Acute Ethanol Inhibits Dopamine Release in the Nucleus Accumbens via $\alpha 6$ Nicotinic Acetylcholine Receptors

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Received November 19, 2013; accepted March 18, 2014

ABSTRACT

Electrophysiology and microdialysis studies have provided compelling evidence that moderate to high ethanol concentrations enhance dopamine (DA) neurotransmission in the nucleus accumbens (NAc) through the mesolimbic DA system. However, with fast-scan cyclic voltammetry, short-term exposure to moderate to high doses of ethanol decreases evoked DA release at terminals in the NAc. The aim of this study was to evaluate the involvement of nicotinic acetylcholine receptors (nAChRs) in modulating the effects of ethanol on DA release in the NAc of C57BL/6 mice ex vivo and in vivo. Local stimulation evoked robust, frequency-dependent DA release in the NAc slice preparation, with maximal release at 40 Hz in the shell and

20 Hz in the core. Nicotine decreased DA release in a concentration-dependent (0.01–10 μ M) manner in the shell and core, with an IC_{50} of 0.1 μ M ex vivo and 0.5 mg/kg in vivo. Nicotine and ethanol inhibition of DA release was blocked by the $\alpha 6^*$ -nAChR antagonist α -conotoxins CtxMII and α -CtxMII [H9A; L15A] ex vivo (100 nM) in the core but not the shell. Furthermore, the nonspecific nAChR antagonist mecamylamine (2 mg/kg) blocked the effects of ethanol in the core in vivo. These findings suggest that DA release is inhibited by ethanol via nAChRs in the NAc and that DA modulation by nAChRs differs in the core versus the shell, with $\alpha 6^*$ -nAChRs affecting DA release in the core but not in the shell.

Introduction

Alcohol abuse is prevalent in today's society, causing an economic burden to the United States of hundreds of billions annually in direct costs alone (Mokdad et al., 2004; Hendrickson et al., 2013). Often taken in concert (Miller and Gold, 1998), tobacco and alcohol are leading causes of preventable death in the United States (Danaei et al., 2009). Approximately 80% of alcoholics are also smokers (Hendrickson et al., 2013; Taylor et al., 2013b), and heavy smokers are 10 times more likely to be alcoholics (DiFranza and Guerrera, 1990). Although smoking rates in the general population have decreased in recent decades, the use of nicotine has remained high (~70%–75%) in individuals who regularly use alcohol (Meyerhoff et al., 2006; Hendrickson et al., 2013).

The mesocorticolimbic system, originating in the ventral tegmental area (VTA) of the midbrain and projecting to the nucleus accumbens (NAc) and other limbic structures, is considered to be the primary neuronal circuit mediating the reinforcing properties of drugs of abuse, including ethanol and nicotine (Laviolette and van der Kooy, 2004; Nashmi et al.,

2007). In this system, dopaminergic neurons are located in the VTA and project to targets in the NAc and prefrontal cortex (Oades and Halliday, 1987; McFarland and Kalivas, 2001; Fields et al., 2007). Elevated dopamine (DA) transmission in the NAc is associated with feelings of euphoria (Kuhar et al., 1991; Kornetsky and Porrino, 1992), whereas lowered transmission of DA in the NAc is characterized by feelings of dysphoria, anxiety, and increased hedonic drive (Heyman, 1983; Lett and Grant, 1989; Stolerman, 1992; Koob, 1996; Koob and Le Moal, 1997; Robinson and Berridge, 2003).

Dopamine release, as measured by microdialysis and fast-scan cyclic voltammetry (FSCV), exhibits disparate results with ethanol and nicotine studies in the NAc. Microdialysis studies typically show a rise in DA levels, whereas voltammetry studies show a fall in evoked DA responses by both short-term nicotine and ethanol (Budygin et al., 2001; Robinson et al., 2009; Zhang et al., 2009; Yorgason et al., 2014). The disparity between these techniques is attributed, in part, to differences in measurements of tonic versus phasic DA release (Robinson et al., 2009). We have recently demonstrated that ethanol preferentially inhibits high-frequency phasic release but not low-frequency "tonic" release (Yorgason et al., 2014). Likewise, nicotinic mechanisms regulate the frequency dependence of DA release, albeit in a different direction, with greater DA release under phasic-like

This work was supported by the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grant R01-AA020919] (to S.C.S.). The authors declare no conflicts of interest.
dx.doi.org/10.1124/jpet.113.211490.

ABBREVIATIONS: ANOVA, analysis of variance; ACSF, artificial cerebrospinal fluid; CFE, carbon fiber electrode; CNS, central nervous system; DA, dopamine; DH β E, dihydro- β -erythroidine hydrobromide; FSCV, fast-scan cyclic voltammetry; MEC, mecamylamine; MLA, methyllycaconitine; NAc, nucleus accumbens; nAChR, nicotinic acetylcholine receptor; VTA, ventral tegmental area.

conditions and decreased release under tonic-like stimulations (Exley et al., 2008).

One population of GABA neurons in the VTA has projections to the NAc (Brown et al., 2012; van Zessen et al., 2012). It has recently been theorized that because of the high percentage of codependence with these drugs, both ethanol and nicotine may act on similar targets in the brain, specifically nicotinic acetylcholine receptors (nAChRs) on presynaptic GABAergic neurons or DAergic terminals (Hendrickson et al., 2013; Taylor et al., 2013b). Ethanol appears to activate DAergic neurons in the VTA through an interaction at nAChRs (Soderpalm et al., 2009). To date, there have not been any studies on the possible regulation of nicotine or ethanol on either of these terminals in the NAc via activation of $\alpha 6^*$ -nAChRs. In this study, we hypothesized that both nicotine and ethanol would modulate evoked DA release in the NAc via activation of nAChRs on presynaptic terminals that inhibit the release of DA at terminals. We demonstrate that specific $\alpha 6^*$ -nAChR antagonist α -conotoxins effectively block inhibition of evoked DA release caused by both nicotine and ethanol, indicating that both drugs of abuse have actions on similar targets in the NAc. Additionally, we show that the $\alpha 6$ subunit is functionally operational in the NAc core, but not the NAc shell, for modulating evoked DA release.

Materials and Methods

Animal Subjects and Surgical Procedure. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Brigham Young University and met or exceeded National Institutes of Health guidelines. Adult male C57BL/6 mice (postnatal days 30–120) from our own breeding colony were used in this study. Once weaned at postnatal day 21, all mice were housed in groups of four per cage and placed on a reverse light/dark cycle with lights on from 8:00 PM to 8:00 AM. For DA FSCV recordings *in vivo*, mice were anesthetized using isoflurane (MWI Veterinary Supply, Boise, ID) 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. None of the experiments required survival procedures; thus, animals were killed after experimentation.

Preparation of Brain Slices. Horizontal brain slices were obtained as described previously (Steffensen et al., 2008). In brief, mice were anesthetized with ketamine (60 mg/kg) and decapitated, and brains were quickly dissected and sectioned into 400- μm -thick horizontal slices in ice-cold artificial cerebrospinal fluid (ACSF), bubbled with 95% O_2 /5% CO_2 . The cutting solution consisted of the following (in mM): sucrose, 220; KCl, 3; NaH_2PO_4 , 1.25; NaH_2CO_3 , 25; MgSO_4 , 12; glucose, 10; and CaCl_2 , 0.2. Slices were immediately placed into an incubation chamber containing normal ACSF bubbled with 95% O_2 /5% CO_2 at 34 – 35°C consisting of the following (in mM): NaCl, 124; KCl, 2; NaH_2PO_4 , 1.25; NaHCO_3 , 24; glucose, 12; MgSO_4 , 1.2; and CaCl_2 , 2 (pH 7.3). Slices were incubated for at least 30 minutes before being transferred to a recording chamber. Once transferred to a recording chamber with continuous normal ACSF flow (2.0 ml/min), the temperature was maintained at 36°C throughout the experiment. Slices were allowed to stabilize for an additional 15 minutes before recordings were made. The striatum was visualized with Nikon Diaphot inverted microscopes in the transmitted light mode, and the NAc core and shell were visualized by microscopic inspection under low magnification at the level of the anterior commissure. A red light filter was placed in the light path to lower the exposure of the slice to short-wavelength light.

Carbon Fiber Electrodes, Calibration, and FSCV. For voltammetry recordings both *in vivo* and *ex vivo*, a 7.0- μm -diameter

carbon fiber was inserted into borosilicate capillary tubing (1.2-mm outer diameter; A-M Systems, Sequim, WA) under negative pressure and subsequently pulled on a vertical pipette puller (Narishige, East Meadow, NY). The carbon fiber electrode (CFE) was then cut under microscopic control with 150 μm of bare fiber protruding from the end of the glass micropipette. The CFE was backfilled with 3 M KCl. The CFEs were regularly calibrated with a known concentration of DA. With the CFE immersed in the solution of ACSF, we superfused a known concentration of DA at a high flow rate (5 ml/min) past the electrode and observed the maximum nA signal produced by DA. Dopamine calibrations were averaged to convert a signal (in nanoamps) of DA to micromolar concentration of DA.

For *ex vivo* voltammetry recordings, electrodes were positioned $\sim 75 \mu\text{m}$ below the surface of the slice in the NAc core. Dopamine release was evoked every 2 minutes by a 1-millisecond, 1- to 10-pulse stimulation (biphasic, 350 μA) from a micropipette tip broken to 5–10 μm , filled with ACSF, and oriented 100–200 μm from the CFE. The electrode potential was linearly scanned as a triangular waveform from -0.4 to 1.2 V and back to -0.4 V versus Ag/AgCl using a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon fiber electrode every 100 milliseconds (i.e., 10 Hz) by means of a ChemClamp voltage clamp amplifier (Dagan Corporation, Minneapolis, MN). Voltammetric recordings were performed and analyzed using LabVIEW (National Instruments, Austin, TX)-based customized software [Demon Voltammetry (Yorgason et al., 2011)]. Stimulations were performed periodically every 2 minutes. Dopamine levels were monitored for a stabilization period typically lasting 1 hour. Once the stimulated DA response was stable for five successive collections and did not vary by $>5\%$, baseline measurements were taken.

For *in vivo* voltammetry recordings, mice were anesthetized with isoflurane and placed in a stereotaxic apparatus (David Kopf Instruments, Tejuanga, CA). Bipolar, coated stainless steel electrodes were stereotactically implanted into the medial forebrain bundle (-1.7 posterior, $+1.1$ lateral, 4.6 – 5.3 ventral), and a capillary glass-based CFE in the NAc shell ($+1.2$ anterior, $+0.6$ lateral, -3.8 to 5.2 ventral) or core ($+1.2$ anterior, $+1.3$ lateral, -3.8 to 4.5 ventral). The medial forebrain bundle was stimulated with 60 biphasic pulses at 60 Hz (4-millisecond pulse width) at 2-minute intervals. Although the frequency of stimulation could be adjusted with *ex vivo* experimentation, effective *in vivo* stimulation is optimal at 60 Hz. Stimulation and recording electrodes were oriented by fine stereotaxic control to optimize the release of DA at 1.0-mA stimulation intensity. Subsequently, the current was adjusted to that stimulus level that evoked DA release at 50% of the maximum signal level (typically 0.2–0.3 mA). Once the stimulated DA response was stable for five successive collections and did not vary by $>5\%$, baseline measurements were taken.

Drug Preparation and Administration. For *ex vivo* experiments, (–)-nicotine hydrogen tartrate salt (Sigma-Aldrich, St. Louis, MO), dihydro- β -erythroidine hydrobromide (DH β E; Tocris Bioscience, Bristol, UK), methyllycaconitine (MLA; Sigma-Aldrich), α -CtxMII (University of Utah), and α -CtxMII [H9A; L15A], synthesized as previously described (McIntosh et al., 2004), were dissolved in stock solutions and then diluted into ACSF for superfusion onto brain slices at a given molar concentration. For *in vivo* experiments, mecamylamine (MEC; Sigma-Aldrich) was dissolved in physiologic saline solution and administered intraperitoneally. Nicotine was dissolved in physiologic saline solution and administered intravenously. Ethanol was dissolved in physiologic saline solution at 16% (w/v) and administered intraperitoneally.

Cerebral Lesion and Verification of Electrode Placement. Following the *in vivo* experimentation, a current of 2.5 mA was then applied through the CFE for 5 seconds with a 5-second interval at both polarities. This current induced an electrolytic lesion in the tissue. The perfused brains were then sliced coronally at 500 μm with a vibratome and visualized under light microscope to confirm placement of the electrode in either the NAc shell or core.

Statistical Analyses. The results for control and drug treatment groups were derived from calculations performed on voltammetry current-versus-time plots. Peak amplitude was determined by a median

Fig. 1. Pharmacology of nicotine inhibition of evoked DA release in the shell of the NAc ex vivo. (A) Superfusion of nicotine ($10 \mu\text{M}$) reduced DA release in this representative recording from the NAc shell slice preparation. Inset shows superimposed DA signals obtained before and after superfusion of nicotine. Calibration bars are nanoamps and seconds. (B) Nicotine significantly reduced DA release with an IC_{50} of $0.1 \mu\text{M}$. (C) Nicotine significantly reduced phasic DA release in the NAc shell across all frequencies tested. (D) Pharmacology of nicotine inhibition of DA release in the shell. While the $\alpha 6^*$ -nAChR antagonist $\alpha\text{-CtxMII}$ had no

filter peak detection algorithm in LabVIEW. Three successive DA peak responses were averaged for control and drug conditions within each experiment. The means were then grand-averaged across animals. Values were expressed as means $\pm$ S.E.M. for cumulated data. Between-subject group comparisons were analyzed via either t tests or one-way analyses of variance (ANOVAs). Within-subject frequency responses were analyzed with a two-way repeated-measures ANOVA, with frequency and drug as the within-subject comparisons. The criterion of significance was set at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) and are visible in Figs. 1 through 5. All statistics were calculated with IBM SPSS Statistics 21 (SPSS, Inc., Armonk, NY).

Results

Nicotine Dose Response, Frequency Response, and Pharmacology in the Shell Region of the Nucleus Accumbens. By use of FSCV, we evaluated the effects of nicotine (0.01 – $10 \mu\text{M}$) on evoked DA release in the NAc shell. Nicotine significantly decreased the peak amplitude of the DA signal with an IC_{50} of $\sim 0.1 \mu\text{M}$ (Fig. 1, A and B; $0.01 \mu\text{M}$: $P = 0.39$, $n = 4$; $0.1 \mu\text{M}$: $P = 0.001$, $n = 4$; $1.0 \mu\text{M}$: $P = 0.001$, $n = 4$; $10.0 \mu\text{M}$: $P < 0.0001$, $n = 7$). We evaluated the frequency dependency for nicotine effects on DA release in the NAc shell. We applied stimulation trains (10 pulses) at varying frequencies ranging from 10 to 200 Hz and compared the response produced by multiple pulses to that obtained by a single pulse. The frequency response followed an inverted-U relationship, with the maximum release occurring at 40-Hz stimulation in the NAc shell (Fig. 1C). Nicotine significantly decreased the amplitude of the DA signal across all frequencies tested. Two-way repeated-measures ANOVA revealed a main effect of frequency for the amplitude of the DA signal ($F_{5,20} = 2.897$, $P = 0.04$) and nicotine ($F_{1,4} = 16.367$, $P = 0.016$). Next we evaluated the effects of various nAChR antagonists on evoked DA release in the NAc shell. We analyzed evoked DA release in the presence of nicotine with the selective $\alpha 6^*$ antagonist $\alpha\text{-CtxMII}$ (100 nM) and also the partially selective $\alpha 4\beta 2^*$ antagonist DH β E ($50 \mu\text{M}$). Superfusion of $\alpha\text{-CtxMII}$ did not block the inhibition caused by $0.1 \mu\text{M}$ nicotine in the NAc shell, whereas DH β E did (Fig. 1D). $\alpha\text{-CtxMII}$ did not significantly affect the DA signal ($F_{1,7} = 0.090$, $P = 0.773$). However, superfusion of DH β E significantly inhibited the DA signal approximately 40% from baseline. There was no statistical significance between $\alpha\text{-CtxMII}$ -exposed nicotine and control nicotine ($P > 0.05$). However, one-way ANOVA comparing effects of DH β E + nicotine to nicotine alone on evoked DA in the NAc shell revealed a significant attenuation in nicotine effects on NAc shell DA transmission ($F_{1,6} = 35.376$, $P = 0.001$). Additionally, DH β E blockade of nicotine-induced DA reductions was significantly increased compared with $\alpha\text{-CtxMII}$ experiments ($F_{1,7} = 27.790$, $P = 0.0012$).

Nicotine Dose Response, Frequency Response, and Pharmacology in the Core Region of the Nucleus Accumbens. By use of FSCV, we evaluated the effects of nicotine (0.01 – $10 \mu\text{M}$) on evoked DA release in the NAc core. Nicotine significantly decreased the peak amplitude of the DA

effect on nicotine inhibition of DA release at $0.1 \mu\text{M}$ (the IC_{50} level), the $\alpha 4\beta 2$ antagonist DH β E significantly reduced nicotine inhibition of DA release in the NAc shell ($P = 0.001$). Significance of $\alpha\text{-CtxMII}$ vs. DH β E ($P = 0.0012$). Values in parentheses represent n values. CON, control; NIC, nicotine.

Fig. 2. Pharmacology of nicotine inhibition of evoked DA release in the core of the NAc ex vivo. (A) Superfusion of nicotine ($10 \mu\text{M}$) reduced DA release in this representative recording from the NAc core slice preparation. Inset shows superimposed DA signals obtained before and after superfusion of nicotine. Calibration bars are nanoamps and seconds. (B) Nicotine significantly reduced DA release with an IC_{50} of $0.1 \mu\text{M}$. (C) Nicotine significantly reduced phasic DA release in the NAc core across

signal with an IC_{50} of $\sim 0.1 \mu\text{M}$ (Fig. 2, A and B; $0.01 \mu\text{M}$: $P = 0.269$, $n = 4$; $0.1 \mu\text{M}$: $P = 0.006$, $n = 4$; $1.0 \mu\text{M}$: $P = 0.001$, $n = 4$; $10.0 \mu\text{M}$: $P = 0.002$, $n = 7$). We noted varying results at higher concentrations of nicotine ($10 \mu\text{M}$) in the NAc shell (Fig. 1B) versus the NAc core (Fig. 2B). Specifically, there was a significant difference between the shell and the core at the $10 \mu\text{M}$ nicotine concentration ($F_{1,12} = 35.765$, $P < 0.0001$). The frequency dependency for nicotine effects on DA release in the NAc core (Fig. 2C) was similar to the shell; however, the maximum release occurred at 20 Hz rather than 40 Hz . Nicotine significantly decreased the amplitude of the DA signal across all frequencies tested. Two-way repeated-measures ANOVA revealed a main effect of frequency for the amplitude of the DA signal ($F_{5,15} = 16.632$, $P = 0.0001$), and nicotine ($F_{1,3} = 592.034$, $P = 0.0002$). We analyzed the effects of the $\alpha 6$ antagonist α -conotoxins α -CtxMII (100 nM) and α -CtxMII [H9A; L15A] on nicotine inhibition of evoked DA release in the NAc core. We used the more specific α -CtxMII [H9A; L15A] to compare with α -CtxMII. However, as α -CtxMII [H9A; L15A] is less potent (McIntosh et al., 2004), a higher concentration (500 nM) was used. Superfusion of α -CtxMII significantly inhibited the DA signal approximately 30% from baseline, while the more specific $\alpha 6^*$ antagonist α -CtxMII [H9A; L15A] only slightly inhibited the DA signal 8% from baseline. Both of the α -conotoxins blocked the inhibition caused by $0.1 \mu\text{M}$ nicotine in the NAc core (Fig. 2D). One-way ANOVA comparing nicotine and MII + nicotine revealed significance ($F_{1,8} = 48.971$, $P = 0.0001$). Given that α -CtxMII in the NAc core completely attenuated the inhibition of nicotine, we determined not to perform any additional experiments with other nAChR antagonists (as we did in the NAc shell), as nicotine was apparently not acting by any other nAChR subunit in the NAc core. One-way ANOVA comparing nicotine and MII [H9A; L15A] + nicotine revealed significant reductions in nicotine effects in MII-pretreated slices ($F_{1,8} = 8.775$, $P = 0.0181$).

Ethanol Inhibition of Dopamine Release in the Nucleus Accumbens: Role of $\alpha 6^*$ -nAChRs. Because nicotine inhibition of evoked DA release in the core was blocked by the $\alpha 6$ antagonist α -CtxMII (100 nM), and we have previously demonstrated that ethanol similarly decreases evoked DA release in the core (Yorgason et al., 2014), we evaluated the effects of ethanol (20 – 160 mM) on DA release, as well as the effects of α -CtxMII (100 nM) on ethanol inhibition of DA release in the NAc core. From our previous experimentation, we have noted no disparities in the effects of ethanol in the NAc shell or core, as we have with nicotine. Ethanol significantly decreased the peak amplitude of the DA signal with an IC_{50} of $\sim 80 \text{ mM}$ (Fig. 3, A and B; 20 mM : $P = 0.001$, $n = 11$; 40 mM : $P = 0.001$, $n = 11$; 80 mM : $P = 0.0002$, $n = 11$; 160 mM : $P < 0.0001$, $n = 10$). Ethanol significantly decreased the amplitude of the DA signal across all frequencies tested, with the exception of single-pulse stimulation (Fig. 3C). This confirms the frequency-dependent nature of ethanol in the NAc reported previously (Yorgason et al.,

frequencies. (D) Pharmacology of nicotine inhibition of DA release in the core. The $\alpha 6^*$ -nAChR antagonist α -conotoxins α -CtxMII and α -CtxMII [H9A; L15A] significantly reduced nicotine inhibition of DA release in the NAc core ($P = 0.0001$ and 0.0181 , respectively). Values in parentheses represent n values. CON, control; NIC, nicotine.

Fig. 3. Nicotinic receptor pharmacology of EtOH inhibition of evoked DA release in the core of the NAc ex vivo. (A) Superfusion of ethanol (80 mM) reduced DA release in this representative recording from the NAc core slice preparation. Inset shows superimposed DA signals obtained before and after superfusion of ethanol. Calibration bars are nanoamps and seconds. (B) Ethanol significantly reduced DA release with an IC_{50} of 80 mM. (C) Superfusion of ethanol significantly reduced phasic DA release across frequencies from 10 to 80 Hz. (D) Pharmacology of ethanol inhibition of DA release in the core. While the $\alpha 7$ -nAChR antagonist MLA did not alter ethanol inhibition of DA release, the $\alpha 6^*$ -nAChR

2014). A two-way repeated-measures ANOVA revealed a main effect of frequency for the amplitude of the DA signal ($F_{4,32} = 25.703$, $P < 0.0001$), ethanol ($F_{1,8} = 6.744$, $P = 0.032$), and interaction of frequency by ethanol ($F_{1,8} = 4.427$, $P = 0.006$). We analyzed evoked DA release in the presence of ethanol with the specific $\alpha 7$ - and $\alpha 6^*$ -nAChR antagonists MLA (100 nM) and α -CtxMII [H9A; L15A] (500 nM), respectively. MLA did not alter ethanol inhibition of evoked DA release in the core at 80 mM ethanol (Fig. 3D; $F_{1,20} = 0.478$, $P = 0.497$). However, superfusion of the specific $\alpha 6^*$ -nAChR antagonist α -CtxMII revealed a significant effect of α -CtxMII [H9A; L15A] on ethanol inhibition of evoked DA release in the NAc (Fig. 3D; $F_{1,30} = 4.296$, $P = 0.047$).

Role of nAChRs in Ethanol Inhibition of Dopamine Release In Vivo. Typically, short-term ethanol administration (2 g/kg) reduces the DA signal $\sim 50\%$ (Fig. 4A) in vivo. It has previously been shown that the nonspecific nAChR antagonist MEC (Peng et al., 2013) reduces ethanol consumption and blocks ethanol-induced DA release in the NAc (Blomqvist et al., 1993; Hendrickson et al., 2013). To understand how nAChRs contribute to ethanol's inhibitory effects on DA in the NAc, we evaluated the effects of MEC on evoked DA signals in anesthetized mice. MEC did not alter the DA signal on its own, consistent with our ex vivo studies (data not shown). However, MEC (1 mg/kg i.p.) effectively blocked the inhibitory effects of ethanol in the NAc (Fig. 4B). Normalizing data to control values, MEC + ethanol demonstrated statistical significance versus ethanol alone (Fig. 4C; $F_{1,24} = 12.279$, $P = 0.002$).

Effects of Nicotine In Vivo in Both the Shell and Core of the Nucleus Accumbens. By use of FSCV, we evaluated the effects of nicotine (0.15–0.5 mg/kg i.v.) on evoked DA release in the NAc shell and core in vivo. Nicotine had a slightly excitatory effect in the NAc shell (Fig. 5, A and C), although it did not prove statistically significant via one-way ANOVA at 0.15 mg/kg ($F_{1,7} = 1.508$, $P = 0.265$) or 0.5 mg/kg ($F_{1,13} = 3.008$, $P = 0.108$). However, nicotine had an inhibitory effect on evoked DA release in the NAc core at both 0.15 mg/kg ($F_{1,9} = 10.435$, $P = 0.012$) and 0.5 mg/kg (Fig. 5, B and C; $F_{1,15} = 27.766$, $P < 0.0001$). Although an IC_{50} is statistically visualized at 0.15 mg/kg, the signals were more inconsistent at that dose than at 0.5 mg/kg. Thus, we used 0.5 mg/kg nicotine as our IC_{50} dose. As observed with the ex vivo experimentation, there was a significant difference between the response of the NAc shell and core with nicotine administration (Fig. 5C). One-way ANOVA revealed significance of 0.15 mg/kg core versus shell ($F_{1,8} = 8.874$, $P = 0.021$) and 0.5 mg/kg core versus shell ($F_{1,14} = 25.448$, $P = 0.0002$). Electrode placements were verified by microscopically visualizing the lesions in coronal slices induced by high current at the tip of the CFE (Fig. 5D).

Discussion

We demonstrated that evoked DA release is reduced by nicotine application in the NAc shell and core ex vivo. These inhibitory effects of nicotine were observed across all frequencies and were blocked by nAChR antagonists. Specifically, in

antagonist α -CtxMII [H9A; L15A] significantly reduced ethanol inhibition of DA release in the NAc core ($P = 0.047$). Values in parentheses represent n values. CON, control.

Fig. 4. Nicotinic receptor pharmacology of EtOH inhibition of evoked DA release in the core of the NAc in vivo. (A) Representative time course of DA release in the NAc core in an anesthetized mouse demonstrating inhibition produced by intraperitoneal administration of ethanol (2 g/kg). Inset shows superimposed DA signals obtained before and 30 minutes after injection of ethanol. Calibration bars are nanoamps and seconds. (B) Representative time course of DA release in the NAc demonstrating the blocking effect of intraperitoneal administration of MEC (1 mg/kg) on the inhibition typically produced by the administration of ethanol. Inset demonstrates representative nanoamp recordings of DA release in control, MEC, and ethanol conditions. Calibration bars are nanoamps and seconds. (C) Pharmacology of ethanol inhibition of DA release in vivo. Ethanol inhibits DA release at an IC_{50} of 2.0 g/kg. With the administration of MEC, ethanol has significantly less effect on DA release ($P = 0.002$). All values of MEC are normalized to control conditions. Values in parentheses represent n values. CON, control.

the shell, nicotine's effects were blocked by the $\alpha 4\beta 2^*$ -selective antagonist DH β E but not by the $\alpha 6^*$ -nAChR antagonist α -CtxMII. In contrast, nicotine modulation of DA signals in the core appears to be more through $\alpha 6^*$ -nAChRs, as α -CtxMII and the more selective $\alpha 6$ antagonist α -CtxMII [H9A; L15A] blocked nicotine's inhibition of evoked DA release. Similar to previous studies, we demonstrated that ethanol reduces stimulated DA release in the core in a frequency-dependent manner, with the greatest inhibition at higher stimulation frequencies. Ethanol's inhibitory effects were blocked by α -CtxMII [H9A; L15A], but not MLA, suggesting that ethanol is acting through heteromeric $\alpha 6^*$ -nAChRs and not $\alpha 7$

homomeric receptors. We also demonstrated that the non-specific nAChR antagonist MEC also blocks ethanol's inhibitory effects on evoked DA release under in vivo conditions.

Region-Dependent Nicotine Modulation of Dopamine Release. Nicotine decreased evoked DA release ex vivo in both the shell and the core of the NAc with an IC_{50} of 0.1 μM . Nicotine behaved similarly in the NAc shell and core, but not at the highest concentration of 10 μM where the NAc core demonstrated significantly less inhibition of DA release (Figs. 1B and 2B), suggesting that the nAChRs in the NAc core desensitize to high concentrations of nicotine compared with nAChRs in the NAc shell. More importantly, this significant variability in response to the higher concentrations of nicotine in the NAc core versus NAc shell demonstrates specific differences in nAChR subunits in the NAc core and the NAc shell. Nicotine decreased evoked DA release in the core in vivo but had no significant effect in the shell. Although these results do not exactly correspond to our findings ex vivo, it is important to note that the ex vivo recordings are influenced less by intact circuit responses but rather the behavior of stimulation at terminals of the neurons in the NAc. More importantly, the in vivo results demonstrate and further verify that there exists a significant variability in the nAChRs present in the NAc shell and core and provide compelling evidence that the ex vivo pharmacology demonstrated was valid.

Neuronal nAChRs are ligand-gated cation (primarily Na^+ or Ca^{2+}) channels of a wide variety in the central nervous system (CNS). These channels can exist in either homomeric or heteromeric form, with the $\alpha 7$ subunit composing the most common homomeric nAChR in the CNS, and pentameric mixtures of α ($\alpha 2$ – $\alpha 10$) and β ($\beta 2$ – $\beta 4$) subunits, with $\alpha 4\beta 2^*$ -nAChRs as the most common heteromeric CNS receptor (Taylor et al., 2013a). Although nAChRs are of a wide variety in the VTA (Wooltorton et al., 2003), heteromeric $\alpha 6^*$ -nAChRs are highly expressed in the mesolimbic DA system (Champtiaux et al., 2003; Yang et al., 2009b, 2011) and $\alpha 6$ is the primary α subunit that plays a prominent role in DA release (Quirk et al., 2011), and they are predominantly expressed in catecholaminergic systems (Brunzell, 2012). $\alpha 6^*$ -nAChR subunits are functional in recombinant systems when paired with β subunits or hybrids of α subunits. In fact, expression of the $\alpha 6^*$ -nAChR subunit is 16-fold higher than other subunit mRNAs in the VTA (Yang et al., 2009a), and they have been implicated in DA transmission and nicotine dependence (Drenan et al., 2008; Exley et al., 2008; Jackson et al., 2009; Drenan et al., 2010; Gotti et al., 2010; Brunzell, 2012).

Nicotine inhibition of evoked DA release in the shell and the core of the NAc were blocked by nAChR antagonists ex vivo. However, the NAc core, but not the NAc shell, was blocked by α -conotoxins, suggesting that nicotine's effects are mediated by $\alpha 6^*$ -nAChRs. Although it was previously determined that cholinergic interneurons excite presynaptic nAChRs on DA terminals (Jones et al., 2001; Yang et al., 2009b; Zhang et al., 2009; Brown et al., 2012), we demonstrate here that short-term nicotine administration causes a decrease in evoked DA release in the NAc, as reported previously by others (Zhang et al., 2009; Perez et al., 2013). Under the previous model, direct activation of the nAChR on the DA terminal would induce increased DA release, as a cationic influx into the presynaptic terminal would induce a higher magnitude of vesicular release. However, our results would suggest that there are interneurons regulating

Fig. 5. Pharmacology of nicotine effects of evoked DA release in the shell and core of the NAc in vivo. (A) Intravenous administration of nicotine (0.5 mg/kg) reduced DA release in this representative recording from the NAc shell in vivo. Inset shows superimposed DA signals obtained before and after injection of nicotine. Calibration bars are nanoamps and seconds. (B) Intravenous administration of nicotine significantly reduced DA release in the NAc core. (C) Summary of nicotine effects on DA release in vivo. At similar doses of nicotine, the release of DA in the NAc shell and core vary significantly, with the core demonstrating significant decrease

DA release in the NAc. It is likely that nAChRs are present on the terminals of these interneurons that then modulate the release of DA from DAergic neurons (Yang et al., 2011; Taylor et al., 2013b). Under this model, activation of the nAChRs on the interneurons would decrease the level of evoked DA release recorded by FSCV. Our experiments with the specific $\alpha 6^*$ -nAChR antagonist α -conotoxins in both the NAc core and NAc shell demonstrate a distinct difference in the nAChR distribution in these anatomic regions. Given that α -CtxMII completely blocks the effects of nicotine in the NAc core and does not block the effects of nicotine in the NAc shell, yet DH β E completely blocks the effects of nicotine in the NAc shell, these results indicate that the NAc core primarily operates via $\alpha 6^*$ -nAChRs and the NAc shell primarily operates via $\alpha 4\beta 2^*$ -nAChRs. This verifies the results of those previously reported (Exley et al., 2012).

Ethanol Inhibits Dopamine Release through nAChR Interactions. Given that ethanol has been shown to decrease evoked DA release, as measured by FSCV, we sought to evaluate the role of ethanol on nAChRs in mediating evoked DA release in the NAc in vivo and ex vivo, as we are not aware of any previous studies that have systemically focused on the involvement of ethanol inhibition via nAChRs at DA terminals. Local stimulation in the NAc slice preparation produces a robust increase in DA release with a single pulse but evinces marked frequency modulation with multiple pulses, with optimal release at 20 Hz (10 pulses). Similar to nicotine, ethanol significantly decreased DA release at concentrations of 20–160 mM and reduced phasic responses across all frequencies equally. Superfusion of the $\alpha 7$ -subunit-specific antagonist MLA had no effect on ethanol inhibition of evoked DA release, suggesting that ethanol is not acting in either the NAc core or NAc shell via $\alpha 7^*$ -nAChRs. However, as α -CtxMII and α -CtxMII [H9A; L15A] significantly reduced ethanol inhibition of DA release in the NAc core, this suggests that ethanol is acting via $\alpha 6^*$ -nAChRs in the NAc core. Thus, as α -CtxMII significantly blocked both nicotine and ethanol in the NAc core, both nicotine and ethanol appear to be acting via the same $\alpha 6^*$ -nAChRs. However, given that the block of α -CtxMII was complete with nicotine and incomplete with ethanol, it is probable that ethanol is acting through more than one mechanism in the NAc, whether intracellular or through an extracellular receptor.

Ethanol reduced evoked DA release in vivo with an IC_{50} of 2.0 g/kg, a moderately intoxicating dose level. We determined this IC_{50} of ethanol in vivo through previous experimentation (data not shown). These studies provide the physiologic relevancy of ethanol inhibition of evoked DA release at terminals in the NAc. As α -CtxMII cannot be administered in vivo due to its inability to cross the blood-brain barrier, we used intraperitoneal administration of MEC to block nAChRs. MEC blocked ethanol inhibition of evoked DA release, thus

from baseline and from the shell at both dose levels. NIC (0.15 mg/kg) shell vs. core ($P = 0.012$). NIC (0.5 mg/kg) shell vs. core ($P = 0.0002$). Control vs. NIC (0.15 mg/kg) core ($P = 0.012$). Control vs. NIC (0.5 mg/kg) core ($P < 0.0001$). Values in parentheses represent n values. (D) Representative coronal brain slice through the NAc. Solid lines represent electrode placements in the NAc core. Dashed lines represent electrode placements in the NAc shell. aca, anterior commissure; AcbSh, accumbens shell; LAcSh, lateral accumbens shell; CON, control; NIC, nicotine.

providing further evidence that the mechanism of ethanol inhibition of DA release occurs through an nAChR subunit. Comparing these physiologically relevant results with our ex vivo experimentation, we conclude that MEC is antagonizing the $\alpha 6^*$ -nAChRs in the NAc against the attenuation of ethanol. Given that $\alpha 6^*$ -nAChRs are located on GABA terminals and known to enhance GABA release (Yang et al., 2011), it is possible that ethanol inhibition of evoked DA release results from activation of $\alpha 6^*$ -nAChRs on GABA terminals that inhibit evoked DA release. However, we acknowledge that the mechanism of nAChRs in the NAc may be much more complex due to the compelling evidence of nAChRs present on DA terminals (Zoli et al., 2002; Exley et al., 2012).

It is noteworthy that $\alpha 6$ knockout mice fail to self-administer nicotine. However, once the $\alpha 6$ subunit is re-expressed via a lentiviral vector, the mice develop sustained short-term self-administration behavior (Pons et al., 2008). The VTA has $\alpha 6^*$ -nAChRs on presynaptic GABA terminals but not on the somata of the DAergic neurons (Yang et al., 2009b; Taylor et al., 2013b). Thus, nicotine can modulate both DA and GABA terminal neurotransmitter release (Yin and French, 2000; Mansvelder et al., 2002), although the majority of endogenous cholinergic inputs into the VTA appear to contact GABA rather than DA neurons (Garzon et al., 1999; Fiorillo and Williams, 2000). More specifically, nAChRs play a crucial role in modulating GABA release onto DA neurons in the VTA (Yang et al., 2011; Taylor et al., 2013b). The majority of GABA neurons in the VTA express $\alpha 4$ - and $\beta 2^*$ -nAChR subunits, which can be blocked by the noncompetitive antagonist MEC or by the competitive antagonist DH β E (Mansvelder et al., 2002). It is the emerging view that the early short-term effects of nicotine in the VTA predominantly affect GABA neurons and that the nAChRs that have been associated with these cells desensitize rapidly, leading to a long-lasting excitation (disinhibition) of DA neurons through removal of the inhibitory influence of GABA (Yang et al., 2011). Recent optogenetic studies have demonstrated that selective activation of VTA GABA neurons drives conditioned place aversion (Tan et al., 2012) and disrupts reward consumption (van Zessen et al., 2012), providing compelling evidence for the importance of GABA neurons in regulating DA transmission in the mesocorticolimbic reward pathway, and in particular interactions between ethanol and nicotine reward signaling. Thus, GABA inhibition is a critical regulator of DA neurotransmission in the mesocorticolimbic system and for the rewarding properties of both ethanol and nicotine.

In conclusion, these experiments verify that both nicotine and ethanol reduced phasic release of DA in the NAc, and that $\alpha 6^*$ -nAChRs are involved in ethanol inhibition of evoked DA release in the NAc core but not the NAc shell of C57BL/6 mice. Future studies will confirm the hodology of GABA interaction with DA terminals in the NAc with combined transmission electron microscopy and immunohistochemistry and will evaluate whether a population of VTA GABA neurons is projecting to the NAc and connects to DA terminals.

Authorship Contributions

Participated in research design: Schilaty, Hedges, Yorgason, Steffensen.

Conducted experiments: Schilaty, Hedges, Jang, Folsom.

Contributed new reagents or analytic tools: McIntosh.

Performed data analysis: Schilaty, Hedges, Yorgason, Steffensen.

Wrote or contributed to the writing of the manuscript: Schilaty, Hedges, Yorgason, Steffensen, McIntosh.

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