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Frequency Dependent Effects of Ethanol on Dopamine Release in the Nucleus Accumbens

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Abstract

Background—Ethanol is known to have excitatory effects on dopamine (DA) release, with moderate to high doses (0.5–2.5 g/kg) of acute ethanol enhancing DA neuron firing rates in the ventral tegmental area (VTA) and DA levels in the nucleus accumbens (NAc). Ethanol has also been shown to reduce DA activity, with moderate doses (1–2 g/kg) attenuating electrically evoked release, and higher doses (5 g/kg) decreasing NAc DA levels, demonstrating a biphasic effect of ethanol on DA release. The purpose of the current study was to evaluate ethanol's inhibitory effects on NAc DA terminal release under low- and high-frequency stimulation conditions.

Methods—Using fast-scan cyclic voltammetry in NAc slices from C57BL/6J mice, we examined ethanol's (40–160 mM) effects on DA release under several different stimulation parameters, varying frequency (5–125 Hz), number of pulses (1–10), and stimulation intensity (50–350 μ A). Additionally, calcium concentrations were manipulated under high-frequency stimulation conditions (20 Hz, 10 pulses, 350 μ A) in order to determine if ethanol's effects were dependent upon calcium concentration, and by extension, the amount of DA release.

Results—Acute ethanol (40–160 mM) inhibited DA release to a greater extent under high-frequency, multiple-pulse stimulation conditions, with increased sensitivity at 5 and 10 pulses and frequencies of 20 Hz or higher. High-frequency, multiple-pulse stimulations also resulted in greater DA release compared to single-pulse release, which was controlled by reducing stimulation intensity. Under reduced DA conditions, high-frequency stimulations still showed increased ethanol sensitivity. Reducing calcium levels also decreased DA release at high-frequency stimulations, but did not affect ethanol sensitivity.

Conclusions—Ethanol appears to inhibit DA release at NAc terminals under high-frequency stimulation conditions that are similar to release events observed during phasic burst firing in DAergic neurons, suggesting that ethanol may provide inhibition of DA terminals selectively during phasic signaling, while leaving tonic DA terminal activity unaffected.

Keywords

voltammetry; ethanol; phasic; dopamine; mouse

Introduction

Mesolimbic dopamine (DA) signaling originating in the ventral tegmental area (VTA) and projecting to the nucleus accumbens (NAc) has been heavily implicated in the reinforcing effects of natural and drug related rewards (for review see Wanat et al., 2009). Supporting a role for this neural circuit in alcohol reward, local injections of DA antagonists into the NAc reduce ethanol self-administration in rats (Samson and Chappell, 2004). Moreover, rats will self-administer ethanol directly into the VTA (Gatto et al., 1994). Also, acute ethanol increases rat VTA DAergic cell firing in the slice preparation, for example increasing firing rates by 20% at 40 mM (Brodie et al., 1990). These increases in firing rates are accompanied by increases in both rapid DAergic release events as measured by voltammetry in freely moving rat preparations (Cheer et al., 2007; Robinson et al., 2009), and increases in DA levels as measured by microdialysis (Imperato and Di Chiara, 1986).

Although the excitatory effects of ethanol on DA transmission have been examined extensively, only a few studies have described ethanol's inhibitory effects on DA transmission. Ethanol's inhibitory effects on DA release, as measured by microdialysis, are mostly associated with its sedating effects, and are only observed after high dose injections (5.0 g/kg IP; Imperato and Di Chiara, 1986). However, in this previous study, it is noteworthy that the peak excitatory effect was at 1.0 g/kg, and that ethanol-evoked DA release was slightly attenuated at the 2.5 g/kg dose, suggesting that ethanol may already be inhibiting DA release at this dose in rats (Imperato and Di Chiara, 1986). *In vivo* voltammetry studies measuring electrically evoked NAc DA release, have also demonstrated the biphasic effects of ethanol, albeit at different dose ranges, with low doses (0.1 g/kg) increasing release in rats (Pelkonen et al., 2010; Yavich and Tiihonen, 2000), but moderate to high doses (1.0–5.0 g/kg) decreasing evoked DA release in rats and mice (Pelkonen et al., 2010; Yavich and Tiihonen, 2000; Jones et al., 2006; Budygin et al., 2001a). *Ex vivo* studies examining ethanol's effects on evoked terminal striatal DA release have only shown inhibitory effects in rats and mice with high concentrations (> 150 mM) of ethanol (Budygin et al., 2001b; Mathews et al., 2006). Although ethanol-induced reductions in DA overflow have been shown in both *in vivo* and *ex vivo* preparations, there are some prominent differences between the results from these studies that create a gap in our current understanding of ethanol's effects on DA terminals. One important difference already mentioned are the large disparities between ethanol concentrations used in these studies. For example, intraperitoneal (IP) injections of 2.5 g/kg ethanol, which produce ~50 mM ethanol brain concentrations (Yoshimoto and Komura, 1993), was shown in *in vivo* voltammetry studies to reduce evoked DA signaling to 34 % of control in rats (Budygin et al., 2001a). However, previous *ex vivo* studies in the NAc failed to demonstrate any effect of ethanol on evoked DA overflow for concentrations below 100 mM, and only modest decreases at higher (150–200 mM), non-physiological concentrations in rats (Budygin et al., 2001b). Ethanol has many targets with varying sensitivities, such that the large concentration

disparities between these *in vivo* and *ex vivo* studies may be engaging different mechanisms. Along these lines, it has been postulated that the differences in ethanol sensitivity for evoked DA release in *in vivo* and *ex vivo* studies are primarily due to differences in available neural circuitry, such that ethanol's inhibitory effects on DA release are thought to be through interactions at the cell body and not the terminals (Budygin et al., 2001b). However, increased distal circuitry in intact preparations may be only partially responsible for observed increases in ethanol potency for *in vivo* preparations. Another explanation for this disparity in sensitivity may be related to the major differences between the stimulation paradigms used for eliciting DA release in these studies. For instance, while the previous *ex vivo* studies used single-pulse stimulations to elicit DA release, the *in vivo* studies used multiple-pulse, high-frequency stimulation trains, which may result in increased autoreceptor activity (Zhang and Sulzer, 2012). Indeed, several previous *ex vivo* studies have demonstrated that high-frequency stimulations produce larger DA changes than single-pulse stimulations (Gonon, 1986; Zhang et al., 2009), which in turn result in increased autoreceptor activity (Bello et al., 2012; Phillips et al., 2002).

In addition to increases in DA release, and subsequent autoreceptor activity, high-frequency stimulations also increase DA terminal sensitivity to heteroreceptor antagonists, suggesting the presence of additional local circuitry activity under high-frequency electrical stimulation conditions (for review see Zhang and Sulzer, 2012; Rice et al., 2011). Indeed, GABA and glutamate antagonists have little to no effect on single-pulse stimulations, but robust effects when applied during high-frequency stimulations (Rice et al., 2011). It has therefore been suggested that these disparate drug effects with high-frequency stimulations are due to recruitment of additional local circuitry and auto-feedback mechanisms (Rice et al., 2011). To test if ethanol has increased potency under different stimulation conditions, using a parametric analysis we examined ethanol's effects at DA terminals in an *ex vivo* preparation.

Material and Methods

Animals

Male C57BL/6J mice (Jackson Labs; aged 6–12 weeks) were given *ad libitum* access to food and water, and were maintained on a reverse 12:12-h light/dark cycle (lights on at 15:00 h). All protocols and animal care procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Wake Forest University Institutional Animal Care and Use Committee.

Brain Slice Preparation

Isoflurane (Patterson Veterinary, Devens, MA) anesthetized mice were sacrificed by decapitation and brains were rapidly removed and transferred into ice-cold, pre-oxygenated (95% O₂/5% CO₂) artificial cerebral spinal fluid (aCSF) consisting of (in mM): NaCl (126), KCl (2.5), NaH₂PO₄ (1.2), MgCl₂ (1.2), NaHCO₃ (25), glucose (11), L-ascorbic acid (0.4), pH adjusted to 7.4. Although endogenous extracellular calcium levels are typically around 1.2 mM, presently, calcium concentrations were maintained at 2.4 mM (except for experiments examining calcium effects on ethanol sensitivity) in order to compare results with previous studies, which use the higher calcium concentrations to obtain reliable

exocytotic DA release (Budygin et al., 2001b; Mathews et al., 2006). Tissue was sectioned into 400 μm -thick coronal striatal slices with a vibratome (Leica VT1000S, Vashaw Scientific, Norcross, GA). Brain slices were placed in a submersion recording chamber, and perfused at 1 ml/min at 32 $^{\circ}\text{C}$ with oxygenated aCSF.

Fast Scan Cyclic Voltammetry

DA release was evoked every 5 min by a bipolar stimulating electrode (Plastics One, Roanoke, VA) placed 100–200 μm from the carbon-fiber electrode in the NAc core, as previously described (Yorgason et al., 2013). For initial experiments examining ethanol effects on various stimulations, DA release was evoked from a single-pulse (4 ms, 350 μA , monophasic +) or high-frequency stimulation (20 Hz, 10 pulses). For additional experiments examining frequency dependent effects of ethanol, pulse number was maintained at either 1 pulse (350 μA), or 10 pulses (5, 20, and 125 Hz; 350 μA). For experiments examining pulse dependent effects of ethanol, we stimulated with 1 pulse (350 μA), or multiple-pulses at a high-frequency (2, 5, and 10 pulses at 20 Hz; 350 μA). In order to examine DA release under different stimulation intensities at high frequencies, stimulations were maintained at 1 pulse (350 μA) or 20 Hz, 10 pulses at varying intensities (50, 100, and 350 μA). Stimulation paradigms were based on high and low frequency stimulations used in previous studies examining local circuitry interactions with DA release (see supplementary methods).

Voltammetry recordings of DA signals were performed and analyzed as previously described, using recently developed in-house software (Demon Voltammetry and Analysis; Yorgason et al., 2011). The carbon fiber electrode (7 μm X ~150 μm) potential was linearly scanned as a triangular waveform from -0.4 V to 1.2 V and back to -0.4 V (Ag vs AgCl) at a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon fiber electrode every 100 msec by means of a potentiostat (Dagan Corporation, Minneapolis, MN). Once the stimulated DA response was stable for three successive collections, baseline measurements were taken, and ethanol bath concentrations were manipulated. Extracellular concentrations of DA were assessed by comparing the current at the peak oxidation potential for DA with electrode calibrations of known concentrations of DA (1–3 μM) as described previously (Yorgason et al., 2011).

Statistical Analysis

Evoked DA release was determined from voltammetry collections, where DA oxidation occurring at ~ 0.6 V produces a faradaic current proportional to the amount of DA present at the electrode. For experiments examining stimulation- and calcium-dependent changes in DA, one-way ANOVAs were performed, with stimulation parameter or calcium concentration as the between-subjects variable. For experiments examining changes in DA release following ethanol, DA signals were compared to baseline where three consecutive stable collections were averaged to determine baseline. For figures showing only one baseline, this baseline represents the third baseline in relation to the averaged baseline value in μM . For ethanol concentration response curves, two-way analysis of variance (ANOVA) with ethanol concentration as the within-subject variable, and stimulation paradigm as the between-subjects variable, were used to examine ethanol interactions with different stimulations. For experiments examining DA across ethanol concentration, Dunnett's tests

were performed comparing each drug concentration to their respective baseline, as well as to single pulse controls, to test for significant differences. For ANOVAs where comparisons were not planned, Tukey's or Bonferroni post-hoc tests were used to test for significance. For experiments examining effects of calcium concentration changes, a one-way repeated measures ANOVA across all time points was initially conducted, and subsequently, Dunnett's post-hoc comparisons were conducted comparing each time point relative to the baseline time point immediately prior to calcium change. All statistics were performed using GraphPad Prism 5 (Graphpad Software, La Jolla CA), and NCSS (NCSS LLC, Kaysville UT).

Results

Low vs High Frequency Evoked Dopamine Release: Effects of Ethanol

First, to evaluate if ethanol had direct effects on the carbon fiber electrode, we tested the effects of 160 mM ethanol on DA detection using a flow cell. Compared to ACSF controls, ethanol had no effect, $t(4)=1.609$, $p=0.1829$, on voltammetric detection of known DA concentrations *in vitro*. Next, ethanol's inhibitory effects on evoked NAc DA release from brain slices were examined under single-pulse, and high-frequency multiple-pulse (20 Hz, 10 pulses) stimulation conditions with bath application and washout of 80 mM ethanol (Figure 1A). Signals were normalized to their respective baselines (1 pulse or 20 Hz, 10 pulses) to control for differences in DA release following the different stimulation parameters, and to specifically test relative reductions after application of ethanol. A two-way repeated measures ANOVA with ethanol across time and stimulation as the within-subject variables revealed a main effect of ethanol across time, $F(11,10)=24.12$, $p<0.0001$, and stimulation, $F(1,10)=34.61$, $p=0.0002$, and an interaction between these variables, $F(11,110)=12.03$, $p<0.0001$. Under high frequency (phasic) stimulation conditions, ethanol reduced DA signals by ~32% (Figure 1B). These effects were also present for raw data that was not normalized: ethanol, $F(11,10)=17.96$, $p<0.0001$, stimulation, $F(1,10)=7.10$, $p=0.05$, and interaction, $F(11,110)=14.76$, $p<0.0001$. To better understand the relationship between ethanol's inhibitory effects on low frequency (tonic) and 'phasic'-like release, we examined ethanol's effects under 1 pulse vs 20 Hz 10 pulse stimulation conditions across increasing ethanol concentrations (Figure 1C). A two-way ANOVA with ethanol concentration as the within subjects comparison, and stimulation as the between-subjects variable, revealed a main effect of stimulation, $F(1,10)=24.89$, $p=0.0005$, and concentration, $F(4,10)=73.91$, $p<0.0001$, and an interaction between these two variables, $F(4,40)=13.05$, $p<0.0001$. Dunnett's test, comparing each concentration to its baseline, showed significant effects of ethanol on single-pulse release for 160 mM ethanol ($p<0.001$), whereas ethanol reduced multiple-pulse stimulated release at 80 ($p<0.001$), and 160mM ($p<0.001$). Additional Bonferroni post-hoc analysis comparing ethanol effects between low and high frequency stimulation paradigms revealed significant differences at 40 ($p<0.05$), 80 ($p<0.001$) and 160 mM ethanol ($p<0.001$). These effects were also present for raw data that was not normalized: stimulation, $F(1,10)=24.89$, $p<0.001$; ethanol concentration, $F(4,10)=73.91$, $p<0.0001$; and interaction, $F(4,40)=13.05$, $p<0.0001$.

Ethanol Inhibits Evoked Dopamine Release at High Frequencies

DA release was measured under several stimulation frequencies (5, 20 and 125 Hz), while holding constant pulse number and intensity (10 pulses @ 350 μ A; Figure 2A). Similar to previous studies examining DA release under high-frequency stimulation conditions (Gonon, 1986; Zhang et al., 2009), we observed an increase in DA release that was frequency-dependent, with peak DA release occurring at 20 Hz, $F(3,20)=10.28$, $p=0.0003$. Tukey's post-hoc test revealed significant increases in DA release at 20Hz ($p<0.01$), and 125 Hz ($p<0.05$), compared to 1 pulse, and significant increases at 20 Hz ($p<0.01$), and 125 Hz ($p<0.05$), compared to 5 Hz stimulations. Normalized DA signals from these various frequency stimulations were examined in the presence of increasing concentrations of ethanol (20, 40, 80 & 160 mM). A two-way repeated measures ANOVA, with ethanol concentration as the within-subjects variable, and stimulation frequency as the between-subjects variable, revealed a main effect of ethanol concentration, $F(4,20)=97.39$, $p<0.0001$, and frequency, $F(3,20)=6.993$, $p=0.0021$, as well as an interaction between these variables, $F(12,80)=7.549$, $p<0.0001$, suggesting that ethanol reduces DA release differently across stimulation frequencies. Dunnett's test revealed significant differences between single-pulse and 20 Hz stimulations at 40 ($p<0.05$), 80 ($p<0.001$), and 160 mM ethanol ($p<0.001$), as well as 125 Hz at 80 ($p<0.001$), and 160 mM ethanol ($p<0.001$), but no significant differences between ethanol effects for single-pulse and 5Hz stimulations. These effects were also present for raw data that were not normalized: ethanol concentration, $F(4,20)=21.38$, $p<0.0001$; and the interaction, $F(12,80)=7.71$, $p<0.0001$.

Ethanol Inhibition of Dopamine Release Requires Several Pulses

Phasic DA release is not only characterized by higher frequencies, but also increased number of action potentials, with a burst containing typically 3–4 action potentials/burst (Grace and Bunney, 1984a; Grace and Bunney, 1984b; Hyland et al., 2002). Therefore, we examined DA release at several different pulse stimulations (1, 2, 5 and 10 pulses) while holding constant frequency and intensity (20 Hz @ 350 μ A). DA release increased in response to multiple-pulses, $F(3,20)=6.788$, $p=0.0024$, with significant differences for single-pulse vs 10 pulses ($p<0.01$), and 2 pulses vs 10 pulses ($p<0.01$). Ethanol's inhibitory effects were examined across increasing concentrations (20, 40, 80 & 160 mM) under these various pulse number conditions (Figure 3B). Two-way repeated measures ANOVA, with concentration as the within-subjects variable, and pulse number as the between-subjects variable, revealed a main effect of pulse number, $F(3,20)=10.71$, $p=0.0002$, and concentration, $F(4,20)=53.44$, $p<0.0001$, as well as an interaction between these variables, $F(12,80)=3.516$, $p=0.0003$ (Figure 3C). Dunnett's post-test revealed significant differences between single-pulse and 5 pulse release at 80 ($p<0.01$), and 160 mM ethanol ($p<0.001$), as well as 10 pulse release at 80 ($p<0.001$), and 160 mM ethanol ($p<0.001$), but no significant differences for ethanol between single-pulse and 2 pulse stimulations. These effects were also present for raw data that were not normalized: ethanol concentration, $F(4,20)=20.58$, $p<0.0001$, and the interaction, $F(12,80)=6.83$, $p<0.0001$.

Ethanol inhibition of Dopamine Release at Different Stimulation Intensities

As illustrated above, increases in frequency and pulses results in increased DA release and increased ethanol sensitivity. DA release was examined under similar low- and high-frequency multiple-pulse stimulation conditions, while varying stimulation intensity (Figure 4A; 1 pulse @ 350 μ A; 20 Hz, 10 pulses @ 50, 100 or 350 μ A). One-way ANOVA revealed a main effect of stimulation intensity, $F(3,20)=5.853$, $p=0.0049$, with peak DA release at 350 μ A conditions significantly increased from single-pulse, $p<0.05$, and multiple-pulse at 50 μ A, $p<0.05$ (Figure 4B). Ethanol's inhibitory effects were examined under these various stimulation conditions, across increasing ethanol concentrations (20, 40, 80 and 160 mM; Figure 4C). Two-way repeated measures ANOVA with ethanol concentration as the within-subjects variable, and stimulation intensity as the between-subjects variable, revealed a main effect of stimulation intensity, $F(3,20)=5.549$, $p=0.0061$, and ethanol concentration, $F(4,20)=140.4$, $p<0.0001$, and an interaction between these variables, $F(12,80)=5.062$, $p<0.0001$. Dunnett's test, comparing ethanol effects at high-frequency stimulations to single-pulse effects, revealed increased ethanol sensitivity for 50 μ A at 80 mM ($p<0.01$), 160 mM ($p<0.01$); and 100 μ A at 40 mM ($p<0.01$), 80 mM ($p<0.001$), 160 mM ($p<0.001$), and 350 μ A at 40 mM ($p<0.05$), 80 mM ($p<0.001$), and 160 mM ($p<0.001$). These effects were also present for raw data that were not normalized: ethanol concentration, $F(4,20)=20.54$, $p<0.0001$; and the interaction, $F(12,80)=5.64$, $p<0.0001$.

Increased Ethanol Sensitivity Under High Frequency Conditions is not Calcium Dependent

Stimulated DA release is calcium-dependent, so that increasing or reducing calcium results in modulation of DA release (Ford et al., 2010). Therefore, to confirm that increased DA release observed under ethanol sensitive high-frequency stimulation is not responsible for the increased sensitivity to ethanol, we examined exocytotic DA responses to ethanol at phasic stimulations (20 Hz 10 pulses 350 μ A) in the presence of various concentrations of calcium (1.2, 2.4, and 4.8 mM) at 80 mM ethanol. Increasing calcium from 2.4 to 4.8 mM resulted in increased DA release, $F(10,50)=17.24$, $p<0.0001$, while decreasing calcium from 2.4 to 1.2 mM resulted in decreased DA release, $F(10,50)=62.05$, $p<0.0001$ (Figure 5A). Two-way ANOVA on normalized DA signals, comparing ethanol's (80 mM) effects on DA release revealed a main effect of ethanol, $F(1,15)=8.720$, $p=0.0099$, but no interaction between calcium concentration and ethanol effects, $F(2,15)=0.02456$, $p=0.9758$ (Figure 5B), further demonstrating that calcium level and the amount of DA release are not responsible for observed increased ethanol sensitivity under high-frequency stimulation conditions.

Discussion

As an important control for our *ex vivo* ethanol experiments, we first demonstrated that ethanol, even at high concentrations (160 mM), does not affect voltammetric detection of evoked DA. For NAc slice experiments examining DA release at various stimulations, in agreement with results from previous studies examining release throughout the striatum of rats and mice, we have shown that evoked DA release is contingent upon a number of factors, including frequency, pulse number, stimulation intensity, and calcium concentration (Gonon, 1986; Patel et al., 1992; Zhang et al., 2009; Ford et al., 2010). Additionally, the present results have also shown for the first time that ethanol inhibits DA release with

greater potency at higher frequency (20 Hz) and pulse number (5 pulses) conditions. Additionally, ethanol potency under high-frequency conditions is not due to increases in DA release, as lower-intensity, high-frequency trains, which produce DA concentrations similar to single-pulse stimulations, still exhibited increased ethanol sensitivity. Similarly, increasing and decreasing aCSF calcium concentrations resulted in increases or decreases in DA release, respectively, but maintained the relative sensitivity to ethanol's inhibitory effects, further confirming that the overall amount of DA release is not responsible for the observed increase in ethanol potency under high stimulation conditions.

Dopamine Release from Low and High Frequency Stimulations

DA signaling can be described by two different modes of DA release, 'tonic' and 'phasic', which are related to neuron firing activity. Tonic firing, which occurs at 1–10 Hz (typically 3–4 Hz) is believed to be largely responsible for controlling basal DA levels in the absence of salient environmental stimuli (Hyland et al., 2002; Grace and Bunney, 1984b; Overton and Clark, 1997; Panin et al., 2012). Basal DA levels are typically measured with microdialysis techniques (Imperato and Di Chiara, 1986; but see Owesson-White et al., 2012), which have consistently shown that DA levels elicited by endogenous tonic activity are between 5–40 nM in the NAc (Wightman and Robinson, 2002; Justice, 1993; Parsons and Justice, 1992). In NAc and dorsal striatum slice voltammetry studies where spontaneous DA release is not a factor, low-frequency 'tonic'-like stimulations are sometimes used to study the direct effects of drugs on DA tone through terminal interactions, although it is of note that stimulated DA release under these 'tonic'-like stimulations is still greater than the estimated DA levels of microdialysis (Zhang et al., 2009). Previous studies examining ethanol's effects on the mesolimbic DA system have shown that ethanol increases tonic firing activity, which increases NAc DA levels (Brodie et al., 1990; Imperato and Di Chiara, 1986). In the present study, we demonstrated that under 'tonic'-like stimulation conditions, ethanol inhibits evoked DA release, but only at supra-physiological concentrations (160 mM). Combined with the aforementioned studies demonstrating increases in tonic and phasic firing activity, and in NAc DA levels, these data suggest that ethanol's elevating effects on DA tone are mainly through excitatory interactions at the cell bodies.

Phasic burst firing is also characteristic of DA neurons (Grace and Bunney, 1984a; Overton and Clark, 1997; Hyland et al., 2002). Phasic firing occurs in the presence of salient stimuli, such as an unexpected reward, or when a cue predicts a reward, and is thought to be responsible for learned associations of motivationally relevant stimuli (for review see Wanat et al., 2009). Phasic burst firing occurs at 14–22 Hz with an average of 3–4, but up to 22.2 (± 13.3) action potentials per burst, that decrease in amplitude across a burst (Grace and Bunney, 1984a; Overton and Clark, 1997; Hyland et al., 2002). Phasic burst firing is largely responsible for the rapid phasic NAc DA release events observed spontaneously in freely moving rats (Robinson et al., 2009; Cheer et al., 2007) as well as those observed during cue-conditioned learning in operant behavioral paradigms (Somers et al., 2009), although it should be mentioned that local NAc acetylcholine interneuron activity can also induce release similar to that observed from a phasic burst (Threlfell et al., 2012; Cachope et al., 2012). Similar to tonic activity, phasic activity is also thought to contribute to NAc DA levels, as increases in phasic burst firing are often associated with increases in DA levels as

measured by microdialysis (Robinson et al., 2009). Ethanol studies examining non-stimulated phasic activity using electrophysiological and voltammetric techniques have shown that ethanol increases phasic firing activity in the VTA (Mereu et al., 1983), as well as increased frequency of phasic release events in the NAc core (Cheer et al., 2007; Robinson et al., 2009). Although phasic release in the NAc may occur spontaneously in *in vivo* anesthetized preparations (Park et al., 2010), it is typically mimicked using high-frequency multiple-pulse stimulations of the midbrain, or the medial forebrain bundle (Yavich and Tiihonen, 2000; Jones et al., 2006). In *in vivo* voltammetry studies, ethanol has a biphasic effect on electrically evoked DA release, slightly increasing evoked DA release at low doses (0.1 g/kg, I.P.) in the NAc of rats, but robustly decreasing evoked release at moderate to high doses (1–5 g/kg) in the NAc and dorsal striatum in rats and mice (Pelkonen et al., 2010; Yavich and Tiihonen, 2000; Jones et al., 2006; Budygin et al., 2001a). We demonstrate here for the first time that similar to *in vivo* studies, ethanol also reduces DAergic signals from high frequency, ‘phasic’-like stimulations in *ex vivo* preparations, suggesting that ethanol’s inhibitory effects on DA involve interactions at DA terminals. Collectively, these different tonic and phasic electrophysiology, microdialysis and voltammetry studies suggest that ethanol has both excitatory and inhibitory effects on DA release, which culminate in overall changes in DA levels, which are then involved in modulating ethanol’s reinforcing effects.

As mentioned earlier, previous freely-moving rat voltammetry studies have demonstrated that ethanol increases the frequency of naturally occurring ‘spontaneous’ NAc DA release events (Cheer et al., 2007; Robinson et al., 2009). This increase in transients is believed to contribute to overall increase in DA levels as measured by microdialysis (Robinson et al., 2009). The present studies demonstrate that ethanol inhibits rapid ‘phasic’-like DA signaling amplitude. In agreement with our current studies, Cheer et al., (2007) reported no decreases in DA release amplitude from a 1 g/kg I.P. injection, which should produce approximately ~20 mM ethanol brain concentrations. Robinson et al., (2009) examined ethanol’s effects with a large range of doses (0.125–2.0 g/kg), and reported increases in transient release frequency and DA levels. However, an investigation as to whether higher doses of ethanol affect DA release amplitude from spontaneously occurring release, has yet to be completed. Indeed, a study examining ethanol’s effects on *in vivo* spontaneous release at higher doses would be very informative given the results from our current study, as well as several studies examining ethanol’s inhibitory effects on electrically evoked DA release in *in vivo* preparations. Additionally, freely moving studies examining ethanol’s effects on cue elicited release would be beneficial for determining the behavioral relevance of ethanol’s inhibitory effects on DA signaling. For example, several previous studies have demonstrated ethanol impairs conditioned learning at concentrations similar to those used in the present study (Hunt et al., 2009; Yttri et al., 2004). Given the role of task-specific DA release to encode conditioned learning, both the ethanol-induced spontaneous release of DA and reduced DA amplitude at higher concentrations could underlie disruptions in conditioned learning.

Presently, we tested inhibition of DA signaling under high frequency conditions for a range of concentrations (20–160 mM) similar to those obtained from I.P. injections from 1–8 g/kg doses. This range of doses represent a wide array of DA related ethanol induced behavioral

effects. For example, in locomotor assays, acute ethanol at 1–2 g/kg transiently increases locomotion in C57BL/6J mice (Cunningham et al., 1991), which appear to be associated with the excitatory effects of ethanol on DA transmission, as DA antagonists decrease ethanol induced hyperactivity (Cohen et al., 1997). As ethanol doses increase (3–4 g/kg), ethanol's sedative effects become clear, with suppression of locomotor activity, as well as loss of righting reflex (LORR; Cunningham et al., 1991; Lisenbardt et al., 2009). Under the higher sedating doses, decreased DA signaling is believed to contribute to sedation, as non-selective DA receptor antagonists increase sensitivity to ethanol's sedating effects in a LORR assay (Cohen et al., 1997). Moreover, in rats, the selective DA transporter inhibitor GBR 12909 elicits faster recovery from ethanol induced LORR, further implicating reduced DA in ethanol's behavioral sedating effects (Budygin et al., 2001a). Importantly, this series of doses (1–4 g/kg) which produce at first activating, and next sedating effects on behavior, are frequently used for establishing conditioned place preference (CPP) in mice, demonstrating that the rewarding and conditioned learning effects of ethanol can occur even under sedating doses (Cunningham et al., 1991; Groblewski et al., 2008). Finally, at higher doses (>6 g/kg, I.P.), acute ethanol results in heavy sedation, as well hypothermic effects, and eventually lethality in mice (Finn et al., 1989).

Possible Mechanism for Ethanol Inhibition of Dopamine Release at Terminals

In order to discover the mechanism for ethanol's inhibitory effects on DA release with 'phasic'-like stimulations, it may be useful to consider what additional activity is being recruited under high-frequency conditions. For instance, electrical stimulations are well known to be non-selective in nature. Thus, it is likely that our stimulation paradigms are enlisting additional circuit activity not present with low-frequency, low-pulse stimulations. This may include changes in activity of diffusable retrograde messengers after ethanol, such as hydrogen peroxide and nitric oxide. For example, while GABA_A and AMPA receptor antagonists have no effect on single-pulse stimulated DA release, under higher frequency conditions (10 Hz, 30 pulses) GABA_A and AMPA antagonists reduce and increase DA release, respectively, through modulation of K_{ATP} channels via hydrogen peroxide retrograde transmission from medium spiny neurons (Rice et al., 2011). In this scenario, ethanol could interact with several different receptors on medium spiny neurons to increase hydrogen peroxide signaling, and subsequently decrease release. Nitric oxide, on the other hand, has mainly excitatory effects on high-frequency striatal DA release (Rice et al., 2011). In this scenario, ethanol would be inhibiting nitric oxide production, or downstream effectors, to reduce DA release under these conditions.

Another possible target of ethanol's effects could be the enhancement of GABAergic activity which may inhibit DA release through GABA_B heteroreceptor activity. In support of this hypothesis, previous studies have shown that VTA DA neurons express GABA_B receptors, and that GABA_B agonists decrease evoked DA release, although it is still unclear as to whether GABA_B receptors are located on DA terminals (Charara et al., 2000; Schmitz et al., 2002). Also, ethanol is known to increase inhibitory post synaptic potentials in DAergic neurons through increased GABA_B activity (Federici et al., 2009). Since ethanol has increased potency under multiple conditions, it is also noteworthy that multiple-pulse stimulations are frequently used to study GABA_B receptor activity (Otis and Mody, 1992;

Federici et al., 2009). Ethanol has been shown to increase GABA release in several regions (for review see Weiner and Valenzuela, 2006). Therefore, ethanol may be enhancing GABA release under high-frequency stimulation conditions, resulting in increased GABA_B activity on DA terminals (Ariwodola and Weiner, 2004; Theile et al., 2008). We are currently pursuing this hypothesis in a pharmacological analysis of ethanol effects on DA release in the NAc.

Lastly, ethanol may be attenuating DA release through interactions with DA release machinery regulating releasable pools under high-frequency stimulation conditions. Readily releasable pools are regulated by several different proteins including snare proteins, synucleins, synapsins, vesicular coat proteins (Rizzoli and Betz, 2005), which ethanol may be interacting with to decrease the amount of available release under 'phasic' conditions. So far, alpha-synucleins have been examined in alcohol-preferring and non-preferring rats, and despite differences in alpha-synuclein expression, and overall evoked DA release, there were no differences in ethanol's inhibitory effects under high-frequency stimulation conditions in an *in vivo* anesthetized preparation (Pelkonen et al., 2010).

Conclusions

We have shown that ethanol preferentially reduces DA from high-frequency, multiple-pulse electrical stimulations that are more similar to 'phasic' DA release, whereas DA from 'tonic'-like stimulations appears relatively insensitive to ethanol's inhibitory effects. Although high-frequency stimulations are typically associated with greater amounts of DA release, the amount of release does not appear to dictate whether a DA signal has increased sensitivity to ethanol. Rather, it appears that these stimulations selectively recruit additional mechanisms that appear to be ethanol-sensitive. Additional studies examining ethanol inhibition of DA release under high frequency and pulse (phasic-like) conditions in animal models of increased risk for alcohol abuse may help us better understand how this effect contributes to ethanol modulation of learning and reinforcement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ariwodola OJ, Weiner JL. Ethanol potentiation of GABAergic synaptic transmission may be self-limiting: role of presynaptic GABA(B) receptors. *J Neurosci.* 2004; 24:10679–10686. [PubMed: 15564584]
- Bello EP, Mateo Y, Gelman DM, Noain D, Shin JH, Low MJ, Alvarez VA, Lovinger DM, Rubinstein M. Cocaine supersensitivity and enhanced motivation for reward in mice lacking dopamine D2 autoreceptors. *Nat Neurosci.* 2012; 14:1033–1038. [PubMed: 21743470]
- Brodie MS, Shefner SA, Dunwiddie TV. Ethanol increases the firing rate of dopamine neurons of the rat ventral tegmental area in vitro. *Brain Res.* 1990; 508:65–69. [PubMed: 2337793]

- Budygin EA, Phillips PE, Robinson DL, Kennedy AP, Gainetdinov RR, Wightman RM. Effect of acute ethanol on striatal dopamine neurotransmission in ambulatory rats. *J Pharmacol Exp Ther*. 2001a; 297:27–34. [PubMed: 11259524]
- Budygin EA, Phillips PE, Wightman RM, Jones SR. Terminal effects of ethanol on dopamine dynamics in rat nucleus accumbens: an in vitro voltammetric study. *Synapse*. 2001b; 42:77–79. [PubMed: 11574942]
- Cachope R, Mateo Y, Mathur BN, Irving J, Wang HL, Morales M, Lovinger DM, Cheer JF. Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. *Cell Rep*. 2012; 2:33–41. [PubMed: 22840394]
- Charara A, Heilman TC, Levey AI, Smith Y. Pre- and postsynaptic localization of GABA(B) receptors in the basal ganglia in monkeys. *Neuroscience*. 2000; 95:127–140. [PubMed: 10619469]
- Cheer JF, Wassum KM, Sombers LA, Heien ML, Ariansen JL, Aragona BJ, Phillips PE, Wightman RM. Phasic dopamine release evoked by abused substances requires cannabinoid receptor activation. *J Neurosci*. 2007; 27:791–795. [PubMed: 17251418]
- Cohen C, Perrault G, Sanger DJ. Evidence for the involvement of dopamine receptors in ethanol-induced hyperactivity in mice. *Neuropharmacology*. 1997; 36:1099–1108. [PubMed: 9294975]
- Cunningham CL, Niehus DR, Malott DH, Prather LK. Genetic differences in the rewarding and activating effects of morphine and ethanol. *Psychopharmacology (Berl)*. 1992; 107:385–393. [PubMed: 1352057]
- Federici M, Nistico R, Giustizieri M, Bernardi G, Mercuri NB. Ethanol enhances GABAB-mediated inhibitory postsynaptic transmission on rat midbrain dopaminergic neurons by facilitating GIRK currents. *Eur J Neurosci*. 2009; 29:1369–1377. [PubMed: 19309316]
- Finn DA, Bejanian M, Jones BL, Syapin PJ, Alkana RL. Temperature affects ethanol lethality in C57BL/6, 129, LS and SS mice. *Pharmacol Biochem Behav*. 1989; 34:375–380. [PubMed: 2622994]
- Ford CP, Gantz SC, Phillips PE, Williams JT. Control of extracellular dopamine at dendrite and axon terminals. *J Neurosci*. 2010; 30:6975–6983. [PubMed: 20484639]
- Gatto GJ, McBride WJ, Murphy JM, Lumeng L, Li T-K. Ethanol self-infusion into the ventral tegmental area by alcohol-preferring (P) rats. *Alcohol*. 1994; 11:557–564. [PubMed: 7865158]
- Gonon FG. Control of dopamine release by dopamine receptors and by impulse flow as studied by in vivo voltammetry. *Ann N Y Acad Sci*. 1986; 473:160–169. [PubMed: 3492168]
- Grace AA, Bunney BS. The control of firing pattern in nigral dopamine neurons: burst firing. *J Neurosci*. 1984a; 4:2877–2890. [PubMed: 6150071]
- Grace AA, Bunney BS. The control of firing pattern in nigral dopamine neurons: single spike firing. *J Neurosci*. 1984b; 4:2866–2876. [PubMed: 6150070]
- Groblewski PA, Bax LS, Cunningham CL. Reference-dose place conditioning with ethanol in mice: empirical and theoretical analysis. *Psychopharmacology (Berl)*. 2008; 201:97–106. [PubMed: 18633601]
- Hunt PS, Levillain ME, Spector BM, Kostelnik LA. Post-training ethanol disrupts trace conditioned fear in rats: effects of timing of ethanol, dose and trace interval duration. *Neurobiol Learn Mem*. 2009; 91:73–80. [PubMed: 18952186]
- Hyland BI, Reynolds JN, Hay J, Perk CG, Miller R. Firing modes of midbrain dopamine cells in the freely moving rat. *Neuroscience*. 2002; 114:475–492. [PubMed: 12204216]
- Imperato A, Di Chiara G. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *J Pharmacol Exp Ther*. 1986; 239:219–228. [PubMed: 3761194]
- Iyer SV, Benavides RA, Chandra D, Cook JM, Rallapalli S, June HL, Homanics GE. α 4-Containing GABA(A) Receptors are Required for Antagonism of Ethanol-Induced Motor Incoordination and Hypnosis by the Imidazobenzodiazepine Ro15–4513. *Front Pharmacol*. 2011; 2:18. [PubMed: 21779248]
- Jones SR, Mathews TA, Budygin EA. Effect of moderate ethanol dose on dopamine uptake in rat nucleus accumbens in vivo. *Synapse*. 2006; 60:251–255. [PubMed: 16752364]
- Justice JB Jr. Quantitative microdialysis of neurotransmitters. *J Neurosci Methods*. 1993; 48:263–276. [PubMed: 8105154]

- Linsensardt DN, Moore EM, Gross CD, Goldfarb KJ, Blackman LC, Boehm SL 2nd. Sensitivity and tolerance to the hypnotic and ataxic effects of ethanol in adolescent and adult C57BL/6J and DBA/2J mice. *Alcohol Clin Exp Res*. 2009; 33:464–476. [PubMed: 19120054]
- Mathews TA, John CE, Lapa GB, Budygin EA, Jones SR. No role of the dopamine transporter in acute ethanol effects on striatal dopamine dynamics. *Synapse*. 2006; 60:288–294. [PubMed: 16786536]
- Mereu G, Fadda F, Gessa GL. Ethanol stimulates the firing rate of nigral dopaminergic neurons in unanesthetized rats. *Brain Res*. 1984; 292:63–69. [PubMed: 6697212]
- Otis TS, Mody I. Differential activation of GABAA and GABAB receptors by spontaneously released transmitter. *J Neurophysiol*. 1992; 67:227–235. [PubMed: 1348084]
- Overton PG, Clark D. Burst firing in midbrain dopaminergic neurons. *Brain Res Brain Res Rev*. 1997; 25:312–334. [PubMed: 9495561]
- Owesson-White CA, Roitman MF, Sombers LA, Belle AM, Keithley RB, Peele JL, Carelli RM, Wightman RM. Sources contributing to the average extracellular concentration of dopamine in the nucleus accumbens. *J Neurochem*. 2012; 121:252–262. [PubMed: 22296263]
- Panin F, Cathala A, Piazza PV, Spampinato U. Coupled intracerebral microdialysis and electrophysiology for the assessment of dopamine neuron function in vivo. *J Pharmacol Toxicol Methods*. 2012; 65:83–92. [PubMed: 22326873]
- Park J, Aragona BJ, Kile BM, Carelli RM, Wightman RM. In vivo voltammetric monitoring of catecholamine release in subterritories of the nucleus accumbens shell. *Neuroscience*. 2010; 169:132–142. [PubMed: 20451589]
- Parsons LH, Justice JB Jr. Extracellular concentration and in vivo recovery of dopamine in the nucleus accumbens using microdialysis. *J Neurochem*. 1992; 58:212–218. [PubMed: 1727431]
- Patel J, Trout SJ, Kruk ZL. Regional differences in evoked dopamine efflux in brain slices of rat anterior and posterior caudate putamen. *Naunyn Schmiedebergs Arch Pharmacol*. 1992; 346:267–276. [PubMed: 1407013]
- Pelkonen A, Hiltunen M, Kiianmaa K, Yavich L. Stimulated dopamine overflow and alpha-synuclein expression in the nucleus accumbens core distinguish rats bred for differential ethanol preference. *J Neurochem*. 2010; 114:1168–1176. [PubMed: 20533994]
- Phillips PE, Hancock PJ, Stamford JA. Time window of autoreceptor-mediated inhibition of limbic and striatal dopamine release. *Synapse*. 2002; 44:15–22. [PubMed: 11842442]
- Rice ME, Patel JC, Cragg SJ. Dopamine release in the basal ganglia. *Neuroscience*. 2011; 198:112–137. [PubMed: 21939738]
- Rizzoli SO, Betz WJ. Synaptic vesicle pools. *Nat Rev Neurosci*. 2005; 6:57–69. [PubMed: 15611727]
- Robinson DL, Howard EC, McConnell S, Gonzales RA, Wightman RM. Disparity between tonic and phasic ethanol-induced dopamine increases in the nucleus accumbens of rats. *Alcohol Clin Exp Res*. 2009; 33:1187–1196. [PubMed: 19389195]
- Samson HH, Chappell AM. Effects of raclopride in the core of the nucleus accumbens on ethanol seeking and consumption: the use of extinction trials to measure seeking. *Alcohol Clin Exp Res*. 2004; 28:544–549. [PubMed: 15100604]
- Schmitz Y, Schmauss C, Sulzer D. Altered dopamine release and uptake kinetics in mice lacking D2 receptors. *J Neurosci*. 2002; 22:8002–8009. [PubMed: 12223553]
- Somers LA, Beyene M, Carelli RM, Wightman RM. Synaptic overflow of dopamine in the nucleus accumbens arises from neuronal activity in the ventral tegmental area. *J Neurosci*. 2009; 29:1735–1742. [PubMed: 19211880]
- Theille JW, Morikawa H, Gonzales RA, Morrisett RA. Ethanol enhances GABAergic transmission onto dopamine neurons in the ventral tegmental area of the rat. *Alcohol Clin Exp Res*. 2008; 32:1040–1048. [PubMed: 18422836]
- Threlfell S, Lalic T, Platt NJ, Jennings KA, Deisseroth K, Cragg SJ. Striatal dopamine release is triggered by synchronized activity in cholinergic interneurons. *Neuron*. 2012; 75:58–64. [PubMed: 22794260]
- Ungless MA, Grace AA. Are you or aren't you? Challenges associated with physiologically identifying dopamine neurons. *Trends Neurosci*. 2012; 35:422–430. [PubMed: 22459161]
- Wanat MJ, Willuhn I, Clark JJ, Phillips PE. Phasic dopamine release in appetitive behaviors and drug addiction. *Curr Drug Abuse Rev*. 2009; 2:195–213. [PubMed: 19630749]

- Weiner JL, Valenzuela CF. Ethanol modulation of GABAergic transmission: the view from the slice. *Pharmacol Ther.* 2006; 111:533–554. [PubMed: 16427127]
- Wightman RM, Robinson DL. Transient changes in mesolimbic dopamine and their association with 'reward'. *J Neurochem.* 2002; 82:721–735. [PubMed: 12358778]
- Yavich L, Tiihonen J. Ethanol modulates evoked dopamine release in mouse nucleus accumbens: dependence on social stress and dose. *Eur J Pharmacol.* 2000; 401:365–373. [PubMed: 10936495]
- Yorgason JT, Espana RA, Jones SR. Demon voltammetry and analysis software: analysis of cocaine-induced alterations in dopamine signaling using multiple kinetic measures. *J Neurosci Methods.* 2011; 202:158–164. [PubMed: 21392532]
- Yorgason JT, Espana RA, Konstantopoulos JK, Weiner JL, Jones SR. Enduring increases in anxiety-like behavior and rapid nucleus accumbens dopamine signaling in socially isolated rats. *Eur J Neurosci.* 2013; 37:1022–1031. [PubMed: 23294165]
- Yoshimoto K, Komura S. Monitoring of ethanol levels in the rat nucleus accumbens by brain microdialysis. *Alcohol Alcohol.* 1993; 28:171–174. [PubMed: 8517887]
- Yttri EA, Burk JA, Hunt PS. Intermittent ethanol exposure in adolescent rats: dose-dependent impairments in trace conditioning. *Alcohol Clin Exp Res.* 2004; 28:1433–1436. [PubMed: 15597074]
- Zhang H, Sulzer D. Regulation of striatal dopamine release by presynaptic auto- and heteroreceptors. *Basal Ganglia.* 2012; 2:5–13. [PubMed: 22712055]
- Zhang L, Doyon WM, Clark JJ, Phillips PE, Dani JA. Controls of tonic and phasic dopamine transmission in the dorsal and ventral striatum. *Mol Pharmacol.* 2009; 76:396–404. [PubMed: 19460877]

Figure 1.

Ethanol decreases high- but not low-frequency stimulated dopamine (DA) release. A) Representative DA traces from single or 20 Hz 10 pulse stimulations. B) Average ($\pm$ SEM) percent baseline DA release elicited from single or multiple-pulse (20 Hz, 10 pulses) stimulations in the presence of 80 mM ethanol (black bar) and post-ethanol washout. Ethanol (80 mM) decreases DA release from multiple-pulses, but not single-pulse DA release. C) Mean ($\pm$ SEM) percent baseline decreases in DA release across several increasing ethanol concentrations for single and multiple-pulse (10 pulses, 20 Hz) stimulations. Ethanol

has increased potency for multiple-pulse stimulations. For data points where no error bars are visible the SEM fell in a range that obscured the symbol. *** $p < 0.001$

Figure 2.

Ethanol's inhibitory effects on dopamine (DA) release are frequency dependent. The number of pulses and stimulation intensity were held constant (1 pulse or 10 pulses) and frequency was varied (5, 20 & 125 Hz). A) Raw DA signals from 'tonic'-like (1 pulse or 5 Hz, 10 pulses) and 'phasic'-like (20 and 125 Hz, 10 pulses) stimulations (duration denoted by black lines below traces). B) Average peak DA release ($\pm$ SEM) evoked from single- or multiple-pulse stimulations with varying frequencies (tonic: 1 pulse or 5 Hz, 10 pulses; phasic: 20 Hz, 10 pulses or 125 Hz, 10 pulses). DA release elicited is increased with high-

frequency stimulation with an optimal frequency of 20 Hz. C) Mean ($\pm$ SEM) percent baseline DA release across increasing concentrations of ethanol, demonstrating increased ethanol potency under high-frequency 'phasic'-like stimulation conditions. For data points where no error bars are visible the SEM fell in a range that obscured the symbol. Significance symbols are in relation to 1 pulse ethanol concentration response curve from post-hoc analysis (*=20 Hz; #=125 Hz). *,# $p<0.05$; ** $p<0.01$; ***,### $p<0.001$.

Figure 3.

Ethanol's inhibitory effects on dopamine (DA) release are pulse dependent. Frequency and stimulation intensity were held constant (20 Hz, 350 μ A) and the number of pulses was varied (1, 2, 5 and 10 pulses). A) Raw DA signals from 'tonic' - (1 pulse) and 'phasic'-like (2, 5 and 10 pulses, 20 Hz) stimulations (duration denoted by black lines below signals). B) Average ($\pm$ SEM) DA release evoked from high-frequency stimulations with varying pulse trains (tonic: 1 pulse; phasic: 2, 5 and 10 pulses). DA release increases with number of pulses for 20 Hz stimulation trains. C) Mean ($\pm$ SEM) percent baseline DA release across

increasing concentrations of ethanol, demonstrating increased ethanol potency under increased pulse stimulation conditions. For data points where no error bars are visible the SEM fell in a range that obscured the symbol. Significance symbols are in relation to 1 pulse ethanol concentration response curve from post-hoc analysis (*=10 pulses; #=5 pulses). **, ## $p<0.01$; ***, ### $p<0.001$.

Figure 4.

Ethanol's inhibitory effects on high-frequency dopamine (DA) release is not due to increased amount of DA released. Frequency and the number of pulses were held constant (1 pulse, 350 μ A, or 20 Hz, 10 pulses) and the current intensity was varied for 'phasic'-like, high-frequency stimulations (50, 100, 350 μ A). A) Raw DA signals from phasic stimulations (duration denoted by black lines below signals) of varying intensity (50, 100 and 350 μ A). B) Mean ($\pm$ SEM) DA release is dependent upon stimulation intensity (50, 100 and 350 μ A) with decreased release at lower intensities. C) Mean ($\pm$ SEM) percent baseline DA release

across increasing concentrations of ethanol. Ethanol decreases DA release to a similar extent despite large differences in baseline stimulated release (shown in B). For data points where no error bars are visible the SEM fell in a range that obscured the symbol. Significance symbols are in relation to 1 pulse ethanol concentration response curve from post-hoc analysis (*=350 μ A; #=100 μ A; +=50 μ A). * $p<0.05$; ##,++ $p<0.01$; ***, ### $p<0.001$.

Figure 5.

Ethanol inhibition of dopamine (DA) release is not calcium dependent. A) Mean ($\pm$ SEM) DA levels expressed in percent baseline (2.4 mM Calcium) before and after calcium (either 1.2 mM or 4.8 mM Calcium) induced changes in electrically stimulated (20 Hz, 10 pulses, 350 μ A) DA release. B) Raw data showing μ M or percent baseline DA release in response to phasic stimulations (20 Hz, 10 pulses) at various calcium concentrations (1.2–4.8 mM), and respective decreases after 80 mM ethanol. C) Average ($\pm$ SEM) percent baseline decreases in DA release after ethanol (80mM). DA release decreases similarly across increasing calcium

concentrations. For data points where no error bars are visible the SEM fell in a range that obscured the symbol. ** $p < 0.01$; *** $p < 0.001$.