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Nicotine Decreases Ethanol-induced Dopamine Signaling and Increases Self-administration via Stress Hormones

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SUMMARY

Tobacco smoking is a well-known risk factor for subsequent alcohol abuse, but the neural events underlying this risk remain largely unknown. Alcohol and nicotine reinforcement involve common neural circuitry, including the mesolimbic dopamine system. We demonstrate in rodents that pre-exposure to nicotine increases alcohol self-administration and decreases alcohol-induced dopamine responses. The blunted dopamine response was due to increased inhibitory synaptic transmission onto dopamine neurons. Blocking stress hormone receptors prior to nicotine exposure prevented all interactions with alcohol that we measured, including the increased inhibition onto dopamine neurons, the decreased dopamine responses, and the increased alcohol self-administration. These results indicate that nicotine recruits neuroendocrine systems to influence neurotransmission and behavior associated with alcohol reinforcement.

Keywords

nicotinic receptors; nAChR; neuroendocrine; HPA axis; alcohol; GABA; reward; mesolimbic; ventral tegmental area; nucleus accumbens

INTRODUCTION

Tobacco (nicotine) and alcohol are the two most abused and costly drugs to society. Epidemiological studies consistently find a positive correlation between nicotine and alcohol use, with alcoholism approximately 10 times more prevalent in smokers than in non-smokers (DiFranza and Guerrera, 1990; Harrison et al., 2008; McKee et al., 2007; Schorling et al., 1994; Weitzman and Chen, 2005). Several studies also show that nicotine exposure increases alcohol self-administration (Barrett et al., 2006; Le et al., 2003; Smith et al., 1999); and smoking, particularly at an early age, is a significant risk factor for subsequent alcohol abuse (Chen et al., 2002; Grant, 1998; Morgen et al., 2008; Sher et al., 1996; Torabi et al., 1993).

In addition to psychosocial and genetic factors (Bobo and Husten, 2000; Schlaepfer et al., 2008), evidence suggests that the interactions between nicotine and alcohol arise from

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shared pharmacological actions (Funk et al., 2006; Hurley et al., 2011; Larsson and Engel, 2004). These drugs activate common neural substrates, including the mesolimbic dopamine (DA) system (De Biasi and Dani, 2011; Di Chiara, 2000; Gonzales et al., 2004) and the hypothalamic-pituitary-adrenal (HPA) axis associated with stress hormone signaling (Armario, 2010; Lutfy et al., 2012; Richardson et al., 2008). Both the DA and HPA systems are centrally linked to drug use and addiction (Koob and Kreek, 2007; Ungless et al., 2010).

Alcohol use disorders involve long-term alterations in the stress hormone systems (Sinha et al., 2011; Vendruscolo et al., 2012). Stress hormones, such as the glucocorticoids, have a profound influence on neural function (Joels and Baram, 2009) and modulate DA transmission (Barrot et al., 2000; Butts et al., 2011). Other stress-related neuroactive hormones also modify GABA transmission (Di et al., 2009; Stell et al., 2003; Wirth, 2011), which may contribute to the pharmacological action of alcohol (Biggio et al., 2007; Helms et al., 2012; Morrow et al., 2009).

To simplify this complex and multifaceted interaction between nicotine and alcohol, we studied how acute nicotine exposure in naïve animals alters subsequent responses to alcohol, including alcohol-induced DA signals and alcohol self-administration. We found that pretreatment with nicotine increased subsequent alcohol self-administration and decreased alcohol-induced dopamine signals in the ventral tegmental area (VTA) and the nucleus accumbens (NAc). The decreased dopamine responses to alcohol arose via two mechanisms: an initial activation of stress hormone receptors in the ventral tegmental area and a subsequent increase in alcohol-induced inhibitory neurotransmission. These results identify the mesolimbic dopamine system as a locus for multiple neurophysiological interactions between nicotine and alcohol.

RESULTS

Nicotine attenuates ethanol-induced DA release

The initial administration of addictive drugs, such as nicotine and ethanol, increases basal DA levels in the nucleus accumbens (NAc) as measured by microdialysis (Di Chiara and Imperato, 1988). We found that simultaneous co-administration of nicotine and ethanol produces an additive increase in NAc DA release relative to the response of each drug alone (Fig. S1). To determine whether prior exposure to nicotine influences ethanol-induced DA release in the NAc, we injected rats with nicotine or saline 3 hrs prior to administering ethanol. Guided by nicotine's metabolic half-life in rats of 45 min (Matta et al., 2007), we chose a 3-hr pretreatment period to decrease any carryover in the pharmacological effects of nicotine.

Microdialysis samples were collected to follow the change in extracellular DA levels induced by ethanol administration (Fig. 1). After pretreatment with saline or nicotine, there was no difference in the basal DA concentrations prior to ethanol exposure: 1.0 ± 0.2 nM after nicotine pretreatment and 1.0 ± 0.1 nM after saline pretreatment. To avoid handling-related stress, ethanol was administered intravenously over a 5-min period (Fig. 1A–C; shaded columns). Ethanol induced a sustained increase in DA release in the saline control group (Fig. 1A–C, black circles). Nicotine pretreatment (0.4 mg/kg, i.p., 3 hrs prior) significantly attenuated the ethanol-induced increase in DA release (Fig. 1A, red circles) (group x time: $F(10,100) = 2.37$, $p < 0.05$). The administered ethanol dose falls within the typical range tested in rodents (Gonzales et al., 2004) and produces brain ethanol concentrations in rodents that humans commonly achieve (Howard et al., 2008). Brain ethanol concentrations peaked 10 min after the ethanol infusion and then decreased to a relatively stable concentration just above 30 mM for more than 30 min (Fig. S2). Blood

ethanol concentrations were 26 ± 4 mM when measured from blood samples taken 95 min after ethanol administration.

To determine the duration of nicotine's effect on ethanol-induced DA release, we increased the interval between the nicotine pretreatment and the ethanol exposure to 15 hrs and 40 hrs, respectively. Remarkably, the DA release induced by ethanol remained significantly (group x time: $F(10,250) = 6.16$, $p < 0.01$) blunted 15 hrs after nicotine pretreatment (0.4 mg/kg, i.p.) (Fig. 1B, red circles) compared to the saline control (Fig. 1B; black circles). This effect was less evident 40 hrs after nicotine pretreatment (group x time: $F(10,150) = 1.31$, $p > 0.05$). However, a post hoc analysis of the first three post-ethanol dialysate samples (plus baseline) revealed a statistical difference between the nicotine and saline pretreatments (group x time: $F(5,75) = 2.63$, $p < 0.05$), suggesting at least some influence of nicotine 40 hrs after administration (Fig. 1C). The distribution of the microdialysis probe placements within the NAc were similar between the cohort of animals pretreated with nicotine and those pretreated with saline (Fig. 1D), indicating that regional differences in DA release do not account for these results.

Although the animals were habituated to needle injections, we further controlled for the stimulus effects of the intraperitoneal injection of nicotine, which could potentially contribute to a stress response. A separate experimental group was pretreated with nicotine administered intravenously by cannula 15 hrs prior to ethanol administration. This group displayed the same attenuated DA response to ethanol compared to the group pretreated with intravenous saline (group x time: $F(10,210) = 5.35$, $p < 0.01$). Thus, physical handling and the stimulus of the needle injections during the pretreatment period did not contribute to the inhibitory effect of nicotine on ethanol-induced DA release.

Nicotine increases ethanol self-administration

Given that the single nicotine pretreatment decreased ethanol-induced DA release, we determined whether that same nicotine pretreatment influenced ethanol self-administration (Smith et al., 1999). To parallel the 40 hr time course of our microdialysis experiments (see Fig. 1), we examined ethanol intake during the early acquisition of drinking behavior. Early acquisition was defined as the first four sessions of ethanol self-administration (1 session/day for 45 min/session). Operant responses to saccharin (0.125%, w/v) were first established, followed by an introduction of ethanol (2–4%) into the drinking solutions over four days (Doyon et al., 2005).

We pretreated the rats with either nicotine (0.4 mg/kg, i.p.) or saline 3 hrs prior to an initial ethanol exposure, as in the microdialysis experiments (see Fig. 1A). Ethanol intake across the first four self-administration sessions was significantly higher after nicotine pretreatment (0.97 g/kg, n = 20) compared to the saline pretreatment control (0.75 g/kg, n = 17) ($p < 0.01$) (Fig. 1E). Rats pretreated with nicotine also initiated significantly more operant responses (44 ± 2) than the saline pretreatment control (36 ± 2).

To confirm that these effects were specific to ethanol and not related to the saccharin in the drinking solution, we included a separate control group that responded for saccharin alone (no ethanol) (n = 10). This group did not drink significantly more fluid following nicotine pretreatment (15.6 ± 1.8 ml/session) across four drinking sessions than the ethanol control rats pretreated with saline (13.9 ± 0.8 ml/session) ($p > 0.05$).

Nicotine attenuates ethanol-induced DA signals via β2* nAChRs

Neuronal nicotinic acetylcholine receptors (nAChRs) consist of many subunit combinations, but nicotine exerts its action primarily through two major receptor subtypes containing either the high-affinity β2 subunit (often with α4 or α6 subunits) or the low-affinity α7

subunit (McGehee and Role, 1995; Nashmi and Lester, 2006; Tapper et al., 2004). To determine which general nAChR subtype contributed to the nicotine-ethanol interaction, we selectively blocked β2-containing (β2*) nAChRs with DHβE or blocked α7* nAChRs with MLA at the time of the nicotine pretreatment. The attenuation of ethanol-induced DA release by nicotine pretreatment (15 hrs before ethanol) was prevented by DHβE pretreatment in a dose-dependent manner (group x time: $F(10,180) = 3.09$, $p < 0.01$) (Fig. 2A, traces with squares), indicating the involvement of β2* nAChRs. By comparison, blocking α7* nAChRs with MLA did not influence the interaction between nicotine and ethanol (group x time: $F(10,160) = 0.38$, $p > 0.05$) (Fig. 2B, trace with squares).

Given that the nicotine-ethanol interaction depended on the β2* nAChRs, it is possible that this acute nicotine pretreatment altered the long-term function of the β2* nAChRs. To test this hypothesis, we examined the effect of nicotine or saline pretreatment (15 hrs prior) on nicotine-induced DA release. The nicotine-induced DA release was not significantly different between those rats pretreated with nicotine and the control rats pretreated with saline (group x time: $F(14,112) = 0.79$, $p > 0.05$) (Fig. 2C). This result suggested that acute nicotine pretreatment did not causatively influence the long-term function of the β2* nAChRs within the DA system. That is, nicotine-induced changes in nAChR function do not likely explain the results obtained with ethanol. The distribution of the microdialysis probe placements for these experiments (Fig. 2D) was similar to the placements shown for the earlier experiments (Fig. 1D).

Nicotine attenuates ethanol-induced VTA DA neuron firing

Evidence indicates that ethanol enhances DA release in the NAc by increasing the firing rate of VTA DA neurons (Foddai et al., 2004; Gessa et al., 1985). Therefore, we examined whether nicotine pretreatment altered the responses of VTA DA neurons to ethanol. Rats were injected with nicotine or saline 15 hrs prior to the experiment. We then cut midbrain slices and performed patch clamp cell-attached recordings to measure the spontaneous firing rate of VTA DA neurons before and after applying ethanol to the bath. DA neurons were recorded from the lateral VTA and were identified by a combination of factors that were verified by immunohistochemistry (Fig. 3A). We chose DA neurons based on their location, low pacemaker-like firing rate (1–5 Hz), long duration action potential, and the presence of a large hyperpolarization-activated inwardly rectifying cation current (I_h) (Chen et al., 2008; Zhang et al., 2010). Thirteen of 14 cells that displayed these electrophysiological properties were positive for tyrosine hydroxylase (Fig. 3A).

The mean basal firing rate prior to ethanol exposure was 2.4 ± 0.3 Hz following saline pretreatment (n = 11) and 2.7 ± 0.2 Hz following nicotine pretreatment (n = 11), indicating that the spontaneous baseline firing of the DA neurons was unchanged by the pretreatment. Bath application of ethanol increased the firing rate of the DA neurons from rats pretreated with saline (Fig. 3B,C), which is consistent with previous studies (Brodie et al., 1990; Okamoto et al., 2006). By comparison, ethanol exposure did not significantly alter the firing rate of DA cells from nicotine-pretreated rats, and in several cases, there was a reduction in activity (Fig. 3C, red circles). ANOVA confirmed a significant group by time interaction between the saline and nicotine pretreatments ($F(9,180) = 3.16$, $p < 0.01$).

Nicotine increases ethanol-induced VTA GABA transmission

Inhibitory synaptic inputs regulate the spontaneous firing rate of VTA DA neurons (Grace et al., 2007). In addition to its excitatory action, ethanol also enhances GABA-mediated inhibitory transmission onto VTA DA neurons (Melis et al., 2002; Theile et al., 2008). We postulated that the combination of nicotine pretreatment and ethanol exposure shifted the balance between the inhibitory and excitatory inputs onto DA neurons.

To test this hypothesis, we pretreated rats with saline or nicotine 15 hrs before performing whole-cell patch clamp recording from VTA DA neurons. We measured spontaneous inhibitory postsynaptic currents (sIPSCs) and separately spontaneous excitatory postsynaptic currents (sEPSCs, described below). To examine GABAergic activity, glutamate receptors were blocked with DNQX (20 μM) and AP5 (50 μM). Nicotine pretreatment did not significantly alter the mean basal sIPSC frequency between the groups (saline pretreatment control, 2.7 ± 0.3 Hz; nicotine pretreatment, 3.4 ± 0.6 Hz; n = 7, 8; $p > 0.05$) or the mean basal sIPSC amplitudes (saline pretreatment control, 27.9 ± 4.2 pA; nicotine pretreatment, 27.9 ± 2.8 pA; n = 7, 8; $p > 0.05$). In control DA neurons from saline-pretreated rats, bath-applied ethanol (50 mM) induced a marginal increase in the sIPSC frequency (black data, Fig. 4A,C; n = 7) (Theile et al., 2008). By contrast, in DA neurons from nicotine-pretreated rats, ethanol caused a much greater potentiation of the sIPSC frequency above the control response (red data, Fig. 4B,C; n = 8; $p < 0.01$) with no change in sIPSC amplitude. We repeated this experiment using a lower bath ethanol concentration (25 mM) and determined that the effect of nicotine and ethanol on sIPSC frequency was still present. Nicotine pretreatment increased the sIPSC frequency induced by 25 mM ethanol by approximately 24% (n = 6/group, $p < 0.05$) compared to the saline pretreatment response, whereas in 50 mM ethanol the percent increase between the nicotine and saline pretreatment was approximately 56% (Fig 4C; n = 7, 8; $p < 0.01$).

An increase in the frequency, but not the amplitude, of the sIPSCs after nicotine pretreatment suggested a presynaptic change in GABA transmission. To investigate whether a presynaptic mechanism rather than a postsynaptic mechanism was at work, we measured the paired-pulse ratio of evoked IPSCs under different pretreatment conditions after ethanol application. Differences in the amplitudes between two consecutively evoked IPSCs (i.e., the paired-pulse ratio) suggest a transient change in the probability of GABA release. Baseline paired-pulse ratios were not different between the saline pretreatment and nicotine pretreatment groups ($p > 0.05$). Application of ethanol decreased the paired-pulse ratio in both the saline pretreatment control (n = 14) and the nicotine pretreatment group (n = 21). The magnitude of this paired-pulse depression, however, was significantly greater ($p < 0.05$) after the nicotine pretreatment (78.6 ± 3.4%) compared to the saline pretreatment (90.0 ± 3.8%) (Fig. 4D), which is consistent with a presynaptic change in GABA transmission.

To confirm that changes in ethanol-induced GABA transmission contribute to changes in DA neuron responses, we blocked $GABA_A$ receptors prior to the bath application of ethanol. We applied picrotoxin, a non-competitive $GABA_A$ receptor antagonist, to slices containing the VTA and then measured the spontaneous firing rate of DA neurons in response to bath-applied ethanol. Picrotoxin (50 μM) did not significantly alter the baseline firing rate of DA neurons between the nicotine and saline pretreatments. However, in the presence of picrotoxin (50 μM), ethanol no longer inhibited DA neuron firing after nicotine pretreatment (red circles compared to dotted line, Fig. 4E). In the presence of picrotoxin, nicotine and saline pretreatment groups showed a similar increase in firing rate in response to ethanol (group x time: $F(9,144) = 0.30$, $p > 0.05$). These results, combined with the increase in spontaneous and evoked GABA IPSCs, indicate that nicotine pretreatment increased ethanol-induced GABA transmission onto DA neurons, thereby reducing DA neuron excitability.

To examine whether the effect of nicotine was selective to the actions of ethanol, we recorded from DA neurons and measured sIPSCs induced by other drugs of abuse following nicotine or saline pretreatment. Bath-applied nicotine (1 μM) increased the sIPSC frequency similarly in both treatment groups (saline pretreatment, 156 ± 24% of basal; nicotine pretreatment, 155 ± 25% of basal; n = 5–6, $p > 0.05$), indicating no causative effect of the nicotine pretreatment. Because our data suggest adaptations in GABA transmission, we also

tested diazepam, a benzodiazepine that positively modulates $GABA_A$ receptors (Tan et al., 2010). Nicotine pretreatment increased the sIPSC frequency induced by diazepam by approximately 63% compared to the saline pretreatment response ($n = 6, 7$/group, $p < 0.01$), indicating that nicotine pretreatment specifically altered the GABAergic responses to drugs such as ethanol and diazepam.

The interaction between nicotine and ethanol could potentially alter the excitatory glutamatergic signals that regulate VTA DA neurons (Xiao et al., 2009). We tested this possibility by performing whole-cell patch clamp recordings of sEPSCs before and after ethanol application to the bath. The basal sEPSC frequency was not different between the saline pretreatment control cells (1.8 ± 0.3 Hz) and the nicotine pretreatment cells (1.2 ± 0.2 Hz; $n = 7$, $p > 0.05$). Subsequent application of ethanol increased the sEPSC frequency to a similar degree in both groups (saline pretreatment control, 165 ± 7%; nicotine pretreatment, 169 ± 8%; $n = 7$, $p > 0.05$).

Interactions between nicotine and ethanol require stress hormones

To understand how nicotine and ethanol interact and impinge on the DA and GABA systems, we considered several possible mechanisms. A functional alteration in nAChRs by nicotine is not likely to contribute to the nicotine-ethanol interaction because the DA release (see Fig 2C) and the sIPSCs induced by nicotine were unaffected by nicotine pretreatment. Moreover, the recovery from desensitization is more rapid than 15 to 40 hrs (Lester and Dani, 1995; Wooltorton et al., 2003). Nicotine can enhance glutamatergic synaptic plasticity onto DA neurons (Gao et al., 2010; Mansvelder et al., 2002; Saal et al., 2003), but the fact that nicotine pretreatment did not alter the baseline DA firing rates and did not alter sEPSCs makes this explanation unlikely. As an alternative, we hypothesized that nicotine administration altered the DA and GABA responses to alcohol through a neuroendocrine signal (Armario, 2010). Stress-related hormones, such as glucocorticoids, cause long-term homeostatic changes in neural function and influence DA and GABA transmission (Barrot et al., 2000; Butts et al., 2011; Joels and Baram, 2009).

Nicotine activates the HPA axis to increase plasma levels of corticosterone (Lutfy et al., 2012), the principle glucocorticoid in rodents, which we confirmed (Fig. S3). To determine whether glucocorticoid receptor activation during nicotine pretreatment contributes to subsequent alterations in ethanol-induced DA release, we systemically blocked glucocorticoid receptors with RU486 (Cadepond et al., 1997) prior to nicotine pretreatment. Pretreatment with RU486 (Fig. 5A, blue circles) prevented the inhibitory effect of nicotine on ethanol-induced DA release (group x time: $F(10,240) = 4.75$, $p < 0.01$). This increased DA response to ethanol following RU486 and nicotine pretreatment was not distinguishable from the control rats pretreated with saline alone or RU486 alone (Fig. 5A, dashed trace).

These results suggested that stress receptor activation within the VTA, following nicotine pretreatment, attenuated the subsequent DA response to ethanol. To test this hypothesis, we blocked glucocorticoid receptors locally in the VTA with RU486 prior to nicotine pretreatment. The control group that received a local intra-VTA microinfusion of vehicle followed by nicotine pretreatment showed a decreased DA response to ethanol 15 hrs later (Fig. 5B, red circles), consistent with our previous data (see Fig. 1). This inhibitory effect of nicotine pretreatment was prevented by intra-VTA microinfusion of RU486 prior to nicotine pretreatment (Fig. 5B, blue circles) (group x time: $F(10,140) = 2.43$, $p < 0.05$). We should note that the intra-VTA RU486 did not completely reverse the effect of nicotine pretreatment. A post hoc comparison indicated a significant difference between the saline control (Fig. 5B, dashed line) and the group pretreated with intra-VTA RU486 + Nic ($F(10,220) = 2.01$, $p < 0.05$). The microinfusion sites were dispersed mainly in the more ventral VTA, including the anterior and posterior regions (Fig. 5C). There was no consistent

relationship between the microinfusion site and the individual DA responses to ethanol in either group. As a negative control, microinfusion of RU486 outside and adjacent to the VTA did not reverse the inhibitory effect of nicotine pretreatment (n =3).

To determine whether stress-related signals also contributed to increased ethanol consumption after nicotine pretreatment (as in Fig 1E), we pretreated rats with RU486 prior to nicotine administration and then monitored early acquisition of ethanol self-administration over the first 4 sessions. RU486 pretreatment prevented the increased ethanol self-administration induced by nicotine pretreatment (Fig. 5D). The mean ethanol intake for the group pretreated with RU486 and nicotine (0.74 ± 0.06 g/kg/session, n = 12) was significantly lower than the nicotine pretreatment alone (0.97 g/kg; n = 17) ($p < 0.01$) and nearly identical to the saline pretreatment control (Fig. 5D, dashed line). Thus, nicotine required the activation of stress hormone receptors to enhance subsequent ethanol self-administration.

Because local infusions of RU486 into the VTA prevented the inhibition of ethanol-induced DA release, we hypothesized that stress hormone action altered ethanol-induced GABA transmission onto DA neurons. Therefore, we pretreated rats with RU486 prior to nicotine pretreatment and measured ethanol-induced sIPSCs 15 hrs later. The nicotine-pretreatment potentiation of the sIPSC frequency by ethanol was prevented by RU486 pretreatment (Fig. 6A,B) ($p < 0.05$). The average sIPSC frequency induced by ethanol (relative to basal) was 187 ± 12% after nicotine pretreatment (Fig. 6B, red bar) and 118 ± 8% after RU486 and nicotine pretreatment (Fig. 6B, blue bar). In addition, the enhanced paired-pulse depression after the nicotine pretreatment (78.6 ± 3.4%; see Fig. 4D) was also prevented by RU486 (92.1 ± 3.0%; n = 15; not shown) ($p < 0.05$). These results and others indicate that stress hormone receptor activation in response to nicotine pretreatment altered ethanol-induced GABA network activity in the VTA.

DISCUSSION

Acute pretreatment with nicotine induced a long-lasting attenuation of ethanol-induced DA signals within the mesoaccumbens pathway. The decreased ethanol-induced DA signals were due to an increase in GABAergic inhibition of DA neurons and a consequent decrease in VTA DA neuron firing. These nicotine-induced neuroadaptations required a stress hormone signal that acted significantly within the VTA. Concomitant with these physiological changes, we also show that increases in ethanol self-administration induced by nicotine were prevented by RU486, a glucocorticoid/progesterone receptor antagonist (Cadepond et al., 1997).

In addition to other interactions with ethanol (Al-Rejaie and Dar, 2006; Collins et al., 1996; Gulick and Gould, 2008; Lopez-Moreno et al., 2008), nicotine exposure influences subsequent ethanol consumption and abuse (Barrett et al., 2006; Grant, 1998; Le et al., 2003; Morgen et al., 2008; Smith et al., 1999). Although the development of drug abuse involves the mesolimbic DA system, there is little mechanistic data indicating how nicotine influences DA responses to ethanol. Our results suggest that nicotine acts through stress hormone signaling pathways in the VTA to enhance subsequent ethanol-induced GABAergic drive onto DA neurons, thereby decreasing ethanol-induced DA signals. The inhibitory effect on DA signals was fully expressed for at least 15 hrs and waned after 40 hrs, which paralleled the time course of changes in ethanol self-administration induced by nicotine. A blunted DA system has been associated with increased impulsivity (Reuter et al., 2005) and increased susceptibility to drug and alcohol abuse (Martinez et al., 2005; Volkow et al., 1996; Zhang et al., 2012). Previous studies have shown that higher ethanol preference in mice corresponds to lower DA neuron responses to ethanol (Brodie and Appel, 2000).

Thus, increased ethanol self-administration in response to nicotine may arise from increased GABAergic inhibition of DA neurons. This hypothesis is consistent with evidence that disruption of GABAergic transmission modulates ethanol consumption (Chester and Cunningham, 2002; Nie et al., 2011; Nowak et al., 1998).

Nicotine pretreatment (15 hrs prior) did not alter the baseline parameters we examined, including basal DA concentration, basal DA neuron firing rate, basal sEPSC frequency, and basal sIPSC frequency, nor did nicotine pretreatment alter nicotine-induced DA release (Fig. 2C) or nicotine-induced sIPSCs. The effects of nicotine pretreatment, however, were revealed in the presence of ethanol (and diazepam), drugs that strongly modulate GABA transmission. Ethanol increases GABA release onto DA neurons (Melis et al., 2002; Theile et al., 2008; Wanat et al., 2009), while also enhancing $GABA_A$-receptor function in many systems (Glykys et al., 2007; Harris, 1999; Kumar et al., 2009). Because glutamatergic transmission was not altered significantly, the present results suggest that the combination of nicotine pretreatment and ethanol exposure shifted the balance between the inhibitory and excitatory input onto DA neurons in favor of inhibition. This effect likely contributed to the blunted DA response to ethanol, which we directly tested by blocking $GABA_A$-mediated inhibition with picrotoxin (Fig. 4E). Nicotine also increased the inhibitory responses to diazepam, suggesting that nicotine altered $GABA_A$ receptor signaling pathways. Adaptations in the inhibitory input onto VTA DA neurons could arise from local GABA neurons and various afferent projections, including prominent GABAergic pathways from the rostromedial tegmental nucleus (Hong et al., 2011), the ventral pallidum (Grace et al., 2007), and the nucleus accumbens (Xia et al., 2010).

We hypothesize that adaptations arising from the stress hormone response to nicotine were revealed by ethanol's potent action on GABAergic transmission. Most drugs of abuse activate the HPA axis, but previous studies have not explicitly linked stress hormone activity to a specific drug interaction. Nicotine induces the release of glucocorticoids and other stress-related hormones that mediate various long-term homeostatic processes (Armario, 2010; Joels and Baram, 2009), including regulation of GABAergic activity (Di et al., 2009; Gunn et al., 2011; Wirth, 2011). We should note that RU486 is an antagonist for both the glucocorticoid and the progesterone receptor (Cadepond et al., 1997). Progesterone and its metabolites are produced in the brain and participate in stress responses (Wirth, 2011), and thus progesterone and glucocorticoid receptors could contribute to interactions between nicotine and ethanol. Our demonstration that nicotine enhances ethanol-induced VTA GABA transmission through a stress hormone signal is consistent with evidence that GABAergic neuroactive hormones contribute to ethanol self-administration (Biggio et al., 2007; Helms et al., 2012; Morrow et al., 2009). These results complement previous studies showing a critical role for glucocorticoids in alcohol reward and in the transition to compulsive alcohol drinking (Rotter et al., 2012; Vendruscolo et al., 2012).

We found no evidence of long-term changes in nAChR function after one nicotine exposure. However, activation of nAChRs in the brain stem may contribute to the initial response of the HPA axis to nicotine (Armario, 2010), which our results suggest involves high-affinity β2* nAChRs (Fig 2A). Blocking the initial stress hormone response locally in the VTA prevented the long-term alterations in ethanol-induced DA release (Fig. 5B), and thus identified the VTA as a locus for mechanistic interactions between nicotine and ethanol. Interestingly, local VTA infusion of RU486 to antagonize stress receptors did not completely reverse the effects of nicotine pretreatment on ethanol-induced DA release compared to the saline control. This incomplete effect could arise from a partial diffusion of RU486 in the VTA, but it is also feasible that nicotine pretreatment acted outside of the VTA to induce neuroadaptations that regulate DA signals.

In summary, we provide evidence that nicotine pretreatment decreases ethanol-induced DA transmission owing to increased GABAergic inhibition onto DA neurons. These responses to nicotine pretreatment, including increased ethanol intake, required an initial stress hormone signal. These results support the hypothesis that the actions of drugs of abuse recruit neuroendocrine pathways (Kenna et al., 2012; Koob, 2008; Richards et al., 2011). Our data suggest a neurophysiological basis for the observation that nicotine use can increase the reinforcing properties of alcohol

EXPERIMENTAL PROCEDURES

Subjects

Long-Evans rats (Harlan Sprague Inc., Indianapolis IN, USA) weighing between 300–500 g were used. The rats were handled and weighed for at least 3 days and commonly more that a week prior to surgery and testing, and the rats were housed in a humidity and temperature-controlled (22°C) environment under a 12-hr light/dark cycle. The rats had food and water available *ad libitum* in the home cage. All procedures complied with guidelines specified by the Institutional Animal Care and Use Committee at Baylor College of Medicine.

Surgical procedures

For the microdialysis experiments, each animal was implanted with an intravenous catheter through the jugular vein and a stainless steel guide cannula (21 gauge) (Plastics One, Inc., Roanoke, VA, USA). The surgery occurred under isoflurane anesthesia (1.5–2.5% in 100% O_2, 1 L/min). The catheters were constructed with Silastic tubing (0.30 mm ID, 0.64 mm OD; Dow Corning, Midland, MI, USA) with one end modified with a 22-gauge cannula (Plastics One, Inc.). The microdialysis guide cannulae were positioned as follows (in mm relative to bregma): +2.1 anterior-posterior, +1.1 medial-lateral, −4.0 ventral to the skull surface (Paxinos and Watson, 2007). The experiments were conducted after a minimum recovery period of 3 days.

Drugs and experimental design

All drugs (Sigma-Aldrich Inc., St. Louis, MO, USA) were dissolved in sterile saline, except Mifepristone (RU486), which was dissolved in dimethyl sulfoxide (DMSO). Pretreatment with nicotine tartrate (0.4 mg/kg, freebase, i.p.), or an equivalent volume of saline, occurred 3–40 hrs prior to the experiments. Dihydro-β-erythroidine (DHβE, 2.5 or 5.0 mg/kg) or methyllycaconitine (MLA, 5.0 mg/kg) were administered (i.p.) simultaneously with nicotine. RU486 was administered 15 min prior to nicotine pretreatment at a dose of 40 mg/kg (Saal et al., 2003). We opted for this dose because of the limited capacity of RU486 to cross the blood brain barrier (Heikinheimo and Kekkonen, 1993). The intra-VTA concentration of RU486 was (10 ng/0.5 μl) and 0.5 μl of the solution was delivered by pump over 1 min (Segev et al., 2012). The microinfusion injector was left in place for 2 additional min and then removed. The infusion cannula was aimed at the following VTA coordinates (in mm relative to bregma): +5.7 anterior-posterior, +1.0 medial-lateral, −7.1 ventral to the skull surface (Paxinos and Watson, 2007). Following the experiments, Chicago Sky blue was injected into the VTA to determine the location of the microinfusion.

Baseline samples were collected (15–30 min), followed by a timed intravenous (i.v.) drug infusion (i.e., ethanol or nicotine). The i.v. administration route circumvents handling-related stress associated with a needle injection (Dong et al., 2010). For the i.v. ethanol experiments, the rats received 1.5 g/kg ethanol (20% in sterile saline, v/v, i.v.) over 5 min. Two hrs prior to the experiment, rats were administered a similar volume of vehicle (sterile saline) to habituate them to the stimulus effects of the infusion. For the i.v. nicotine

experiments (Fig. 2C), the rats were infused with saline or nicotine (0.07 mg/kg) over 5 min (Palmatier et al., 2008).

In vivo microdialysis

The active dialysis membrane (2.0 mm), was made of hollow cellulose fiber (inner diameter = 200 μm; molecular weight cutoff = 18,000; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The inlet and outlet to the membrane was composed of fused-silica tubing (inner diameter = 40 μm; Polymicro Technologies, Phoenix, AZ, USA). The microdialysis probes were perfused with artificial cerebral spinal fluid (ACSF): 149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.25 mM ascorbic acid, 5.4 mM D-glucose. At least 14 hrs before the experiment, we lowered the probes into the brain through the guide cannula. The perfusion flow rate was set to 2.0 μl/min. Each sample vial was manually changed and immediately stored at −80° C until analyzed.

Dopamine analysis

The HPLC system included a pump (Model 582; Thermo Scientific, West Palm Beach, FL, USA), an autosampler (Model 542; Thermo Scientific), and a HR-3.2 × 80 mm column (3-μm particle size; Thermo Scientific). A coulometric cell (5014B; Thermo Scientific) was connected to a Coulochem II detector. The mobile phase comprised of citric acid (4.0 mM), sodium dodecyl sulfate (3.3 mM), sodium dihydrogen phosphate dehydrate (100.0 mM), and ethylenediaminetetraacetic acid (0.3 mM), acetonitrile (15%), and methanol (5%). The autosampler mixed 9.5 μl of the dialysate with ascorbate oxidase (EC 1.10.3.3; 162 units/mg; Sigma-Aldrich Inc.) prior to injection. DA signals were acquired with 501 chromatography software and Chromeleon Software (Thermo Scientific). Quantification of dialysate DA concentration was carried out by comparing the peak area to external standards (0–2.5 nM).

Histology

The rats were overdosed with pentobarbital (120 mg/kg, i.v.). Saline was perfused through the heart, followed by 10% formalin (v/v). The brains were removed and immersed in 10% formalin for at least 2 days. The brains were cut into 75-μm coronal sections (Leica Microsystems Inc., Buffalo Grove, IL, USA) and stained with cresyl violet as indicated by the figures defining anatomical placements.

Midbrain slices and electrophysiology

Horizontal slices (220 μm) containing the VTA were cut from Long-Evans rats (21–30 days old) and placed in ice-cold, oxygenated artificial cerebral cerebrospinal fluid (ACSF in mM): 205 sucrose, 2.5 KCl, 21.4 $NaHCO_3$, 1.2 NaH_2PO_4, 0.5 $CaCl_2$, 7.5 $MgSO_4$, 11.1 dextrose, and 95% O_2/5% CO_2. The slices were maintained at 32°C in ACSF buffer for 20–40 min, then at room temperature for 40–60 min, and transferred to a holding chamber and perfused (~2 ml/min at 32°C) with the following (in mM): 120.0 NaCl, 3.3 KCl, 25.0 $NaHCO_3$, 1.2 NaH_2PO_4, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 10.0 dextrose, 20.0 sucrose. Patch electrodes made of thin-walled borosilicate glass had resistances of 1.5–2.5 MΩ when filled with the internal solution (in mM): 135.0 KCl, 12.0 NaCl, 2.0 Mg-ATP, 0.5 EGTA, 10.0 HEPES, and 0.3 Tris-GTP, pH 7.2–7.3. The firing rates of VTA DA neurons were recorded in a cell-attached configuration in passive voltage-follower mode.

For the whole-cell recordings, the cutting and recording solutions were similar to those used for the cell-attached recordings, with the exception of 20.0 mM sucrose and the addition of 120.0 NaCl in the ACSF. IPSCs and EPSCs were recorded in voltage clamp mode while holding the cells at −60 mV. While recording IPSCs glutamatergic synaptic transmission

was inhibited by 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM) and DL-2-amino-5-phosphonopentanoic acid (AP5, 50 μM) (Tocris Bioscience, Ellisville, MO, USA). Ethanol-induced sIPSCs were blocked by the $GABA_A$-receptor antagonist, picrotoxin (50 μM; Sigma-Aldrich Inc.). For the paired-pulse evoked IPSC recordings, a bipolar tungsten stimulating electrode was placed 50–100 μm rostral to the recording electrode. Pairs of constant-current pulses (100-μs duration, 20–200 μA amplitude) were applied every 10 s at an interstimulus interval of 70 ms. To better isolate sEPSCs, the composition of the internal solution was adjusted as follows (in mM): 136.0 K-gluconate, 1.0 NaCl, 10.0 HEPES, 0.5 EGTA, 2.0 Mg-ATP and 0.3 Tris-GTP, pH 7.2–7.3.

DA neurons were identified in the lateral VTA by their morphology, low firing frequency (1–5 Hz), and the presence of a large I_h current, which together correlate (>95%) with tyrosine hydroxylase (TH)-positive cells (Chen et al., 2008; Zhang et al., 2010). Recordings were made using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), filtered at 10 kHz, digitized at 20 kHz using pClamp 9.2 (Digidata Interface, Molecular Devices), and analyzed off-line using Clampfit 9.2.

Immunohistochemistry

To validate the identification of DA neurons, neurobiotin backfills and TH double labeling were used. The recording pipette contained 0.3% neurobiotin (Vector Laboratories, Burlingame, CA, USA). Slices were fixed with 10% neutral formalin phosphate buffer for 12–24 hrs, incubated in a blocking solution containing 3% normal goat serum solution and 0.3% triton X-100 for 2 hrs, and then incubated overnight with primary anti-TH (1:100; Chemicon, Billerica, MA, USA) at 4 °C. The slices were then rinsed with PBS and treated with the secondary antibody Cy3-conjugated donkey antirabbit IgG (1:200) and AMCA-conjugated streptavidin (1:1000; Jackson ImmunoResearch, West Grove, PA, USA).

Operant ethanol self-administration

Standard operant chambers (Med Associates Inc., St. Albans, VT, USA) were used for the self-administration experiments. Activation of an interior chamber light and presentation of a retractable lever accompanied the start of each session. Depression of the lever triggered the entry of a retractable drinking spout on the opposite side of the wall. Each lever press resulted in 15 sec of access to the drinking spout (a fixed ratio-1 reinforcement schedule). Each session lasted 45 min. The rats lived in the same quiet room in which daily training sessions occurred, and the rats were typically trained one at a time to avoid any auditory distractions from activity in neighboring chambers.

The rats were trained to lever press for saccharin reinforcement (0.125%, w/v). Consistent responses for saccharin occurred in ~4–8 days. Three hrs prior to their first ethanol exposure, the animals were injected with nicotine (0.4 mg/kg) or saline. The rats were exposed to ethanol by gradually adding ethanol (2–4 %, v/v) into their saccharin solution over a 4-day period (Doyon et al., 2005; Roberts et al., 1999). Consumption was monitored by measuring the volume of liquid in the drinking bottle before and after the session. Body weights were measured each day.

Statistical analysis

Analysis of variance (ANOVA) with repeated measures (in SPSS for Windows) was used to analyze the dialysate DA concentrations, the DA neuron firing rates, and the daily ethanol intake. For analysis of action potential firing, the raw data (in Hz) were converted into a percentage of basal, and the last three bins (2-min each) were used as the baseline. A two-tail *t*-test assuming equal variance was used to assess differences between mean sIPSC and

sEPSC frequency, as well as mean behavioral responses. Significance for all analyses was determined by $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Nicotine pre-exposure attenuates alcohol-induced dopamine signals
- Nicotine pre-exposure enhances alcohol-induced inhibition of dopamine neurons
- Nicotine pre-exposure enhances alcohol self-administration
- Interactions between nicotine and alcohol require activation of stress hormones

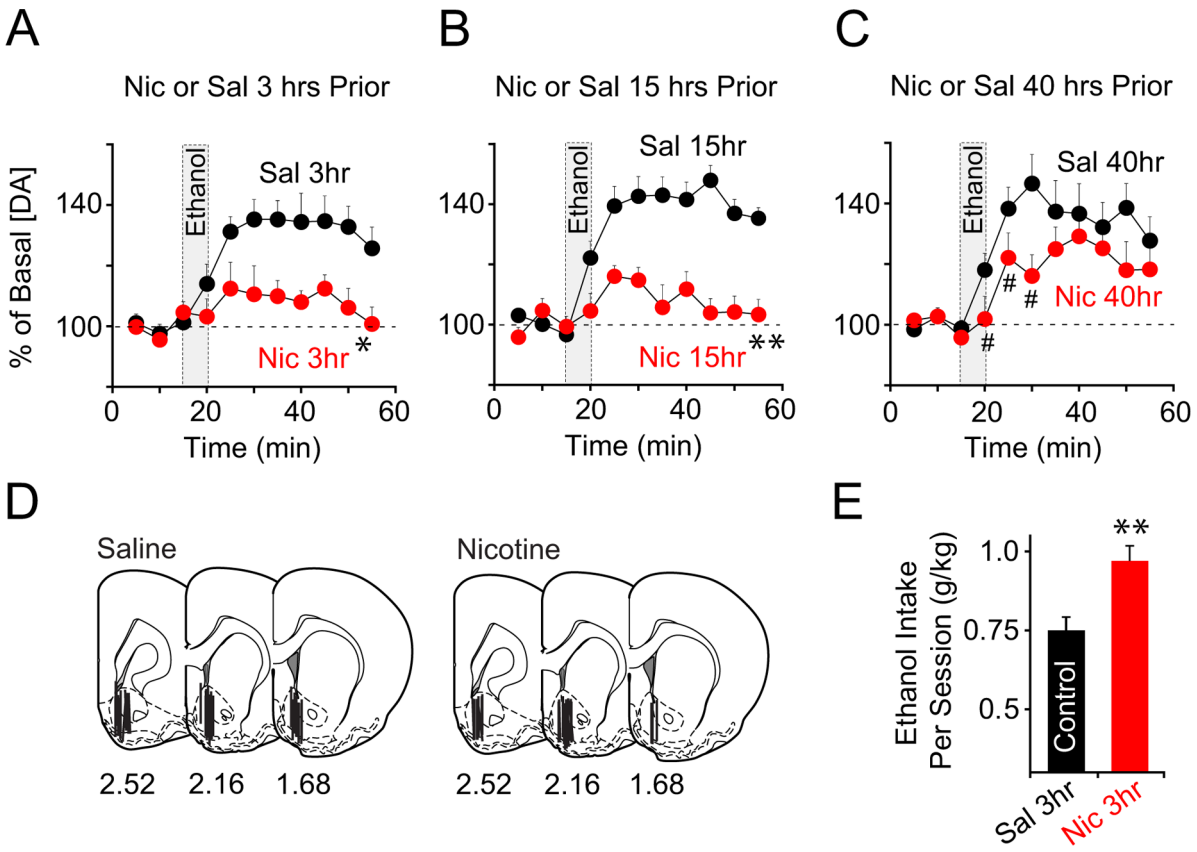

Figure 1.

Nicotine pretreatment attenuates ethanol-induced DA release and increases ethanol self-administration. Rats were injected once with nicotine (0.4 mg/kg) or saline, which occurred either (A) 3 hrs prior to in vivo ethanol administration, (B) 15 hrs prior to in vivo ethanol administration, or (C) 40 hrs prior to in vivo ethanol administration. Changes in [DA] were measured in 5-min intervals using microdialysis with HPLC. Ethanol (1.5 g/kg) was infused i.v. over a 5-min period (shaded vertical bars). * Significantly different from the control by ANOVA with repeated measures ($p < 0.05$); ** $p < 0.01$; n = 6–16 rats/group. # Significantly different from the control by *posthoc* ANOVA with repeated measures. (D) Microdialysis probe placements in the NAc for all rats pretreated with saline (left) or nicotine (right) (Paxinos and Watson, 2007). (E) Nicotine increases acquisition of ethanol self-administration. Rats were pretreated once with either saline (black bar) or nicotine (0.4 mg/kg, i.p.; red bar) 3 hrs prior to an initial ethanol exposure. The mean ethanol intake was then measured over the first four self-administration sessions (45 min/session). ** Significantly different from the control by t-test ($p < 0.01$); n = 20, 17. Data presented as the mean ± SEM.

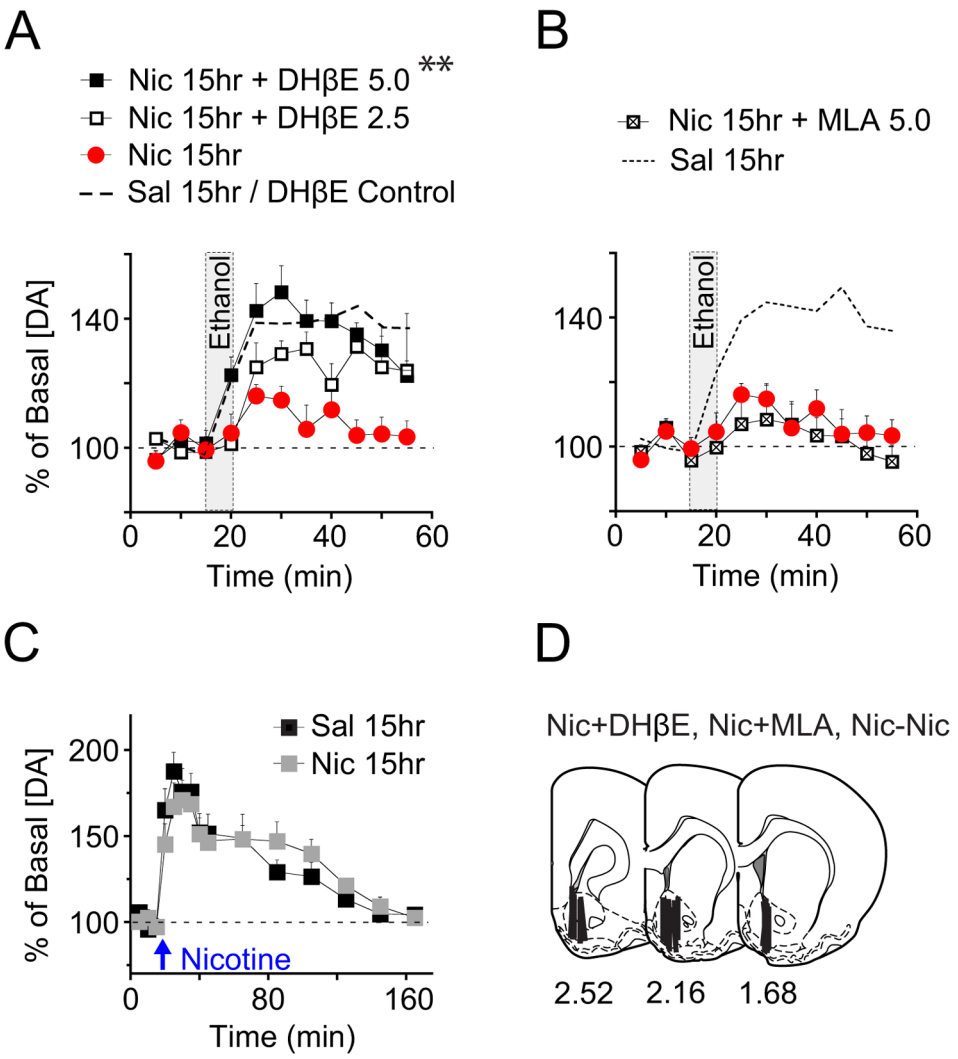

Figure 2.

Nicotine initially activates β2-nAChRs to alter ethanol-induced DA release. (A) Pretreatment with DHβE (a β2-nAChR antagonist; 2.5–5.0 mg/kg), at the same time as nicotine (Nic 15hr), prevented the attenuation of ethanol-induced DA release 15 hrs later. The control response to ethanol (Sal 15hr) and the control response to DHβE pretreatment (no nicotine; n =4) were combined and shown for comparison (dashed traces). (B) Pretreatment with MLA (an α7-nAChR antagonist; 5.0 mg/kg), at the same time as nicotine, was not different from the inhibitory effect of nicotine alone. ** Significantly different from the Nic 15hr by ANOVA with repeated measures ($p < 0.01$). (C) Alterations in [DA] in response to nicotine (0.07 mg/kg, i.v.) were not different between groups following saline or nicotine pretreatment, suggesting normal nAChR function after nicotine pretreatment. n = 5–20 rats/group. (D) Microdialysis probe placements in the NAc for all three data sets shown above. Data presented as the mean ± SEM.

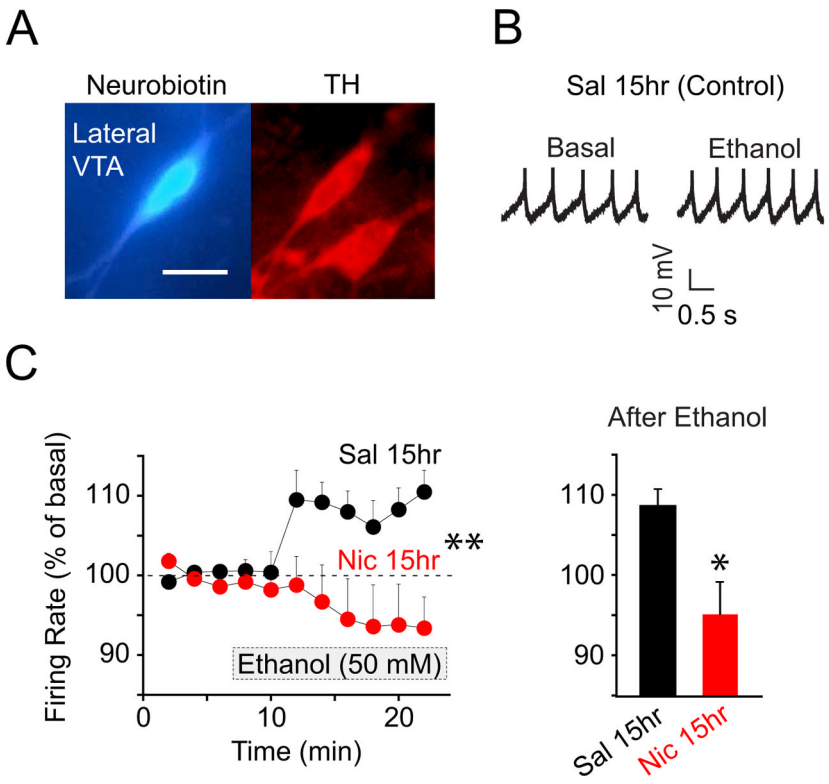

Figure 3.

Nicotine pretreatment attenuates DA neuron firing rates in VTA brain slices. (A) The DA phenotype was confirmed in a separate group of VTA neurons. Neurobiotin-labeled neurons selected from the lateral VTA possessed low firing rates (< 5 Hz), large Ih currents (>100 pA), and were immunoreactive for tyrosine hydroxylase (red stain). Scale bar = 20 μm. (B) A cell-attached patch electrode recording of a representative DA neuron before (basal) and after bath ethanol application (50 mM). Rats were pretreated 15 hrs before with saline (Sal 15hr) or nicotine (Nic 15hr). (C) Percent of basal firing rates in response to ethanol application in the saline control and the nicotine-pretreated group. n = 11 cells/group. ** Significantly different from the control by ANOVA with repeated measures ($p < 0.01$); * $p < 0.05$ by t-test. Data presented as the mean ± SEM.

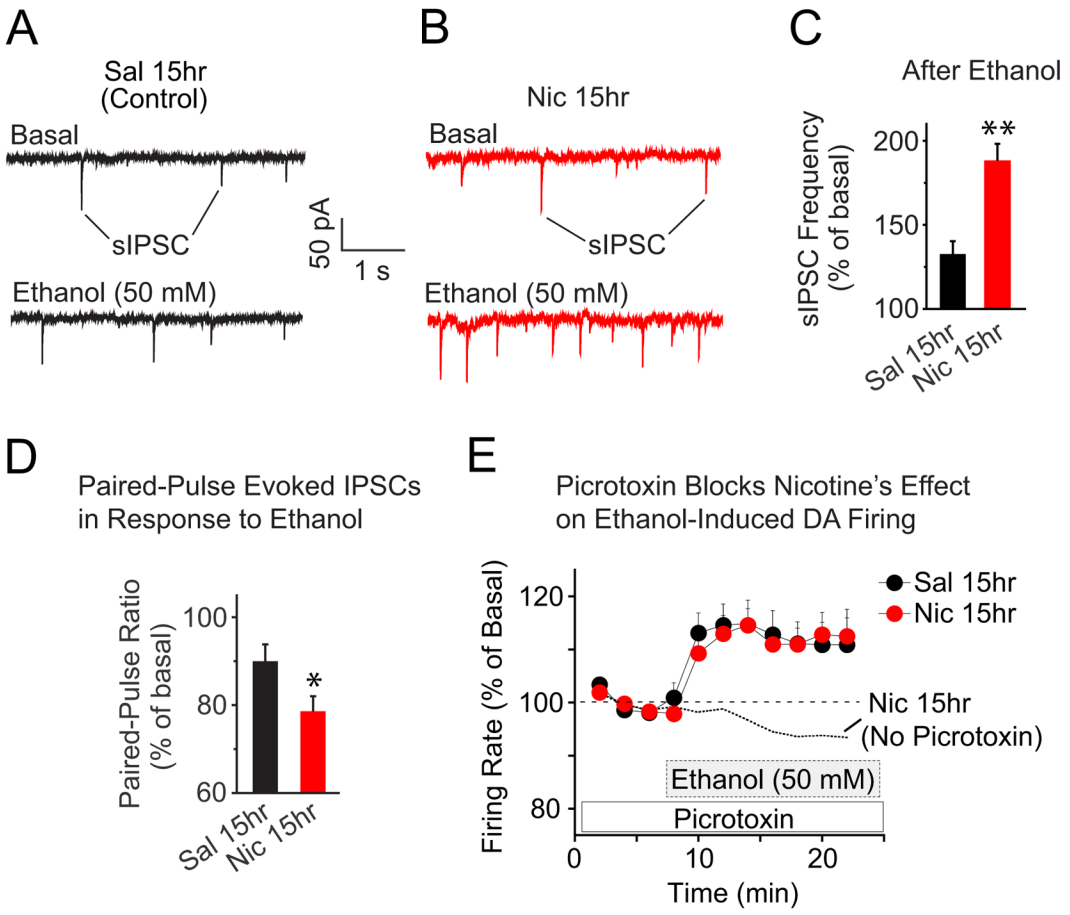

Figure 4.

Nicotine increases ethanol-induced GABAergic transmission onto VTA DA neurons. (A) Cells from rats pretreated with saline (15 hrs before ethanol) showed a small increase in the sIPSC frequency after bath ethanol application. (B) Nicotine pretreatment increased the sIPSC frequency induced by ethanol. (C) Mean changes in the sIPSC frequency after ethanol application following pretreatment with saline or nicotine. n = 7, 8 cells/group (D) Nicotine pretreatment decreased the paired-pulse ratio induced by ethanol. Interstimulus interval = 70 ms. n = 14, 21 cells/group. (E) In the presence of the $GABA_A$ receptor antagonist picrotoxin (50 μM), ethanol-induced DA neuron firing was not inhibited by nicotine pretreatment and was similar to the response of the saline control. The effect of nicotine pretreatment and ethanol application in the absence of picrotoxin from Fig. 3C is shown for comparison (dotted line). Recordings were in a cell-attached configuration. n = 7, 11 cells/group. ** Significantly different by t-test ($p < 0.01$); * $p < 0.05$. Data presented as the mean ± SEM.

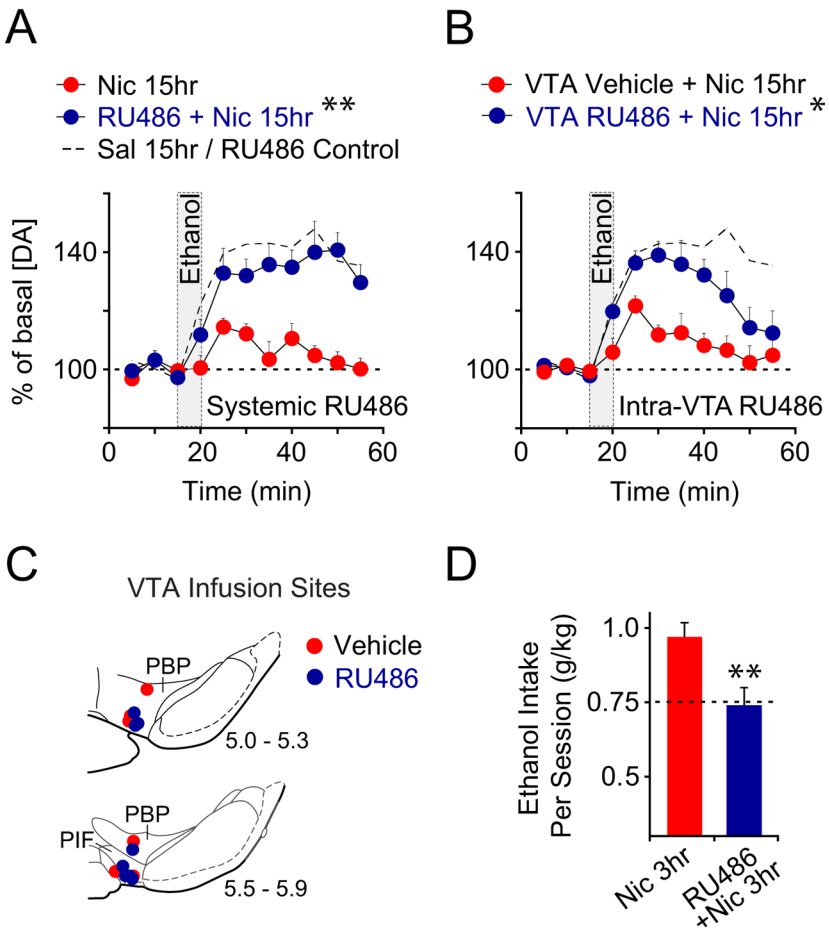

Figure 5.

Nicotine requires glucocorticoid signaling to alter ethanol-induced DA release and ethanol self-administration. (A) Rats were injected with RU486 (40 mg/kg) 15 min prior to nicotine (0.4 mg/kg) or saline pretreatment. 15 hrs later, changes in extracellular [DA] in response to ethanol (shaded vertical bar) were measured. Control subjects that received RU486 alone were combined with the saline controls from Fig. 1B as these groups were not statistically different. n = 9–16 rats/group. (B) The effect of microinfusion of RU486 or vehicle into the VTA prior to nicotine pretreatment on the DA response to ethanol 15 hrs later. ** Significantly different from the nicotine pretreatment by ANOVA with repeated measures ($p < 0.01$), * $p < 0.05$, n = 8/group. (C) VTA microinfusion sites for all rats pretreated with RU486 (blue circle) or vehicle (red circle. PIF: parainterfascicular nucleus, PBP: parabrachial pigmented nucleus. (D) Systemic RU486 blocked the increase in ethanol self-administration induced by nicotine pretreatment, as shown in Fig. 1E. Ethanol intake in the saline pretreatment control is indicated by the dashed line. ** Significantly different from the nicotine pretreatment by t-test ($p < 0.01$), n = 20, 12. Data presented as the mean ± SEM.

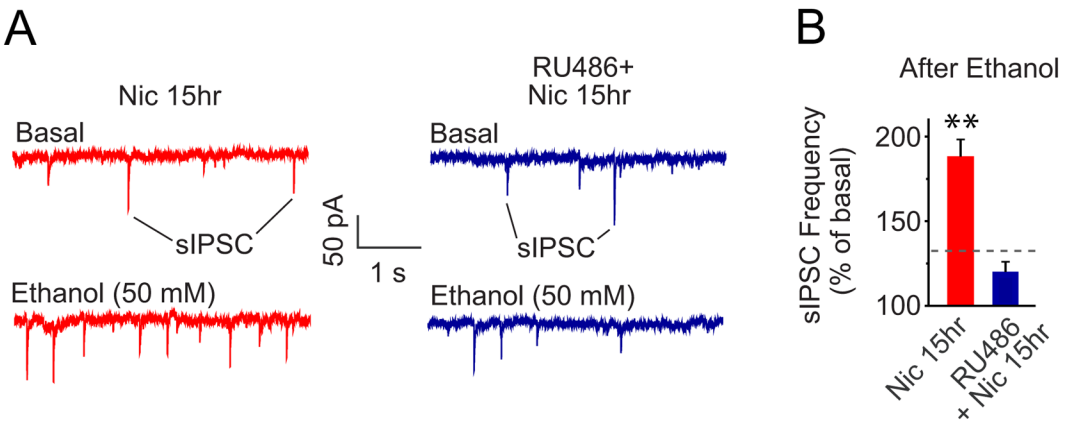

Figure 6.

Nicotine requires glucocorticoid signaling to increase ethanol-induced GABA transmission onto VTA DA neurons. (A) Compared to cells from rats pretreated with nicotine alone (15 hrs before ethanol), those pretreated with the glucocorticoid antagonist RU486 prior to nicotine did not show a potentiation of the sIPSC frequency after bath ethanol application. (B) Mean changes in the sIPSC frequency after ethanol application following pretreatment with nicotine or RU486 plus nicotine. The dashed line indicates the saline control response from Fig. 4C. n = 7, 8 cells/group. ** Significantly different by t-test ($p < 0.01$). Data presented as the mean ± SEM.