HHS Public Access

Author manuscript

*Neuropharmacology*. Author manuscript; available in PMC 2020 December 01.

Published in final edited form as:

*Neuropharmacology*. 2019 December 01; 160: 107773. doi:10.1016/j.neuropharm.2019.107773.

# High ethanol sensitive glycine receptors regulate firing in D1 medium spiny neurons in the nucleus Accumbens

**S. Gallegos**[1], **B. Muñoz**[2], **A. Araya**[1], **LG. Aguayo**[1,*]

[1]Laboratory of Neurophysiology, Department of Physiology, Universidad de Concepcion, Concepcion, Chile

[2]Present address: Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN, 46202, USA

## Abstract

Inhibitory glycine receptors (GlyRs) are widely expressed in spinal cord and brain stem. They are also expressed in the nucleus Accumbens (nAc) where they have been implicated in the release of dopamine from the ventral tegmental area to the nAc in the presence of ethanol. One of the major types of neurons in the nAc are the Dopamine 1 receptor-expressing (D1+) medium spiny neurons (MSNs) that are activated when addictive drugs, like ethanol, are administrated. Thus, D1(+) MSNs are a relevant target for the study of ethanol effects. Here, using electrophysiological recordings, we report that GlyRs in D1(+) MSNs are highly sensitive to ethanol, with potentiation starting at 5 mM (26±5%). Single channel recordings in D1(+) MSNs showed that 10 mM ethanol increased the open probability of the channel (0.22±0.05 versus 0.66±0.16), but did not affect channel conductance (~40 pS). A glycinergic mediated tonic current in D1(+) MSNs was potentiated by 10 and 50 mM ethanol causing a reduction in the excitability of these cells. A 34±7% reduction in action potential firing was observed in these neurons in the presence of 50 mM ethanol. Interestingly, no effects of ethanol were detected in the presence of strychnine or in D1(−) MSNs in the nAc.

These results indicate that GlyRs present in D1(+) MSNs are sensitive to low concentrations of ethanol, and that potentiation of this inhibitory current regulates the activation of nAc, acting as a homeostatic signal that would prevent over-activation of the reward system when drugs like ethanol are consumed.

### Keywords

ethanol; glycine receptor; nucleus Accumbens; D1 MSNs

*Corresponding Author: Luis G. Aguayo, Universidad de Concepción, PO Box 160-C, Phone: 56 41 2203380 Fax: 56 41 2245975, laguayo@udec.cl, Concepcion, 4030000, Chile.

Conflict of Interest

The authors declare no conflicts of interest.

## Introduction

The nucleus Accumbens (nAc) is one of the most critical regions in the brain reward system because it receives abundant inputs that control its activity. For example, it receives dopaminergic signaling from the ventral tegmental area (VTA) and glutamatergic inputs from the prefrontal cortex (PFC), amygdala, and hippocampus (Russo and Nestler 2013, Volkow and Morales 2015). The principal neurons in the nAc are medium spiny neurons (MSNs) that are inhibitory because they release GABA at their projecting terminals. Two main types of MSNs have been described: D1(+) MSNs that express D1-type dopamine receptors and form part of the direct projection pathway of the nAc, and D2 MSNs (referred to as D1(−) MSNs in this paper) that express the D2-type dopamine receptor and contribute to the indirect pathway (Russo and Nestler 2013). In addition, these pathways have distinctive roles. For example, the direct pathway is primarily associated with reward, whereas the indirect pathway is associated with aversion (Hikida, Kimura et al. 2010, Nakanishi, Hikida et al. 2014). This reward circuit regulates natural rewarding inputs, however, drugs of abuse also activate this system (Koob and Volkow 2016). Independent of the associated mechanism, drugs of abuse including ethanol, cause an increase in the dopamine level released from VTA into the nAc (Chiara and Imperato 1988), and this has been associated to addictive behaviors (Di Chiara 2000). In cellular and molecular terms, addiction can be described as a form of neuroplasticity induced by drugs in different brain regions (Nestler 2001, Volkow and Morales 2015). For example, cocaine produces long term potentiation in glutamatergic synapses especially in D1(+) MSNs (Dobi, Seabold et al. 2011, Bock, Shin et al. 2013), as well as the formation and maintenance of new dendritic spines (Lee, Kim et al. 2006). Interestingly, permanent functional and structural changes have also been reported after prolonged administration or consumption of ethanol in D1(+) MSNs in the dorsomedial striatum (Wang, Cheng et al. 2015, Cheng, Huang et al. 2017), and in D1(+) MSNs in the nAc after chronic intermittent ethanol vapor exposure (Jeanes, Buske et al. 2011, Renteria, Maier et al. 2017). For this reason, D1(+) MSNs are an important subtype of neurons to study the effects of clinically relevant acute concentrations of ethanol.

Glycine receptors (GlyRs) are inhibitory receptors widely expressed in the spinal cord and brain stem. In these regions, the main roles of GlyRs are the control of pain transmission, respiratory rhythms, motor coordination, reflex responses and sensory processing (Burgos, Muñoz et al. 2015). However, the presence of different GlyR subunits (α1–3 and β) has also been reported in upper brain regions in rats (Delaney, Esmaeili et al. 2010, Jonsson, Morud et al. 2012). Additionally, *in vivo* studies demonstrated that activation of GlyRs in nAc, either by application of the agonist or a glycine transporter 1 (GlyT1) inhibitor (Org24598), increased dopamine release to nAc and lowered ethanol consumption, whereas application of strychnine (STN), a highly selective GlyR antagonist, led to a decrease in the dopamine level and an increase in ethanol consumption (Molander, Löf et al. 2005, Molander and Söderpalm 2005, Lidö, Ericson et al. 2011).

We recently reported that GlyRs are expressed in mouse nAc. D1(+) MSNs expressed α1, α2 and β mRNA and these neurons displayed higher current density and sensitivity to glycine than D1(−) MSNs. In addition, we showed that a tonic current induced by GlyR activation in D1(+) MSNs was potentiated by a high (100 mM) concentration of ethanol

resulting in a decrease in action potential firing in these neurons (Förstera, Muñoz et al. 2017). However, it is likely that the addictive actions of ethanol are initiated with much lower concentrations, for instance, those found with 2–3 drinks, namely 10 to 20 mM of ethanol (Pizon, Becker et al. 2007, Abrahao, Salinas et al. 2017). Therefore, in the present study, we wanted to examine if D1(+) MSNs were sensitive to low concentrations of ethanol in the range of those reported to potentiate GlyRs in spinal neurons (Aguayo, Tapia et al. 1996). For this, we used a transgenic mouse that expresses the green fluorescent protein (GFP) under the promoter for the D1 receptor (D1-GFP) and performed electrophysiological recordings to test if the glycine-mediated tonic currents and action potential firing were affected by pharmacological concentrations of ethanol (10 and 50 mM) in D1(+) MSNs. Our findings indicate that GlyRs from D1(+) MSNs are highly sensitive to ethanol. The potentiation of GlyRs by low and moderate concentrations of ethanol reduced neuronal firing in the nAc, suggesting that GlyRs can modulate the excitability of D1(+) MSNs in this brain region.

## Materials and Methods

### Animals

Animal care and experimental protocols for this study were approved by the Institutional Animal Care and Use Committee at the University of Concepción and followed the guidelines for ethical protocols and care of experimental animals established by NIH (National Institutes of Health, Maryland, USA). DRD1-GFP (Tg(Drd1a-EGFP)x60Gsat/Mmmh) transgenic mice with a C57BL/6J background were obtained from the facilities of Dr. David M. Lovinger (National Institutes of Health, Maryland, USA). Female and male mice from 6–8 weeks of age were used for experiments. Mice were housed in groups of 2–4 mice on a 12-h light/dark cycle and given food and water *ad libitum*.

### Preparation of brain slices

Coronal slices containing the nAc 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, Glucose 10, saturated with 95% $O_2$ and 5% $CO_2$ and adjusted to pH 7.4). The brain was cut and glued with the cut surface to the chilled stage of a VT1200S vibratome (Leica, Germany) and sliced to a thickness of 300 μm. Slices containing the nAc were transferred to aCSF solution (in mM: NaCl 124, KCl 4.5, $MgCl_2$ 1, $NaHCO_3$ 26, $NaH_2PO_4$ 1.2, Glucose 10, $CaCl_2$ 2, saturated with 95% $O_2$ and 5% $CO_2$ at 32°C and adjusted to pH 7.4 and 310–320 mOsm). Brain slices were allowed to rest in $O_2$–perfused aCSF at 32°C for at least 1 h before recording or enzymatic treatment for dissociation.

### Preparation of acutely dissociated neurons

Acutely dissociated neurons were prepared from acute brain slices. The nAc, including the core and shell region but not the fascicle of the anterior commissure, was dissected from acute brain slices and incubated for 30 min with 0.5 mg/ml pronase (Calbiochem/EMD Bioscience, Darmstadt, Germany) in oxygenated aCSF (95% $O_2$/5% $CO_2$) at 37°C. Accumbal neurons were dissociated by mild mechanical trituration (10 times each with a 1000 μl and 200 μl micropipette and with a fire polished self-drawn glass-pipette) in

trituration buffer (in mM: NaCl 20, N-methyl-D-glucamine (NMG) 130, KCl 2.5, $MgCl_2$ 1, HEPES 10, Glucose 10, adjusted to pH 7.4 and 340 mOsm) and allowed to settle for 15 to 20 min before recording in a 35-mm diameter culture dish (Nunc, ThermoFisher Scientific, Waltham, MA, USA).

## Electrophysiology

For electrophysiological recordings, acute brain-slices were transferred to the recording chamber with aCSF solution saturated with 95% $O_2$ and 5% $CO_2$ at 30–32°C. The slices were observed in a DIC-IR microscope using 10x and 40x objectives (Nikon Eclipse FN1,Tokyo, Japan) and perfused with oxygenated aCSF (95% $O_2$/5% $CO_2$) at 2 ml/min at 30–32 °C.

Whole-cell recordings of accumbal dissociated neurons and brain slice neurons were performed using the voltage-clamp technique. Patch pipettes with a resistance of 4–5 MΩ were prepared from filament containing borosilicate micropipettes (World Precision Instruments, Sarasota, Florida, USA) using a P-1000 micropipette puller (Sutter Instruments, Novato, California, USA) and filled with internal solution (in mM: 120 KCl, 4.0 $MgCl_2$, 10 BAPTA, 0.5 NaGTP and 2.0 MgATP, adjusted to pH 7.4 and 290–310 mOsm). For current-clamp recordings in brain slices an internal solution of potassium gluconate was used (in mM: 120 KGluc, 10 KCl, 10 HEPES, 10 BAPTA, 2 MgATP, 0.5 NaGTP, adjusted to pH 7.4 and 305 mOsm). Action potentials (AP) were evoked with current injections of 0–400 pA for 200 ms. The number of AP was evaluated before and after perfusion with ethanol and strychnine (STN). The tonic current was measured at its baseline after 5 minutes of bath application of a cocktail containing receptor antagonists (bicuculline for $GABA_A$ receptors, 10 μM; CNQX for AMPA receptors, 10 μM; D-APV for NMDA receptors, 50 μM; TTX for voltage-gated sodium channel, 0.5 μM; and mecamylamine for nicotinic acetylcholine receptors, 10 μM). The current shift was calculated as the mean holding current during a recording period of 30 seconds. The all-points holding current histograms were fit with a Gaussian curve. The difference between the peaks of these Gaussian curves in the presence and absence of ethanol and STN was calculated to determine the change in the holding current. The glycine-evoked current was recorded at −60 mV using an internal solution containing (in mM): 120 CsCl, 4.0 $MgCl_2$, 10 BAPTA, 0.5 NaGTP and 2.0 MgATP (pH 7.4, 290–310 mOsmol). The external solution contained (in mM) 150 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose and 10 HEPES (pH 7.4, 315–320 mOsm). Neurons were perfused with an $EC_{10}$ of glycine (15 μM) in the presence or absence of 10 mM ethanol. Single channel recordings were performed in the outside-out configuration in dissociated neurons. The pipette resistance for the singe channel recording was 10–15 MΩ. Outside-out patches were held at −60 mV, and single channel recordings were made as previously reported by Hamill et al. (Hamill, Marty et al. 1981). Data were pre filtered at 10 kHz with a Digidata 1322A (Molecular Devices), low-pass filtered at 2 kHz, and recorded on a computer hard drive using pClamp 9 software (Molecular Devices). Data were analyzed off-line using the single channel analysis sub routine in Clampfit 9.0, that allowed preprocessing, open/closed dwell-time analysis and chopping of data into clusters. Tracings with activity clearly representing closed or open channel states were selected by eye and fit to Gaussian curves using the amplitude modeling subroutine. The conductance in each patch was determined by

subtracting the mean closed current from the mean open current and then dividing that number by the holding voltage (−60 mV).

D1(+) MSNs were identified by the presence of green fluorescence and D1(−) MSNs by their electrophysiological properties and lack of green fluorescence. Signals were captured using an Axopatch 200B amplifier (Axon Instruments, Berkeley, California, USA) at a holding potential of −60 mV, recorded and stored on a personal computer using a 1322A Digidata (Axon Instruments) and analyzed with Clampfit 10.1 (Axon Instruments). All compounds and reagents were acquired from Merck or Sigma-Aldrich, unless otherwise noted.

### Statistical data analysis

Slice and dissociated neuron recordings were analyzed by Student's t test. Data was analyzed by one-way or two-way ANOVA test followed by a Bonferroni post hoc test using Origin 6.0 (Microcal, Inc., Northampton, Massachusetts, USA) or GraphPad Prism 6 Software. Data are shown as mean ± SEM unless otherwise noted. n.s. not significant, *p<0.05, **p<0.01, ***p<0.001.

## Results

### GlyRs present in D1(+) MSNs are potentiated by low concentrations of ethanol

A study showed the presence of α1, α2 and β GlyR subunits in the rat nAc (Jonsson, Morud et al. 2012). In addition, a recent study reported differential expression of these subunits in D1 and D2 MSNs in mice (Förstera, Muñoz et al. 2017). Furthermore, the glycine-activated current in D1(+) MSNs displayed a higher sensitivity to glycine together with a higher current density as compared to D1(−) MSNs (Förstera, Muñoz et al. 2017). In the present study, using a D1-GFP mouse, we examined the effect of lower and pharmacologically active concentrations of ethanol on the glycine-activated current in acutely dissociated MSNs. Figure 1A shows representative traces of two D1(+) MSNs exposed to low (1–10 mM) and moderate (50 mM) concentrations of ethanol. The comparative analysis done in D1(+) and D1(−) MSNs showed that D1(+) MSNs were significantly more sensitive to all of the ethanol concentrations examined. For instance, the GlyR potentiation in D1(+) MSNs was evident with 1–5 mM ethanol (Fig. 1B, black squares). On the other hand, GlyRs in D1(−) neurons were only minimally affected by 10 and 50 mM ethanol (n=14). The comparison between the potentiation obtained with the application of 10 mM ethanol in D1(+) and D1(−) MSNs showed statistically different results: 30±3% (n=10) and 4±3% (n=14), respectively ($F_{(1,22)}$= 12.31; p= 0.0020 Two-way ANOVA). The scatter analyses in D1(−) MSNs showed that few neurons were sensitive to ethanol (Fig. 1C) supporting the notion that ethanol potentiates glycinergic inhibition preferentially in D1(+) MSNs.

### Low ethanol increased the open probability of the glycine-activated channel in D1(+) MSNs

To evaluate the effect of a low ethanol concentration on the unitary properties of the glycinergic current, we examined single-channel recordings in D1(+) MSNs using the outside-out configuration (Fig. 2A). The single-channel analyses showed that 10 mM ethanol increased the open probability of the channel (nPO) in a statistically significant

manner (control: 0.22±0.05, n=7; ethanol 10 mM: 0.66±0.16, n=6; unpaired Student t test, t(11)= 2.463; *p= 0.0315. Fig. 2B). This increased on nPO caused by a low concentration of ethanol was not associated with a higher open channel dwell time (Fig. 2C), but there is a tendency to decrease the inter-event interval time (time between burst) (Fig. 2D). Thus, the properties of GlyRs were similar in control and with ethanol (unpaired Student t test, t(8)= 0.635; p= 0.5427 for dwell time. unpaired Student t test t(8)= 1.965 p= 0.0850 for inter-event interval, n= 5). Similarly, ethanol did not alter the channel conductance (control: 40±2 pS, n=6; ethanol 10 mM: 41±2 pS, n=6, unpaired Student t test t(10)= 0.4450; p=0.6658, Fig. 2E). Also, a channel conductance of ~40 pS that we observed in the nAc correlates with the presence of heteromeric GlyRs (Lynch 2004, Lynch 2009). Taken together, these results support the idea of highly ethanol-sensitive heteromeric GlyRs in D1(+) MSNs.

## A glycine-activated tonic current was potentiated with low concentrations of ethanol in D1(+) MSNs

It was recently reported that the glycine-activated tonic current in nAc was potentiated with 100 mM of ethanol (Förstera, Muñoz et al. 2017). Interestingly, the glycinergic synaptic currents, activated by either optogenetic or electrical stimulation, were not affected by low and high ethanol concentrations (Muñoz, Yevenes et al. 2018). In the present study, we wanted to examine if the tonic current in D1(+) MSNs was potentiated by lower concentrations of ethanol. Figure 3 shows the recording of a GlyR-mediated tonic current obtained from the nAc in a brain slice of a D1-GFP mouse in control conditions and in the presence of two concentrations of ethanol and STN (1 μM) (Fig. 3A). The tonic current is detected as a shift from the red dotted line that corresponds to the baseline. An all-point histogram shows the analysis of every sampling point in 30 seconds of the current trace. The trace shows that the strychnine sensitive current was potentiated with low (10 mM) and moderate (50 mM) concentrations of ethanol (Fig. 3A). The average current shift in the presence of ethanol and STN is shown in figure 3B (ethanol 10 mM: −9±2 pA, ethanol 50 mM: −10±3 pA, STN: 6±1 pA, ($F_{(2,35)}$= 14.92; p= 0.0001 One-way ANOVA, n=14). These data show that the glycine-mediated tonic current in D1(+) MSNs is sensitive to low concentrations of ethanol.

## Ethanol decreased neuronal firing in D1(+) MSNs

It was important to examine whether the potentiation of the glycinergic tonic current with a low concentration of ethanol was enough to change the neuronal excitability of D1(+) MSNs. Thus, we analyzed the frequency of action potentials (AP) in control conditions and in the presence of 10 and 50 mM of ethanol. For this, we performed whole-cell recordings in the current clamp mode and applied current pulses to cause repetitive firing in D1(+) MSNs. The data showed that the number of AP elicited by a 200 pA current was reduced in a concentration-dependent fashion when brain slices were perfused with 10 or 50 mM ethanol (Fig. 4A and B). While application of 10 mM ethanol caused a small reduction in the number of AP (16±7%), ($F_{(3,34)}$= 4.392; p= 0.0102 One-way ANOVA, Bonferroni post hoc test control versus ethanol 50 mM **p<0.01, n=12) a much larger and statistically significant reduction of AP frequency was found with 50 mM ethanol (34±6%). To confirm that this reduction in spike firing was mediated by potentiation of the tonic current activated by GlyRs in D1(+) MSNs, we performed the experiment in slices pre-treated with STN (4

μM) to block GlyRs. The data showed that under this condition, the effect of ethanol on the frequency of AP was abolished (Figure 4C). Figure 4D shows the number of AP evoked with different current pulses. In the presence of ethanol, higher current pulses are needed to evoke the same number of AP than in control conditions. Thus, the data show that ethanol decreases the excitability of D1(+) MSNs ($F_{(1,161)}$= 14.64; p= 0.0002 Two-Way ANOVA for control versus ethanol 50 mM, n=12). Supporting a role of GlyRs on this effect, when the neurons were pre treated with STN, the excitability of D1(+) MSNs was not affected by 50 mM ethanol (gray triangles, n=9). The voltage traces in figure 4A and B show values for the membrane resting potential and AP trigger threshold. These values did not change significantly in the presence of ethanol (Table 1). Figure S1 shows that the membrane resistance of the recorded neurons also did not change significantly when ethanol was applied.

### Ethanol did not affect neuronal firing in D1(−) MSNs

The previous results were recorded in D1(+) MSNs detected as GFP positive cells. To confirm that the effect was specific for D1(+) MSNs and not for other types of neurons in nAc, we tested if ethanol also affected the firing of AP in D1(−) neurons. For this, we performed recordings using 50 mM of ethanol applied alone or with 4 μM of STN. The data shows that contrary to D1(+) MSNs, the firing of AP in D1(−) MSNs was not affected by ethanol and did not show any difference in the AP number recorded in control conditions (figure 5A and B) suggesting that D1(+) MSNs are the primary accumbal targets that are under the major inhibitory regulation mediated by GlyRs ($F_{(2,15)}$= 0.0164; p= 0.9837 One-way ANOVA, n=6). Table 1 shows the features of AP recorded in D1(+) and D1(−) MSNs in the presence of a concentration of ethanol (50 mM) that reduced the firing frequency. The data shows that despite changes in firing with ethanol, the intrinsic properties of the action potentials were not affected.

## Discussion

### Potentiation of the GlyR by ethanol

It was previously reported that GlyRs in spinal cord neurons are potentiated by ethanol, starting at a concentration of 10 mM (Aguayo, Tapia et al. 1996). Similarly, GlyRs in hypoglossal motoneurons were affected by concentrations above 10 mM (Eggers and Berger 2004, Aguayo, Castro et al. 2014). In other studies, different concentrations of ethanol have been examined in upper brain regions (Ye, Tao et al. 2001, Maguire, Mitchell et al. 2014); and in the nAc, the effects of a high concentration of ethanol (i.e. 100 mM) were recently evaluated (Förstera, Muñoz et al. 2017). At this concentration, we were unable to discriminate a neuron-specific effect of ethanol between the MSNs. Therefore, in the present study, we tested lower concentrations of ethanol (1–50 mM) because these levels can be found clinically in blood samples (Pizon, Becker et al. 2007). The present results show that similar to spinal cord neurons, GlyRs in D1(+) MSNs, but not in D1(−) MSNs, are potentiated by low concentrations of ethanol, starting from 5–10 mM.

### Tonic inhibition mediated by $GABA_A$Rs and GlyRs in nAc

It was reported that activation of $GABA_A$ receptors having an α4βδ composition generated a sustained tonic current in nAc neurons that was able to affect firing of these neurons (Maguire, Macpherson et al. 2014). Similarly, it was recently reported that GlyRs can also generate a tonic current in nAc neurons contributing to the control of neuronal excitability. These GlyRs in D1 and D2 MSNs were composed of α1, α2 and β subunits, and were potentiated by a high concentration of ethanol (100 mM) (Förstera, Muñoz et al. 2017). In the present study, we found that GlyRs in D1(+) MSNs were more sensitive to low concentrations of ethanol, and in addition, the glycinergic-mediated tonic current was also potentiated with low concentrations of ethanol, equivalent to 1–2 drinks (Pizon, Becker et al. 2007). These results are relevant because, until now, it was believed that $GABA_A$ receptors provided exclusive inhibition for brain mesolimbic regions involved in reward; however, GlyRs appear to also have a regulatory role in nAc, specifically in D1(+) MSNs.

### Presence of high ethanol-sensitive GlyR subunits in D1(+) MSNs

Depending on the conformation, GlyRs have different glycine and ethanol sensitivities (Aguayo, van Zundert et al. 2004, Sánchez, Yévenes et al. 2015). GlyRs have a pentameric structure made up of α1, α2, or α3 and β subunits. While α subunits can assemble into functional ion channels, the β subunit cannot because its acts as a structural component (Burgos, Yévenes et al. 2016). Previous studies have shown that the α1, but not the α2/α3 subunits, are sensitive to ethanol (Yevenes, Moraga-Cid et al. 2010, Sánchez, Yévenes et al. 2015). Therefore, the present study suggests that D1(+) MSNs likely contain more α1 subunits as compared to D1(−) MSNs. We found that low concentrations of ethanol potentiated the STN-sensitive tonic current in D1(+) MSNs. The analysis of single channel recordings showed that 10 mM ethanol increased channel opening by increasing the open probability and slightly, but consistently, reducing the inter event interval time of the GlyRs. This data is in agreement with a previous study that showed that even a higher concentration of ethanol (50 mM) did not significantly affect open and closed dwell times and likehood of homomeric α1 GlyRs in oocytes. In addition, they found that ethanol had subtle effects on burst durations and number of channel openings per burst. Further kinetic analysis indicated that ethanol increases burst durations by decreasing the rate of glycine unbinding from the site (Welsh, Goldstein et al. 2009). Altogether, our data indicate that ethanol affected neuronal excitability by potentiating the function of these receptors. Indeed, the results showed that ethanol was able to reduce spike firing only in D1(+) MSNs, and this effect was blocked by STN. These results highlight the importance of GlyRs in regulating the excitatory/inhibitory balance in nAc.

### Differential expression of receptors in nAc MSNs

Recent studies have identified that MSNs in nAc are not completely segregated as in dorsal striatum. In nAc, for example, the direct and indirect pathways are not exclusively composed of D1 and D2 MSNs, respectively, and both D1 and D2 MSNs project to the ventral pallidum. However, it was also reported that only D1(+) MSNs project directly to VTA (Smith, Lobo et al. 2013, Kupchik, Brown et al. 2015). Another difference between these two groups of neurons is the type of receptors that they express. For example, D1(+) MSNs

express muscarinic M4 cholinergic and adenosine A1 receptors, and co-release dynorphin, and substance P with GABA, whereas D2-MSNs express adenosine A2a receptors, and co-release enkephalin and neurotensin with GABA (Smith, Lobo et al. 2013). The differences found in D1(+) and D1(−) cells in the present study suggest that the GlyRs expressed in nAc are also formed by different subunits, most likely a higher expression of α1 in D1(+) MSNs and α2 or α3 in D1(−) MSNs because of the different responses to ethanol in these two groups of neurons. Interestingly, a recent study from our laboratory showed that a genetically modified mouse expressing an ethanol insensitive α1 GlyR displayed higher conditioned place preference and drinking behavior than the WT mice (Muñoz, Gallegos et al. 2019), once again highlighting the importance of GlyR subunits in ethanol addictive behaviors. Additionally, the values for channel conductance that were found in this study suggest that GlyRs are most likely expressed in a heteromeric form in nAc because homomeric receptors have higher conductances (Yevenes, Moraga-Cid et al. 2008, Lynch 2009, Yevenes, Moraga-Cid et al. 2010).

### Potential impact of GlyRs on addiction phenomenon in accumbal neurons.

D1(+) MSNs form part of the direct pathway that is believed to play a critical role in positive reward and neuroplasticity related to addiction. These neurons are stimulated by dopamine released by the VTA, and also by glutamatergic inputs from several brain regions (Russo and Nestler 2013), and even higher levels of dopamine when ethanol is administered (Chiara and Imperato 1988). Therefore, the presence of GlyRs having high sensitivity to ethanol in these neurons should serve as excitability regulators, especially in the presence of ethanol because they can enhance inhibition by potentiating the glycine-tonic current and depress spike firing, as shown in the present study. For this reason, a reduced sensitivity to ethanol in mutated GlyRs would cause a higher reward because D1 activation would not be opposed by the potentiated $Cl^-$ current thereby producing an increase in intake and preference to ethanol (Muñoz, Gallegos et al. 2019). This idea is in agreement with the results of Söderpalm and co-workers because when they applied STN in the nAc in their working model, ethanol intake increased (Molander, Löf et al. 2005), and when an inhibitor of glycine transporter GlyT1 (Org 25935) was used, ethanol consumption and preference was reduced (Molander, Lidö et al. 2007, Lidö, Ericson et al. 2011). Thus, the data shows that ethanol has complex cellular and behavioral effects. In MSNs, ethanol increases dopamine release and potentiates GlyRs that in turn would regulate ethanol intake changing the excitability/inhibition ratio of the reward circuitry.

In conclusion, this study provides new data indicating that GlyRs in D1(+) MSNs are potentiated by low concentrations of ethanol by increasing single channel opening, and that this contributes to inhibition of neuronal excitability in nAc.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgment

We thank Dr. David M. Lovinger for providing the D1-GFP mice and commenting on the paper. Also, we appreciate the assistance of Lauren Aguayo and Carolina Benitez for technical assistance.

Aníbal Araya is a student of the Pharmacology PhD program of the Universidad de Chile. Scarlet Gallegos and Aníbal Araya are supported by CONICYT scholarships 21150416 and 21190807, respectively.

Funding

This work was supported by the National Institute on Alcohol Abuse and Alcoholism, NIH grant R01AA025718.

## Abbreviations.

| | |
|---|---|
| **aCSF** | artificial cerebral spinal fluid |
| **AMPA** | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| **AP** | action potentials |
| **CNQX** | 6-Cyano-7-nitroquinoxaline-2,3-dione |
| **D1(+) MSNs** | dopamine 1 receptor-expressing medium spiny neurons |
| **D2 MSNs** | dopamine 2 receptor-expressing medium spiny neurons |
| **D-APV** | D-2-Amino-5-Phosphonovaleric acid |
| **GABA** | gamma-aminobutyric acid |
| **GABAAR** | type A GABA receptor |
| **GFP** | green fluorescent protein |
| **GlyR** | glycine receptor |
| **GlyT1** | glial glycine transporter 1 |
| **mM** | milli molar |
| **MSNs** | medium spiny neurons |
| **mV** | milli volt |
| **nAc** | nucleus Accumbens |
| **NMDA** | N-methyl-D-aspartate |
| **pA** | pico ampere |
| **PFC** | prefrontal cortex |
| **pS** | pico Siemens |
| **STN** | strychnine |
| **TTX** | tetrodotoxin |

| | |
|---|---|
| **VTA** | ventral tegmental area |

## References


Abrahao KP, Salinas AG and Lovinger DM (2017). "Alcohol and the Brain: Neuronal Molecular Targets, Synapses, and Circuits." Neuron 96(6): 1223–1238. [PubMed: 29268093]

Aguayo LG, Castro P, Mariqueo T, Muñoz B, Xiong W, Zhang L, Lovinger DM and Homanics GE (2014). "Altered sedative effects of ethanol in mice with α1 glycine receptor subunits that are insensitive to Gβγ modulation." Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39(11): 2538–2548. [PubMed: 24801766]

Aguayo LG, Tapia JC and Pancetti FC (1996). "Potentiation of the glycine-activated Cl- current by ethanol in cultured mouse spinal neurons." The Journal of pharmacology and experimental therapeutics 279(3): 1116–1122. [PubMed: 8968332]

Aguayo LG, van Zundert B, Tapia JC, Carrasco MA and Alvarez FJ (2004). "Changes on the properties of glycine receptors during neuronal development." Brain research. Brain research reviews 47(1–3): 33–45. [PubMed: 15572161]

Bock R, Shin HJ, Kaplan AR, Dobi A, Markey E, Kramer PF, Gremel CM, Christensen CH, Adrover MF and Alvarez VA (2013). "Strengthening the accumbal indirect pathway promotes resilience to compulsive cocaine use." Nature neuroscience 16(5): 632–638. [PubMed: 23542690]

Burgos CF, Muñoz B, Guzman L and Aguayo LG (2015). "Ethanol effects on glycinergic transmission: From molecular pharmacology to behavior responses." Pharmacological Research 101: 18–29. [PubMed: 26158502]

Burgos CF, Yévenes GE and Aguayo LG (2016). "Structure and Pharmacologic Modulation of Inhibitory Glycine Receptors." Molecular Pharmacology 90(3): 318–325. [PubMed: 27401877]

Cheng Y, Huang C, Ma T, Wei X, Wang X, Lu J and Wang J (2017). "Distinct Synaptic Strengthening of the Striatal Direct and Indirect Pathways Drives Alcohol Consumption." Biological Psychiatry 81(11): 918–929. [PubMed: 27470168]

Chiara DG and Imperato A (1988). "Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats." Proceedings of the National Academy of Sciences of the United States of America 85(14): 5274–5278. [PubMed: 2899326]

Delaney AJ, Esmaeili A, Sedlak PL, Lynch JW and Sah P (2010). "Differential expression of glycine receptor subunits in the rat basolateral and central amygdala." Neuroscience Letters 469(2): 237–242. [PubMed: 19995593]

Di Chiara G (2000). "Role of dopamine in the behavioural actions of nicotine related to addiction." European journal of pharmacology 393(1–3): 295–314. [PubMed: 10771025]

Dobi A, Seabold GK, Christensen CH, Bock R and Alvarez VA (2011). "Cocaine-induced plasticity in the nucleus accumbens is cell specific and develops without prolonged withdrawal." The Journal of neuroscience : the official journal of the Society for Neuroscience 31(5): 1895–1904. [PubMed: 21289199]

Eggers ED and Berger AJ (2004). "Mechanisms for the modulation of native glycine receptor channels by ethanol." Journal of neurophysiology 91(6): 2685–2695. [PubMed: 14762156]

Förstera B, Muñoz B, Lobo MK, Chandra R, Lovinger DM and Aguayo LG (2017). "Presence of ethanol-sensitive glycine receptors in medium spiny neurons in the mouse nucleus accumbens." The Journal of Physiology 595(15): 5285–5300. [PubMed: 28524260]

Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ (1981). "Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches." Pflugers Arch 391(2): 85–100. [PubMed: 6270629]

Hikida T, Kimura K, Wada N, Funabiki K and Nakanishi S (2010). "Distinct Roles of Synaptic Transmission in Direct and Indirect Striatal Pathways to Reward and Aversive Behavior." Neuron 66(6).

Jeanes ZM, Buske TR and Morrisett RA (2011). "In Vivo Chronic Intermittent Ethanol Exposure Reverses the Polarity of Synaptic Plasticity in the Nucleus Accumbens Shell." Journal of Pharmacology and Experimental Therapeutics 336(1): 155–164. [PubMed: 20947635]

Jonsson S, Morud J, Pickering C, Adermark L, Ericson M and Söderpalm B (2012). “Changes in glycine receptor subunit expression in forebrain regions of the Wistar rat over development.” Brain research 1446: 12–21. [PubMed: 22330726]

Koob GF and Volkow ND (2016). “Neurobiology of addiction: a neurocircuitry analysis.” The lancet. Psychiatry 3(8): 760–773. [PubMed: 27475769]

Kupchik YM, Brown RM, Heinsbroek JA, Lobo M, Schwartz DJ and Kalivas PW (2015). “Coding the direct/indirect pathways by D1 and D2 receptors is not valid for accumbens projections.” Nature Neuroscience 18(9): 1230–1232. [PubMed: 26214370]

Lee K-W, Kim Y, Kim AM, Helmin K, Nairn AC and Greengard P (2006). “Cocaine-induced dendritic spine formation in D1 and D2 dopamine receptor-containing medium spiny neurons in nucleus accumbens.” Proceedings of the National Academy of Sciences of the United States of America 103(9): 3399–3404. [PubMed: 16492766]

Lidö H, Ericson M, Marston H and Söderpalm B (2011). “A Role for Accumbal Glycine Receptors in Modulation of Dopamine Release by the Glycine Transporter-1 Inhibitor Org25935.” Frontiers in Psychiatry 2: 8. [PubMed: 21556278]

Lynch JW (2004). “Molecular structure and function of the glycine receptor chloride channel.” Physiological reviews 84(4): 1051–1095. [PubMed: 15383648]

Lynch JW (2009). “Native glycine receptor subtypes and their physiological roles.” Neuropharmacology 56(1): 303–309. [PubMed: 18721822]

Maguire EP, Macpherson T, Swinny JD, Dixon CI, Herd MB, Belelli D, Stephens DN, King SL and Lambert JJ (2014). “Tonic Inhibition of Accumbal Spiny Neurons by Extrasynaptic α4βδ GABAA Receptors Modulates the Actions of Psychostimulants.” The Journal of Neuroscience 34(3): 823–838. [PubMed: 24431441]

Maguire EP, Mitchell EA, Greig SJ, Corteen N, Balfour DJ, Swinny JD, Lambert JJ and Belelli D (2014). “Extrasynaptic glycine receptors of rodent dorsal raphe serotonergic neurons: a sensitive target for ethanol.” Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 39(5): 1232–1244. [PubMed: 24264816]

Molander A, Lidö HH, Löf E, Ericson M and Söderpalm B (2007). “The glycine reuptake inhibitor Org 25935 decreases ethanol intake and preference in male wistar rats.” Alcohol and alcoholism (Oxford, Oxfordshire) 42(1): 11–18.

Molander A, Löf E, Stomberg R, Ericson M and Söderpalm B (2005). “Involvement of Accumbal Glycine Receptors in the Regulation of Voluntary Ethanol Intake in the Rat.” Alcoholism: Clinical and Experimental Research 29(1): 38–45.

Molander A and Söderpalm B (2005). “Glycine Receptors Regulate Dopamine Release in the Rat Nucleus Accumbens.” Alcoholism: Clinical & Experimental Research 29(1): 1726.

Muñoz B, Gallegos S, Peters C, Murath P, Lovinger DM, Homanics GE and Aguayo LG (2019). “Influence of nonsynaptic α1 glycine receptors on ethanol consumption and place preference.” Addiction biology.

Muñoz B, Yevenes GE, Förstera B, Lovinger DM and Aguayo LG (2018). “Presence of Inhibitory Glycinergic Transmission in Medium Spiny Neurons in the Nucleus Accumbens.” Frontiers in molecular neuroscience 11: 228. [PubMed: 30050406]

Nakanishi S, Hikida T and Yawata S (2014). “Distinct dopaminergic control of the direct and indirect pathways in reward-based and avoidance learning behaviors.” Neuroscience 282.

Nestler EJ (2001). “Molecular Neurobiology of Addiction.” The American Journal on Addictions 10(3): 201–217. [PubMed: 11579619]

Pizon AF, Becker CE and Bikin D (2007). “The clinical significance of variations in ethanol toxicokinetics.” Journal of Medical Toxicology 3(2): 63–72. [PubMed: 18072163]

Renteria R, Maier EY, Buske TR and Morrisett RA (2017). “Selective alterations of NMDAR function and plasticity in D1 and D2 medium spiny neurons in the nucleus accumbens shell following chronic intermittent ethanol exposure.” Neuropharmacology 112: 164–171. [PubMed: 26946430]

Russo SJ and Nestler EJ (2013). “The brain reward circuitry in mood disorders.” Nature Reviews Neuroscience 14(9): 609–625. [PubMed: 23942470]

Sánchez A, Yévenes GE, San Martin L, Burgos CF, Moraga-Cid G, Harvey RJ and Aguayo LG (2015). “Control of ethanol sensitivity of the glycine receptor α3 subunit by transmembrane 2, the

intracellular splice cassette and C-terminal domains." The Journal of pharmacology and experimental therapeutics 353(1): 80–90. [PubMed: 25589412]

Smith RJ, Lobo M, Spencer S and Kalivas PW (2013). "Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways)." Current Opinion in Neurobiology 23(4): 546–552. [PubMed: 23428656]

Smith RJ, Lobo M, Spencer S and Kalivas PW (2013). "Cocaine-induced adaptations in D1 and D2 accumbens projection neurons (a dichotomy not necessarily synonymous with direct and indirect pathways)." Current Opinion in Neurobiology 23(4).

Volkow ND and Morales M (2015). "The Brain on Drugs: From Reward to Addiction." Cell 162(4): 712725.

Wang J, Cheng Y, Wang X, Hellard E, Ma T, Gil H, Hamida S and Ron D (2015). "Alcohol Elicits Functional and Structural Plasticity Selectively in Dopamine D1 Receptor-Expressing Neurons of the Dorsomedial Striatum." The Journal of Neuroscience 35(33): 11634–11643. [PubMed: 26290240]

Welsh BT, Goldstein BE and Mihic JS (2009). "Single-Channel Analysis of Ethanol Enhancement of Glycine Receptor Function." Journal of Pharmacology and Experimental Therapeutics 330(1): 198–205. [PubMed: 19380602]

Ye JH, Tao L, Ren J, Schaefer R, Krnjevic K, Liu PL, Schiller DA and McArdle JJ (2001). "Ethanol potentiation of glycine-induced responses in dissociated neurons of rat ventral tegmental area." The Journal of pharmacology and experimental therapeutics 296(1): 77–83. [PubMed: 11123365]

Yevenes GE, Moraga-Cid G, Avila A, Guzmán L, Figueroa M, Peoples RW and Aguayo LG (2010). "Molecular requirements for ethanol differential allosteric modulation of glycine receptors based on selective Gbetagamma modulation." The Journal of biological chemistry 285(39): 30203–30213. [PubMed: 20647311]

Yevenes GE, Moraga-Cid G, Peoples RW, Schmalzing G and Aguayo LG (2008). "A selective G betagamma-linked intracellular mechanism for modulation of a ligand-gated ion channel by ethanol." Proceedings of the National Academy of Sciences of the United States of America 105(51): 20523–20528. [PubMed: 19074265]

## Highlights

- Glycine receptors in D1(+) MSNs of Nucleus Accumbens are more sensitive to low concentrations of ethanol than D1(− ) MSNs.
- A low ethanol concentration increases the open probability of the glycine receptor channel; however, it does not affect the channel conductance.
- Low ethanol potentiates the glycine receptor-mediated tonic current in D1(+) MSNs.
- Ethanol reduced action potential firing of D1(+) MSNs in a manner dependent on GlyR activation.

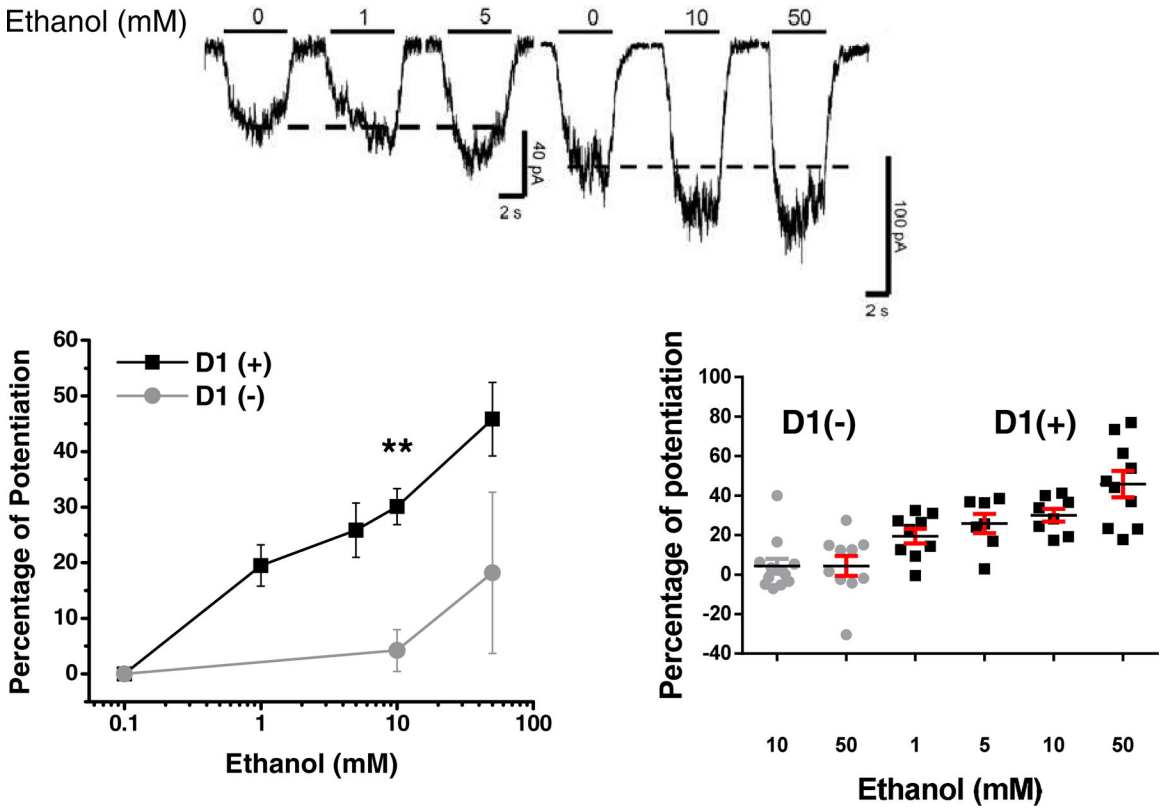


**Figure 1.**

A) Representative traces of evoked glycine currents for two D1(+) MSNs and the effect of 1, 5, 10 and 50 mM of ethanol. B) Graph summarizes the effects of different concentrations of ethanol (1–50 mM) in D1(+) (black squares) and D1(−) (gray circles) MSNs. C) Scatter graph of the percentage of potentiation of ethanol in D1(+) and D1(−) MSNs. Data represent mean ± SEM (shown in red) of D1(+) and D1(−) MSNs (n=10 and 14, respectively), $F_{(1,22)}$= 12.31; **p= 0.0020 Two-way ANOVA.

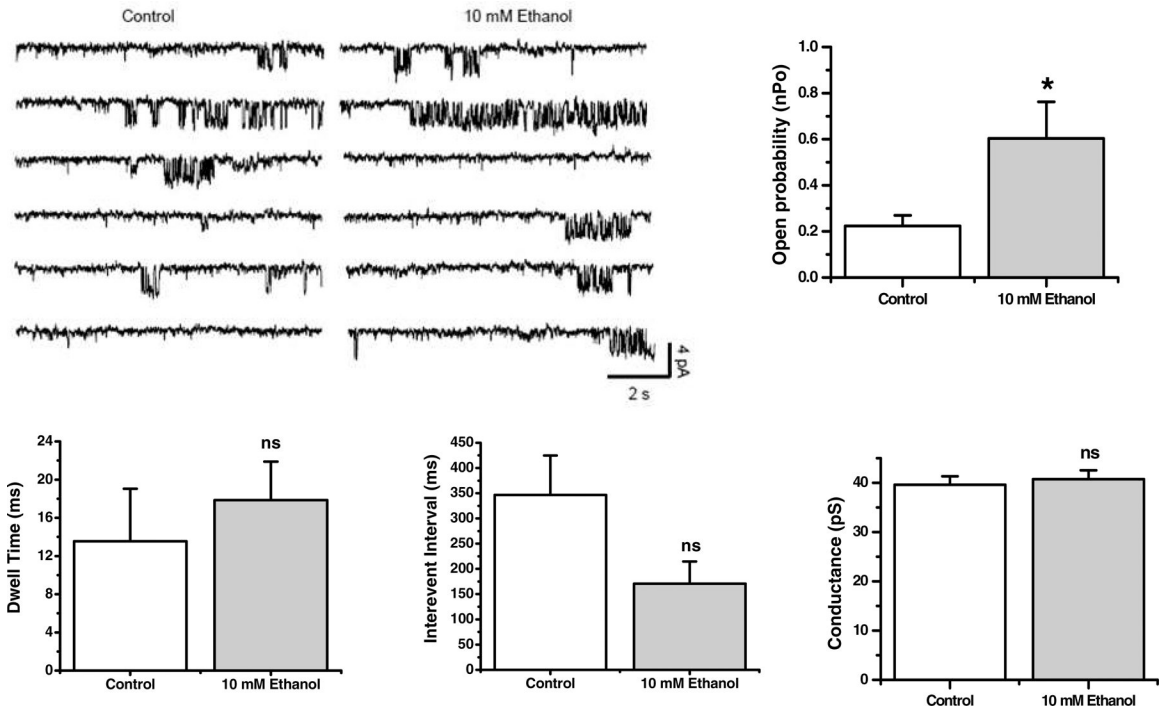


**Figure 2.**

A) Representative traces of single-channel recordings (out-side out) in dissociated D1(+) MSNs perfused with 10 μM of glycine before and after adding 10 mM ethanol. B) Graph shows the open probability (nPo) of the receptor after the perfusion of 10 mM ethanol in D1(+) neurons. C) Graph of the dwell time of the channel in the open state before and after the perfusion of 10 mM ethanol. D) Graph shows the inter-event interval time is reduced in the presence of a low concentration of ethanol. E) Graph summarizes the data for conductance of the GlyRs in D1(+) which was not affected by 10 mM ethanol. Data represent mean ± SEM, n=7, *p= 0.0315 (unpaired Student t test), ns not significant p>0.05.

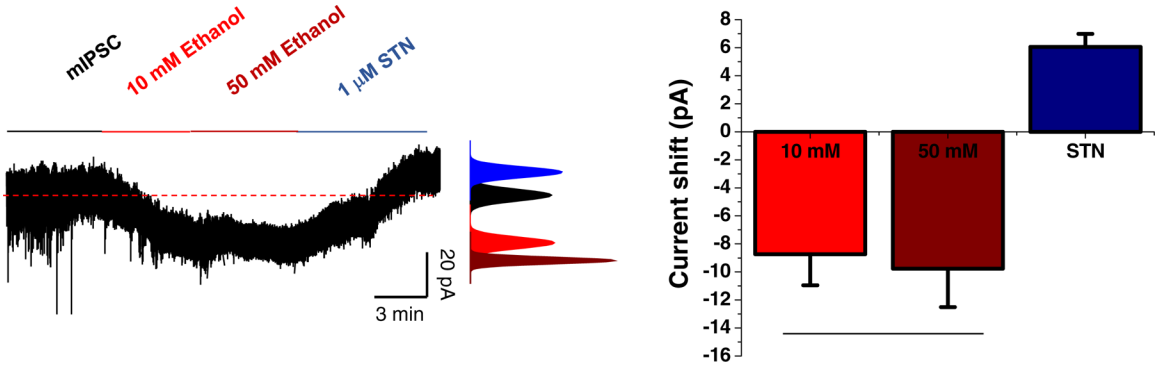


**Figure 3.**

A) Representative electrophysiological trace of a glycinergic tonic current in a D1(+) MSN in the presence of 10 and 50 mM of ethanol and 1 μM of STN. The red dotted line indicates the baseline. The histogram shows the analysis of every sampling point in 30 seconds of the current trace. Ethanol increased the GlyR-mediated current in the nAc and STN produced a positive shift in the holding current. B) Graph shows the average response of tonic currents in D1(+) neurons recorded in the presence of ethanol and STN. Both concentrations of ethanol caused a significant shift in the tonic current. Data show mean ± SEM (n= 14), $F_{(2,35)}$= 14.92; ***p= 0.0001 One-way ANOVA.

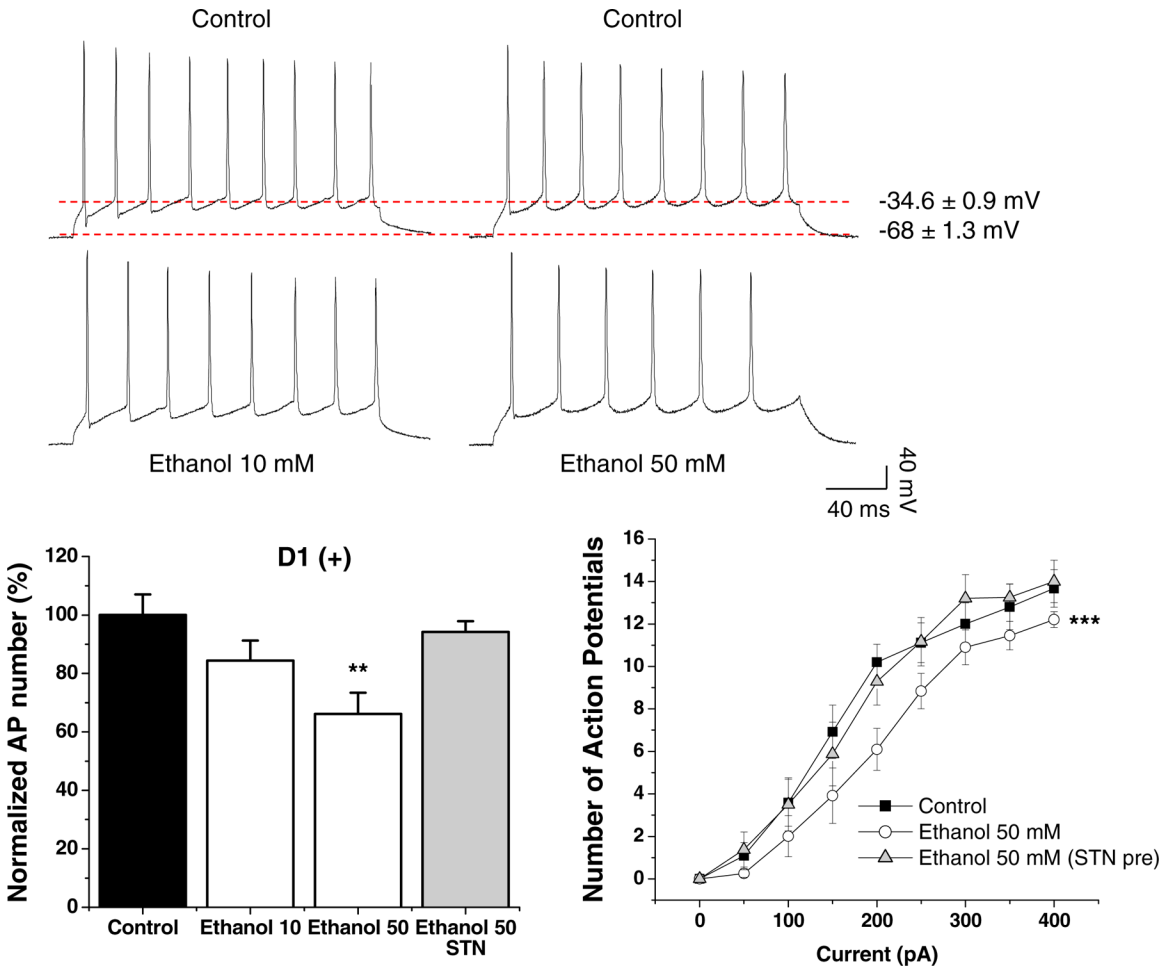


**Figure 4.**

A-B) Representative traces of action potentials (AP) in D1(+) neurons from the nAc elicited with a current pulse of 200 pA and 200 ms in control conditions and in the presence of 10 and 50 mM of ethanol, respectively. The red dotted lines show the membrane resting potential (−68 ± 1 mV) and the threshold voltage (−34.6 ± 1 mV) for AP generation; these values did not change between the conditions. C) The graph summarizes the percentage of AP measured in D1(+) neurons in the presence of 10 and 50 mM of ethanol, and also in the presence of 50 mM of ethanol in cells pre-treated with strychnine (STN 4 μM). D1(+) MSNs pre-treated with STN did not show a reduction in AP frequency in the presence of ethanol, supporting the importance of GlyRs in mediating these effects ($F_{(3,34)}$= 4.392; p= 0.0102 One-way ANOVA, Bonferroni post hoc test **p<0.01). D) The curves show the number of AP evoked with 50–400 pA. In the presence of 50 mM ethanol, D1(+) neurons (white circles) evoked fewer AP than in control conditions (black squares). And when the slices were pre-treated with STN, ethanol did not reduce the number of AP (gray triangles). $F_{(1,161)}$= 14.64; ***p= 0.0002 Two-Way ANOVA for control versus ethanol 50 mM. Data represent the mean ± SEM (n= 12 for control and 50 mM ethanol; n= 6 for 10 mM ethanol; n=9 for ethanol pre-treated with STN).

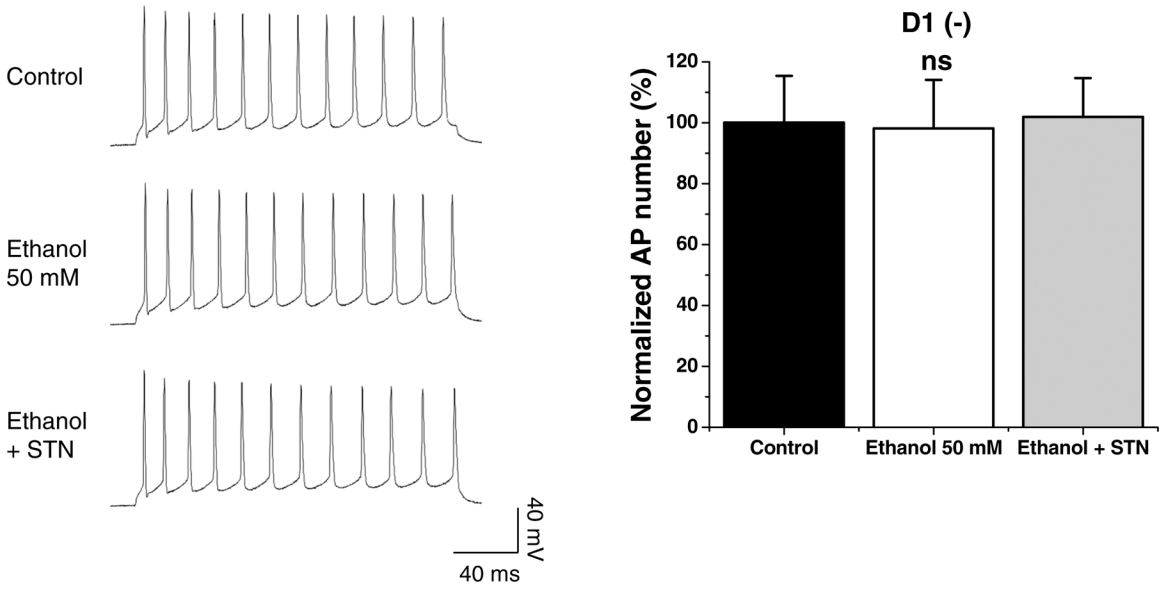


**Figure 5.**

A) Representative traces of AP in D1(−) neurons from the nAc elicited with a current pulse of 200 pA and 200 ms in control conditions and in the presence of 50 mM of ethanol, and the co-application of ethanol and 4 μM of STN. B) Graph summarizes the percentage of AP registered in D1(−) neurons in the presence of 50 mM of ethanol and 50 mM of ethanol plus STN. D1(−) MSNs did not show a reduction in AP frequency in any of the conditions mentioned above. ($F_{(2,15)}$= 0.0164; p= 0.9837 One-way ANOVA. Data represent mean ± SEM, n=6, n.s. p>0.05

**Table 1.**

Features of action potentials in nAc in control conditions and in the presence of 50 mM ethanol.

| | Resting Potential (mV) | Threshold (mV) | Amplitude (mV) | Input Resistance (MΩ) | Half Width (ms) | n |
|---|---|---|---|---|---|---|
| D1(+) Control | − 68.03 ± 1.3 | − 34.6 ± 0.9 | 89.53 ± 3.1 | 103.4 ± 11.3 | 1.37 ± 0.14 | 12 |
| D1(+) Ethanol 50 mM | − 69.28 ± 1.5 | − 36.2 ± 1.3 | 90.86 ± 2.7 | 96.1 ± 10.9 | 1.51 ± 0.18 | 12 |
| D1(−) Control | − 68.68 ± 1.7 | − 35.8 ± 0.7 | 93.7 ± 1.2 | 99.7 ± 8.7 | 1.23 ± 0.07 | 6 |
| D1(−) Ethanol 50 mM | − 69.25 ± 1.7 | − 36.3 ± 0.4 | 90.9 ± 1.6 | 95.9 ± 10.1 | 1.18 ± 0.07 | 6 |

Values are given as mean ± SEM. Data from each neuron was obtained from the analysis of the action potentials using the program Clampfit 10.1 (Axon Instruments) and then analyzed as group with the software Origin 6 (Microcal, Inc., Massachusetts, USA).

(n)=number of cells