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Acute and Chronic Ethanol Modulate Dopamine D2-Subtype Receptor Responses in Ventral Tegmental Area GABA Neurons

Kimberly H. Ludlow, Katie D. Bradley, David W. Allison, Seth R. Taylor, Jordan T. Yorgason, David M. Hansen, Christine H. Walton, Sterling N. Sudweeks, and Scott C. Steffensen

Department of Psychology and Center for Neuroscience, Brigham Young University, Provo, Utah

Abstract

Background—Ventral tegmental area (VTA) γ -aminobutyric acid (GABA) neurons appear to be critical substrates underlying the acute and chronic effects of ethanol on dopamine (DA) neurotransmission in the mesocorticolimbic system implicated in drug reward. VTA GABA neuron firing rate is reduced by acute ethanol and enhanced by DA via D2 receptor activation. The objective of this study was to evaluate the role of D2 receptors in acute ethanol inhibition of VTA GABA neuron activity, as well as the adaptation of D2 receptors by chronic ethanol consumption.

Methods—Using electrophysiological methods, we evaluated the effects of intraperitoneal ethanol on DA activation of VTA GABA neurons, the effects of DA antagonists on ethanol inhibition of their firing rate, as well as adaptations in firing rate following chronic ethanol consumption. Using single cell quantitative RT-polymerase chain reaction (PCR), we evaluated the expression of VTA GABA neuron D2 receptors in rats consuming ethanol versus pair-fed controls.

Results—In acute ethanol studies, microelectrophoretic activation of VTA GABA neurons by DA was inhibited by acute intraperitoneal ethanol, and intravenous administration of the D2 antagonist eticlopride blocked ethanol suppression of VTA GABA neuron firing rate. In chronic ethanol studies, while there were no signs of withdrawal at 24 hours, or significant adaptation in firing rate or response to acute ethanol, there was a significant down-regulation in the expression of D2 receptors in ethanol-consuming rats versus pair-fed controls.

Conclusions—Inhibition of DA activation of VTA GABA neuron firing rate by ethanol, as well as eticlopride block of ethanol inhibition of VTA GABA neuron firing rate, suggests an interaction between ethanol and DA neurotransmission via D2 receptors, perhaps via enhanced DA release in the VTA subsequent to ethanol inhibition of GABA neurons. Down-regulation of VTA GABA neuron D2 receptors by chronic ethanol might result from persistent DA release onto GABA neurons.

Keywords

GABA; VTA; Ethanol; D2 Receptors; Adaptation; Dopamine

The mesocorticolimbic dopamine (DA) system originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAcc) is considered to be a key area in reward from natural behaviors such as feeding (Phillips et al., 2003), drinking (Agmo et al., 1995), and drug reward, including alcohol reward (Koob, 1996). Support for a role for this

neural circuit in alcohol reward hinges on the evidence demonstrating that local injections of DA antagonists into the NAcc and/or afferent circuit systems prevent ethanol self-administration in rats (Hyytia and Koob, 1995; Pettit et al., 1984; Roberts et al., 1996; Vaccarino et al., 1985). Moreover, rats will self-administer ethanol directly into the VTA (Gatto et al., 1994), and an ethanol-induced increase of DA release in the NAcc, detected by microdialysis, has been reported extensively (Di Chiara and Imperato, 1988; Weiss et al., 1993; Wozniak et al., 1991; Yoshimoto et al., 1992). Acute ethanol increases the firing rate of midbrain DA neurons both in vivo and in vitro (Brodie et al., 1990a; Gessa et al., 1985), and chronic ethanol reduces both DA activity and release of DA in the NAcc during withdrawal (Diana et al., 1993).

Although mesencephalic DA neurons in the midbrain are excited by ethanol, it has been suggested that their excitation may be attributed to disinhibition produced by a primary inhibitory effect on γ -aminobutyric acid (GABA)-containing neurons of the midbrain (Mereu and Gessa, 1985). Accordingly, we have demonstrated that acute ethanol reduces VTA GABA neuron firing rate (Gallegos et al., 1999) and cortico-tegmental excitatory synaptic responses (Stobbs et al., 2004) in vivo, with an IC_{50} of 1.0 g/kg (100 mg percentage blood alcohol level), a moderately intoxicating dose, at a substantial fraction of the EC_{50} for ethanol excitation of DA neurons in vitro (Brodie et al., 1999). Moreover, VTA GABA neurons become hyperexcitable during ethanol withdrawal and evince tolerance to ethanol inhibition of firing rate during chronic ethanol (Gallegos et al., 1999), suggesting that GABA neurons in the VTA constitute a critical substrate for the acute and chronic effects of ethanol in the mesocorticolimbic DA system (Diana et al., 2003). Theoretically, inhibition of VTA GABA neurons by acute ethanol would result in hyperexcitability of DA neurons and an increased amount of DA release, while chronic ethanol would result in hypoexcitability of DA neurons due to hyperexcitability of GABA neurons.

Dopamine neurons in the midbrain are inhibited by DA via D2 autoreceptor activation (for review see Adell and Artigas, 2004). GABA neurons in the VTA are activated by DA (Lassen et al., 2007; Stobbs et al., 2004) via D2 receptors (Steffensen et al., 2008). As ethanol inhibits and DA enhances VTA GABA neuron activity, the aim of this study was to evaluate the role of DA in acute ethanol effects, as well as the effects of chronic ethanol consumption on the potential adaptation of VTA GABA neuron activity and D2 receptors. As others have shown that critical DA-related gene products in the VTA, such as tyrosine hydroxylase are up-regulated (Ortiz et al., 1995), and D2 receptors are down-regulated (Rommelspacher et al., 1992), during withdrawal from chronic ethanol, and we have shown that VTA GABA neurons become hyperexcitable with chronic ethanol (Gallegos et al., 1999), we hypothesized that D2 receptor expression in VTA GABA neurons would adapt in association with chronic ethanol consumption.

Materials and Methods

Animal Subjects

Mature male Wistar rats (Charles River Laboratory, Hollister, CA) weighing 250 to 400 g (P50 to P80) were used in the electrophysiological and molecular studies of VTA GABA and DA neurons. Rats were housed 2 to a cage from the time of weaning (P25), with ad libitum access to food and water. The room temperature was controlled (22°C to 25°C) and maintained on a reverse 12 hours light/dark cycle (off 8 AM and on 8 PM). Animal care, maintenance, and experimental procedures were in accordance with the Brigham Young University Animal Research Committee and met or exceeded National Institutes of Health guidelines for the care and use of laboratory animals.

Ethanol Liquid Diet

Rats weighing 140 to 160 g (34- to 38-day old) were housed separately and given ad libitum access to solid food, water, and a commercially available liquid diet (Dyets, Bethlehem PA), known as the Lieber–DeCarli diet (Lieber and DeCarli, 1989). The liquid diet was made up fresh every few days in a blender and stored at 4°C. The solid diet and water was removed and rats were randomly assigned to either a control or chronic ethanol group. Pair-fed rats received the standard liquid diet and the chronic ethanol group received an ethanol-containing diet. Due to the natural aversion to ethanol, the rats in the chronic ethanol group were introduced to ethanol by increasing the percentage of ethanol from 3% to 5% in 3 increments over a 5-day period. To ensure that the control and chronic ethanol rat group diets were iso-caloric, the chronic ethanol rats were pair-fed with the control group. Pair-fed rats received the same volume of liquid diet as their ethanol paired rats consumed the previous day. In addition, maltose dextrin was added to the control diet in place of ethanol to ensure an iso-caloric diet compared with the ethanol diet. Once the chronic ethanol group reached an ethanol level of 5% in their liquid diet, they were maintained at this concentration for 2 to 3 weeks. Both control and ethanol liquid diets were changed daily at 5 PM. Prior to in vivo and in vitro electrophysiology experiments, chronic ethanol rats were withdrawn from ethanol for a period 24 hours.

Surgical Procedure and Single Cell Electrophysiology

For acute electrophysiological recordings of VTA GABA neurons, mature male rats weighing 250 to 400 g were anesthetized using isoflurane and placed in a stereotaxic apparatus. Anesthesia level was maintained at 1% throughout the experiments. Body temperature was maintained at $37.4 \pm 0.4^\circ\text{C}$ by a feedback regulated heating pad. With the skull exposed, holes were drilled for placement of stimulating and recording electrodes. Extracellular potentials were recorded by 3.0 M KCl-filled micropipettes (2 to 4 M Ω ; 1 μm inside diameter). Potentials were amplified with an Axon Instruments Multiclamp 700A amplifier (Union City, CA). Microelectrodes were oriented, via stereotaxic coordinates, into the VTA [from bregma: 5.6 to 6.5 posterior (P), 0.5 to 1.0 lateral (L), 6.5 to 7.5 ventral (V)] with a piezoelectric microdrive (EXFO Burleigh 8200 controller and Inchworm, Victor, NY). Single cell activity was filtered at 0.3 to 10 kHz (–3 dB) with the Multiclamp 700A amplifier and displayed on Tektronix (Beaverton, OR) digital oscilloscopes. Potentials were sampled at 20 kHz (12 bit resolution) with National Instruments data acquisition boards in Macintosh computers (Apple Computer, Cupertino, CA). Extracellularly recorded action potentials were discriminated with a World Precision Instruments WP-121 Spike Discriminator (Sarasota, FL) and converted to computer-level pulses. Single-unit potentials, discriminated spikes, and stimulation events were captured by National Instruments NB-MIO-16 digital I/O and counter/timer data acquisition boards (Austin, TX) in Macintosh computers.

Characterization of VTA GABA Neurons In Vivo

Ventral tegmental area GABA neurons were identified by previously established stereotaxic coordinates and by spontaneous and stimulus-evoked electrophysiological criteria (Steffensen et al., 1998). They included: relatively fast firing rate (>10 Hz); on–off phasic nonbursting activity; spike duration less than 200 microseconds; and multiple poststimulus spike discharges produced by stimulation of the internal capsule (IC; coordinates: –1.0 to 1.3 P, 2.3 to 3.0 L, and 5.0 to 6.0 V). Activation of the IC was accomplished by stimulating with insulated, bipolar stainless-steel electrodes with square-wave constant current stimulus pulses (50 to 2,000 μA ; 0.15 ms duration; average frequency, 0.1 Hz) that was generated by an AMPI IsoFlex isolation unit controlled by an AMPI MASTER-8 Pulse Generator (Jerusalem, Israel). We evaluated only those spikes that had greater than 4:1 signal-to-noise ratio.

Preparation of Slices

Following the in vivo electrophysiological recordings, the rats were anesthetized with ketamine (60 mg/kg) and decapitated. The brains were quickly removed and sectioned in ice-cold artificial cerebrospinal fluid (ACSF), bubbled with 95% O₂/5% CO₂. This cutting solution consisted of (in mM): 220 sucrose, 3 KCl, 1.25 NaH₂PO₄, 25 NaH₂CO₃, 12 MgSO₄, 10 glucose, 0.2 CaCl₂, and 0.4 ketamine. VTA-targeted horizontal slices (~200 μ m thick) were immediately placed into normal ACSF, bubbled with 95% O₂/5% CO₂ at 36°C consisting of (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 12 glucose, 1.5 MgSO₄, 2 CaCl₂, pH 7.3, and allowed to incubate for at least 45 minutes. Slices were then transferred to a recording chamber (Warner Instruments RC27, Hamden, CT) with continuous flow (2.0 ml/min) and maintained at 34°C to 35°C throughout the experiment. The location of the VTA in the slice was visually identified using infrared differential interference contrast (IR-DIC) microscopy at low power (4 $\times$ objective) by triangulation from anatomical landmarks including the mammillothalamic tract, oculomotor nerve, and the substantia nigra pars compacta. Recording/aspiration electrodes were made from filamented glass (Sutter Instruments, Novato, CA) using a P-97 micropipette puller (Sutter Instruments) to a tip diameter of approximately 3 μ m. Intra-pipette solution consisted of (in mM): K-gluconate 115, NaCl 9, KCl 25, HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] 10, EGTA (ethylene glycol tetraacetic acid) 0.2, MgCl₂ 1.2, Na-ATP 3, and Na-GTP 1 and had a resistance of 2 to 4 M Ω . Voltage clamp recordings were filtered at 2 kHz while current-drive spikes were filtered at 6 kHz using an Axon Instruments Multiclamp 700A or 700B amplifier, digitized at 5 to 20 kHz, respectively, using an Axon 1440A digitizer, and collected and analyzed using pClamp10 and Igor Pro (Wavemetrics, Lake Oswego, OR) software packages. Cells were visualized with either a Nikon (Melville, NY) Eclipse FN1 or E600FN microscope in the transmitted de Sénarmont DIC/IR configuration.

Characterization of VTA GABA Neurons In Vitro

Neurons in the VTA of the horizontal slice were characterized by several criteria, depending on the experiment type; specifically, the presence of (DA neuron), or lack of (GABA neuron), the noncation specific inward rectifying current (I_h), spike width, spike accommodation, and input resistance (Allison et al., 2006; Johnson and North, 1992; Margolis et al., 2006). DA neurons are typically large in size, have lower input resistances, have a prominent I_h , and exhibit spike accommodation. GABA neurons are typically smaller in size, have higher input resistances, are I_h negative, and do not exhibit spike accommodation.

Single Cell Quantitative RT-PCR

Following electrophysiological characterization, VTA GABA neurons from control and ethanol treated rats were aspirated under visual observation by application of suction using a 10 cc syringe attached to the recording pipette, and were then added to a reverse transcription (RT) reaction mixture. The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) was used for a total volume of 10 μ l per reaction. Reactions were run at 25°C for 10 minutes, 42°C for 60 minutes, and 95°C for 5 minutes in a PTC-200 thermal cycler (MJ Research Inc., Watertown, MA). Reactions were then stored at -20°C until running the polymerase chain reaction (PCR). A preamplification round of multiplex PCR was performed by adding iTaq Supermix with ROX (Bio-Rad) and a cocktail of primers to the completed RT reaction, for a final volume of 50 μ l. The reactions were held at 94°C for 30 seconds and then cycled 20 times. Each cycle consisted of 92°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. One microliter samples of the initial multiplex PCR were then used as substrate for each reaction in the subsequent real-time quantitative PCR. Real-time quantitative PCR using gene-specific primers with FAM-TAMRA TaqMan® probes

(Applied Biosystems, Foster City, CA) for the DA D2 receptor and 18s rRNA were performed using the iTaq Supermix with ROX (Bio-Rad) with an iCycler IQ (Bio-Rad) real-time PCR System. Samples were amplified in triplicate, together with a negative control for each subunit (an ACSF-only aspiration taken from the brain slice recording chamber when the cells were aspirated). The amplification protocol was 50°C for 2 minutes, 95°C for 5 minutes, then 50 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. Cycle threshold (C_t) values were calculated automatically by the iCycler IQ software, with threshold values set between 5 and 20. Relative fold expression was calculated using the $2^{-\Delta\Delta C_t}$ method as described in (Livak and Schmittgen, 2001). The results were then compared between groups using the unpaired two-sample for mean t -test.

Drug Preparation and Administration In Vivo

Systemic administration of 1.0 g/kg ethanol (16% w/v ethanol in saline) was accomplished by intraperitoneal injections. For in situ microelectrophoretic application of DA in the VTA, DA (100 mM) was dissolved in 1 M KCl (pH = 7.0) and iontophoresed by current injection (+40 nA) through the recording electrode with the Axon Instruments 700A amplifier. Eticlopride hydrochloride was dissolved in saline at 1 mg/ml and administered intravenously at a volume corresponding to each rat's weight in μ l/g. An equal volume of saline was administered intraperitoneally to a paired rat for comparisons of ethanol effects on VTA GABA neuron firing rate. Eticlopride was obtained from Sigma Chemical (St Louis, MO).

Analysis of Responses and Statistics

Discriminated spikes and stimulation events were processed with National Instruments LabVIEW and Igor Pro software (Wavemetrics, Lake Oswego, OR). Extracellularly recorded single-unit action potentials were discriminated by a peak detector digital processing LabVIEW algorithm. To determine changes in VTA GABA neuron activity produced by microelectrophoretic DA, current pulses (+40 nA) were applied for 1 minute every 2 minutes. DA effects on VTA GABA neuron activity were determined by rectangular activation of ratemeter records over the 1 minute DA iontophoretic current pulse, less the baseline-firing rate with Igor Pro software. To determine changes in VTA GABA neuron firing rate produced by eticlopride and ethanol administration, firing rate was determined by averaging 5 minutes epochs of activity before and 5 minutes epochs at 10 minutes after ethanol injection, by rectangular integration of ratemeter records with Igor Pro software. The results for control and drug treatment groups were derived from calculations performed on ratemeter records and expressed as mean $\pm$ SEM. A paired two-sample for mean t -test was performed to determine statistical significance for within drug versus saline comparisons with Microsoft Excel Statistical Analysis Toolpak (Microsoft, Redmond, WA) and Igor Pro (Wavemetrics) Stat Pak with $\alpha = 0.05$ and level of confidence of 95%. A simple one-way ANOVA was used to compare firing rate and weights between rats on the chronic ethanol diet versus pair-fed controls. A two-way ANOVA ($\alpha < 0.05$), was used to compare the expression of gene products in ethanol-treated versus control rats. Figures were compiled by using Igor Pro Software.

Results

Effects of Ethanol on Dopamine Activation of VTA GABA Neurons in Ethanol-Naïve Rats

VTA GABA neurons were characterized by previously established criteria including firing properties, waveform properties, and response to IC stimulation (Steffensen et al., 1998). Of 13 neurons tested for sensitivity to DA, 46% of them were activated by microelectrophoretic current, as previously reported (Lassen et al., 2007; Stobbs et al., 2004). Thus, we evaluated the effects of acute ethanol on 6 VTA GABA neurons that were activated by in situ DA. A representative DA-activated VTA GABA neuron is shown in Fig. 1A. Microelectrophoretic

application of DA (+40 nA) significantly enhanced the firing rate of VTA GABA neurons [$151 \pm 8.4\%$; $p < 0.001$, $t_{(2,5)} = 4.8$; $n = 6$; mean baseline firing rate = 30.6 ± 3.7 Hz]. We then tested the effects of acute intraperitoneal ethanol (1.0 g/kg) on DA activation of VTA GABA neurons. As shown in Fig. 1A, ethanol suppressed DA activation of this VTA GABA neuron with recovery in about 20 minutes. For all cells studied, ethanol significantly decreased [$72.5 \pm 6.9\%$; $p < 0.001$, $t_{(2,5)} = 6.6$; $n = 6$] DA activation of VTA GABA neurons evaluated 5 to 10 minutes after the ethanol injection (Fig. 1B). Although there was a tendency for ethanol to increase DA activation of VTA GABA neurons at 30 minutes after ethanol injection, it was not statistically significant [$48.5 \pm 40.1\%$; Fig. 1B; $p > 0.05$, $t_{(2,5)} = 0.95$; $n = 6$].

Effects of Dopamine D2 antagonists on Ethanol Inhibition of Firing Rate in Ethanol-Naïve Rats

Dopamine activation of VTA GABA neurons is mediated by DA D2 receptors (Steffensen et al., 2008). Thus, as DA excites and ethanol inhibits VTA GABA neuron firing rate, we evaluated the effects of intravenous administration of the DA D2 antagonist eticlopride (1.0 mg/kg) on ethanol inhibition of firing rate of 10 VTA GABA neurons, and compared with intravenous saline injections on ethanol inhibition of firing rate. Figure 2A shows the firing rate of a representative VTA GABA neuron following intraperitoneal 1.0 g/kg ethanol preceded by an intravenous dose of saline (0.3 ml). Intravenous saline (0.3 ml) had a mild activating effect on this VTA GABA neuron, but did not affect the ability of ethanol to reduce firing rate, as previously demonstrated (Gallegos et al., 1999). In a separate experiment with a VTA GABA neuron recorded in a different rat, intravenous administration of eticlopride (1.0 mg/kg; 0.3 ml) mildly enhanced VTA GABA neuron firing rate, but markedly reduced the typical inhibition of VTA GABA neuron firing rate by ethanol (Fig. 2B). Compared with intravenous administration of saline (saline + ethanol = $53.7 \pm 2.0\%$), eticlopride significantly reduced ethanol inhibition of VTA GABA neuron firing rate [Fig. 2C; eticlopride + ethanol = $2.8 \pm 11.1\%$; $p < 0.002$, $t_{(2,9)} = 3.7$; $n = 10$].

Effects of Ethanol Liquid Diet on Growth Rate

Rats consuming the liquid ethanol diet had body weights that did not differ significantly from their pair-fed controls [$p = 0.85$, $F_{(1,19)} = 0.036$], suggesting that the chronic ethanol treatment did not adversely affect their dietary intake of calories (Fig. 3) or overall health. This is consistent with previous studies using the Lieber–DeCarli diet and the average daily growth rate for both groups was approximately 5 g/d. The average consumption of ethanol during the 3-week period for the ethanol diet rats was 10.1 g/kg/d. There were no overt behavioral signs of withdrawal at 24 hours after discontinuance of the ethanol diet, suggesting that the constant low-dose ethanol consumption does not evoke as strong of a withdrawal in the rat than the less frequent high-dose ethanol injections previously used (Gallegos et al., 1999).

Effects of Ethanol Liquid Diet on VTA GABA Neuron Firing Rate

We evaluated the effects of an acute injection of 1.0 g/kg ethanol on VTA GABA neuron firing rate in rats on the chronic ethanol diet compared with their pair-fed control rats at 24 hours after ethanol withdrawal. While the mean baseline firing rate of VTA GABA neurons in ethanol-consuming rats during withdrawal (46.4 ± 10.6 Hz) was greater than their pair-fed controls (30.0 ± 3.29 Hz), it was not statistically significant [$p = 0.15$, $F_{(1,20)} = 2.19$; $n = 11$]. Moreover, there was no significant difference between pair-fed controls (Fig. 4A) versus ethanol-consuming rats (Fig. 4B) for acute ethanol inhibition of VTA GABA neuron firing rate [Fig. 4C; mean acute ethanol inhibition in ethanol-consuming rats = $55.1 \pm 8\%$ vs. pair-fed control rats = 35.4 ± 10.1 Hz; $p = 0.78$, $F_{(1,20)} = 0.08$; $n = 11$]. Blood alcohol levels were measured before and 2 hours after the 1.0 g/kg ethanol injection. There was no detectable

ethanol in ethanol-consuming rats 24 hours after withdrawal. The mean blood alcohol level (BAL) at 2 hours after the 1.0 g/kg injection of ethanol was 48.61 ± 1.31 mg%, indicating that the injections of ethanol were systemically effective in both ethanol-consuming and pair-fed rats.

Effects of Chronic Ethanol Treatment on Expression of Select Genes in VTA GABA Neurons: Quantitative Single Cell RT-PCR

To determine if molecular adaptation occurred with chronic ethanol, we patched VTA GABA neurons in the horizontal slice preparation, characterized the neurons electrophysiologically, aspirated them and evaluated the expression of D2 receptors in chronic ethanol-consuming rats and compared them to pair-fed controls. Figure 5 summarizes the differences between VTA GABA neurons from ethanol-consuming rats and pair-fed controls for the expression of D2 receptors. The expression of D2 in ethanol-consuming rats was significantly less than pair-fed controls [$p = 0.0068$, $t_{(1,16)} = 3.1$; mean pair-fed D2 expression = 1.13 ± 0.14 and mean ethanol diet D2 expression = 0.52 ± 0.06 , $n = 18$]. The C_t for the standard housekeeping gene 18s was not significantly different between groups [$p = 0.08$, $t_{(1,16)} = 1.85$; mean pair-fed 18s $C_t = 12.63 \pm 0.35$ and mean ethanol diet 18s $C_t = 12.02 \pm 0.16$].

Discussion

Systemic administration of an acute intoxicating dose of ethanol suppressed DA activation of VTA GABA neuron firing rate. DA markedly enhances the firing rate of VTA GABA neurons via D2 receptor activation (Steffensen et al., 2008). DA activation may occur directly via D2 receptors located on GABA neurons, as has been observed in substantia nigra (Ruffieux and Schultz, 1981; Sesack et al., 1994; Waszczak and Walters, 1986), or indirectly, through some unknown mechanism involving D2 autoreceptor inhibition of DA neurons and subsequent activation of VTA GABA neurons. DA inhibition of DA neurons via D2 autoreceptors is a well-known mechanism for limiting DA neural activity in the midbrain (for review see Adell and Artigas, 2004). However, as evidenced by RT-PCR studies, VTA GABA neurons express D2 transcripts (Steffensen et al., 2008), albeit at lower levels than DA neurons, suggesting that DA activation could be due to direct activation of D2 receptors on VTA GABA neurons, although D2 excitatory effects have been the subject of much controversy (Waddington, 1997). Regardless, our finding that ethanol inhibits DA activation of VTA GABA neuron firing rate suggests a physiological interaction between ethanol and DA on the excitability of these neurons.

An acute intoxicating dose of ethanol suppressed DA activation of VTA GABA neuron firing rate, and the D2 antagonist eticlopride blocked acute ethanol inhibition of firing rate, providing further evidence in support of an interactive effect between ethanol and DA on VTA GABA neuron excitability. Although not statistically significant, there was a tendency for DA activation of VTA GABA neurons to be enhanced 30 minutes after ethanol injection and following the earlier suppression of DA activation by ethanol. This late enhancement of DA activation of VTA GABA neuron firing rate may be due to rebound excitation through inhibition of the long-loop VTA–NAcc–VTA negative-feedback system (Kohl et al., 1998), as previously reported with acute ethanol alone on VTA GABA neuron firing rate (Gallegos et al., 1999). Nonetheless, there appears to be a strong interaction between DA and ethanol for modulating the excitability of VTA GABA neurons via D2 receptors. Given that local circuit GABA neurons inhibit DA neurons in the VTA, DA activation of VTA GABA neurons might lead to inhibition, while ethanol inhibition of VTA GABA neurons might lead to enhancement, of DA activity and subsequent DA release in limbic structures such as the NAcc.

These acute studies implicated DA and D2 receptor activation in ethanol effects on VTA GABA neuron firing rate, and provided the impetus for evaluating D2 receptor expression in chronic ethanol studies. DA D2 receptor mRNA expression was significantly lower in ethanol-consuming rats compared with their pair-fed controls. An adaptive reduction of D2 receptors in the midbrain 24 hours after ethanol withdrawal has been reported previously (Rommelspacher et al., 1992); however, this study did not address which specific class of neurons in the midbrain had reduced D2 receptor expression. While D2 receptors in DA neurons would seem to be the likely neuronal substrate, VTA GABA neurons also express D2 receptor transcripts as demonstrated here and previously (Steffensen et al., 2008). As ethanol inhibits DA activation of VTA GABA neurons acutely, down-regulation of D2 receptors by chronic ethanol seems somewhat counterintuitive, as one might expect that chronic ethanol would result in a compensatory increase in D2 receptor expression, at least in VTA GABA neurons, given that they inhibit VTA DA neurons. A plethora of explanations are plausible for the down-regulation of D2 receptors, considering that the potential adaptations may be occurring in parallel with D2 receptors on DA neurons. However, it seems that the most parsimonious explanation might be that the neuroadaptations that result in hyperexcitable VTA GABA neurons in dependent rats may be separate from those in nondependent rats. While rats consumed more than 10 g/kg/d of ethanol, they showed no signs of overt withdrawal and thus can be considered “nondependent.” Indeed, there was no increase in baseline activity or tolerance to ethanol inhibition of VTA GABA neuron firing rate, as we have reported previously after 2 to 3 weeks of twice-daily 2.0 g/kg injections (Gallegos et al., 1999). Although 10 g/kg/d of ethanol consumed by the rats in this study was more than twice the amount of ethanol exposure in the previous study, it appears that higher BALs are essential for producing ethanol dependence. We did not observe any rat that drank to obvious intoxication. Notwithstanding the lack of dependence or significant GABA neuron physiological adaptations in ethanol-consuming rats, there was a significant down-regulation in the expression of D2 receptor mRNA in these neurons compared with pair-fed controls. A role for D2 receptors in modulating VTA GABA neurons is not totally unexpected, as D2 receptors are known to regulate ethanol effects on mesolimbic DA neurotransmission and ethanol self-administration in alcohol-preferring rats (Eiler and June, 2007; Eiler et al., 2003; McBride et al., 2005). It is possible, and likely, that the different methods of ethanol consumption, whether high or low dose, each lead to different modulations of the brain to account for the corresponding types of ethanol consumption. Thus, future studies will address neuroadaptations in D2 receptors as well as other critical VTA GABA neuron genes in ethanol dependent and nondependent rats.

In conclusion, D2 receptor activation appears to be an important regulator of VTA GABA neuron excitability, as well as response to acute ethanol. Although D2 autoreceptor inhibition of DA neurons is a well-known feedback mechanism for controlling DA excitability, D2 regulation of VTA GABA neurons is not inconsequential. As both DA and GABA neurons in the VTA appear to be regulated by D2 receptors, determining the critical role of specific mesolimbic neurons in mediating ethanol reward might come down to what specific class of neurons or neural substrate is most sensitive to acute ethanol. Accordingly, we have shown previously that VTA GABA neuron firing rate and afferent-evoked synaptic activity are more sensitive to acute and chronic ethanol than DA neurons. For example, ethanol inhibition of VTA GABA neuron firing rate (Gallegos et al., 1999) and evoked synaptic activity (Stobbs et al., 2004) are nearly an order of magnitude greater than ethanol excitation of DA neuron firing rate (Brodie et al., 1990b, 1999). Inhibition of DA activation of VTA GABA neuron firing rate by ethanol, as well as eticlopride block of ethanol inhibition of VTA GABA neuron firing rate, suggests an interaction between ethanol and DA neurotransmission via D2 receptors, perhaps via enhanced DA release locally in the VTA subsequent to ethanol inhibition of GABA neurons. Down-regulation of VTA GABA

neuron D2 receptors by chronic ethanol might result from persistent DA release onto GABA neurons. Adaptation of D2 receptors on VTA GABA neurons by chronic ethanol may lead to dysregulation of VTA GABA neuron excitability and, ultimately, to reduction in DA neurotransmission, and to the cravings that result from ethanol withdrawal.

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Fig. 1.

Effects of acute ethanol on dopamine (DA) activation of VTA GABA neuron firing rate in ethanol naïve rats. **(A)** This ratemeter record shows the effects of systemic administration of saline and 1.0 g/kg ethanol on DA activation of VTA GABA neuron firing rate. The baseline firing rate of this neuron was approximately 22 Hz. Microelectrophoretic DA markedly enhanced the firing rate of this VTA GABA neuron which was repeatable with little or no acute sensitization or tolerance. Intraperitoneal saline had no effect on DA activation, while intraperitoneal ethanol modestly decreased the baseline firing rate of VTA GABA neurons and markedly decreased DA activation of VTA GABA neurons. Horizontal bars indicate time of DA current ejection from the micropipette. The # symbol indicates the DA current epoch at approximately 5 to 10 minutes after ethanol injection wherein ethanol exerted its greatest inhibition on DA activation of VTA GABA neuron firing rate. Note that at 30 minutes after ethanol the baseline firing rate of VTA GABA neurons is somewhat elevated from baseline, which is typical of some VTA GABA neurons, and that there is a slight increase in the DA activation of firing rate. **(B)** This graph summarizes the effects of saline and ethanol, 10 and 30 minutes after injection on DA activation of VTA GABA neuron firing rate. Compared with saline injection, ethanol significantly decreased DA activation of VTA GABA neuron firing rate 73% at 10 minutes. Although there was a tendency towards increased activation of firing rate by DA at 30 minutes, it was not statistically significant. Asterisk * represents significance level $p < 0.001$.

Fig. 2.

Effects of a dopamine antagonist on ethanol inhibition of VTA GABA neuron firing rate in ethanol naïve rats. **(A)** This ratemeter record shows the effects of intravenous saline administered approximately 5 minutes before intraperitoneal 1.0 g/kg ethanol on the firing rate of a VTA GABA neuron. The baseline firing rate of this neuron was approximately 18 Hz. While intravenous saline had a slight activating effect, intraperitoneal ethanol produced a prolonged decrease in VTA GABA neuron firing rate, as previously reported (Gallegos et al., 1999). Intravenous saline appeared to have no effect on the ability of ethanol to produce its typical inhibition of firing rate at this dose. **(B)** This ratemeter record shows the effects of intravenous administration of the D2 antagonist eticlopride administered approximately 5 minutes before 1.0 g/kg ethanol on the firing rate of a VTA GABA neuron. The baseline firing rate of this neuron was approximately 17 Hz. Eticlopride produced a slight enhancement while 1.0 g/kg ethanol was without much effect on VTA GABA neuron firing rate. **(C)** This graph summarizes the effects of intravenous saline and eticlopride on ethanol inhibition of VTA GABA neuron firing rate. Prior administration of eticlopride blocked ethanol inhibition of firing. Asterisk * represents significance level $p < 0.002$.

Fig. 3.

Chronic ethanol consumption rates: effects on weight. Ethanol-treated rats were fed a 5% v/v solution of ethanol in a liquid diet. They consumed approximately 10.1 g/kg of ethanol/d ($n = 12$). Pair-fed control and alcohol-fed rats maintained similar weight gains throughout the 3-week feeding period. Both groups had normal growth rates of approximately 5 g/d while on the liquid diet. There was no significant difference in weight gain between chronic ethanol-treated rats and their pair-fed controls.

Fig. 4. Effects of chronic liquid ethanol diet on acute ethanol inhibition of VTA GABA neuron firing rate. **(A)** This rate-meter record shows the effects of intraperitoneal 1.0 g/kg ethanol on the firing rate of a VTA GABA neuron in a pair-fed control rat. As demonstrated in naïve rats (Fig. 2) and previously (Gallegos et al., 1999), ethanol reduced VTA GABA neuron firing rate in pair-fed rats. **(B)** This rate-meter record shows the effects of intraperitoneal 1.0 g/kg ethanol on the firing rate of a VTA GABA neuron in a rat on the liquid ethanol diet. Chronic consumption of ethanol did not affect the ability of an acute ethanol challenge to reduce VTA GABA neuron firing rate. **(C)** This graph summarizes the effects of ethanol diet on acute ethanol inhibition of VTA GABA neuron firing rate. There was no significant

difference between ethanol treated rats and their pair-fed controls for acute ethanol inhibition of VTA GABA neuron firing rate.

Fig. 5.

Effects of chronic ethanol treatment on expression of D2 receptors in VTA GABA neurons. Single cell quantitative RT-PCR was performed on aspirated VTA GABA neurons recorded in ethanol-consuming rats and their pair-fed control rats. There was a significant decrease in D2 receptor expression in VTA GABA neurons of chronic ethanol-treated rats versus pair-fed controls. All other gene expressions were comparable for ethanol diet and pair-fed control rats.