



Published in final edited form as:

Cell Rep. 2018 April 03; 23(1): 68–77. doi:10.1016/j.celrep.2018.03.030.

Adolescent Nicotine Exposure Alters GABA_A Receptor Signaling in the Ventral Tegmental Area and Increases Adult Ethanol Self-Administration

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SUMMARY

Adolescent smoking is associated with pathological drinking later in life, but the biological basis for this vulnerability is unknown. To examine how adolescent nicotine exposure influences subsequent ethanol intake, nicotine was administered during adolescence or adulthood, and responses to alcohol were measured 1 month later. We found that adolescent, but not adult, nicotine exposure altered GABA signaling within the ventral tegmental area (VTA) and led to a long-lasting enhancement of alcohol self-administration. We detected depolarizing shifts in GABA_A reversal potentials arising from impaired chloride extrusion in VTA GABA neurons. Alterations in GABA signaling were dependent on glucocorticoid receptor activation and were associated with attenuated dopaminergic neuron responses to alcohol in the lateral VTA. Importantly, enhancing chloride extrusion in adolescent nicotine-treated animals restored VTA GABA signaling and alcohol self-administration to control levels. Taken together, this work suggests that adolescent nicotine exposure increases the risk profile for increased alcohol drinking in adulthood.

In Brief

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SUPPLEMENTAL INFORMATION

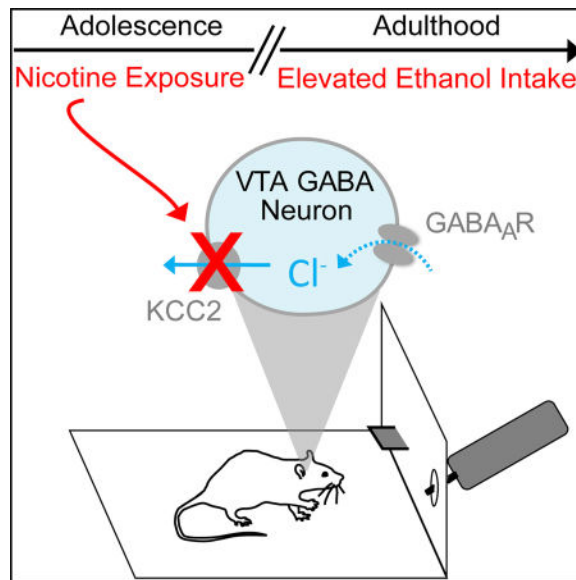
Supplemental Information includes four figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.03.030>.

AUTHOR CONTRIBUTIONS

A.M.T. designed and performed *in vivo* experiments, assisted by K.K. and T.B.-M. A.O. designed and performed *ex vivo* experiments, assisted by B.A.K., M.B.T., and W.M.H. J.A.D. originated, planned, and oversaw the experiments. Led by J.A.D., A.M.T. and A.O. wrote the manuscript, assisted by all the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.



Thomas et al. show that nicotine treatments during adolescence, but not adulthood, cause a long-term increase in alcohol self-administration in adult rodents. Adolescent nicotine exposure shifts GABA_A receptor signaling within the ventral tegmental area circuitry, thereby altering subsequent responses to alcohol.

INTRODUCTION

Nearly 90% of smokers start smoking by the age of 18 (U.S. Department of Health and Human Services, 2017), and tobacco use is strongly predictive of pathological alcohol consumption (Grant, 1998; Harrison and McKee, 2011). This association is thought to originate in adolescence, with early tobacco exposure acting as a gateway for subsequent alcohol use and abuse (Cross et al., 2017; Torabi et al., 1993). Adolescence is a neurodevelopmental window marked by major reorganization of limbic brain regions important for reward processing (Casey et al., 2008). Nicotine exposure may, therefore, alter brain development to promote pathological drug use later in life. Evidence from rodent models shows that exposure to nicotine during adolescence, but not post-adolescence, is associated with enhanced drug reinforcement in adulthood (Adriani et al., 2003; Lárraga et al., 2017). Although long-term alterations in brain function have been reported following adolescent nicotine exposure (Counotte et al., 2009; Doura et al., 2010), the specific neuroadaptations that give rise to excessive alcohol consumption in adulthood remain unknown.

Our previous work suggested that nicotine-ethanol interactions could arise via altered GABA_A receptor (GABA_AR) signaling within the ventral tegmental area (VTA) (Doyon et al., 2013). GABA_ARs are positively modulated by ethanol and are implicated in ethanol reinforcement (Boyle et al., 1993; Rassnick et al., 1993). The strength and polarity of GABA_AR signaling are predominantly dictated by cellular chloride (Cl⁻) gradients and maintained by the K⁺, Cl⁻ transporter, KCC2 (Doyon et al., 2016). Impaired KCC2 function and intracellular Cl⁻ accumulation leads to depolarizing shifts in the Cl⁻ reversal potential

(E_{GABA}) and compromised GABA_AR-mediated inhibition (Hewitt et al., 2009; Ostroumov et al., 2016). Within VTA GABA neurons, functional KCC2 downregulation and the consequent decreased GABA_AR-mediated inhibition are thought to promote alcohol self-administration following glucocorticoid receptor activation (Ostroumov et al., 2016).

Based on that previous work, we hypothesized that adolescent nicotine exposure promoted ethanol consumption in adulthood via persistent adaptations in Cl⁻ homeostasis. We found that rats treated with nicotine as adolescents, but not those treated as adults, showed elevated ethanol self-administration and excitatory shifts in GABA_AR signaling a month later that were dependent on glucocorticoid receptor activation. Adolescent nicotine exposure impaired Cl⁻ extrusion via KCC2 downregulation and altered mesolimbic responses to ethanol. Enhancing Cl⁻ extrusion in adolescent nicotine-treated rats returned GABAergic inhibitory signaling to normal and returned alcohol consumption to control levels. These results indicate that adolescent nicotine exposure produces long-lasting alterations in Cl⁻ homeostasis and GABA_AR signaling and contributes to elevated ethanol self-administration in adulthood.

RESULTS

Adolescent Nicotine Increases Adult Ethanol Self-Administration

Adolescent rats were administered daily injections of saline or nicotine (0.4 mg/kg, intraperitoneal [i.p.] injections) on post-natal day (p)28–p42, and ethanol self-administration was measured approximately 1 month later (p70–p100; 1-hr session per day) (Figure 1A). Operant responses to saccharin were first established, followed by the introduction of 2%–4% ethanol into the drinking solution (Ostroumov et al., 2016). Saccharin consumption was measured 3 days immediately prior to ethanol exposure and the average intake was not statistically different between adolescent nicotine-treated animals (10 ± 1 mL) and adolescent saline controls (8 ± 1 mL) (Figure S1B): $p > 0.05$, $n = 8$, 10 rats per group. Upon adding ethanol to the drinking solution, adolescent nicotine-treated animals showed significantly increased self-administration compared to adolescent saline controls (Figure 1B): for group, $F(1, 16) = 14.48$, $p < 0.01$. Analysis of total fluid consumption also revealed greater intake among adolescent nicotine-treated rats compared to controls (Figure S1C): for group, $F(1, 16) = 14.69$, $p < 0.01$. Mean ethanol intake across the first 7 days was 1.03 ± 0.05 g/kg for the nicotine-pretreated group and 0.75 ± 0.05 g/kg for the saline-pretreated group (Figure 1C): $p < 0.01$, $n = 8$, 10 rats per group.

Next, we examined self-administration of higher ethanol concentrations. The average intake of 8% ethanol was significantly increased in the adolescent nicotine group compared to adolescent saline controls (Figure 1D): for nicotine, 1.83 ± 0.11 g/kg; for saline, 1.30 ± 0.16 g/kg; $p < 0.05$; $n = 9$, 5 rats per group. To confirm the specificity of this effect to ethanol, saccharin was removed, and the intake of 8% ethanol was measured. Elevated drinking was observed in adolescent nicotine-treated animals compared to saline-treated controls after the removal of saccharin (Figure 1E): for nicotine, 0.88 ± 0.05 g/kg; for saline, 0.60 ± 0.05 g/kg; $p < 0.01$, $n = 11$, 9 rats per group. The effects of adolescent nicotine exposure persisted throughout the duration of self-administration experiments (25 days), revealing long-lasting

alterations in the acquisition and maintenance of ethanol self-administration in adult animals.

Adult Nicotine Exposure Does Not Increase Adult Ethanol Self-Administration

To determine the effect of post-adolescent nicotine exposure on subsequent ethanol consumption, young adult animals were administered comparable nicotine or saline injections over a 2-week period (p60–p74). Self-administration was assessed in these animals approximately 1 month later (Figure 2A). Adult nicotine pretreatment failed to increase daily ethanol self-administration compared to adult saline controls (Figure 2B): for group, $F(1, 9) = 0.01$, $p > 0.05$. The average intake in the adult nicotine group (0.76 ± 0.08 g/kg; Figure 2C, gray bar) was not statistically different from that in adult saline controls (0.68 ± 0.06 g/kg; Figure 2C, black bar) or adolescent saline controls (0.71 ± 0.05 g/kg; dotted line of Figure 2C: $p > 0.05$, $n = 9, 14, 9$ rats per group). These results suggest that adolescent, but not adult, nicotine exposure produces long-lasting elevations in subsequent ethanol self-administration.

Adolescent Nicotine Alters GABA Transmission and Chloride Homeostasis in the VTA

Enhanced ethanol self-administration has been previously associated with depolarizing shifts in the $GABA_{A}$ reversal potential (E_{GABA}) of VTA GABA neurons (Ostroumov et al., 2016). To examine whether adolescent nicotine exposure altered E_{GABA} , midbrain slices were prepared from adult rats treated with nicotine or saline during adolescence (Figure 3A). Next, gramicidin perforated patch-clamp recordings were performed in VTA GABA neurons to preserve the intracellular anion concentrations (Figure 3B). We measured $GABA_{A}$ currents at different membrane potentials following electrical stimulation (Figure 3C). VTA GABA neurons from adult rats receiving adolescent nicotine showed a significantly more depolarized E_{GABA} value compared to saline-treated controls (Figures 3C and 3D): -66 ± 2 mV after nicotine (indicated in red) versus -87 ± 4 mV in controls (indicated in black), $n = 6, 7$ cells per group, $p < 0.01$. Adolescent nicotine did not alter the membrane resting potential (measured as zero holding current): -56 ± 2 mV after nicotine versus -61 ± 3 mV in controls, $n = 6, 7$ cells per group, $p > 0.05$. Analogous experiments were conducted to determine whether adolescent nicotine altered E_{GABA} in VTA dopaminergic (DA) neurons (Figures S2A and S2B). The E_{GABA} in VTA DA neurons from adult rats receiving adolescent nicotine were indistinguishable from that in saline-treated controls (Figure S2B): -76 ± 3 mV after nicotine (indicated in red) versus -77 ± 2 mV in controls (indicated in black), $n = 6$ cells per group, $p > 0.05$.

We previously showed that nicotine targets glucocorticoid receptors to alter GABAergic signaling within the VTA (Doyon et al., 2013). To examine whether glucocorticoid receptor activation is necessary for adolescent nicotine to alter E_{GABA} , adolescent animals were injected with RU486 (40 mg/kg, i.p. injections) 15 min prior to injections with saline or nicotine. Systemic injection of RU486 prior to nicotine prevented the adolescent nicotine-mediated alterations in E_{GABA} (Figure 3D, blue): -84 ± 2 mV, $n = 6$ cells, $p > 0.05$. These animals did not differ significantly from controls treated with RU486 and saline (data not shown): -83 ± 4 mV, $n = 6$ cells, $p > 0.05$.

We next examined the effect of post-adolescent nicotine exposure on E_{GABA} . Young adult animals were administered nicotine or saline injections over a 2-week period (p60–p74), and slice electrophysiology experiments were conducted approximately 1 month later. No differences in E_{GABA} were observed after adult nicotine exposure compared to adolescent or adult saline-treated controls (Figure 3D, gray): -87 ± 4 mV, $n = 6$ cells, $p > 0.05$. Therefore, long-lasting adaptations in E_{GABA} occur after adolescent, but not adult, exposure to nicotine.

A depolarizing shift in E_{GABA} reflects higher intracellular anion concentration and is often mediated by a decrease in Cl^- extrusion capacity. To test whether adolescent nicotine weakens Cl^- extrusion in VTA GABA neurons, we applied repetitive $GABA_A$ R stimulation during whole-cell recordings under a holding potential that favors chloride influx ($V_h = 0$ mV). Faster rates of inhibitory postsynaptic current (IPSC) amplitude reduction during repetitive stimulation are indicative of impaired chloride extrusion (Hewitt et al., 2009). Adolescent nicotine-treated animals showed significantly faster rates of IPSC amplitude reduction compared to adolescent saline controls (Figures 3E and 3F): $F(2;5) = 9.30$, $p < 0.05$. No differences in synaptic depression were observed between groups when the Cl^- driving force was outward ($V_h = -90$ mV; Figure S2C). These data confirm that the differential effects on short-term depression are derived from Cl^- extrusion efficacy.

Adolescent Nicotine Downregulates KCC2 Expression

Reductions in Cl^- extrusion capacity are often mediated by downregulation of the K^+ , Cl^- cotransporter, KCC2 (Sarkar et al., 2011). To examine adolescent nicotine-induced alterations in KCC2 protein expression, we performed western blot analysis using an antibody against KCC2 protein. Immunoblots revealed two prominent bands (~140 and ~270 kDa), indicating the presence of monomeric and dimeric structures of KCC2 protein (Figure 3G). A significant reduction in the expression of KCC2 was observed after adolescent nicotine pretreatment (Figures 3G and 3H): $74\% \pm 6\%$ for monomer, $76\% \pm 6\%$ for dimer, $n = 12$, $p < 0.01$. As reported previously (Taylor et al., 2016), immunolabeling analysis suggested that KCC2 protein was expressed on non-DA, GABAergic neurons within the VTA (Figure S2D), which is consistent with the presence of another chloride extrusion mechanism in DA neurons (Gulácsi et al., 2003). These data indicate that adolescent nicotine exposure induces long-lasting alterations in KCC2 expression, chloride homeostasis, and $GABA_A$ R signaling within VTA GABA neurons.

Adolescent Nicotine Increases Ethanol-Induced Inhibition of Dopamine Neurons *Ex Vivo*

VTA GABA transmission influences mesolimbic DA neuron activity and is modulated upon exposure to ethanol (Tan et al., 2012; Theile et al., 2008). Depolarizing shifts in E_{GABA} within the VTA GABA neurons were previously associated with greater ethanol-induced inhibition of lateral VTA DA neurons (Ostroumov et al., 2016). To determine whether adolescent nicotine exposure altered ethanol-induced GABA release onto DA neurons, we performed whole-cell patch-clamp recordings of VTA DA neurons and measured spontaneous IPSCs (Figures 4A and 4B). In control rats, bath-applied ethanol (50 mM) produced a small increase in spontaneous IPSC (sIPSC) frequency (Figures 4C and 4D): $114\% \pm 4\%$ of basal (indicated in black), $n = 8$. In contrast, lateral VTA DA neurons from rats treated with nicotine during adolescence showed significantly greater ethanol-induced

potentiation of sIPSC frequency compared to the control response (Figures 4C and 4D): for nicotine-treated rats, $177\% \pm 6\%$ of basal, (indicated in red), $n = 13$, $p < 0.01$. No significant differences in baseline sIPSC frequency were detected between saline and nicotine groups: 4 ± 1 Hz in saline-treated rats versus 4 ± 1 Hz in nicotine-treated rats ($p > 0.5$). Importantly, neither group showed alterations in sIPSC amplitude upon exposure to ethanol (45 ± 5 pA in saline-treated versus 38 ± 6 pA in nicotine-treated rats, $p > 0.05$). An increase in the frequency, but not the amplitude, of sIPSCs suggests enhanced presynaptic GABA release onto the DA neurons.

Adolescent Nicotine Attenuates Ethanol-Induced Dopamine Activity *In Vivo*

Ethanol application typically increases the firing of VTA DA neurons, and a more depolarized E_{GABA} in VTA GABA neurons has been shown to attenuate DA responses to ethanol (Foddai et al., 2004; Ostroumov et al., 2016). We therefore hypothesized that adolescent nicotine exposure would also attenuate adult ethanol-induced DA responses from the lateral VTA *in vivo* (Figure 4E). To test this, we first conducted *in vivo* single-unit recordings of lateral VTA DA neurons in anesthetized adult rats. DA neurons were recorded in the lateral VTA and were identified based on their electrophysiological and pharmacological properties (see Experimental Procedures). The spontaneous firing rate of these VTA DA neurons was measured before and after intravenous infusion of ethanol (0.3 mg/kg), and this dose was increased every 3 min until a final concentration of 1.5 mg/kg was achieved. Basal spontaneous firing rate of these DA neurons was not altered after exposure to adolescent nicotine: 8.0 ± 0.7 Hz after nicotine and 7.4 ± 0.5 Hz in control. Ethanol administration (0.6–1.5 mg/kg) induced a significant increase in the spontaneous firing rate of lateral VTA DA neurons in saline-treated controls (Figures 4F and 4G, indicated in black): $124 \pm 5\%$ of basal, $n = 17$. In contrast, DA neurons from adolescent nicotine-treated animals did not show significant increases in DA firing (Figures 4F and 4G, indicated in red): $98 \pm 2\%$ of basal, $n = 7$, $p < 0.01$. The VTA recording sites from adolescent saline-treated (black) and adolescent nicotine-treated (red) rats were similar across experiments (Figure 4H).

The *in vivo* electrophysiology results suggest that adolescent nicotine pretreatment attenuates DA responses to ethanol in lateral VTA. To determine whether adolescent nicotine treatment influences ethanol-induced DA signaling in freely moving rats, we conducted *in vivo* microdialysis and measured ethanol-induced DA release in the more medial nucleus accumbens near the border of the medial shell and core (Figure 4J). A sustained increase in DA was observed in saline-treated controls upon ethanol administration (Figure 4I, black trace). Adolescent nicotine-treated rats, in comparison, showed blunted DA responses to ethanol in this medial shell region (Figure 4I, red trace): for Group \times Time, $F(9, 117) = 2.90$, $n = 8$, 7 rats per group, $p < 0.01$. No significant differences in baseline DA levels were detected between saline and nicotine groups: 1.02 ± 0.21 nM in saline-treated rats versus 1.06 ± 0.13 nM in nicotine-treated rats. The placements of the microdialysis probes in the nucleus accumbens did not differ between saline (indicated in black) and nicotine (indicated in red) treatment groups (Figure 4J; also see an example placement in Figure S3A). Taken together, these data indicate that adolescent nicotine exposure produced long-lasting, circuit-wide adaptations in mesolimbic responses to ethanol.

Restoring KCC2 Function Normalizes GABA Signaling and Alcohol Self-Administration

Intracellular Cl^- accumulation can be reversed by enhancing Cl^- extrusion with the KCC2 agonist CLP290 (CLP) (Gagnon et al., 2013; Ostroumov et al., 2016). To determine whether CLP290 restored Cl^- extrusion in VTA GABA neurons, we applied repetitive GABA_AR stimulation to slices incubated in CLP290 (>1 hr, 10 μM) under a holding potential (V_h) of 0 mV to induce chloride influx (Figures 5A and 5B). Following CLP290 incubation, the rates of IPSC amplitude reduction were indistinguishable between adolescent nicotine-treated animals (indicated in red) and adolescent saline-treated controls (indicated in black; Figure 5C, $p > 0.05$).

Because CLP incubation prevented intracellular chloride accumulation, we hypothesized that CLP incubation would also prevent enhanced GABA release onto DA neurons in adolescent nicotine-treated animals (Figure 5D). Indeed, the nicotine-mediated potentiation of the sIPSC frequency by ethanol was prevented by CLP290 incubation (Figure 5E) ($p > 0.05$). The average sIPSC frequency induced by ethanol (relative to basal) was $123\% \pm 4\%$ in adolescent nicotine-treated animals (Figure 5E, red bar with CLP treatment) and $123\% \pm 9\%$ in saline-treated controls (Figure 5E, black bar). These results indicate that the effects of adolescent nicotine pretreatment on altered ethanol-induced GABA network activity in the VTA could be reversed by CLP290.

We next examined whether enhancing Cl^- extrusion would prevent elevated ethanol self-administration following adolescent nicotine exposure. Adolescent-treated rats received bilateral intra-VTA infusions of CLP290 (1 μL , 50 μM) or vehicle prior to ethanol self-administration on non-consecutive days (Figures 5F and 5G, blue arrows). Compared to adolescent nicotine-treated rats that received intra-VTA infusion of vehicle (Figures 5G and 5H, red), intra-VTA infusion of CLP290 significantly decreased daily ethanol consumption (Figures 5G and 5H, blue): for group, $F(1, 19) = 18.43$, $n = 9$, 13 rats per group, $p < 0.01$. Mean ethanol intake over 7 days was also significantly lower in rats (Figure 5H) that received infusions of CLP290 (0.79 ± 0.04 g/kg, blue bar), compared to animals that received infusions of vehicle (1.04 ± 0.03 g/kg, red bar); $n = 9$, 13 rats per group, $p < 0.01$. Bilateral VTA cannulation and vehicle infusions did not significantly alter ethanol consumption in adolescent saline-treated controls compared to controls without surgery (Figures 5G and 5H, dotted gray lines): 0.75 ± 0.04 g/kg, $n = 6$, $p > 0.05$. In addition, intra-VTA infusions of CLP290 did not significantly alter ethanol consumption in adolescent saline-treated animals compared to vehicle infusions (Figure S4, blue bar): 0.63 ± 0.05 g/kg, $n = 5$, $p > 0.05$. Infusion sites were similarly distributed throughout the anterior/posterior axis of the VTA (Figures S3B and S4).

DISCUSSION

Adolescent tobacco exposure is predictive of pathological drinking later in life (Jensen et al., 2003; Riala et al., 2004), but the neuronal adaptations underlying this interaction have not been well delineated. We demonstrated in rats that repeated exposure to nicotine during adolescence, but not adulthood, promotes long-lasting increases in ethanol self-administration. In VTA GABA neurons, adolescent nicotine treatment functionally downregulated KCC2, attenuated Cl^- extrusion, and caused a depolarizing shift in E_{GABA} .

These alterations were associated with enhanced GABAergic inhibition of DA neurons in the lateral VTA upon ethanol exposure and diminished DA responses to ethanol. Most importantly, enhancing chloride extrusion in adolescent nicotine-treated animals prevented alterations in VTA GABA signaling and normalized ethanol self-administration to control levels.

Adolescence is thought to be a critical period of neural development, and drug exposure during this time is known to induce persistent neurobehavioral alterations (Chambers et al., 2003; Spear, 2016). Here, we show that adolescent, but not adult, nicotine exposure increases ethanol self-administration and induces depolarizing shifts in E_{GABA} . These findings support previous work suggesting that a unique vulnerability to nicotine may exist during adolescence (Adriani et al., 2003; Counotte et al., 2009; Lárraga et al., 2017) and point to differential regulation of GABAergic transmission as a factor mediating adolescent vulnerability to substance use.

Within the VTA, GABA_ARs on GABA and DA neurons can show differential responses to drugs of abuse (Tan et al., 2012). We previously demonstrated that GABA_AR signaling onto VTA GABA neurons is positively modulated by ethanol and contributes to ethanol reinforcement (Ostroumov et al., 2016). Depolarizing shifts in GABA_AR signaling within the VTA have been reported in adult animals under conditions of stress, drug dependence, and withdrawal (Laviolette et al., 2004; Taylor et al., 2016), but our results demonstrate that these adaptations can persist into adulthood following an adolescent drug exposure. These long-lasting alterations in GABA_AR signaling may, therefore, represent a neurobiological mechanism by which adolescent nicotine exposure acts as a gateway for subsequent alcohol use and abuse (Chen et al., 2002; Cross et al., 2017; Torabi et al., 1993).

The strength of GABAergic inhibition is dynamically regulated in the adult nervous system through changes in the transmembrane chloride gradient (Doyon et al., 2016). We provide evidence that changes in E_{GABA} following adolescent nicotine treatment arise from impaired chloride extrusion and downregulation of the K^+ , Cl^- transporter KCC2. This neuron-specific transporter is differentially expressed across development and differentially regulated by environmental exposures (Williams et al., 1999). In mature neurons, KCC2 downregulation is known to occur in pathological conditions like epilepsy and chronic pain (Kaila et al., 2014). More recently, KCC2 downregulation has also been observed in VTA GABA neurons following acute stress and opiate withdrawal (Ostroumov et al., 2016; Taylor et al., 2016). Thus, KCC2 is a key molecular target underlying changes in GABAergic signaling across the CNS.

Our findings suggest the involvement of glucocorticoid receptors, which have previously been shown to reduce KCC2 activity via S940 dephosphorylation (Ostroumov et al., 2016). It is known that dephosphorylation at S940 precedes KCC2 internalization and decreased membrane expression (Lee et al., 2007), which suggests that adolescent exposure to nicotine more potently drives KCC2 downregulation compared to acute stress. Future work should examine the intermediate signaling molecules from glucocorticoid receptors to changes in KCC2 expression, because these mechanisms may differ between adolescent and adult populations.

Downstream of the KCC2 and VTA GABA neuron alterations, there was increased ethanol-induced inhibition of lateral VTA DA neurons and attenuated ethanol-induced DA responses measured in the medial nucleus accumbens from adolescent nicotine-treated animals. These electrophysiological data were collected in an anesthetized preparation and correspond to a blunted, but still present, increase in DA release to ethanol in freely moving animals. The DA release was measured by microdialysis sampling from the border between the medial shell and core of the nucleus accumbens. Because DA signaling is heterogeneous from its source in the midbrain to its targets throughout the striatum and elsewhere (Lammel et al., 2012; Yang et al., 2018), the amplitude and temporal DA signal throughout the nucleus accumbens in response to ethanol is not anticipated to be homogeneous. Furthermore, ethanol acts at multiple targets within the midbrain to elicit DA release, including glutamatergic, GABAergic, and direct actions on DA neuron cell firing (Harris, 1999). We observed elevated DA responses during ethanol administration in all animals, but intermediate responses (from the areas we recorded) were associated with greater ethanol intake. This correlation between intermediate DA responses to ethanol and enhanced ethanol self-administration is consistent across multiple studies (Brodie and Appel, 2000; Doyon et al., 2013; Ostroumov et al., 2016; Ramachandra et al., 2007) and illustrates how impaired chloride extrusion can influence broader mesolimbic responses to ethanol.

Direct modulation of KCC2 is a favored therapeutic strategy to restore GABA_A function under conditions involving impaired Cl⁻ transport (Gagnon et al., 2013). We showed that, following adolescent nicotine exposure, elevated drinking in adulthood could be prevented by intra-VTA infusion of the KCC2 agonist, CLP290. These results suggest that chloride extrusion enhancers may serve as a therapeutic strategy to mitigate excessive alcohol consumption in smoking populations. Taken together, these results reveal that adolescent nicotine exposure induces long-lasting alterations in mesolimbic responses to ethanol and promotes ethanol consumption in adulthood. Thus, adolescent tobacco use may increase the risk for excessive alcohol drinking in adulthood.

EXPERIMENTAL PROCEDURES

Animals

Male Long-Evans rats (Harlan-Envigo) were singly housed in a quiet temperature- and humidity-controlled satellite facility under a 12-hr:12-hr light:dark cycle. Rats had food and water available *ad libitum* in their home cages. All procedures were carried out in compliance with guidelines specified by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Drugs and Experimental Design

All drugs (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in sterile saline unless otherwise specified. Rats were administered daily injections of saline or nicotine tartrate (0.4 mg/kg, freebase, i.p. injections) during adolescence (p28–p42), and responses to ethanol were assessed in adulthood beginning 4 weeks later. The p28–p42 window represents a conservative time frame within adolescence (Spear, 2000). A separate cohort of animals was pretreated with RU486 (40 mg/kg, i.p. injections) 15 min prior to each adolescent nicotine

injection to examine the contribution of glucocorticoid receptor signaling. Comparable injections were carried out in post-adolescent animals (>p60 or 300 g), and responses to ethanol were assessed 4 weeks post-nicotine exposure. Intra-VTA microinfusions of CLP290 or vehicle occurred 30 min prior to ethanol self-administration over 3 non-consecutive days. Animals that received vehicle infusions were indistinguishable from non-surgerized controls and were thus combined. The intra-VTA concentration of CLP290 was 50 μ M delivered at 0.5 μ L/min to 1 μ L (Ostroumov et al., 2016) at the following VTA coordinates (in millimeters relative to bregma): +5.7 anterior-posterior, +1.0 medial-lateral, -7.1 ventral to the skull surface (Paxinos, 2007). A separate group of rats treated with 45 μ M CLP290 before the first day of self-administration were indistinguishable from the 50- μ M treatment group, and these results were thus combined.

Operant Ethanol Self-Administration

Standard operant chambers (Med Associates) were used for the self-administration experiments. Illumination of an interior chamber light and presentation of a retractable lever cued 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 s of access to the drinking spout (a fixed-ratio reinforcement schedule of 1). Animals initially had restricted access to water overnight and were trained to press a lever for a saccharin solution (0.125%, w/v). Once trained, the animals no longer had restricted access to water, and their basal saccharin intake was monitored until intake appeared stable for 3 consecutive days (one 60-min session per day). If the animals underwent surgery, saccharin intake was re-established. The effects of nicotine or saline pretreatment on 2%–4% ethanol self-administration were then measured (Ostroumov et al., 2016). We previously confirmed that this self-administration protocol produces pharmacologically relevant blood alcohol levels ranging from 40 to 160 mg/dL (Ostroumov et al., 2016). In comparison, the legal limit of alcohol intoxication in humans is 80 mg/dL. For 8% ethanol studies, initial ethanol fading (2%–8%) occurred over the first 8 days in a saccharin solution (0.15%, w/v), and intake of 8% ethanol consumption was then monitored for 7 consecutive days (Doyon et al., 2005). A separate cohort of animals followed the same 8-day ethanol-fading procedure (2%–8%), but saccharin was then gradually removed over 6 days. Intake of 8% ethanol without saccharin was subsequently monitored for 7 consecutive days.

Ex Vivo Electrophysiology

Horizontal slices (230 μ m) containing the VTA were cut (Leica Microsystems) from adult Long-Evans rats in ice-cold, oxygenated (95% O₂, 5% CO₂), high-sucrose artificial cerebrospinal fluid (ACSF) (in millimolar): 205.0 sucrose, 2.5 KCl, 21.4 NaHCO₃, 1.2 NaH₂PO₄, 0.5 CaCl₂, 7.5 MgCl₂, and 11.1 dextrose. Immediately after cutting, slices were transferred to normal ACSF buffer (in millimolar): 120.0 NaCl, 3.3 KCl, 25.0 NaHCO₃, 1.2 NaH₂PO₄, 2.0 CaCl₂, 1.0 MgCl₂, 10.0 dextrose, and 20.0 sucrose. The slices were constantly oxygenated (95% O₂, 5% CO₂) and maintained at 32°C in ACSF for 40 min, then at room temperature for at least 60 min. For incubation experiments, slices were bathed in CLP290 (10 μ M) for an additional hour. To perform electrophysiological recordings, slices were transferred to a holding chamber and perfused with normal ACSF at a constant rate of 2–3 mL/min at 32°C. Patch electrodes made of thin-walled borosilicate glass (1.12 mm inner

diameter [ID], 1.5 mm outer diameter [OD]; World Precision Instruments [WPI]) had resistances of 1.0–2.0 M Ω when filled with the internal solution (in millimolar): 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).

For E_{GABA} perforated-patch recordings, gramicidin was first dissolved in methanol to a concentration of 10 mg/mL and then diluted in a pipette solution to a final concentration of 150 μ g/mL. For synaptic stimulation recordings, a bipolar tungsten-stimulating electrode (World Precision Instruments) was placed 100–150 μ m away from the recording electrode. To determine E_{GABA}, evoked IPSCs were measured under voltage clamp at different holding potentials. Amplitudes of IPSCs were plotted against voltage to estimate the reversal potential. After each perforated-patch experiment, recordings were converted to the whole-cell configuration, and I_h current was measured. When necessary, recordings were performed in the presence of DNQX, AP5, CGP55845, and tetrodotoxin (0.5 μ M) to isolate GABAergic currents.

VTA GABA neurons in the lateral VTA were identified by a combination of factors, including small somata size, high firing rate (>7 Hz), and the lack of I_h current. Cells with these properties were consistently tyrosine hydroxylase (TH) negative (> 95%) (Korotkova et al., 2006; Ostroumov et al., 2016). Basal spontaneous firing rate of GABA neurons was not altered after exposure to the nicotine treatment: 16 \pm 3 Hz after nicotine and 14 \pm 3 Hz in control. In contrast, DA neurons were identified in the lateral VTA by their morphology (>20 μ m soma size), their low firing frequency (<5 Hz), and the presence of a large I_h current, which, together, was shown to correlate (95%) with TH-positive cells using immunocytochemistry approaches (Doyon et al., 2013; Ostroumov et al., 2016; Zhang et al., 2010). Basal spontaneous firing rate of DA neurons *ex vivo* was not altered after exposure to the adolescent nicotine treatment: 1.9 \pm 0.3 Hz after nicotine treatment and 1.6 \pm 0.1 Hz in control. Spontaneous inhibitory postsynaptic currents onto DA neurons were recorded in voltage-clamp mode in the whole-cell configuration. Synaptic GABA_A inputs were isolated pharmacologically. To measure activity-dependent depression of evoked IPSCs, whole-cell recordings were performed during repetitive stimulation (Hewitt et al., 2009). The estimated GABA reversal was approximately –70 mV, and the internal solution contained (in millimolar): 123.0 K⁺-gluconate, 8.0 NaCl, 2.0 Mg-ATP, 0.2 EGTA, 10.0 HEPES, and 0.3 Tris-GTP (pH 7.2–7.3). Synaptic GABA_A input was isolated using DNQX, AP5, and CGP55845. The liquid junction potential between the bath and the pipette solutions was corrected.

Western Blots

The VTA was harvested in horizontal brain slices from adult rats, and the VTA slices were prepared as described previously in the *Ex Vivo* Electrophysiology section. Membrane fractions were prepared using the Mem-PER Plus Membrane Protein Extraction Kit (Model #89842; Thermo Scientific, Rockford, IL, USA). Samples (30 μ g protein) in 2.5% 2-mercaptoethanol were run through a 4%–15% Precast Protein Gel (#4561083; Bio-Rad). The sample was transferred to nitrocellulose membrane (Bio-Rad). Primary antibodies used were rabbit anti-KCC2 antibody at 1:400 (#07-432; Millipore, Temecula, CA, USA) and mouse anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) antibody (#MAB374;

Millipore) at 1:400. Secondary antibodies used were goat anti-rabbit immunoglobulin (Ig)G secondary antibody (#T2191; Applied Biosystems, Foster City, CA, USA) or goat anti-mouse IgG/IgM (#T2192, Applied Biosystems). All antibodies were diluted in SignalBoost solution (#407207; EMD Millipore, Billerica, MA, USA). Membranes were developed using Tropix CDP-Star solution (T2218; Applied Biosystems) for 5 min and then scanned using the Protein Simple FluorChem R chemiluminescence detector and analyzed using AlphaView SA software. The optical densities of KCC2-specific bands were measured and normalized to the loading control GAPDH values.

To analyze KCC2 immunolabeling in the VTA, rats were perfused with PBS (Chemicon), followed by 4% paraformaldehyde (Boston BioProducts). Brains were post-fixed for additional 2 hr in 4% paraformaldehyde and then kept in 30% sucrose for 24–48 hr. VTA sections were cut at 30 μ m and processed with antibodies against KCC2 (1:500, Millipore, #07-432) and TH (1:1,000, Millipore, #MAB318) or GAD-67 (1:150, Santa Cruz Biotechnology, #sc-5602) overnight at 4°C. After washing in PBS, immunofluorescence reactions were visualized using secondary antibodies labeled with Alexa Fluor 488 or Alexa Fluor 594 (1:1,000; Invitrogen) and confocal microscopy.

***In Vivo* Electrophysiology**

Rats were anesthetized with isoflurane and implanted with a catheter in the jugular vein. Animals were positioned on a stereotaxic apparatus, and burr holes were drilled into the skull to accommodate recording and ground electrodes. Rat body temperature was maintained throughout the experiment at 37°C using an isothermal pad. Glass electrodes backfilled with 0.5 M Na⁺-acetate and 2% Chicago Sky Blue (5–15 MU) were positioned in the lateral VTA (coordinates: 5.3–6.0 mm posterior from bregma, 0.8–1.4 mm lateral to midline, and 7.5–8.5 mm ventral to brain surface). Electrical signals were filtered at 0.3–5 kHz. DA neurons in the lateral VTA were identified *in vivo* using established electrophysiological and pharmacological criteria (Ostroumov et al., 2016). After 6–20 min of stable baseline recording, we infused 0.3 g/kg of ethanol (by intravenous [i.v.] injection) every 3 min to a final dose of 1.5 g/kg over 15 min. Drug-induced changes were detected by the second infusion period and were comparable across all dose ranges (0.6–1.5 mg/kg). Following ethanol administration, the D2 receptor agonist quinpirole and the D2 receptor antagonist eticlopride were infused (i.v. injection, 0.25 mg/kg) to aid in the identification of VTA DA neurons. Chicago Sky Blue injections were used to identify the recording sites.

***In Vivo* Microdialysis**

Microdialysis studies were carried out as previously reported (Doyon et al., 2013). Animals were habituated to tethering and the microdialysis chambers 1 day prior to testing. Baseline DA samples were collected (15–30 min), followed by a timed i.v. infusion of ethanol (1.5 g/kg, 20% in sterile saline, v/v) over 5 min. The i.v. route (using a cannula) was chosen to circumvent handling-related disturbances in DA levels associated with i.p. injections (Dong et al., 2010). Dialysis samples were analyzed for DA content using high-pressure liquid chromatography (HPLC) coupled to an electrochemical detector.

Statistical Analysis

An ANOVA with repeated measures (in SPSS for Windows) was used to analyze the daily ethanol intake and dialysate DA concentrations. Exponential fittings were compared by F test. For western blot analysis, a paired t test was used to compare protein levels from saline- and nicotine-treated animals that were run on the same gel. A two-tailed t test assuming equal variance was used to assess differences between the mean GABA reversal potential, sIPSC frequency, ethanol intake, and DA firing rate. Significance for all analyses was determined by $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Y. De Koninck and A. Castonguay from Laval University for the generous gift of CLP290. This work was supported by grants NS021229, DA036572, and DA009411 from the NIH (to J.A.D.).

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Highlights

- Adolescent nicotine exposure increases alcohol self-administration in adulthood
- Adolescent nicotine exposure increases alcohol-induced VTA GABA release
- Adolescent nicotine exposure impairs chloride extrusion in VTA GABA neurons
- Enhancing Cl⁻ extrusion restores alcohol self-administration to control levels

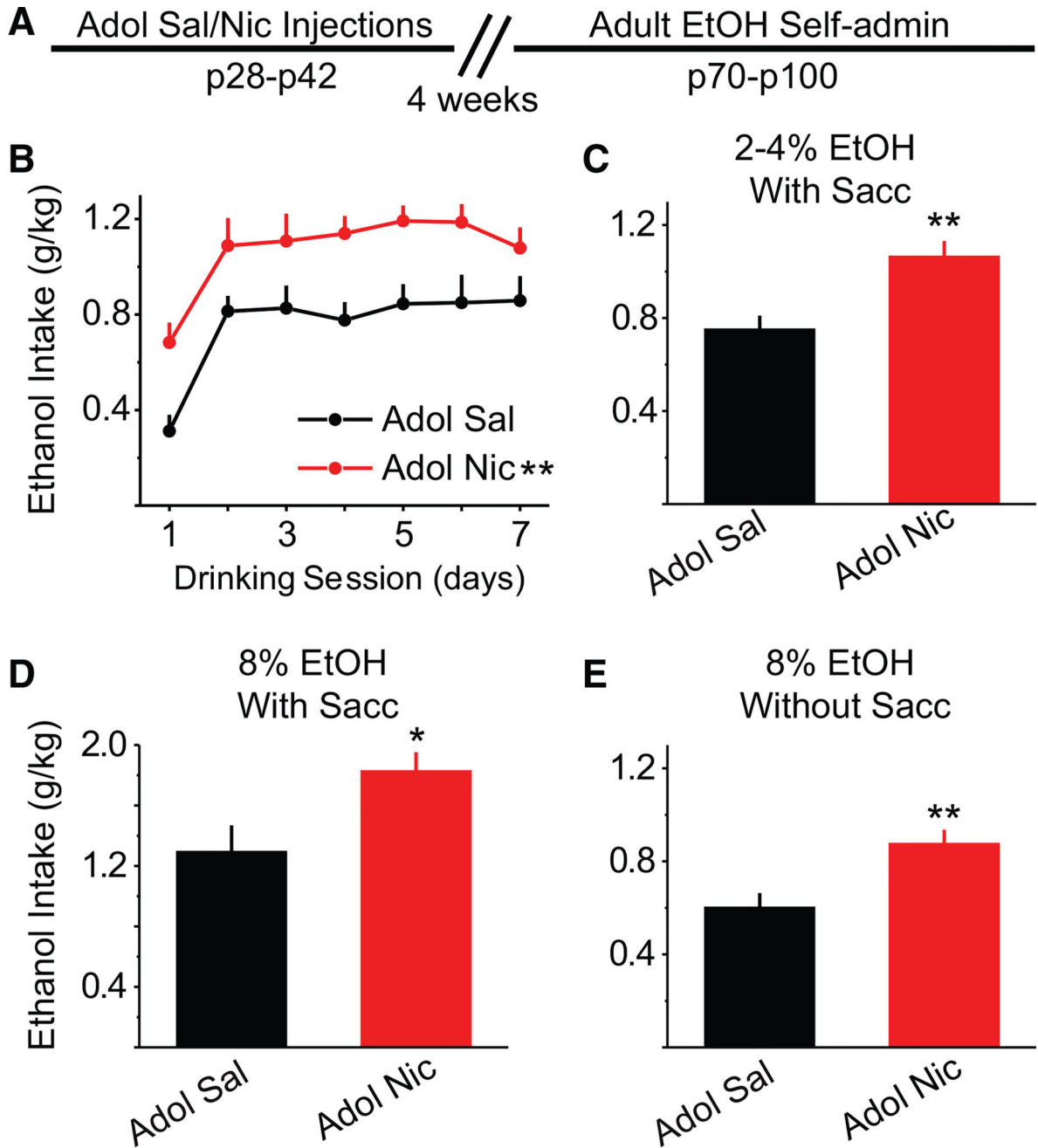


Figure 1. Adolescent Nicotine Exposure Increases Adult Ethanol Self-Administration
 (A) Adolescent animals were administered daily injections of saline or nicotine from p28 to p42, and adult ethanol self-administration was assessed beginning 4 weeks later.
 (B) Mean daily ethanol intake for the first seven self-administration sessions. Adolescent nicotine-treated rats (red) showed greater daily ethanol intake compared to saline-treated controls (black). ** $p < 0.01$, significantly different by ANOVA with repeated measures, $n = 8, 10$ rats per group.

(C) Average intake of 2%–4% ethanol with saccharin over the first seven self-administration sessions. Adolescent nicotine-treated rats showed greater ethanol intake compared to saline-treated controls. $**p < 0.01$, significantly different by t test, $n = 8$, 10 rats per group.

(D) Average intake of 8% ethanol with saccharin over seven self-administration sessions was significantly elevated in adolescent nicotine-treated animals compared to saline-treated controls. $*p < 0.05$, significantly different by t test, $n = 5$, 8 rats per group.

(E) After saccharin removal, intake of 8% ethanol over seven self-administration sessions was significantly elevated in adolescent nicotine-treated animals compared to saline-treated controls. $**p < 0.01$, significantly different by t test, $n = 9$, 11 rats per group.

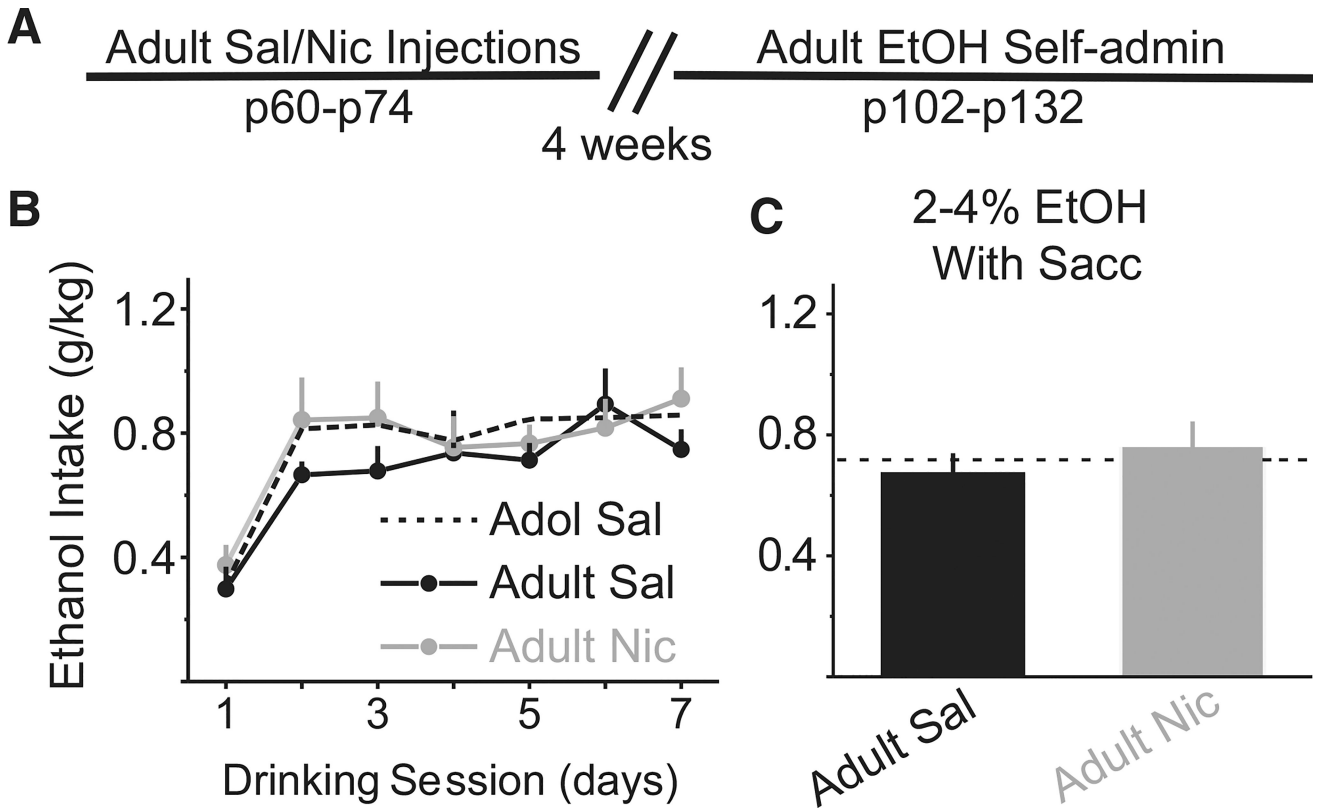


Figure 2. Adult Nicotine Exposure Does Not Increase Adult Ethanol Self-Administration

(A) Adult animals were administered daily injections of saline or nicotine (p60–p74), and ethanol self-administration was assessed beginning 4 weeks later.

(B) Mean daily ethanol intake for the first seven self-administration sessions. No differences in ethanol consumption were observed between adult nicotine-treated (gray) and saline-treated (black) groups, $p > 0.05$, $n = 5, 7$ rats per group. Ethanol intake in adolescent saline-treated controls is shown for comparison (dotted line).

(C) Average intake of 2%–4% ethanol with saccharin over the first seven self-administration sessions. No differences in ethanol consumption were observed between adult nicotine-treated and saline-treated groups, $p > 0.05$, $n = 5, 7$ rats per group. Ethanol intake in adolescent saline controls is shown for comparison (dotted horizontal line).

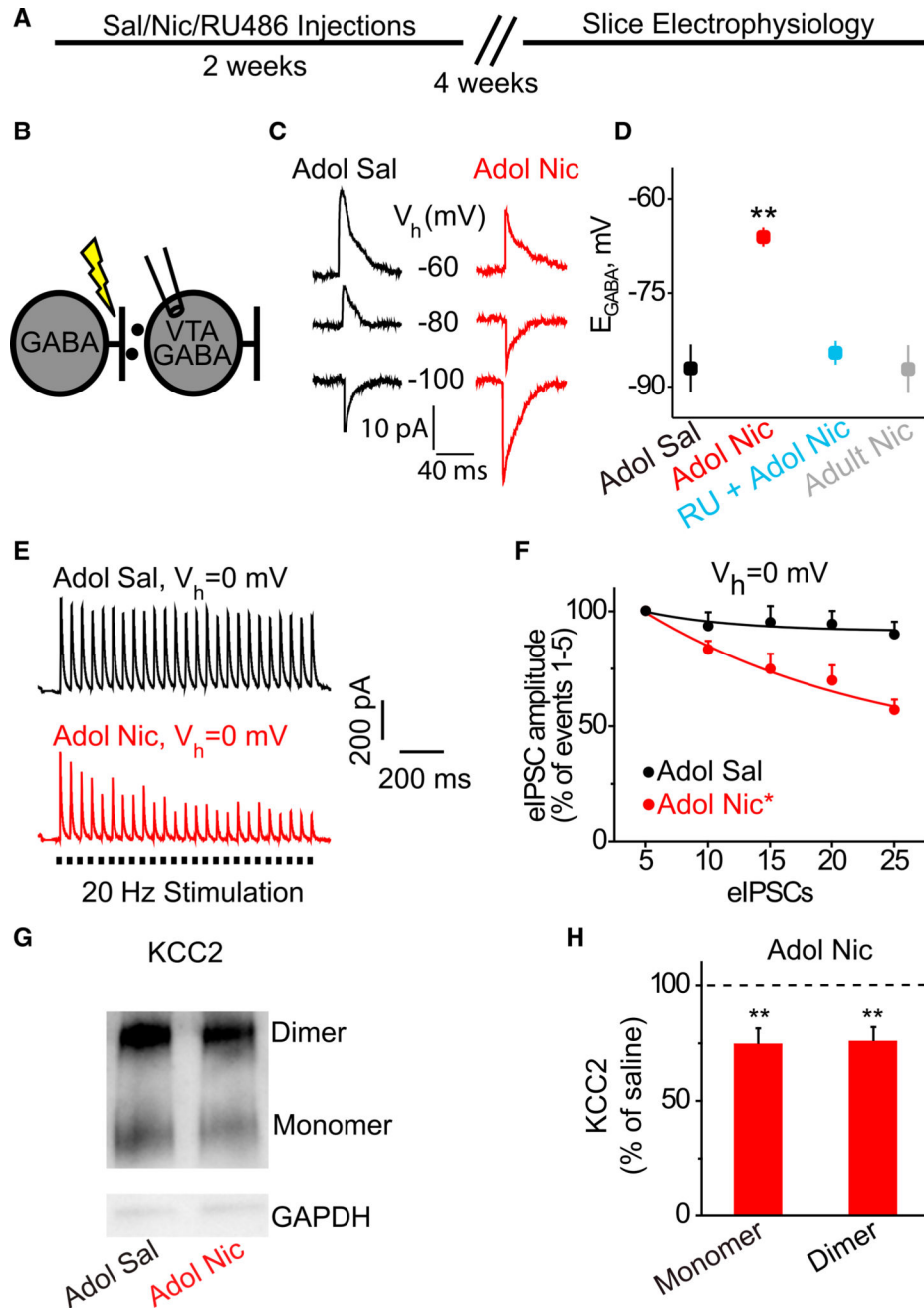


Figure 3. Adolescent Nicotine Exposure Alters GABA Transmission and Chloride Homeostasis (A) Animals were administered saline or nicotine throughout adolescence (p28–p42), and *in vitro* electrophysiology experiments were conducted beginning 4 weeks later. (B) GABAergic input onto VTA GABA neurons was measured using gramicidin perforated-patch whole-cell recordings at different holding potentials. GABA_A IPSCs were evoked by electrical stimulation in the presence of glutamate and GABA_B receptor antagonists. (C) Representative IPSC recordings from saline- and nicotine-treated animals at the given holding potentials (V_h). The IPSCs reverse direction at the E_{GABA} . For display, the traces were filtered, and stimulus artifacts were removed.

(D) VTA GABA neurons from adolescent nicotine-treated animals (red) demonstrated a significantly more positive E_{GABA} value compared to neurons from saline-treated control animals (black). No significant differences in E_{GABA} were observed if animals were pretreated with RU486 during adolescent nicotine (blue) or if animals were treated with nicotine as adults (gray). $**p < 0.01$, significantly different by t test, $n = 6, 7$ cells per group.

(E) Cl^- accumulation was estimated by stimulating repetitive GABA_AR input. Upon stimulation (20 Hz, $V_h = 0$ mV), a representative GABA neuron from a saline-treated animal (black) demonstrated a minor depression of IPSC amplitude compared to the significantly greater depression seen in a GABA neuron from a nicotine-treated animal (red).

(F) At 0 mV, VTA GABA neurons from nicotine-treated animals demonstrated a significantly higher rate of evoked IPSC amplitude depression compared to saline-treated controls. $**p < 0.01$, significantly different from the control by F test, $n = 7, 9$ cells per group.

(G) Western blot analysis was conducted for KCC2 protein expression, with GAPDH as a loading control. A representative western blot shows reduced expression of KCC2 in nicotine-treated animals.

(H) Densiometric analysis showed a significant reduction in KCC2 protein in nicotine-treated animals (red bars) compared to saline-treated controls (horizontal dashed line). $**p < 0.01$, significantly different from the control by t test, $n = 12$ rats per group.

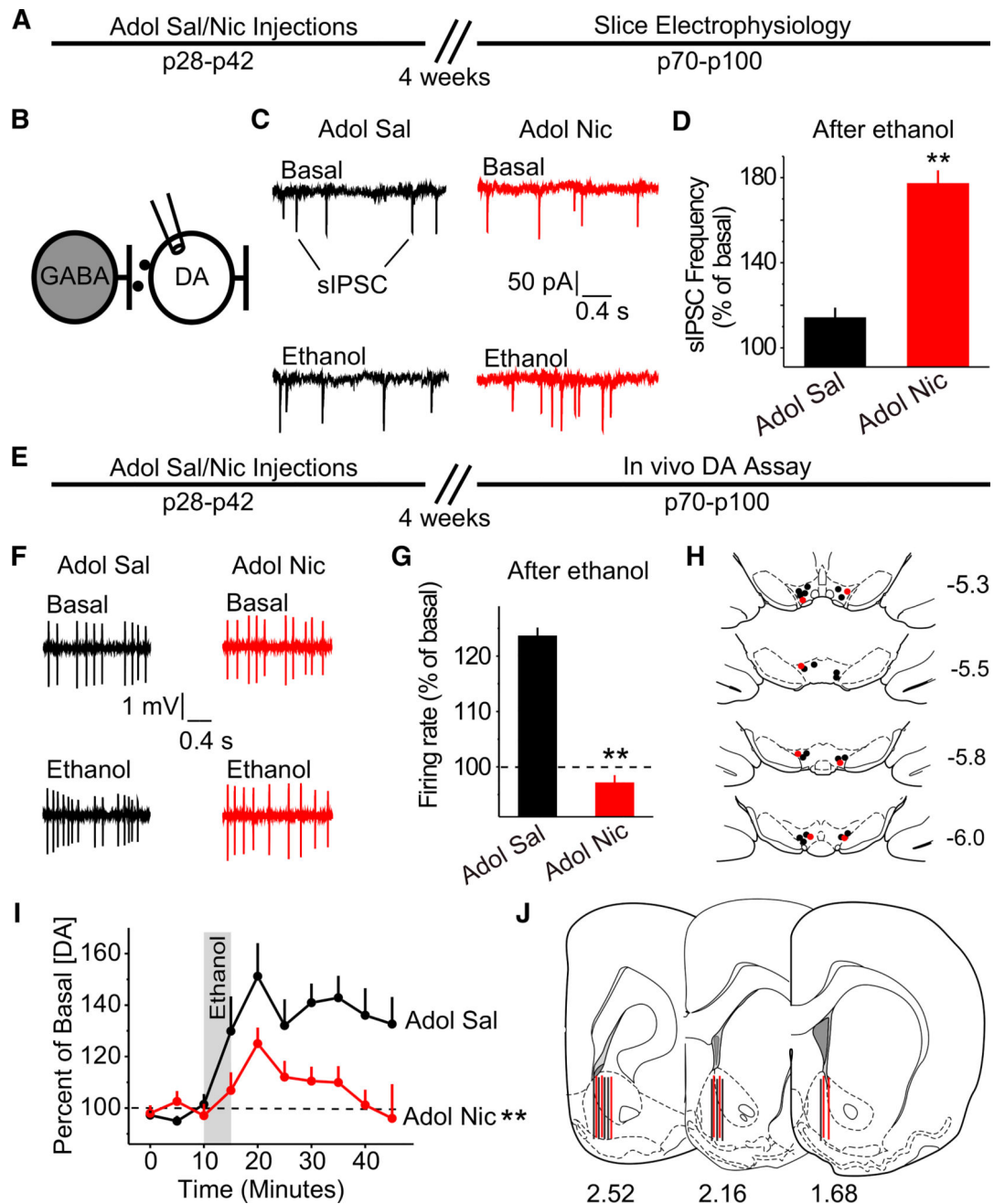


Figure 4. Adolescent Nicotine Exposure Increases Ethanol-Induced Inhibition of Dopamine Neurons

(A) Animals were administered saline or nicotine throughout adolescence (p28–p42), and *in vitro* electrophysiology experiments were conducted beginning 4 weeks later.

(B) Spontaneous inhibitory postsynaptic currents (sIPSCs) onto VTA DA neurons were recorded using the whole-cell patch-clamp configuration in the presence of glutamate receptor antagonists.

(C) Representative recordings of sIPSCs before and after ethanol administration in the adolescent saline-treated (black) and adolescent nicotine-treated (red) animals.

(D) Mean changes in the sIPSC frequency after ethanol (50 mM) application in VTA DA neurons. DA neurons from nicotine-treated animals demonstrated a significantly increased ethanol-mediated sIPSC frequency compared to neurons from saline-treated controls. $**p < 0.01$, significantly by t test, $n = 8$, 13 cells per group.

(E) Animals were administered saline or nicotine throughout adolescence (p28–p42), and *in vivo* DA responses to ethanol were measured beginning 4 weeks later.

(F) Representative *in vivo* recordings from putative DA neurons before and after ethanol administration in the saline- and nicotine-treated groups. No significant differences in the mean basal firing rate were detected.

(G) In saline-treated controls, ethanol increased the firing rate of putative DA neurons. In the nicotine-treated group, ethanol failed to increase the firing rate of putative DA neurons. $**p < 0.01$, significantly different from the control group by t test, $n = 7$, 19 rats per group.

(H) Putative DA neuron recording sites in the VTA from adolescent saline-treated (black) and nicotine-treated (red) animals.

(I) Time course of DA release in the nucleus accumbens measured via microdialysis following ethanol administration in saline-treated and nicotine-treated rats. Ethanol (1.5 g/kg) was administered by i.v. injection over a 5-min period (shaded vertical gray bar). $*p < 0.05$, significantly different by ANOVA with repeated measures, $n = 7$, 9 rats per group.

(J) Anatomical distribution of microdialysis probe locations in the nucleus accumbens. Black indicates saline-treated animals, and red indicates nicotine-treated animals.

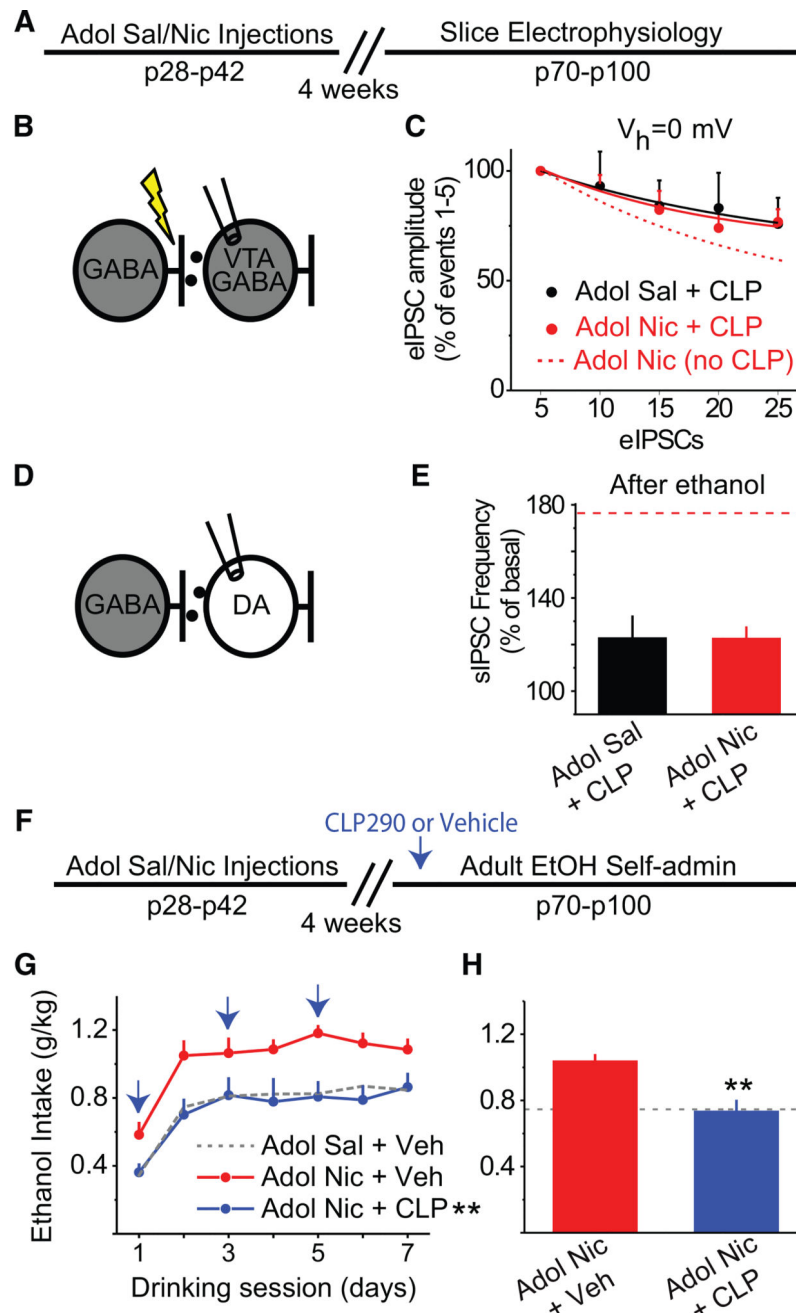


Figure 5. Enhancing VTA Chloride Extrusion Normalizes GABA Signaling and Ethanol Self-Administration

(A) Animals were exposed to saline or nicotine throughout adolescence (p28–p42), and effects of CLP290 on GABAergic signaling were evaluated using slice electrophysiology 4 weeks later (p70–p100).

(B) The effect of CLP290 on Cl^- accumulation was estimated by stimulating repetitive GABA_A input and measuring eIPSC amplitude in VTA GABA neurons.

(C) Following CLP290 incubation, no differences were observed in the rate of evoked amplitude depression in VTA GABA neurons between saline-treated (black) and nicotine-treated (red) animals. $n = 6, 9$ cells per group.

(D) The effect of CLP290 on spontaneous inhibitory postsynaptic currents (sIPSCs) onto VTA DA neurons were recorded using the whole-cell patch-clamp configuration in the presence of glutamate receptor antagonists.

(E) Following CLP290 incubation, no differences were observed between saline-treated and nicotine-treated animals in the sIPSC frequency. $n = 6, 5$ cells per group.

(F) Animals were exposed to saline or nicotine throughout adolescence (p28–p42), and the effects of CLP290 on adult ethanol self-administration were measured.

(G) Effects of CLP290 on mean daily ethanol intake for the first seven self-administration sessions. Vehicle (red) or CLP290 (blue) was locally infused bilaterally intra-VTA prior to ethanol self-administration on non-consecutive days (blue arrows). Adolescent nicotine-treated animals that were also treated with CLP290 showed significantly reduced ethanol consumption compared to vehicle-treated controls. Ethanol consumption in adolescent saline vehicle-treated control rats is shown for comparison (dotted line). $**p < 0.01$, significantly different by ANOVA with repeated measures.

(H) Effects of CLP290 on mean ethanol intake over the first seven self-administration sessions. Adolescent nicotine-treated animals that were also treated with CLP290 showed significantly reduced ethanol consumption compared to vehicle-treated controls. Ethanol consumption in adolescent saline vehicle-treated control rats is shown for comparison (dotted horizontal line). $**p < 0.01$, significantly different from the VTA vehicle group by t test, $n = 9, 13$ rats per group.