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Moderate ethanol drinking is sufficient to alter Ventral Tegmental Area dopamine neurons activity via functional and structural remodeling of GABAergic transmission

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Abstract

Earlier studies have shown a major involvement of Ventral Tegmental Area (VTA) dopamine (DA) neurons in mediating the rewarding effects of ethanol (EtOH). Much less is known on the role of this system in mediating the transition from moderate to excessive drinking and abuse. Here we sought to explore the hypothesis that early stage drinking in rodents, resembling recreational EtOH use in humans, is sufficient to dysregulate VTA DA transmission thus increasing the propensity to use over time. To this purpose, midbrain slice recordings in mice previously exposed to an escalating (3, 6 and 12%) 18-day voluntary EtOH drinking paradigm was used. By recording from DA and γ-aminobutyric acid (GABA) VTA neurons in midbrain slices, we found that moderate EtOH drinking leads to a significant suppression of the spontaneous activity of VTA DA neurons, while increasing their response to acute EtOH application. We also found that chronic EtOH leads to the enhancement of GABA input frequency onto a subset of DA neurons. Structurally, chronic EtOH induced a significant increase in the number of GABA axonal boutons contacting DA neurons, suggesting deep rewiring of the GABA network. This scenario is consistent with a downmodulation of the reward DA system induced by moderate EtOH drinking, a neurochemical state defined as “hypodopaminergic” and previously associated with advanced stages of drug use in humans. In this context, increased sensitivity of DA neurons towards acute EtOH may represent

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CRediT authorship contribution statement

A. Ilari: electrophysiology, behavioral tests, data analysis. **L. Curti:** electrophysiology, behavioral tests, data analysis. **M. Petrella:** electrophysiology, behavioral tests, data analysis. **N. Cannella:** behavioral tests, data analysis. **A. La Rocca:** immunocytochemistry, confocal imaging, stereological count. **G. Ranieri:** behavioral tests, data analysis. **E. Gerace:** electrophysiology, behavioral tests, data analysis. **D. Iezzi:** electrophysiology, behavioral tests, data analysis. **L. Silvestri:** immunocytochemistry, confocal imaging, stereological count. **G. Mannaioni:** manuscript preparation. **R. Ciccocioppo:** research design and Supervision, data analysis, manuscript preparation. **A. Masi:** research design and Supervision, data analysis, manuscript preparation.

Appendix A. Supplementary data
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the neurophysiological correlate of increased unitary rewarding value, possibly driving progression to addiction.

Keywords

Voluntary alcohol drinking; Ventral tegmental area; Dopaminergic system; GABAergic transmission; Patch clamp

1. Introduction

The activation of Ventral Tegmental Area (VTA) dopamine (DA) neurons and the consequent increase in extracellular DA levels in the ventral striatum, the target region of the mesolimbic system, is considered a key mechanism responsible for EtOH rewarding properties (Di Chiara and Imperato, 1986; Yoshimoto et al., 1992; Koob and Volkow, 2010). Maladaptive changes in the mesolimbic DA pathway underlie the transition from recreational EtOH use to excessive drinking and, ultimately, dependence. This transition is common to all major substances of abuse (Wise and Robble, 2020; Adinoff, 2004; Volkow et al., 2019), however, a precise understanding of the neural basis underlying this process is lacking. Several lines of evidence indicate that prolonged stimulation of the DA system by a rewarding substance may lead to a persistent functional downregulation of mesolimbic DA transmission (hypodopaminergic state) and surge of negative emotional response, craving and relapse (Hirth et al., 2016). Many rodent models have been developed to investigate the effects of EtOH at behavioral, molecular and neurophysiological levels (Spanagel, 2017; Ciccocioppo et al., 2006). Pioneering electrophysiological studies in anesthetized rats revealed that systemic acute EtOH application enhances the firing of putative VTA DA neurons (Gessa et al., 1985). This early finding was then confirmed with brain slice recordings from both rats (Brodie et al., 1990) and mice (Okamoto et al., 2006; Brodie and Appel, 2000; Mrejeru et al., 2015). While there is general agreement on the activating effect of EtOH on the DA system, it is still unclear whether this is mediated by direct activation of intrinsic neuronal ionic mechanisms (Okamoto et al., 2006; Brodie et al., 1999, 2007; Nelson et al., 2018) or alteration of the balance between excitatory and inhibitory inputs to DA neurons (Melis et al., 2002; Theile et al., 2008, 2011; Morikawa and Morrisett, 2010). Considering the abundance of GABA interneurons in the VTA (Margolis et al., 2012; Olson and Nestler, 2007) and the strength of GABA innervation onto local DA neurons (Bouarab et al., 2019; Creed et al., 2014; van Zessen et al., 2012), particular attention has been focused on this system. In vivo recordings from putative VTA GABA neurons in freely-moving, EtOH-naïve rats show transient inhibition of firing in response to intraperitoneal (ip) EtOH injections, while prolonged (7–14 days) EtOH exposure leads to long-lasting upregulation of the basal firing rate and suppression of the response to acute challenge (Gallegos et al., 1999). In agreement, dose-dependent suppression of the firing activity of putative VTA GABA neurons was reported with single-unit recordings in awake mice (Burkhardt and Adermark, 2014). The contribution of GABA transmission to the rewarding properties of EtOH has also been addressed with brain slice recordings. A few studies described increase in GABA transmission onto VTA DA neurons following acute EtOH application (Melis et al., 2002; Theile et al., 2008, 2011; Federici et al., 2009),

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thus pointing to a facilitation of GABA neuron firing or local GABA release. Interestingly, the response of GABA transmission to EtOH is affected by the anatomical location of the recorded DA neuron, with the frequency of GABA inputs being, respectively, increased and reduced in anterior and posterior DA neurons (Guan et al., 2012; Melis et al., 2002). A relatively small number of slice electrophysiology studies have addressed the long-lasting effects of previous in vivo EtOH exposure on the VTA network and most of them were based on passive EtOH administration paradigms such as systemic injection and vapor chamber inhalation (Melis et al., 2002; Nelson et al., 2018; Williams et al., 2018; Perra et al., 2011). Passive administration paradigms are often preferred as they allow better control of experimental conditions, the achievement of addictive EtOH levels and the expression of a withdrawal syndrome, which include emotional (anxiety), affective (depression), neurological (convulsions) and behavioral (alcohol deprivation effect, or ADE) symptoms (Perez and De Biasi, 2015). Conversely, voluntary drinking paradigms such as the two-bottle choice (TBC) test, which results in non-toxic EtOH intake, is better suited to model the hedonic and behavioral aspects of human drinking (Spanagel, 2017). However, very few slice electrophysiology studies have been performed on such paradigms (Juarez et al., 2017; Spiga et al., 2014; Avegno et al., 2016), none of which focusing on the response to chronic exposure to sub-toxic EtOH doses, a situation that mimics recreational EtOH use in humans. Here, we performed cell-attached and whole-cell patch clamp recordings from genetically-identified VTA DA neurons as well as from morphologically- and physiologically-identified GABA neurons in midbrain slices obtained from adult mice sacrificed at the end of an 18-day TBC protocol ("exposed" group) and from age-matched, EtOH-naïve littermates ("naïve" group). We found that voluntary drinking of moderate EtOH doses, not leading to emotional or behavioral signs of abstinence upon deprivation, depresses the basal firing rate of DA neurons and enhances GABA transmission, while substantially sparing glutamate transmission. At morphological level, we found that this effect was associated with an increase in the number of GABA axonal boutons contacting DA neurons. We argue that engagement of a specific population of local GABA afferents, exhibiting high EtOH sensitivity in early-stage drinking, may promote the onset of a hypodopaminergic state through the inhibition of specific subsets of VTA DA neurons.

2. Methods

2.1. Animals

All in vivo and ex vivo procedures were conducted in compliance with the Council Directive of the European Community (2010/63/EU), Decreto Legislativo Italiano 26 (March 13, 2014), and authorized by the local Animal Care Committee and the Ministry of Health (authorization number 545/2018). Tg (Th-EGFP)21-31Koba (Matsushita et al., 2002) were housed in a temperature-controlled (24 °C) animal facility under a 12 h light/dark cycle with ad libitum access to food and water. Both male and female mice at postnatal day 40–60 were used in the present study.

2.2. Two-bottle choice test

Five days before TBC drinking, mice were individually housed in standard non ventilated Plexiglas mouse cages with a mouse house (Tecniplast), bedding and nesting materials

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and two 50 mL-tubes filled with water and equipped with rubber stoppers and a metal sipper tube. Then, mice were randomly assigned to the "naive" (no change in conditions) or "exposed" group: in the "exposed" group one of the tubes was filled with a solution of EtOH at 3% v/v. Mice and tubes were weighed daily to monitor intake and the tubes switched in order to avoid the development of a side preference. EtOH concentration was increased from 3% to 6% on day 4 and to 12% on day 8. EtOH intake was calculated as the amount of EtOH consumed in 24 h/mouse weight (g/kg). EtOH preference was calculated as the volume of EtOH solution consumed in 24 h/total fluid intake (%). For the evaluation of withdrawal effects, at the end of the TBC (day 19th), exposed animals were deprived for 24 h by replacing the EtOH tube with a water-filled tube.

2.3. Behavioral tests

Sucrose preference.—Mice were individually housed in single cages with continuous access to water and a solution of sucrose at 5% (w/v) for 4 days. The bottles were weighed and switched every day. Sucrose preference was calculated as the intake of sucrose solution/ total fluid intake (%).

Elevated Plus Maze (EPM).—Mice were trained to walk on a smooth and elevated surface for 5 min/day for 4 days. The test was performed in a sound-attenuated room under dim light conditions. Briefly, mice were placed in the intersection of the four arms of the EPM facing one of the two open arms and activity and movements recorded for 5 min in order to measure the time spent in open arms (% of total) and the number of entries in each arm.

EtOH Deprivation Effect (ADE).—ADE is a phenomenon mimicking relapse drinking and is expressed as a transient increase in the voluntary intake over baseline drinking values when access to EtOH is reinstated following a period of EtOH deprivation (Rodd-Henricks et al., 2000; Vengeliene et al., 2014). Following the 24-h EtOH deprivation, mice were re-exposed to 12% EtOH for 2 days in the same conditions as during the TBC in order to evaluate the EtOH consumption and preference and compare measurements to those obtained before EtOH deprivation. EtOH naive mice with access to water only were used as controls.

2.4. Preparation of midbrain slices and electrophysiological recordings

At the end of the drinking protocol, mice were anesthetized with isoflurane and decapitated for brain collection. Midbrain horizontal slices (200 μm) containing the VTA were cut with a vibroslicer (Leica VT1000S, Leica Microsystem, Wetzlar, Germany) in a slicing solution composed of (in mM): NMDG (92), HEPES (20), glucose (25), NaHCO3 (30), NaH2PO4 (1.25), KCl (2.5), MgSO4 (10), CaCl2 (0.5). The solution was ice-cold and carbo-oxygenated with a 95% O2 + 5% CO2 gas mixture. Slices were allowed to recover in a low-calcium artificial cerebrospinal fluid (aCSF) solution, composed of (in mM): NaCl (130), KCl (3.5), NaH2PO4 (1.25), NaHCO3 (25), glucose (10), CaCl2 (1) and MgSO4 (2), at 34–35 °C with constant oxygenation for about 1 h before the experiment. Slices were individually transferred to the recording chamber of the patch clamp set up and continuously perfused with warm (32–33 °C), carbo-oxygenated aCSF solution, composed of (in mM):

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NaCl (130), KCl (3.5), NaH2PO4 (1.25), NaHCO3 (25), glucose (10), CaCl2 (2) and MgSO4 (1). Patch pipettes were made from thin-walled borosilicate capillaries (Harvard Apparatus, London, UK) with a vertical puller (Narishige PP830, Narishige International Ltd, London, UK) and, back-filled with K+ gluconate- or KCl-based intracellular solutions for the recording of spontaneous excitatory postsynaptic currents (sEPSCs) or spontaneous inhibitory synaptic currents (sIPSCs), respectively. Signals were sampled at 10 kHz and low-pass filtered at 3 kHz with an Axon Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA). The slice was visualized with an inverted microscope (Nikon Eclipse E600FN) equipped for infrared videomicroscopy. The VTA was identified by its position relative to the medial terminal nucleus of the accessory optic tract (MT; Neuhoff et al., 2002; Margolis et al., 2006). VTA DA neurons were visually identified with GFP fluorescence and, during recordings, by the presence of specific physiological hallmarks: slow regular firing (1–2 Hz), broad action potential (AP), prominent hyperpolarization-activated current (Ih; Neuhoff et al., 2002; Masi et al., 2015). GFP-negative neurons exhibiting fast firing (5–6 Hz), narrow action potential and absence of Ih were classified as GABA neurons. During voltage-clamp, Ih current was obtained by imposing 2-s voltage steps with amplitude ranging from 0 mV to −60 mV, from a −60 mV holding potential. The typical DA and/or GABAergic signatures were obtained by a series of hyperpolarizing and depolarizing current steps with amplitude ranging from −100 pA to +100 pA with 50 pA increments, shortly after patch rupture. Cell-attached recordings were made in voltage clamp (V_{HOLD} = 0 mV), “loose patch” (R < 100 MΩ) configuration in order to minimize perturbations of the neuronal activity. Pipette was filled with the following internal solution (mM): K+ gluconate (120), KCl (15), HEPES (10), EGTA (1), MgCl2 (2), Na_2 phosphocreatine (5), NaGTP (0.3), MgATP (4), pH 7.3–7.4, 295–305 mOsm and resulting in a bath resistance of 3–5 MΩ. The same pipette solution was used in whole-cell recordings to measure basic electrophysiological parameters of DA and GABA neurons and sEPSCs. For the recording of sIPSCs, a KCl-based solution was used with the following composition (mM): KCl (140), MgCl2 (1.6), MgATP (2.5), NaGTP (0.5), EGTA (2), HEPES (10), pH = 7.3–7.4, 295–305 mOsm and resulting in a bath resistance of 3–5 MΩ. To isolate sEPSCs and sIPSCs, 10 μM SR95531 ($GABA_A$ receptor blocker), 10 μM NBQX (AMPA glutamate receptor blocker) and 50 μM D/L-APV (NMDA glutamate receptor blocker) were included in the recording solution, respectively. A recording solution containing all the above antagonists plus the $GABA_B$ receptor blocker CGP55845 (1 μM) was used to record synaptically-isolated VTA DA firing activity. The response to acute EtOH was assessed by perfusing the slice with 40 mM EtOH in standard recording aCSF. Neurons responding to acute EtOH were defined as neurons exhibiting a stable increase (fold change >1.40) in the average frequency measured in 30 s-time bins after EtOH perfusion.

2.5. Immunocytochemistry

Mice were anesthetized with Avertin and transcardially perfused with cold phosphate buffered saline with 4% paraformaldehyde. The following day, brains were transferred to a 30% sucrose solution before storage at −80 °C. 30-μm, horizontal slices of the midbrain were obtained with a Leica CM1900 cryostat and stored at 4 °C in a phosphate buffered saline with sodium azide 0,02%. Sections were used for immunostaining with a rabbit vesicular GABA transporter (VGAT) antibody (Synaptic System, 1:1000) and a mouse

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Tyrosine Hydroxylase antibody (Boster Biological Technology; 1:100). Immunolabeled slices where imaged with a confocal microscope (Nikon Eclipse TE300) with a 60X oil-immersion objective (NA 1.4, Nikon). Images were subsequently processed with FIJI/ImageJ (https://imagej.net/software/fiji/) to quantify the number of putative VGAT clusters close to the soma of a TH-positive neuron. In brief, raw images were convolved with a 2D gaussian filter (sigma = 1.5 mm); afterwards, red spheres touching the green cell body were manually annotated as VGAT clusters. These were defined as red spots with intensity at least double with respect to the background, and in close contact with the soma of the DA neuron. Analysis was performed off-line by an operator blinded to the experimental condition.

2.6. Reagents

Unless otherwise specified, reagents were purchased from Merck (Saint-Louis, MO, USA). When specified, the following antagonists were used: NBQX (10 μM), D/L-APV (50 μM), SR95531 (10 μM) and CGP55845 (1 μM; Tocris Bioscience, Bristol, UK). All drugs were diluted from 100–1000X stock solutions to the final concentration in the recording aCSF.

2.7. Data elaboration and statistical analysis

Cell-attached and whole-cell patch-clamp recordings were analyzed using Clampfit 10.7 (Molecular Devices, Sunnyvale, CA, USA) and Origin 2019 (OriginLab, Northampton, MA, USA). Mean values of firing rate, sIPSCs and sEPSCs frequency and amplitude relative to specific experimental condition were calculated offline from 1-min bins of the corresponding recording section (i.e.: aCSF; aCSF + blockers; ACSF + blockers + EtOH 40 mM) by using Clampfit 10.7 (Molecular Devices, Sunnyvale, CA, USA). All data were tested for normality (D'Agostino-Pearson and Shapiro-Wilk, GraphPad Prism 7.0), before using parametric tests. According to the experimental design, statistical significance was assessed with a Student's t-test (paired or unpaired samples, GraphPad Prism 7.0), two-way ANOVA followed by Newman/Keuls post hoc test (STATISTICA 7) to compare means, or two-sample proportion test (Origin, 2019) to compare percentages. When normality test failed, a non-parametric Mann-Whitney test was used. Significance at the $p < 0.05$, 0.01, 0.001, and 0.0001 level are indicated with *, **, ***, and **** respectively, in figures. Cohen's d value (effect size) was calculated as follows $d = (\text{mean}_2 - \text{mean}_1)$/pooled standard deviation. In the text, data are reported in brackets with the following format: unit; mean ± standard error (SEM), p value referring to either t-test, "main effects", interaction or multiple comparison when two-way ANOVA followed by post hoc analysis was used. For simplicity, only relevant statistical parameters are reported in the main text. For each dataset, all experimental values, sample size (N/n = animals/cells), statistical test, p value, Cohen's d and other relevant parameters are reported in detail in the Supporting Information (SI) table. When two-way ANOVA is used, asterisks in histograms refer to p values obtained with multiple comparison. Graphs were generated with GraphPad Prism 7.0, and representative traces were generated with Origin 2019. Example traces represent typical observations.

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3. Results

3.1. TBC results in moderate EtOH intake which does not induce dependence

TH-GFP mice of both sexes initiated the TBC drinking protocol at approximately eight weeks of age (N = 141). In the exposed group (N = 82), EtOH concentration was progressively increased from 3 to 12% during 18 day as schematized in Fig. 1a. Before entering the TBC protocol, the basal hedonic tone of our TH-GFP mouse colony was assessed by measuring the preference for a 5% sucrose solution on a small sample of animals (SI, Fig. 1a). We found no differences with reported values of preference in the same conditions in C57BL/6 mice (Frazier et al., 2008). During TBC drinking, individual intake doubled as the EtOH percentage was raised from 3 to 6% (g/Kg; 3% = 3.84 ± 0.20, 6% = 7.65 ± 0.5), while the trend decreased at 12% (11.69 ± 0.69 g/kg). Preference versus water peaked at 6%, then decayed at 12% (3% = 55.6 ± 2.28%, 6% = 59.9 ± 3.34%, 12% = 55.74 ± 3.37%; Fig. 1b). No difference in either preference or consumption were found between male and female mice (SI, Fig. 1b). We then tested the presence of affective and behavioral signs of withdrawal after 24 h of EtOH deprivation (Fig. 1c and d). Exposed mice did not show increased anxiety levels compared to naive controls, as indicated by the total number of entries (naïve = 10.3 ± 2.24, withdrawal = 7.04 ± 1.23, $p = 0.27$) or time spent in open arms (% of time; naïve = 11.30 ± 3.64, withdrawal = 13.23 ± 2.78, $p = 0.67$) in the EPM test (Fig. 1c). A separate group of mice was tested for the expression of ADE by re-exposure to 12% EtOH solution and assessment of relapse drinking. When mice were re-exposed to 12% EtOH we did not observe increased intake (g/Kg; before = 4.76 ± 0.21, after = 5.48 ± 0.35; $p = 0.10$) or preference (%; before = 47.96 ± 2.54, after = 46.29 ± 2.06; $p = 0.062$) compared to pre-deprivation (Fig. 1d).

3.2. Voluntary EtOH drinking suppresses basal firing activity and increases EtOH sensitivity of VTA DA neurons

In order to explore the effects of chronic moderate EtOH drinking on the activity of VTA DA neurons, we performed electrophysiological recordings on midbrain slices obtained from exposed and naive mice at the end of the TBC. We recorded from both medial and lateral GFP-positive VTA neurons and since we found no effect of soma location on the measured parameters, recordings were pooled together. We first studied basic electrophysiological parameters with whole cell recordings (SI, Fig. 1c) and found no effect of chronic EtOH in membrane capacitance (pF; naïve = 75.03 ± 7.59; exposed = 102.04 ± 10.69; $p = 0.051$), input resistance (MΩ; naïve = 406.23 ± 43.12, exposed = 469.78 ± 35.90; $p = 0.28$) or average subthreshold potential (mV; naïve = −49.72 ± 1.11, exposed = −48.95 ± 1.41; $p = 0.68$). AP threshold is slightly but significantly depolarized in exposed mice (mV; naïve = −39.32 ± 1.09, exposed = −35.73 ± 0.72; $p = 0.009$; SI, Fig. 1c), although this alteration does not affect intrinsic excitability as indicated by the number of APs elicited by a +100 pA square pulse (naïve = 2.40 ± 0.40, exposed = 3 ± 0.41; $p = 0.33$; SI, Fig. 1d). Chronic EtOH did not affect Ih amplitude (pA at −120 mV; naïve = −365.77 ± 57.35, exposed = −337.2 ± 44.97; $p = 0.71$; SI, Fig. 1e). With cell-attached configuration, we then measured the firing frequency of VTA DA neurons in basal conditions and in response to 40 mM EtOH applied with bath perfusion (Fig. 2). Of note, 40 mM is a medium-low concentration in the range of doses used in previous studies (Mrejeru et al., 2015; Theile et al., 2008). In

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basal conditions, firing rate was significantly depressed and less regular in exposed mice, as shown by difference in mean frequency and interevent interval distribution (Hz; naive = 2.22 ± 0.20, exposed = 1.29 ± 0.13; "chronic EtOH": $p = 0.01$; Cohen's $d = -1.35$; Fig. 2b–d). Acute EtOH application significantly enhanced firing rate in both groups, but with greater effect size in VTA DA neurons from exposed mice (fold increase; naive = 1.31 ± 0.05, exposed = 1.79 ± 0.14; "acute EtOH": $p < 0.0001$; "chronic EtOH" × "acute EtOH": $p = 0.024$; Fig. 2d). The effects of chronic or acute EtOH were similar in male and female mice (SI, Fig. 1f). Linear regression analysis revealed no significant correlation between basal firing rate and EtOH intake or preference (SI, Fig. 1g). Incubation with glutamate (NBQX + D/L-APV) and $GABA_{A/B}$ (SR 95531 + CGP 55845) receptor blockers ("synaptic block"; Fig. 2e) did not affect the firing of VTA DA neurons from naive animals, while it normalized baseline firing rate of exposed neurons (Hz; naive = 2.61 ± 0.38, exposed = 2.08 ± 0.25; "chronic EtOH": $p = 0.14$; Fig. 2f) and abolished their sensitivity to acute EtOH (fold increase; naive = 1.13 ± 0.04, $p = 0.0007$; exposed = 1.06 ± 0.05, $p = 0.66$; "chronic EtOH" × "acute EtOH": $p = 0.025$; Fig. 2f). Overall, slice recordings suggest that EtOH has both acute and long-lasting effects on the activity of VTA DA neurons and that expression of these effects requires the integrity of the local synaptic network.

3.3. Glutamate transmission onto VTA DA neurons is substantially unaffected by voluntary EtOH drinking

Based on the evidence that intact synaptic activity is required for the expression of EtOH effects, we recorded sEPSCs in basal and EtOH-stimulated conditions (SI, Fig. 2). We found that basal sEPSC frequency was not affected by chronic EtOH (Hz; naive = 1.36 ± 0.37, exposed = 1.12 ± 0.41; "chronic EtOH": $p = 0.14$), while acute EtOH reduced frequency in the naïve group (fold increase; naive = 0.97 ± 0.16, $p = 0.025$; exposed = 0.86 ± 0.30, $p = 0.099$; "acute EtOH": $p = 0.001$; SI, Fig. 2b). Consistently, event analysis reveals that chronic EtOH causes a right shift on the cumulative frequency distribution curve (SI, Fig. 3). sEPSC amplitude was also substantially insensitive to either chronic (pA; naive = −15.55 ± 1.21, exposed = −18.14 ± 1.04; "chronic EtOH": $p = 0.370$) or acute EtOH (fold increase; naive = 1.03 ± 0.05, exposed = 0.94 ± 0.07, "acute EtOH": $p = 0.13$; SI, Fig. 2c). Although a significant reduction of EPSC rise time was measured in the exposed group (ms; naïve = 3.63 ± 0.74, exposed = 1.77 ± 0.22; $p = 0.03$), there was no effect of chronic EtOH on EPSC area (pA*ms; naïve = 33.62 ± 5.96, exposed = 28.06 ± 1.21; $p = 0.38$; SI, Fig. 3a).

3.4. Voluntary EtOH drinking leads to long-lasting facilitation of GABA release to a subset of VTA DA neurons

When we examined GABA transmission to VTA DA neurons we found a non-significant increase in the basal frequency of IPSCs in the exposed group (Hz; naive = 1.36 ± 0.23, exposed = 1.93 ± 0.33; "chronic EtOH": $p = 0.48$, Fig. 3b). In addition, acute EtOH significantly increased event frequency in naïve, but not in exposed mice (fold increase; naive = 1.62 ± 0.2, $p = 0.0007$; exposed = 1.26 ± 0.07, $p = 0.81$; Fig. 3b). This is consistent with the greater enhancing effect of EtOH on firing in exposed mice. Of note, during dataset analysis we noticed that, in both groups, only a subset of neurons responded to acute EtOH. When we restricted the analysis to neurons responding by a fold increase exceeding that produced by acute EtOH in the entire dataset (+1.4; naive = 16/28 = 57.14%, exposed =

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23/44 = 52.23%; $p = 0.68$; Fig. 3c), a significant difference in basal sIPSC frequency was revealed between naive and exposed group (Hz; naïve = 1.17 ± 0.25, exposed = 2.86 ± 0.79; $p = 0.04$, Mann-Whitney test; Cohen's $d = 0.56$; Fig. 3d). In consideration of the influence of sex on the electrophysiological effects of EtOH in multiple brain areas (Levine et al., 2021; Kirson et al., 2021; Vandegrift et al., 2020), we examined the sex proportion in the dataset and found that the percentage of responding neurons was equal in male and female mice (naïve: males = 62.5%, females = 50%, $p = 0.51$; exposed: males = 52%, females = 52.63%, $p = 0.97$). Classification based on the response to acute EtOH was applied to the sEPSC dataset (SI, Fig. 2b) but no difference in basal frequency between the two groups was revealed (Hz; naive = 1.72 ± 0.48, exposed = 1.90 ± 0.75; "chronic EtOH": $p = 0.99$; SI, Fig. 3b). Finally, chronic EtOH did not affect basal IPSC amplitude (pA; naive = −30.28 ± 4.08, exposed = −26.94 ± 1.84; "chronic EtOH": $p = 0.15$), but abolished the enhancement caused by acute EtOH (fold increase; naïve = 1.52 ± 0.19, $p = 0.0001$; exposed = 1.24 ± 0.04, $p = 0.11$; Fig. 3d), indicating an interaction between chronic and acute EtOH ($p = 0.05$).

3.5. Chronic EtOH induces moderate facilitation of firing activity in VTA GABA neurons

Spontaneous inhibitory currents reflect activity-dependent neurotransmitter release from GABA neurons present in the slice. Therefore, we tested the hypothesis that a subset of VTA GABA neurons responded to EtOH during the TBC by undergoing a long-lasting potentiation of firing activity, as previously demonstrated with in vivo recordings following passive intraperitoneal EtOH administration (Gallegos et al., 1999). To test this hypothesis, we targeted GFP-negative neurons showing physiological signatures typically associated to GABA phenotype, which include fast spontaneous and stimulated firing, narrow AP and lack of Ih (Margolis et al., 2006; Grace and Onn, 1989; Johnson and North, 1992; Chieng et al., 2011; Marino et al., 2001). Cell-attached firing recordings from putative VTA GABA neurons revealed the existence of a subset of neurons (naïve = 13/16 = 81.2%, exposed = 21/27 = 77.8%; $p = 0.78$) responding to acute EtOH with acceleration of firing rate (fold increase; naive = 1.38 ± 0.13, exposed = 1.30 ± 0.06; "acute EtOH": $p < 0.0001$). This subset exhibited elevated basal firing rate compared to naive counterpart, although not significantly (Hz; naïve = 4.20 ± 0.56, exposed = 5.55 ± 0.59; "chronic EtOH": $p = 0.15$; Fig. 4b). Altogether, these results may, at least in part, explain the depression of VTA DA firing and the elevated frequency of GABA currents in the exposed group.

3.6. Chronic EtOH causes formation of new GABA synaptic contacts onto the soma of DA neurons

Based on the evidence that the enhancement of GABA transmission induced by chronic EtOH is pre-synaptically expressed, we evaluated whether structural remodeling of GABA afferents could contribute to facilitation of GABA release onto DA neurons. We performed a double immunostaining for the GABA vesicular transporter (VGAT) and the dopaminergic marker Tyrosine Hydroxylase (TH) on histological sections from exposed and naïve mice (Fig. 5a). The stereological count of VGAT clusters located in close contact with the soma of TH-positive neurons within single confocal sections (Fig. 5a and b) revealed that chronic EtOH causes a significant increase in the number of GABA axosomatic synaptic boutons onto DA neurons (naive = 9.79 ± 1.14, exposed = 14.68 ± 1.39; $p = 0.009$; Cohen's $d = 0.84$).

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4. Discussion

The aim of the present study was to investigate the effects of voluntary EtOH drinking on the physiological properties of VTA DA neurons. We performed extracellular and patch clamp recordings in brain slices from mice exposed to a TBC protocol and from naïve controls. Values of daily intake and preference versus water indicate that the TBC paradigm we implemented produces moderate EtOH consumption. Both intake and preference were consistent with previous studies, with highest EtOH concentration revealing inter-individual variability (Juarez et al., 2017; Yoneyama et al., 2008). Although we did not directly measure the blood EtOH concentration (BEC) of mice during TBC, average intake was similar to that inducing BEC values well below 1 mg/ml (Juarez et al., 2017), the critical threshold for the expression of behavioral effects (Rhodes et al., 2005). Consistently, we did not detect emotional and behavioral signs of withdrawal following 24 h of substance deprivation. Nevertheless, with ex vivo slice recordings, we found that chronic EtOH influences a number of basal electrophysiological parameters and alters the sensitivity to acute stimulation. VTA DA neurons from exposed mice exhibit lower firing rate and enhanced response to acute EtOH application compared to naive controls, suggesting that chronic EtOH activates tonic inhibitory mechanisms while inducing a sensitization to the acute stimulating effect of the substance. Such sensitization was reported previously, although following passive EtOH administration and in the absence of basal effects (Brodie, 2002). Both chronic and acute effects described here are sustained by network activity, as synaptic silencing obtained with simultaneous block of $GABA_{A/B}$ and AMPA/NMDA receptor transmission normalizes the firing rate and abolishes acute response of VTA DA neurons in the exposed group. It was previously reported that the facilitating effect of acute EtOH on the activity of VTA DA neurons is insensitive to synaptic isolation (Okamoto et al., 2006). In our synaptic isolation recordings, while naive DA neurons responded normally, the effect of acute EtOH was absent in VTA DA neurons from exposed mice, suggesting that intrinsic mechanisms mediating acute response were downregulated by chronic EtOH. The effect of chronic EtOH on DA neuron activity was not linearly correlated to intake or preference, suggesting the existence of different individual sensitivity thresholds to the EtOH-induced physiological effects.

The effect of chronic EtOH on the activity of VTA DA neurons observed here must be viewed in light of previous work reporting that EtOH drinking does not modify basal firing rate (Avegno et al., 2016). We propose a number of experimental conditions as the possible explanation for this discrepancy, such as the drinking paradigm (intermittent versus continuous access), brain slicing procedure (coronal versus horizontal) and target area (medial versus entire VTA). Another effect of chronic EtOH that we observed is a significant depolarization of the AP threshold. Threshold depolarization did not seem to affect intrinsic excitability, since the number of current-stimulated spikes was unaltered.

Following up on the evidence that EtOH effects on VTA DA activity require network integrity, we studied how chronic and acute EtOH modulates excitatory and inhibitory transmission to DA neurons. We found that the strength of glutamate transmission to DA neurons, expressed as EPSC frequency and amplitude, is largely insensitive to chronic exposure. The significant acceleration of EPSC rise kinetics induced by chronic EtOH

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is unlikely to affect post-synaptic response to glutamatergic transmission, as EPSC area does not change. Of note, EPSC amplitude was slightly, but significantly, depressed by acute EtOH in both conditions. Facilitation of glutamate transmission to VTA DA neurons by acute EtOH was reported previously, although in slices from juvenile rats and using different neuron selection criteria (Xiao et al., 2009). In contrast, when we studied GABA inputs we observed significant effects of both exposure modalities. Acute EtOH increased the frequency of GABA inputs. This was previously reported and attributed to presynaptic release facilitation (Theile et al., 2011). However, acute EtOH had no effect in the exposed group, in which a stable enhancement of basal GABA release was measured. Importantly, such enhancement was specifically recorded in the subset of DA neurons in which GABA transmission was sensitive to acute EtOH. Furthermore, acute EtOH enhanced GABA current amplitude in the naive group, as previously shown in similar conditions (Theile et al., 2011), but not in the exposed group. This finding is consistent with an EtOH-dependent rearrangement of $GABA_A$ receptor subunit expression (Kumar et al., 2009) and may be interpreted as a postsynaptic homeostatic mechanism to offset increased GABA input. Of note, our recordings of pharmacologically-isolated excitatory and inhibitory synaptic inputs failed to reveal the mechanism underlying firing enhancement induced by acute EtOH in the exposed group. One possible explanation is that GABA and AMPA/NMDA receptor-mediated transmission must be simultaneously functional to sustain EtOH enhancement, at least in the exposed condition, in which intrinsic sensitivity is lost. Remarkably, facilitation of GABA release is paralleled by significant increase in the number of axo-somatic GABA synapses onto DA neurons, as indicated by the count of VGAT positive clusters in close contact with the soma of DA neurons. This finding is especially relevant as it suggests that moderate EtOH drinking is able to induce structural, possibly long-lasting, rewiring of GABA inputs onto VTA DA neurons, in addition to the modulation of release frequency and post-synaptic response.

To test the hypothesis that increased frequency of GABA inputs onto VTA DA neurons induced by chronic EtOH involved local GABA interneurons, we performed cell-attached recordings from GFP-negative VTA neurons exhibiting GABA-like electrophysiological profile. We found that 81.2% and 77.8% neurons from, respectively, naive and exposed animals were sensitive to acute EtOH. In these subgroups, previous chronic EtOH indeed slightly increased basal firing rate, although not significantly, suggesting that EtOH-induced enhancement of GABA transmission to DA neurons may be partially mediated by long-lasting activation of local GABA interneurons. Additional EtOH-sensitive GABA afferents may originate from the rostromedial tegmental nucleus (RMtg), an adjacent nucleus also known as the “VTA tail”, populated by VTA-projecting GABA neurons involved in the processing of multiple aversive stimuli, including those associated to EtOH withdrawal (Fu et al., 2019; Jhou et al., 2009).

Of note, when we examined the influence of sex on the effects exerted by EtOH on key behavioral (EtOH intake and preference) and electrophysiological parameters (firing rate, spontaneous inhibitory activity), we did not find significant differences. This finding marks a difference with multiple studies reporting a significant effect of sex on major behavioral and electrophysiological parameters in multiple brain areas (Levine et al., 2021; Kirson et al., 2021; Vandegrift et al., 2020). We speculate that this divergence may correlate to the

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lower values of EtOH intake achieved with the TBC paradigm implemented here compared to those used in the cited studies.

Overall, the functional and structural effects of chronic EtOH on VTA DA activity and GABA transmission observed in the present study lead to the following conclusions: first, the existence of EtOH-sensitive GABA neurons which adapt to chronic consumption of moderate EtOH amounts by intensifying their inhibitory drive to VTA DA neurons. Increased basal GABA transmission to VTA DA neurons would explain the tonic depression of firing activity observed in EtOH-exposed mice; second, modulation of DA neurons by EtOH occurs through segregated pathways involving specific subpopulations of EtOH-sensitive GABA neurons. Both conclusions are consistent with previous studies demonstrating that VTA DA neurons are divided into distinct populations with specific connectivity and function in reward-related behaviors (Lammel et al., 2008; Yang et al., 2018); third, the finding that acute EtOH produces a significantly higher enhancement of DA firing in EtOH-exposed mice leads to conclude that, although voluntary EtOH drinking produces a GABA-mediated decrease in basal activity of VTA DA neurons, re-exposure to acute EtOH re-equilibrates firing activity thus rescuing depression. This is a particularly relevant finding as it points to the possibility that the hypodopaminergic state elicited by EtOH exposure may emerge from early stage drinking characterized by consumption of moderate amounts of alcohol. If this is the case, it is tempting to speculate that, in recreational EtOH consumers, the combination of the depressed DA tone and increased sensitivity of the reward system for EtOH drives the motivation to drink and the escalation of EtOH consumption.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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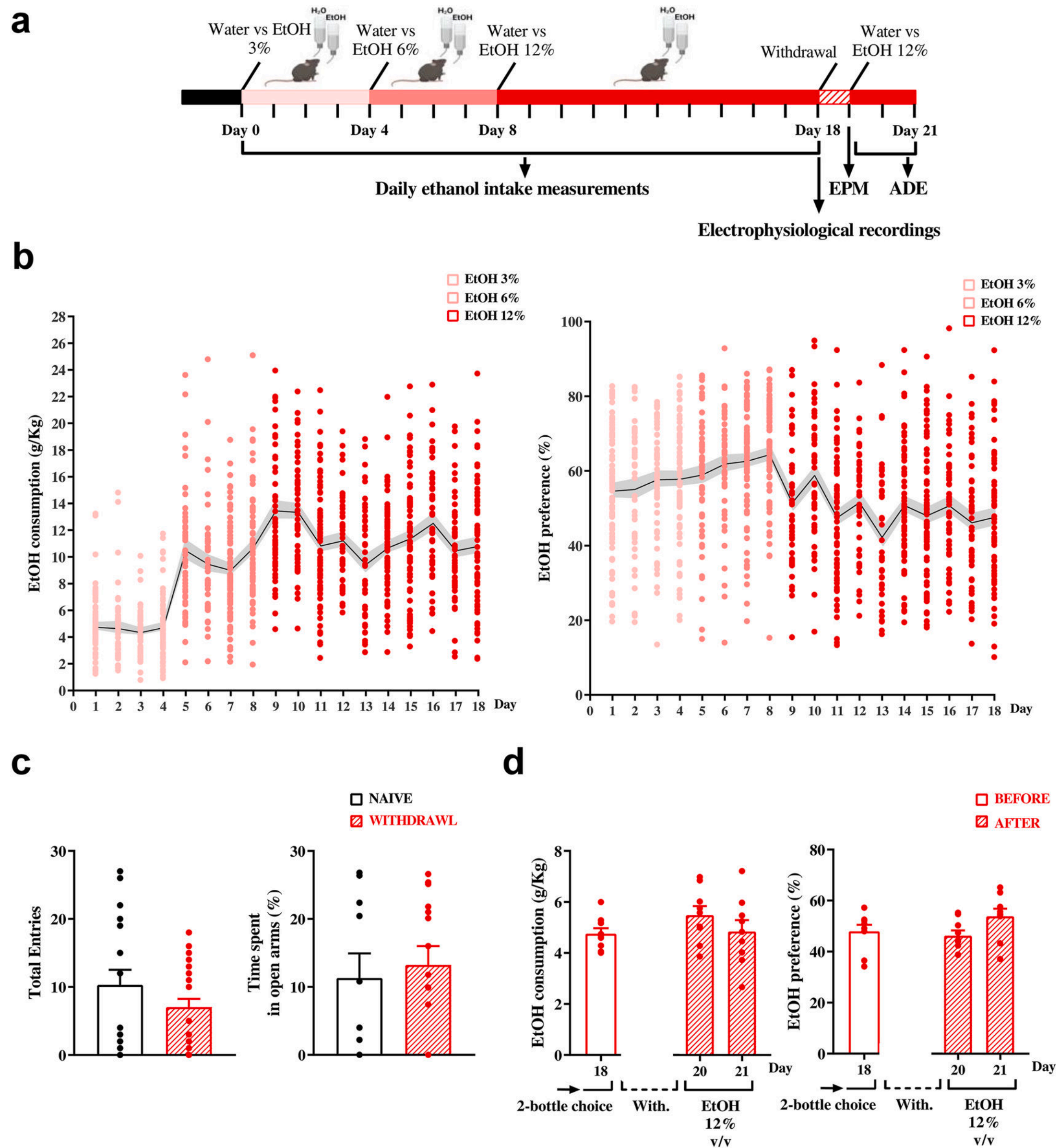

Fig. 1.

TBC results in moderate EtOH intake which does not induce dependence (a) TBC paradigm scheme. (b) Plot diagrams reporting the raw (dots) and mean (black line) ± SEM (gray shading) values of EtOH consumption (left), and preference (right; N = 82). (c) Effect of withdrawal from chronic alcohol exposure on the EPM test. Average number of total entries and time spent in open arms (N; naïve = 10, withdrawal = 14). (d) Effect of re-exposure to 12% EtOH following 24 h of deprivation. Dots and bars represent, respectively, absolute and average values of intake (left) and preference (right) one day before and two days after 24 h of deprivation (N = 9).

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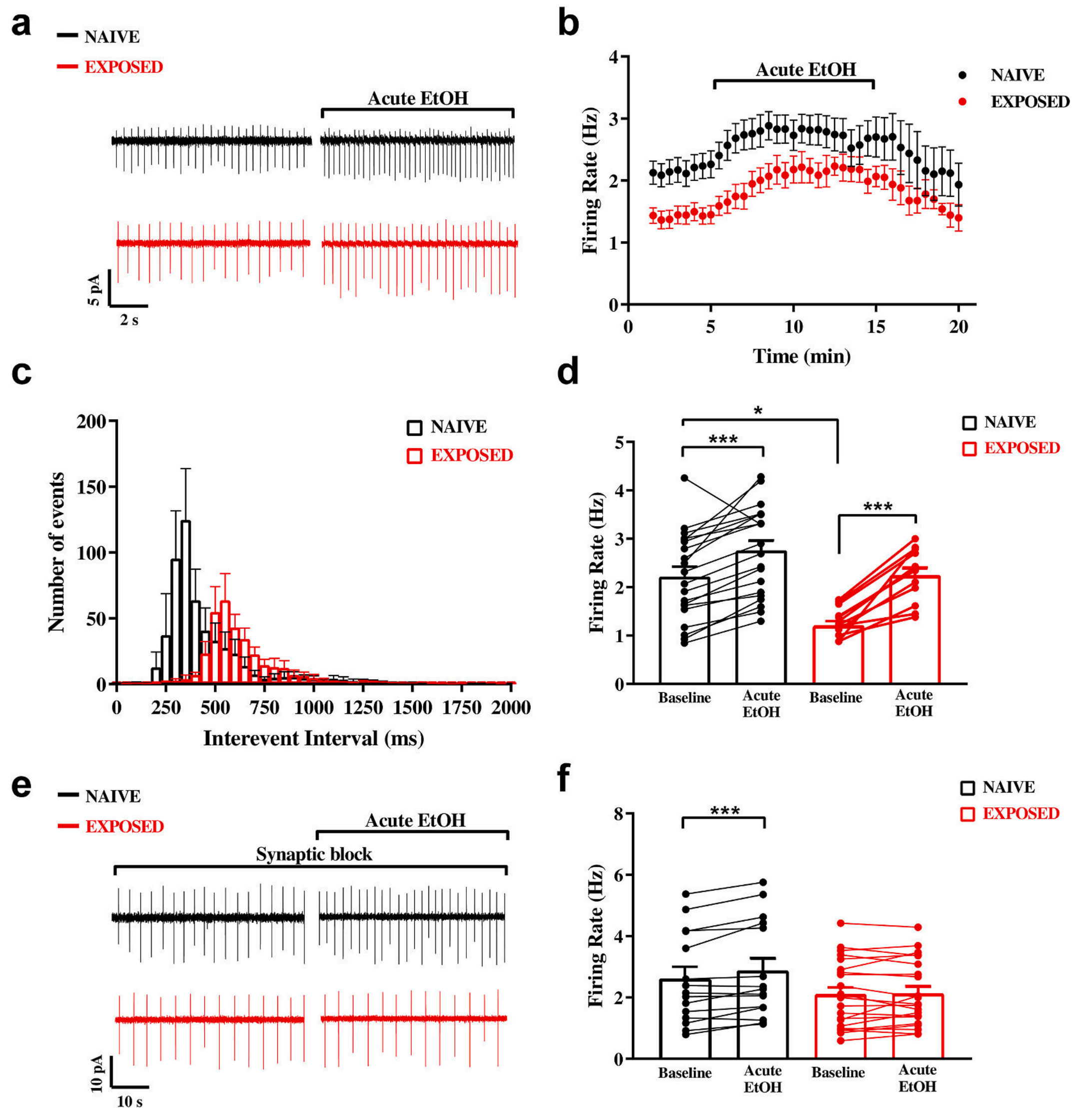

Fig. 2.

Voluntary EtOH drinking suppresses basal firing activity and increases EtOH sensitivity of VTA DA neurons. (a) Example cell-attached recordings of spontaneous firing of VTA DA neurons from naive (black) and exposed (red) mice in baseline (left) and in presence of 40 mM EtOH (right). (b) Time course of EtOH-induced stimulation of firing activity (N/n; naïve = 14/20, exposed = 10/13). (c) Event frequency distribution (N/n; naive = 10/20; exposed = 10/13). (d) Absolute (dots) and average (bars) values of firing frequency before and after 40 mM EtOH application (N/n; naive = 10/20; exposed = 10/13). (e, f) Same as (a, d) but in the presence of antagonists of AMPA/NMDA and $GABA_{A/B}$ receptors (N/n; naive = 6/15; exposed = 9/21). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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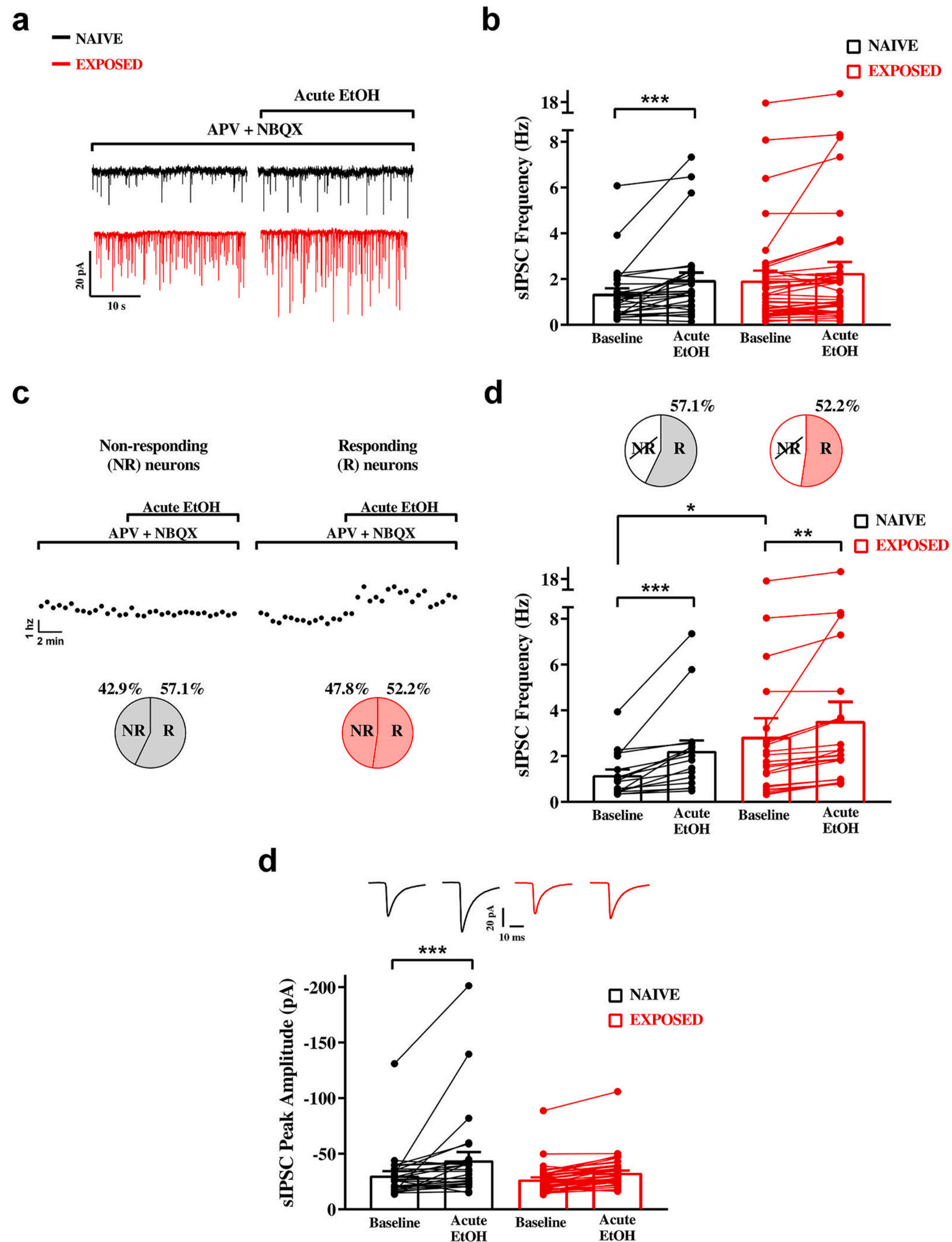

Fig. 3.

Voluntary EtOH drinking leads to long-lasting facilitation of GABA release to a subset of VTA DA neurons. (a) Example of whole-cell voltage-clamp recordings of pharmacologically-isolated sIPSCs from naive (black) and exposed (red) mice before (left) and following (right) stimulation with 40 mM EtOH. (b) Absolute (dots) and average (bars) values of sEPCS frequency before and after acute EtOH application in naive and exposed VTA DA neurons (N/n; naive = 10/28; exposed = 16/44). (c) Representative frequency plots showing difference sensitivity to acute EtOH and percentage of responding versus

non-responding neurons. (d) Same as (b) but limited to EtOH-responding (R) neurons (N/n; naive = 9/16; exposed = 13/23). (d) Same as (b) but for sIPSC amplitude (N/n; naive = 10/28; exposed = 16/44). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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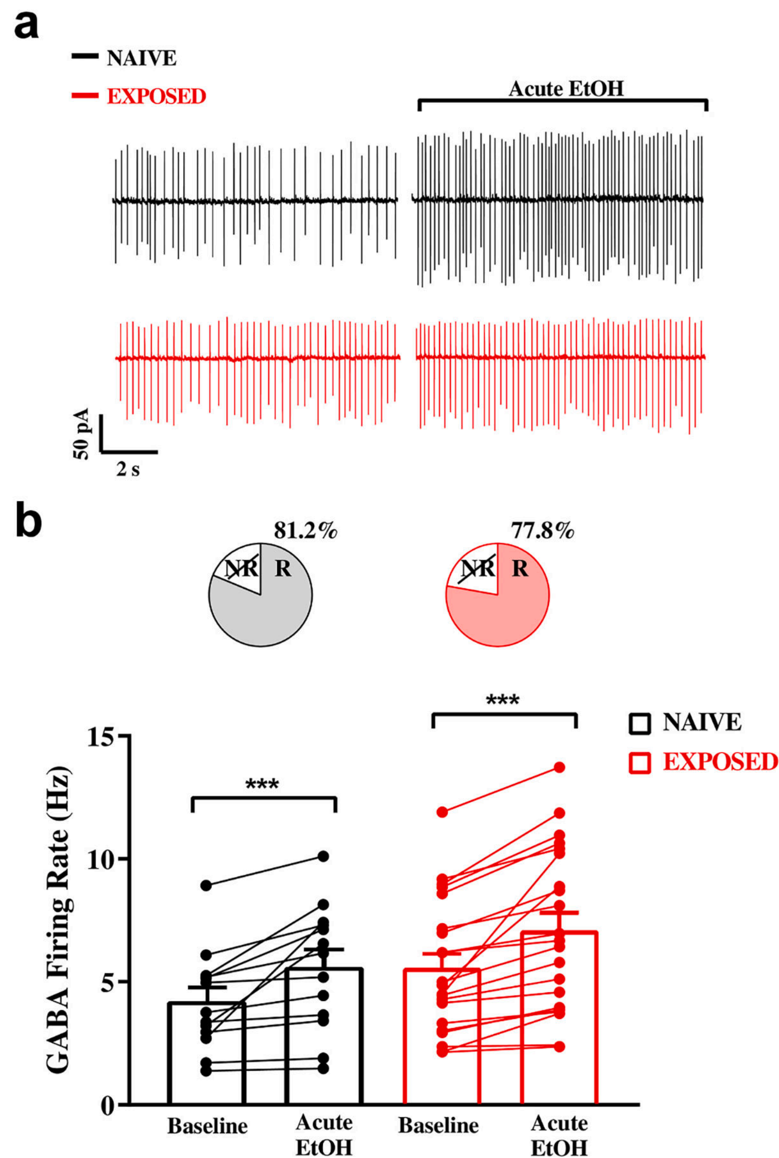

Fig. 4.

Chronic EtOH induces moderate facilitation of firing activity in VTA GABA neurons.

(a) Representative traces from cell-attached recordings of firing rate from putative GABA neurons in naive (black) and exposed (red) mice before (left) and after (right) acute EtOH.

(b) Absolute (dots) and average (bars) values of firing frequency before and after acute EtOH application in the fraction of EtOH-responding putative GABA neurons in slices from naive and exposed mice (N/n; naive = 8/13; exposed = 16/21). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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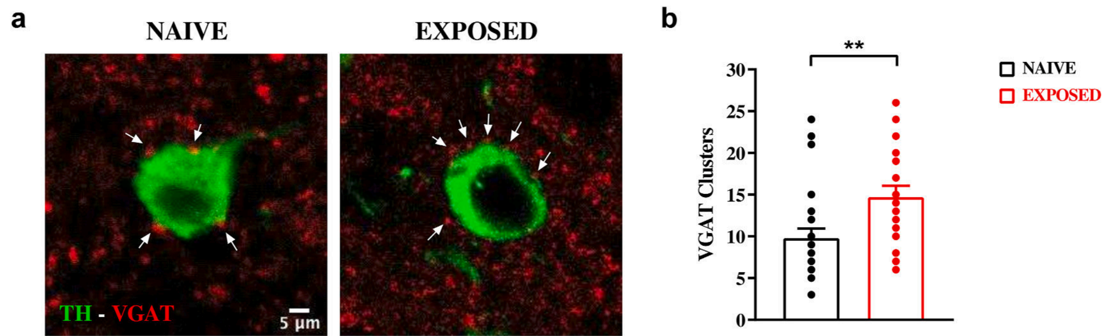

Fig. 5.

Chronic EtOH increases the number of GABA synaptic contacts onto the soma of VTA DA neurons (a) Representative confocal images showing somatic innervation of TH-positive cells (green) by GABAergic axonal boutons (red) identified by VGAT immunoreactivity from naive (left) and exposed (right) mice. (b) Quantitative analysis of axo-somatic VGAT cluster density in the two groups (animals/slices/neurons; naïve = 3/10/24, exposed = 3/9/19). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)