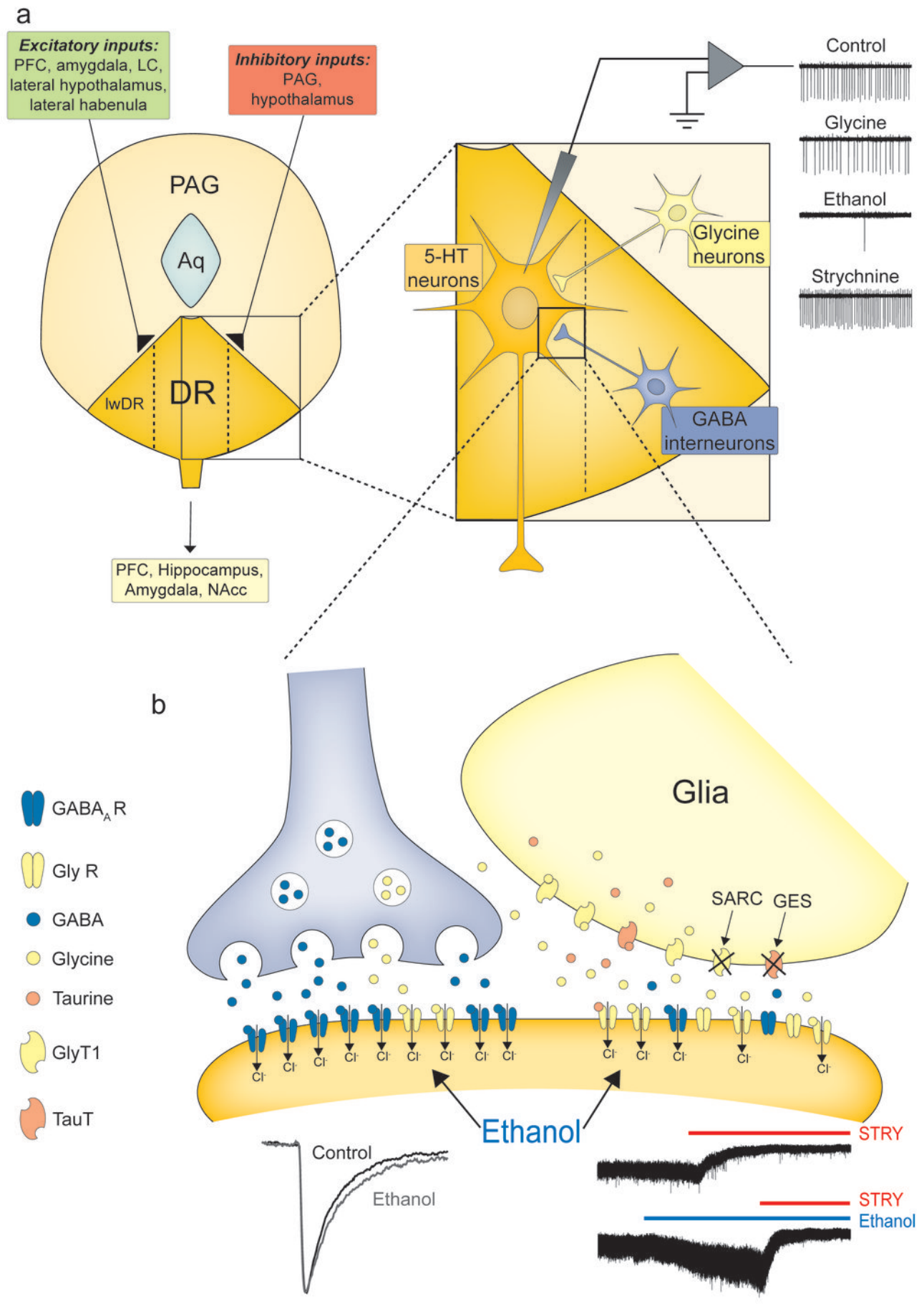

a
0.5 μM STRY
110 pA
30 μM GLY
0.5 μM STRY
52 pA
100 pA
120 s
b
50
0
-50
-250
-300
-350
30 μM GLY
100 μM GLY
0.5 μM STRY
Δ Current (pA)


a
TPH GlyR
b
TPH NL2
c
Overlay
d
DARPP-32 GlyR
e
DARPP-32 NL2
f
Overlay


a
300 μM GLY
20 pA
1 s
b
**
Inter-event Interval (s)
1.0
0.8
0.6
0.4
0.2
0.0
Control
Glycine
c
Control
0.5 μM
STRY
20 mV
0.1 s
60 pA
80 pA
100 pA
d
Control
+ STRY
Normalised Action
Potential Number (%)
140
120
100
80
60
40
20
0
**
**
*
0
20
40
60
80
100
120
140
160
Injected Current (pA)


a
30 mM EtOH
0.5 μM STRY
108 pA
119 pA
100 pA
120 s
b
30 μM GLY
30 mM EtOH
0.5 μM STRY
44 pA
69 pA
100 pA
240 s
c
EtOH induced current
100
80
60
40
20
0
No GLY
30μM GLY
d
300 μM GLY
Control
30 mM EtOH
20 pA
1 s
e
GlyR mIPSC
Control
30 mM EtOH
GABA$_A$R mIPSC
10 pA
5 ms
f
30 mM EtOH
1 μM STRY
100 μM GLY
Frequency (Hz)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35
Time (min)


a
300 μM GES
30 mM EtOH
0.5 μM STRY
25 pA
30 pA
50 pA
120 s
b
Normalised Δ Current
2.5
2.0
1.5
1.0
0.5
0
GLY+
30 mM EtOH
GLY+
100 μM CMZ
GES+
30 mM EtOH
c
300 μM SARC
0.5 μM STRY
184 pA
100 pA
120 s
d
Δ Current (pA)
120
100
80
60
40
20
0
300 μM SARC
300 μM GES


a
Excitatory inputs:
PFC, amygdala, LC,
lateral hypothalamus,
lateral habenula
Inhibitory inputs:
PAG,
hypothalamus
PAG
Aq
DR
lwDR
PFC, Hippocampus,
Amygdala, NAcc
5-HT
neurons
Glycine
neurons
GABA
interneurons
Control
Glycine
Ethanol
Strychnine
b
GABA$_A$R
Gly R
GABA
Glycine
Taurine
GlyT1
TauT
Glia
SARC
GES
Cl-
Ethanol
Control
Ethanol
STRY
STRY
Ethanol