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Combined nicotine and ethanol age-dependently alter neural and behavioral responses in male rats

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Abstract

Use of alcohol (EtOH) and nicotine (Nic) typically begins during adolescence. Smoking and drinking often occur together and lead to higher consumption of alcohol. Although we have shown that Nic+EtOH is reinforcing in self-administration tests in adolescent male rats, whether Nic +EtOH affects other behaviors or neuronal activity in an age-dependent manner is unknown. To address this, adolescent and adult male rats were given intravenous injections of Nic (30 μg/kg) + EtOH (4 mg/kg) and evaluated for locomotor and anxiety-like behaviors. Regional neuronal activity, assessed by *cFos* mRNA expression, was measured and used to evaluate functional connectivity in limbic regions associated with anxiety and motivation. Nic+EtOH increased locomotor activity and was anxiolytic in adolescents, but not adults. The posterior ventral tegmental area (pVTA), a critical regulator of drug reward, was selectively activated by Nic+EtOH in adults, while activity in its target region, the NAc-shell, was decreased. Drug-induced alterations in functional connectivity were more extensive in adults than adolescents, and may act to inhibit behavioral responses to Nic+EtOH that are seen in adolescence. Overall, our findings suggest that brief, low-dose exposure to Nic+EtOH produces marked, age-dependent changes in brain and behavior, and that there may be an ongoing maturation of the pVTA during adolescence that allows increased sensitivity to Nic+EtOH's reinforcing, hyperlocomotor, and anxiolytic effects. Furthermore, this work provides a potential mechanism for high rates of co-use of nicotine and alcohol by teenagers: this drug combination is anxiolytic and recruits functional networks that are unique from protective, inhibitory networks recruited in the mature, adult brain.

Keywords

adolescence; alcohol; anxiety; functional connectivity; stress; tobacco

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Introduction

Alcohol and nicotine, via tobacco or electronic cigarettes (e-cigarettes), are the two most commonly co-used substances. Concurrent use is associated with severe health consequences (Van Skike et al. 2016), and it is estimated that the economic costs associated with alcohol and tobacco use in the U.S. totals almost 500 billion dollars annually (National Institute on Drug Abuse 2015). The majority of individuals with alcohol use disorder also smoke (Falk et al. 2006), and dependent smokers are approximately ten times more likely than non-dependent smokers to have alcohol use disorder (DiFranza and Guerrera 1990).

Initiation of tobacco and alcohol use, either individually or concurrently, typically begins during adolescence. An assessment of 12th grade patterns of use from 1976-2010 has shown that, although overall drug use has declined, tobacco and alcohol co-use has increased (Daw et al. 2013). Moreover, almost 90% of current adult smokers began smoking before the age of 18 (SAMHSA 2011) and alcohol is typically first consumed before the age of 16 (Behrendt et al. 2009). Early adolescent onset of smoking is associated with the greatest risk of excessive alcohol consumption and alcohol abuse disorders (Weitzman and Chen 2005). E-cigarettes, which are now the most commonly used tobacco product among middle and high school students (Jamal et al. 2017), are also associated with increased alcohol use and misuse among adolescents (Hershberger and Cyders 2017; Hershberger et al. 2020).

Adolescence is a transition period between childhood and adulthood conservatively estimated to last from 12 to 18 years of age in humans and postnatal days (P) 28-42 in rodents (Spear 2000). It is highly conserved across mammalian species, and marked by major reorganization of limbic and cortical regions important for learning and memory, executive function, and reward processing (Spear 2000; Yuan et al. 2015). These changes are necessary for the transition to adult autonomy, but do leave the adolescent brain uniquely vulnerable to the detrimental effects of nicotine and alcohol. Consistent with prior work from our lab showing that brief, low-dose nicotine pretreatment during early adolescence (P28-31) enhances acquisition of alcohol self-administration (Dao et al. 2011), more recent work demonstrates that adolescent male rats find concurrent intravenous self-administration of nicotine and alcohol significantly more reinforcing than either drug alone (Lárraga et al. 2017). This enhanced reinforcing effect of co-administered drugs is not evident in adult males or females of either age, and may be due to developmental differences in drug-induced neural activation in reward- and stress-related brain regions. Nicotine exposure in adolescent males, but not adults, also produces long-lasting increases in oral alcohol consumption (Lárraga et al., 2017), which is in line with epidemiological findings described above.

Since preclinical research examining the behavioral and neuronal effects of concurrent nicotine and ethanol (Nic+EtOH) across development is limited, the goal of the present study was to understand the effects of Nic+EtOH on locomotor and anxiety-like behaviors in adolescent and adult male rats. Based on our prior work demonstrating greater sensitivity to the reinforcing, motivational effects of Nic+EtOH, we hypothesized that adolescents would be more sensitive to the locomotor stimulating and anxiolytic effects of Nic+EtOH than adults. We also examined *cFos* mRNA expression, a marker of neuronal activity (Flavell and

Greenberg 2008), after acute Nic+EtOH treatment to test the hypothesis that drug-induced regional neuronal activation would be higher in adolescents than adults in regions mediating drug reward (e.g., NAc, VTA), and lower in adolescents than adults in regions involved in anxiety-like behaviors. We also adapted graph theory methods (Dwyer and Leslie 2016) to construct and evaluate networks of coordinated gene expression (CGE) – analogous to functional connectivity – as increasing evidence indicates maturational and drug-induced changes in functional network activity (Dwyer and Leslie 2016; Ruiz et al. 2020). We demonstrate that Nic+EtOH uniquely influences locomotor activity, anxiety-like behavior, regional neuronal activity, and functional connectivity in adolescent rats. Our findings add to the growing body of literature showing anomalous effects of combined exposure to nicotine and alcohol, and provide a potential mechanism for high rates of co-use of these drugs observed among teenagers and young adults via alterations in anxiety and functional neural circuitry.

Materials and Methods

Animals

Male Sprague Dawley rats were obtained from Charles River at P17 or P75 and juveniles were housed with a dam until weaning (P21). Females were not included as age-specific Nic +EtOH reinforcing effects were not observed in females in our prior study (Lárraga et al. 2017). Weaned juveniles and adults (P76) were group housed in an AALAC-accredited vivarium on a 12-hour light-dark cycle with food and water available *ad libitum*. No more than one animal per litter per experimental group was used to avoid potential litter effects (Lárraga et al. 2017). All procedures were in compliance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. All animals were handled for 2 min daily for three days prior to surgery and thereafter. All behavioral testing occurred during the light cycle. Two cohorts of animals were used; the first was behaviorally tested only and the second was behaviorally tested before sacrifice for tissue collection.

Catheterization

Adolescent and adult rats underwent surgery at P28 and P86, respectively. Animals were anesthetized with Equithesin (0.035 mg/kg, i.p.), and were surgically implanted with a catheter into their right jugular vein (Belluzzi et al. 2005). Rats were given three days to recover before drug exposure. Cannulae were flushed daily with heparinized saline solution to maintain catheter patency. Propofol (5 mg/kg, i.v.) was injected the day before behavioral testing; animals that did not display rapid (5-10 sec) anesthesia were excluded from further analysis.

Drug exposure and behavioral testing

Nicotine hydrogen tartrate was purchased from Glentham Life Sciences (London, UK), 100% ethanol was purchased from Sigma (St. Louis, MO), and propofol was purchased from Abbot Laboratories (Chicago, IL). Nicotine was dissolved in sterile saline and adjusted to pH 7.2-7.4. Ethanol was prepared at concentrations no greater than 20% (v/v). Adolescent (P32) and adult (P90) rats were allowed to habituate to the experimental room for 30 min in

their home cages. Animals then received two injections of saline (1 ml/kg, i.v.) or a low dose of Nic+EtOH (2 x 15 μg/kg nicotine plus 2 x 2 mg/kg EtOH, i.v.) spaced 1 min apart. The injection procedure and chosen drug doses mirror the average responses on the reinforced lever during the first minute of self-administration of Nic+EtOH (Lárraga et al. 2017). Immediately after treatment, animals were placed in a novel open-field activity chamber (43.2 x 43.2 x 30.5 cm) connected to a common interface and computer (Med Associates Inc., St. Albans, VT) for 30 min. Ambulatory counts and time spent in the center of the open-field were recorded automatically. The center area was defined based on the average body size at each age, with the center zone for adults comprising ~30% of the total chamber area and the center zone for adolescents comprising ~50% of the total chamber. Immediately following behavioral testing, the second cohort of animals were decapitated, brains collected and flash frozen in 2-methylbutane at −20°C, and stored at −70°C until processing. The time chosen for tissue collection (i.e., 30 min after drug injection) corresponds to the peak of stimulus-induced *cFos* mRNA expression (Sharp et al. 1993; Cullinan et al. 1995).

Measurement of cFos *mRNA expression*

Tissue preparation and anatomical analysis—Twenty μm sections were cut and processed for *cFos in situ* hybridization. [^{35}S]-labeled UTP (Perkin Elmer, Boston, MA) was used to synthesize cRNA riboprobes for *cFos* in the sense and antisense orientation from a pGEM-3Z plasmid containing a 680bp fragment of *cFos* cDNA between T7 and SP6 promoter sites. *cFos* cDNA was kindly provided by Dr. Stanley J. Watson, University of Michigan. Tissue sections were pretreated with Proteinase K, acetylated, dehydrated through ascending concentrations of ethanol and then air dried. Sections were incubated for 16-18 hr at 60°C, with hybridization solution containing the [^{35}S]-labeled riboprobe (10^7 cpm/ml). Following hybridization, sections were incubated with RNase A and washed with descending concentrations of SSC before being dehydrated in ethanol and apposed in light-tight cassettes to Kodak BioMax film with [^{14}C] standards of known radioactivity for one day. Film was developed and rapidly fixed for quantitative analysis with a MicroComputer Imaging Device (MCID, Imaging Research, St. Catherine, Ontario, Canada), as described earlier (Dwyer and Leslie 2016). A calibration curve of optical density against radioligand concentration (dpm/mg tissue) was constructed using [^{14}C] brain paste standards. Optical densities in each region of interest were measured and values of radioactivity were calculated by interpolation from the calibration curve and averaged to give the value for a single region. Specific hybridization was calculated by subtracting values of radioactivity in sections hybridized with sense probe from those hybridized with antisense. Regional averages were obtained from readings of the left and right hemispheres from two comparable sections for every region. Brain areas in autoradiograms were identified with well-defined anatomical landmarks and with reference to adjacent brain sections processed for Nissl-staining.

Regions were selected *a priori*, based on their involvement in drug reward, anxiety-like behaviors, and previous work demonstrating age- and region-specific effects of nicotine exposure on neuronal activity (reviewed in Marchand 2010; Yuan et al. 2015; Volkow et al. 2016; Xia and Kheirbek 2020). Areas where measurements for *cFos* expression were taken and included for network analysis are listed in Table 1 and include prefrontal cortex [ventral

orbital (VO), lateral orbital (LO), cingulate (Cg1), prelimbic (prL), infralimbic (IL), dorsal peduncular (DP), and dorsal tenia tecta (DTT)], striatum [dorsomedial caudate putamen (dmCPu), dorsolateral caudate putamen (dlCPu), ventromedial caudate putamen (vmCPu), ventrolateral caudate putamen (vlCPu), nucleus accumbens shell (NAc-shell), and nucleus accumbens core (NAc-core)], bed nucleus of the stria terminalis [anterior (aBNST) and posterior (pBNST)], medial preoptic area (mPOA), hypothalamus [lateral (LH) and paraventricular (PVH)], amygdala [medial nucleus (MeA), central nucleus (CeA), and basolateral (BLA)], thalamus [paraventricular nucleus (PVT), centromedial nucleus (CMT), and anteroventral nucleus (AVT)], substantia nigra (SN), ventral tegmental area [anterior (aVTA) and posterior (pVTA)], interpeduncular nucleus (IPN), superior colliculus (SC), periaqueductal gray (PAG), raphe nucleus [dorsal (DR) and median (MR)], and hippocampus [CA1, CA2, CA3, and dentate gyrus (DG)].

Analysis of coordinated gene expression (CGE)—In order to determine network-level CGE, we used a methodology developed by our lab (Dwyer and Leslie 2016) and adapted from Rubinov and Sporns (2010). An adjacency matrix was constructed for each drug treatment and age group, yielding four matrices. Matrices were composed of undirected, weighted Pearson coefficients (r) from the intersubject correlation of *cFos* expression between each pair of brain regions analyzed (36×36 regions). Matrices were thresholded at $p<0.05$ and non-significant r-values were set to zero for visualization purposes. Visual maps of CGE were constructed by importing thresholded matrices into UCINET Netdraw (UCINET 6.0, Analytic Technologies, Lexington, KY). Analyzed brain regions were arranged relative to their anatomical location in a mid-sagittal section of the brain. Significant r-values denoting association between two brain regions (CGE, analogous to functional connectivity) were displayed as lines (edges) connecting two brain regions (nodes). The thickness of the line denotes the strength of the r-value, while color distinguishes r-values that were negative (red) or positive (black).

Properties of each functional network were characterized statistically using a number of measures from the open-source brain connectivity toolbox (Rubinov and Sporns 2010); Matlab R2016b, MatWorks, Natick, MA). *(I) Modularity.* Modularity quantifies and separates networks into optimized community structure comprised of subcommunities with maximal within-group connections and minimal between-group connections. The modularity function (modularity_und.m) assigns each node to an individual community, and functional subcommunities are denoted by color in network visualizations. *(II) Degree.* The degree of a node represents the number of significant correlations, or functional relationships, that a given node has with other nodes in the network. The undirected and unweighted degree function was used (degrees_und.m). *(III) Betweenness Centrality.* Betweenness centrality is a nodal property that reflects the amount of control a node has over the interactions of other nodes within the network, favoring nodes that join communities rather than simply exist within communities. It represents the fraction of shortest paths in the network passing through a given node, and was assessed using the weighted betweenness centrality function (betweenness_wei.m) and an inverse mapping of weighted matrices (i.e., mapping where tighter functional relationships are interpreted as shorter distances). *(IV) Hub analysis.* Hubs are nodes that are poised to contribute strongly

to global network function. Nodes were designated as hubs based on two criteria: degree and betweenness centrality. If a node's degree or betweenness centrality value was at least two standard deviations from the network mean, it was denoted as a hub (Bassett et al. 2008). Hubs are denoted in network visualizations by node size and solid color fill. *Clustering Coefficient.* The clustering coefficient is a measure of functional segregation, or the ability for specialized processing to take place among highly interconnected groups of brain regions. It is equivalent to the fraction of node's neighbors that are neighbors of each other, and was assessed using the binary, undirected clustering coefficient function (clustering_coef.bu.m). *(VI) Global efficiency.* Global efficiency is a measure of functional integration, or how well a network is able to exchange information, and is the average inverse shortest path length in the network. The characteristic path length (charpath.m) function was used to assess global efficiency.

Data analyses

Behavioral data—Data from both cohorts are presented together, and are expressed as mean ± SEM. Locomotor and center time data were analyzed by two-way ANOVA for Age x Drug. All significant main or interaction effects were further analyzed by one-way ANOVA with Bonferroni-adjusted *post hoc* comparisons (GraphPad Prism 6.0, GraphPad Software, San Diego, CA). Outliers (2 SD from group mean) were removed prior to analysis as follows: adolescent saline (n=2); adult saline (n=4); adolescent Nic+EtOH (n=1); adult Nic+EtOH (n=5).

Regional mRNA expression—Data are expressed as mean + SEM and were analyzed by two-way ANOVA for Age x Treatment. All significant main or interaction effects were further analyzed by one-way ANOVA with Bonferroni-adjusted *post hoc* comparisons. All analyses were completed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA).

Network differences in CGE—Drug-treated adolescent and adult *cFos* mRNA CGE networks were compared with networks of their saline-treated counterparts to determine differences in functional relationships. While thresholded networks were used for visualization, all r-values were included for network comparisons so that significant differences between strong and weak networks could be evaluated. R-values for each interregional correlation were transformed to z scores in order to improve normality. Differences between treatment groups were determined by dividing the subtracted z scores by the standard error of the difference. Z difference scores were then converted to p-values, which were corrected using false discovery rate (Genovese et al. 2002). A moderate false discovery rate (q=0.10) was chosen to minimize Type II errors that may be increased when conservative thresholds are used in networks with low signal-to-noise ratios, while still providing correction against Type I errors. For visualization, the brain regions analyzed were arranged relative to their anatomical location in a mid-sagittal section of the brain. Significant changes in CGE between two brain regions as a result of drug treatment were displayed as lines (edges) connecting two brain regions (nodes). The color and style were used to distinguish between a gain (dash, black) or a loss (dash, red) of significant CGE.

Results

Nic+EtOH increases locomotor activity in adolescents, but not adults

We observed significant effects of age and Nic+EtOH exposure on locomotor activity (Figure 1A). Since overall ANOVA indicated significant main effects of Age ($F_{1,66}=24.647$, p<0.001) and Drug ($F_{1,66}=18.993$, p<0.001), as well as a significant Age*Drug interaction ($F_{1,66}=13.042$, p=0.001), data were split by age to analyze the effects of Nic+EtOH. Compared to saline-treated controls, Nic+EtOH increased locomotor activity in adolescents (p<0.001), but not adults. Furthermore, Nic+EtOH-treated adolescents had higher locomotor activity than Nic+EtOH-treated adults (p<0.001).

Nic+EtOH is anxiolytic in adolescents, but not adults

In a measure of anxiety-like behavior, time spent in the center of an open-field, we found a significant main effect of Age ($F_{1,66}=5.333$, p=0.024) and a trend for a main effect of Drug ($F_{1,66}=3.774$, p=0.056). Data were split by age to examine our *a priori* hypothesis that Nic +EtOH would decrease anxiety-like behavior in adolescents. As hypothesized, Nic+EtOH increased time spent in the center zone in adolescents (p=0.035), but not adults. Nic+EtOH-treated adolescents also spent more time in the center than Nic+EtOH-treated adults (p=0.012; Figure 1B).

Regional neuronal activity

To explore which brain regions may be involved in mediating age differences in the behavioral effects of Nic+EtOH, regional *cFos* expression was measured. There were significant main effects of Age in the aBNST ($F_{1,60}=7.302$, p=0.009), pBNST ($F_{1,67}=4.089$, p=0.047), mPOA ($F_{1,50}=14.693$, p<0.001), SN ($F_{1,63}=7.436$, p=0.008), IPN ($F_{1,60}=5.746$, p=0.020), MR ($F_{1,62}=6.222$, p=0.015), and CA2 ($F_{1,57}=7.965$, p=0.007), but no significant effects of Drug or Age*Drug interactions. In all of these areas except the mPOA, neuronal activation was higher in adults than adolescents (Figure 2).

Significant main effects of Drug were found for NAc-core ($F_{1,65}=6.312$, p=0.014), PVT ($F_{1,64}=6.970$, p=0.010), AVT ($F_{1,64}=6.254$, p=0.015), CMT ($F_{1,66}=4.311$, p=0.042), MeA ($F_{1,64}=12.157$, p=0.001), CeA ($F_{1,52}=4.367$, p=0.042), BLA ($F_{1,66}=7.636$, p=0.007), PAG ($F_{1,56}=10.458$, p=0.002), DR ($F_{1,59}=5.171$, p=0.027), CA1 ($F_{1,57}=12.982$, p=0.001), and DG ($F_{1,57}=4.597$, p=0.036), but there were no significant effects of Age or Age*Drug interactions. Nic+EtOH decreased *cFos* mRNA levels in all of these regions, except the CeA where Nic+EtOH increased *cFos* (Figure 3).

Age and Nic+EtOH also interacted to influence *cFos* expression in some regions (Figure 4). Significant Age*Drug interactions were found in the pVTA ($F_{1,57}=5.419$, p=0.023), NAc-shell ($F_{1,68}=6.111$, p=0.016), LO ($F_{1,63}=7.579$, p=0.008), CA3 ($F_{1,52}=7.100$, p=0.010) and aVTA ($F_{1,55}=5.911$, p=0.018). In all of these regions, except pVTA, Nic+EtOH selectively decreased *cFos* expression in adults with no significant effect in adolescents. In the pVTA, the drug combination selectively increased *cFos* expression in adults (p=0.047) with no effect in adolescents.

Age differences in baseline and Nic+EtOH-mediated CGE networks

To examine baseline developmental differences in functional connectivity, networks of coordinated *cFos* gene expression were created and network properties were assessed for saline-treated adolescents and adults (Figure 5). Adolescents had unique community structure, with each community representing highly interconnected and segregated functional subgroups, as well as unique network hubs poised to exert greater influence over global network function (i.e., DP, SC, and PAG) compared to adults (i.e., DP and dlCPu; Table 2). Adolescents (Figure 5A) had significantly fewer functional relationships than adults (p=0.005), and adults had significantly higher clustering coefficients (p<0.001), a measure of functional segregation, than adolescents (Figure 5B).

As with baseline networks, Nic+EtOH-mediated networks displayed unique community structure and network hubs in adolescents compared to adults. In Nic+EtOH-treated adolescents (Figure 5C), network hubs included the Cg1, VO, and DP, while network hubs in adults (Figure 5D) included VO, DP, hippocampal CA2, and BLA (Table 2).

Age-dependent effects of Nic+EtOH on functional connectivity

Drug-induced CGE changes were assessed by comparing Nic+EtOH networks to saline networks at each age (Figure 6). The number of functional relationships was significantly decreased by Nic+EtOH in adolescents (p<0.001), but not adults (p=0.09). However, drug treatment did significantly and uniquely alter functional connectivity at both ages. In adolescents, the dominant effect of Nic+EtOH was a significant loss of positive CGE between midbrain and striatal nuclei (Figure 6A). Specifically, positive CGE was lost between pVTA and striatal nuclei (i.e., dmCPu, vmCPu, vlCPu, and NAc-core), as well as DG. Positive CGE between SN and vlCPU, as well as from the IPN to DR, MR, and CA1 was also lost as a result of adolescent Nic+EtOH exposure, as was connectivity between MR and CA1. There were no significant gains of functional interactions resulting from adolescent Nic+EtOH exposure.

In adults, drug-induced connectivity changes were more extensive, with both gains and losses of positive CGE (Figure 6B). Nic+EtOH produced a profound, significant loss of positive CGE within striatal nuclei, as well as some loss in connectivity within midbrain nuclei (i.e., aVTA with SN, IPN, and MR; IPN with MR). Numerous functional connections with the PAG (i.e., with hippocampal CA3, SN, NAc-core, NAc-shell, dmCPu, dlVPu, and vmCPu) were also lost after adult Nic+EtOH exposure. There was a significant loss of positive CGE between hypothalamic (i.e., PVH) and amygdalar regions (i.e., MeA and BLA), as well as between the AVT and NAc-shell, dlCPu, and PVH. Nic+EtOH exposure also resulted in multiple gains of positive CGE, primarily between midbrain and extended amygdala nuclei. Specifically, Nic+EtOH led to a gain of connectivity between the pVTA and the aBNST and pBNST, as well as between pBNST and both SN and MR. Connections between the LH and pVTA, aBNST, and hippocampal CA2 were also gained after Nic +EtOH exposure in adults.

Discussion

Consistent with our prior behavioral observation that the combination of nicotine and ethanol is reinforcing in adolescent, but not adult, male rats (Lárraga et al. 2017), we now show that Nic+EtOH treatment has unique, age-specific effects on both behavior and neuronal function. This drug combination increases locomotion and decreases anxiety in adolescent males, but not adults. In contrast, neuronal activity, as measured by *cFos* mRNA expression, was selectively altered by drug exposure in a subset of regions in adult brain, being increased in the adult pVTA, but decreased in the aVTA, NAc-shell, LO, and hippocampal CA3. CGE was disrupted by Nic+EtOH in adolescents, as indicated by a large decrease in the number of functional connections, while the organization of functional networks in adults was more profoundly altered by Nic+EtOH. Our findings indicate that even brief, low-dose exposure to Nic+EtOH can produce marked changes in neuronal function that differ widely based on age.

Age-dependent effects of Nic+EtOH on locomotor and anxiety-like behavior

In this study, we have shown that acute Nic+EtOH increases ambulatory activity in adolescents, but not adults. Although prior studies have shown that the addition of ethanol reverses nicotine-induced locomotion increases (Abreu-Villaça et al. 2008; Gulick and Gould 2009), our data are in line with other reports highlighting adolescents' differential responses to nicotine or ethanol individually (White et al. 2002; Cao et al. 2010).

We have also shown that Nic+EtOH increases the amount of time that adolescents, but not adults, spend in the center of an open field, indicating an anxiolytic effect. This Nic+EtOH effect on anxiety-like behavior is unlikely to be driven by drug-induced increases in ambulatory activity, as these behaviors are not correlated (r=0.386, p=0.113; data not shown) and we have shown previously that nicotine-induced alterations in center time are unrelated to locomotor activity (Cao et al. 2010). Indeed, prior research from our lab and others has shown that nicotine alone is similarly anxiolytic in adolescents (Elliott et al. 2004; Cao et al. 2010). In contrast, recent work suggests that adolescents require higher doses of ethanol to experience its anxiolytic effects (Sakharkar et al. 2014). However, our data adds to the growing body of literature highlighting the unique effects of combined nicotine and ethanol on both behavior and neurochemistry (Cross et al. 2017), and further underline the importance of including age comparisons in experimental studies.

Regional neuronal activity in response to acute Nic+EtOH

We also assessed how concurrent nicotine and ethanol treatment affects expression of *cFos* mRNA, an immediate early gene widely used as a marker of neuronal activity, in both adolescents and adults (Flavell and Greenberg 2008). For the majority of the brain regions analyzed, the data did not support our hypothesis that Nic+EtOH would have differential effects on *cFos* induction depending on age. Instead, adolescents had lower baseline *cFos* mRNA expression in the BNST, SN, IPN, MR, and CA2, but higher *cFos* expression in the mPOA, independent of drug treatment, suggesting maturational changes in the activity of these brain areas. Furthermore, neuronal activity was altered by Nic+EtOH at *both* ages in the NAc-core, thalamus, amygdala, PAG, DR, CA1, and CA2.

Interactions between age and drug treatment on regional *cFos* induction were limited, with Nic+EtOH decreasing neuronal activity in the NAc-shell, aVTA, LO, and CA3 of adults, but not adolescents. These adult-specific alterations in neuronal activity contrast with prior studies of nicotine treatment alone, in which *cFos* expression has been shown to be increased in adolescents more than adults in reward-related brain areas, including the NAc, BNST, amygdala, and VTA (Shram et al. 2007; Dao et al. 2011). Acute ethanol, on the other hand, induces similar regional *cFos* expression in both adolescents and adults (Faria et al. 2008), and has been shown to attenuate nicotine's effects on IEG induction in adult mice (Bachtell and Ryabinin 2001).

Of note, however, Nic+EtOH increased *cFos* mRNA expression in the adult pVTA, a region highly implicated in drug reinforcement (Hendrickson et al. 2010; Li et al. 2011; Zhao-Shea et al. 2011; Guan et al. 2012; Sanchez-Catalan et al. 2014), that may act as a key locus for nicotine and alcohol's effects on motivated behavior (Lárraga et al. 2017). Our finding that *cFos* expression in adults was increased by low dose Nic+EtOH in the pVTA, but decreased in its major target region, the NAc-shell (Ikemoto et al. 1997; Ikemoto 2007), without significant changes in adolescents, suggests that maturational changes occur in these regions during adolescence. Indeed, very little is known about adolescent development of the pVTA. Given its important role in modulating nicotine and alcohol responses, more research is needed to better understand how the pVTA matures during adolescence.

Functional connectivity is age-dependently influenced by Nic+EtOH exposure

Discrete brain regions can coordinate activity with one another to form functional networks, or functional connectivity, even in the absence of direct anatomical connections (Sporns and Honey 2006; Rubinov and Sporns 2010). Functional connectivity, therefore, represents deviation from statistical independence between brain regions (Sporns 2002), and connectivity changes during the course of adolescence (Crews et al. 2007; Fair et al. 2009; Hwang et al. 2010; Satterthwaite et al. 2013). Methods in human imaging (e.g., fMRI, PET, EEG) that are typically used for functional network analysis are difficult to use in rodents (Liang et al. 2013). Instead, measurement of immediate early gene expression (Pevzner et al. 2012; Dwyer and Leslie 2016; Ruiz et al. 2020) or cytochrome oxidase activity (Conejo et al. 2010; González-Pardo et al. 2012) allows for high spatial resolution readouts of neuronal activity and functional connectivity. Although studies are limited, these have provided evidence for developmental- and drug-related alterations in functional connectivity in the rodent brain.

In the present study, we used *cFos* as the readout of functional network activity. In comparing baseline networks of adolescents and adults, we found unique community structures and hubs, as well as significantly fewer functional relationships, in adolescents compared to adults. This was unexpected given previous work from our lab (Dwyer and Leslie 2016) and human imaging data (Stevens et al. 2009) suggesting that functional connectivity shifts from local, highly coordinated networks in adolescence to more concentrated and efficient networks in adulthood. However, adults did have significantly higher clustering coefficients (p<0.001), a measure of functional segregation, or the ability for specialized processing to take place among interconnected groups of brain regions

(Rubinov and Sporns 2010). Global efficiency, a measure of functional integration, was not significantly different between adolescents and adults. Together, our findings demonstrate developmental shifts in functional network composition and properties.

Although there were few age and drug interactions in *cFos* mRNA expression in discrete brain areas, we observed robust, age-dependent alterations in functional networks after acute, low-dose Nic+EtOH exposure. The number of functional relationships was decreased by Nic+EtOH in adolescents, but not adults. In adolescents, the predominant effect of drug exposure was a loss of positive connectivity between the pVTA and striatal nuclei, and between IPN/MR and hippocampus. Adults, on the other hand, exhibited a considerable loss of positive intra-striatal and intra-midbrain connectivity, as well as a loss of functional relationships between the PAG and striatal nuclei, and between hypothalamic and extended amygdala nuclei. There were also multiple gains of positive CGE in Nic+EtOH-treated adults, primarily between the BNST and pVTA, SN, or MR.

Of note, functional relationships with the pVTA were influenced by drug exposure at both ages, but in opposite directions. Whereas Nic+EtOH inhibited CGE between pVTA and striatal subnuclei in adolescents, it increased CGE between pVTA and LH, BNST, SN and MR in adults. These data suggest that functional networks involving the pVTA are differentially recruited by Nic+EtOH, and that this age-dependent network recruitment may modulate reinforcement and anxiety-like behavior in adolescence versus adulthood. For example, protective mechanisms, such as BNST glutamate inputs to non-dopaminergic neurons in the VTA that drive aversion and block reward (Jennings et al. 2013; Vranjkovic et al. 2017) may be recruited by Nic+EtOH exposure in adults to increase pVTA cFos expression and subsequently inhibit exploration in a novel environment and reinforcement (Lárraga et al. 2017), while they are not recruited in adolescence. Similarly, reduction of functional connectivity both within and between extended amygdala and corticostriatal regions in Nic+EtOH-treated adolescents is suggestive of maturational changes in circuitry regulating mood-associated behaviors. Indeed, anxiety-like behavior in the open-field and elevated plus maze is correlated with functional connectivity involving the prefrontal cortex, amygdala, and hippocampus in adult mice (Johnson et al. 2018; Xia and Kheirbek 2020). Thus, the low-dose Nic+EtOH exposure may have reduced connectivity in functional circuits that drive anxiety-like behaviors in the mature brain. Although future studies are needed to test these hypotheses and gain a better understanding of the circuitry mediating the effects of concurrent Nic+EtOH on anxiety-like behaviors, our data highlight the complex maturation of limbic circuitry involved in regulating the acute effects of Nic+EtOH.

Further, our findings complement recent human imaging data reporting greater and more diffuse hypoconnectivity in resting state functional networks of individuals who smoke and drink compared to smokers alone or drinkers alone (Vergara et al. 2017). The age-specific alterations in functional connectivity, and the relative insensitivity of adolescents to Nic +EtOH-induced neuronal activation in the pVTA, reported here, again highlight the importance of including age comparisons in preclinical research on nicotine and alcohol, as well as including age of initiation in clinical and epidemiological studies.

Limitations

Our study does have limitations. Although we previously demonstrated no significant sex differences in the reinforcing effects of Nic+EtOH in adolescents or adults, it is possible that other behavioral responses or neuronal responses to acute Nic+EtOH treatment differ based on sex. We also did not examine multiple Nic+EtOH doses, and it is reasonable to predict that functional connectivity could differ with a higher dose of this drug combination. Another limitation is the use of a single measure of anxiety-like behavior, and future studies should include measures such as the elevated plus maze or light-dark box to more fully assess Nic+EtOH's effect on anxiety across development. Finally, the neuronal and functional connectivity changes presented are correlational in nature. While these data suggest that particular regions (e.g., pVTA) and the functional networks containing them may influence behavioral responses to Nic+EtOH, more research is needed to establish a causal relationship.

Conclusion

In the present study, we have demonstrated unique behavioral and neuronal responses to acute Nic+EtOH treatment in adolescent and adult male rats. Analysis of drug-induced *cFos* expression in individual brain areas showed that the pVTA, a critical regulator of drug reward, is selectively activated by Nic+EtOH in adults, whereas activity in its target region, the NAc-shell, was decreased. Drug-induced alterations in CGE are more extensive in adults than adolescents, and may act to inhibit behavioral responses to Nic+EtOH in adults that are seen in adolescence. Both *cFos* expression and network analysis suggest that there may be an ongoing maturation of the pVTA and its connections during adolescence that allows increased sensitivity to the reinforcing, hyperlocomotor, and anxiolytic effects of Nic+EtOH.

Our data add to the growing body of literature illustrating the unique effects of concurrent nicotine and alcohol (Cross et al. 2017), particularly during adolescence. Whether changes in brain or behavior following Nic+EtOH exposure are long-lasting or predictive of subsequent maladaptive behaviors requires further study. However, these findings provide a potential mechanism for the high rates of co-use of nicotine and alcohol by teenagers and young adults. That is, nicotine and alcohol combinations recruit functional networks in adolescence that are unique from protective, inhibitory networks recruited in the mature, adult brain. Given recent increases in e-cigarette use among teens and its association with alcohol consumption (Hershberger and Cyders 2017; Jamal et al. 2017; Hershberger et al. 2020), these data support greater emphasis of future research, as well as policy and public health strategies, on concurrent nicotine and alcohol intake.

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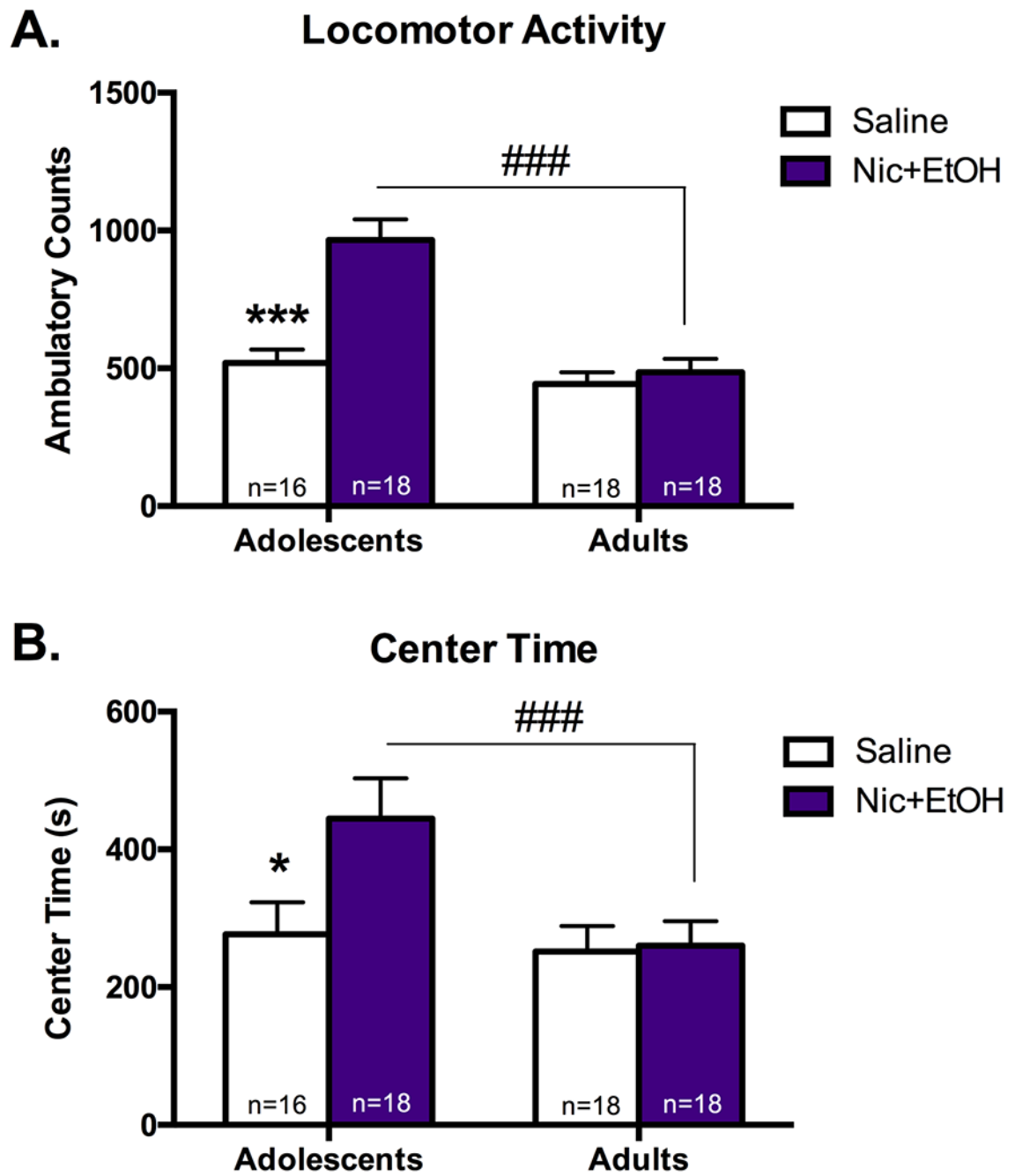
Figure 1. Nic+EtOH increases locomotor activity and center time in adolescent, but not adult, rats.
(A) Total ambulatory counts and (B) time spent in the center zone of a novel open-field chamber. *, $p<0.05$; ***, $p<0.001$ vs. adults; ###, $p<0.001$ vs. Nic+EtOH. Data represent mean + SEM. n = 16-18/group.
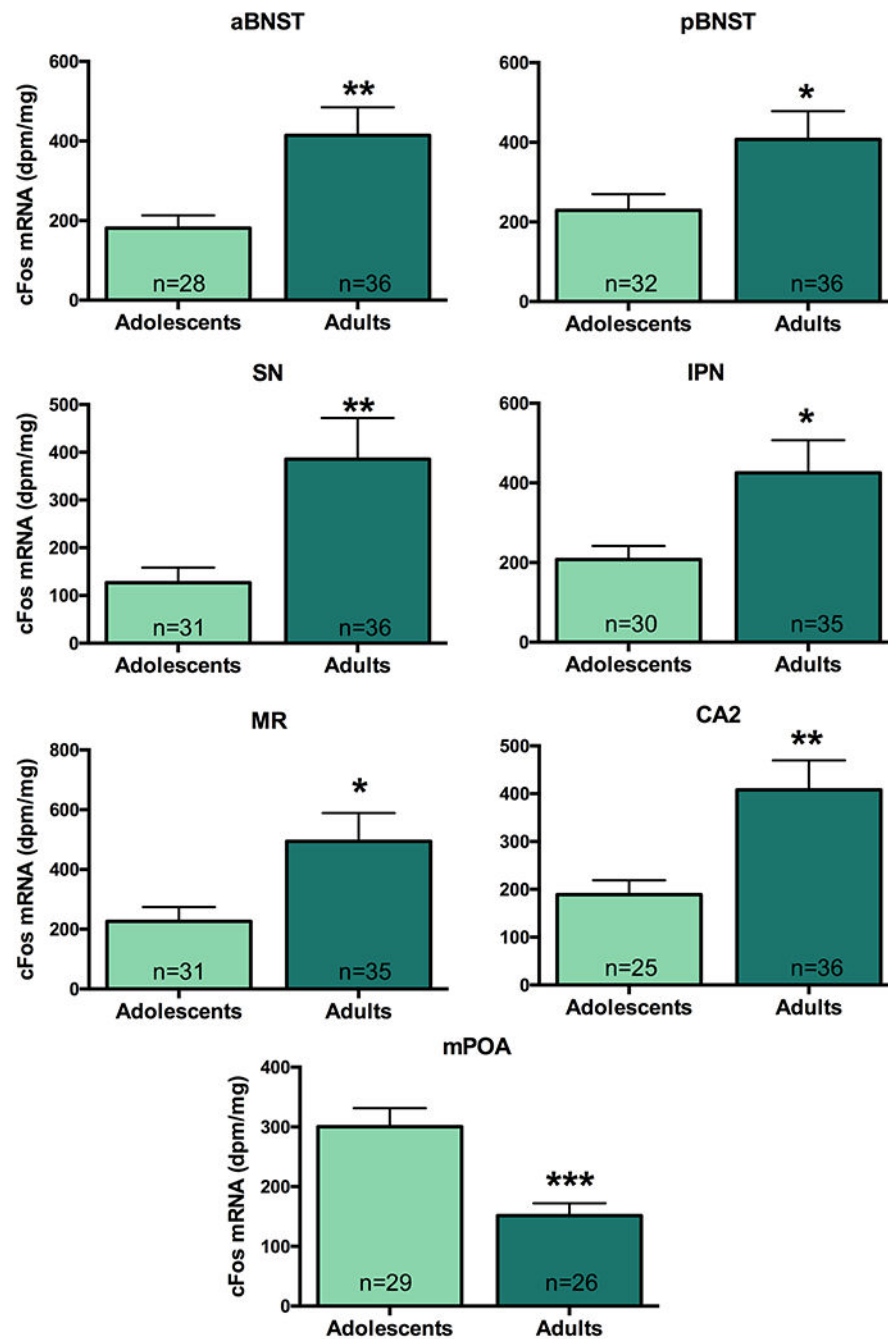
Figure 2. Age differences in regional *cFos* mRNA expression.
Adults have higher *cFos* mRNA expression in extended amygdala, midbrain, and hippocampal areas, but lower *cFos* expression in the mPOA compared to adolescents, independent of drug exposure. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs. adolescents. Data represent mean + SEM and are collapsed across drug group. n = 25-39/age.
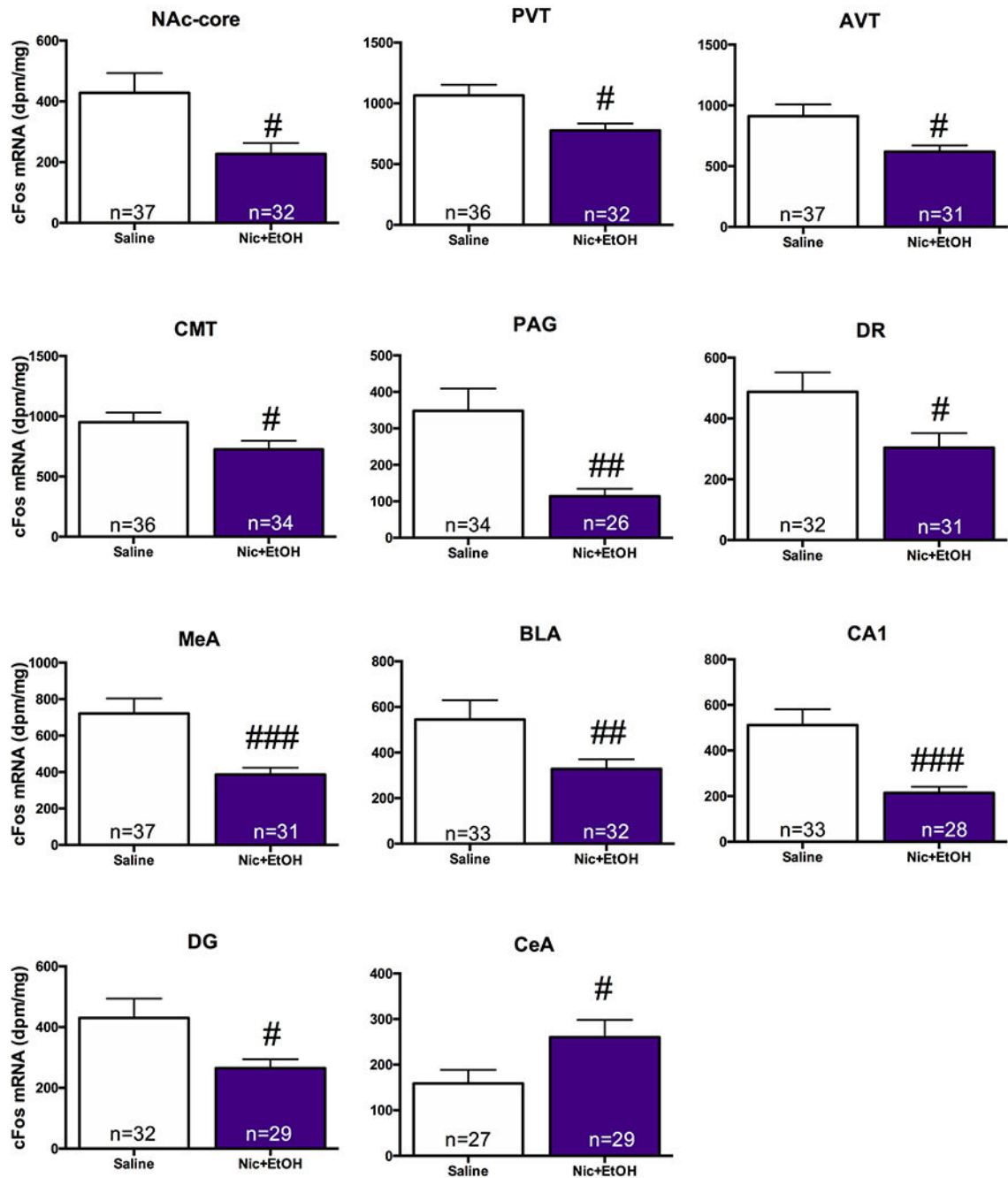
Figure 3. Drug differences in regional *cFos* mRNA expression.
Nic+EtOH had region specific effects on *cFos* mRNA expression, independent of age. Drug-induced differences in regional *cFos* mRNA expression. #, p<0.05, ##, p<0.01, ###, p<0.001 vs. saline. Data represent mean + SEM and are collapsed across age. n = 27-38/drug.
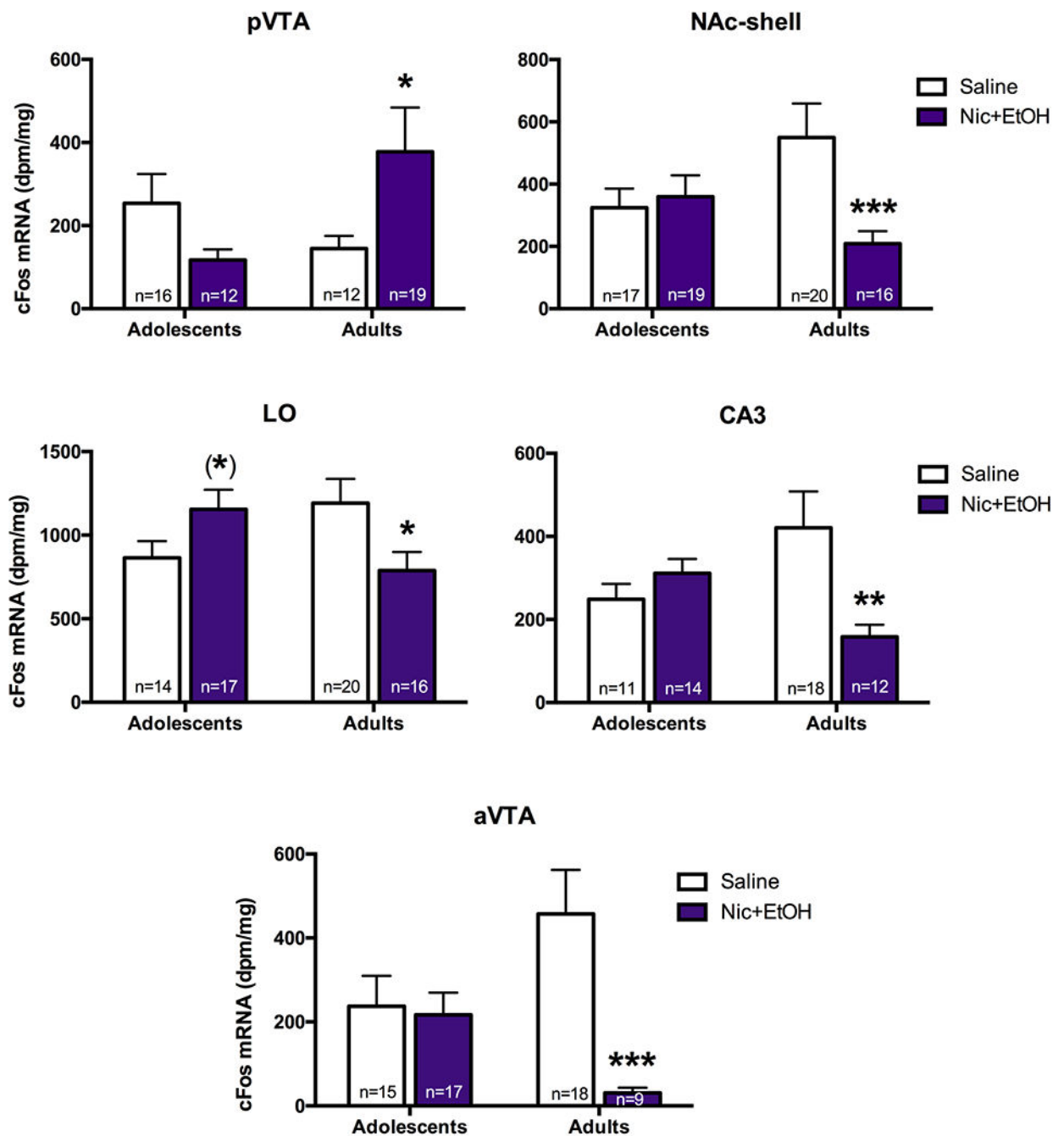
Figure 4. Age-specific effects of Nic+EtOH on regional cFos mRNA expression.
(A) Nic+EtOH increases regional neuronal activity in the pVTA of adults, but not adolescents. In the NAc-shell (B), LO (C), CA (D), and aVTA (E), Nic+EtOH decreased *cFos* mRNA expression in adults compared to saline. *, p<0.05; **, p<0.01; ***, p<0.001 vs. saline. Data represent mean + SEM. n = 12-20/group.
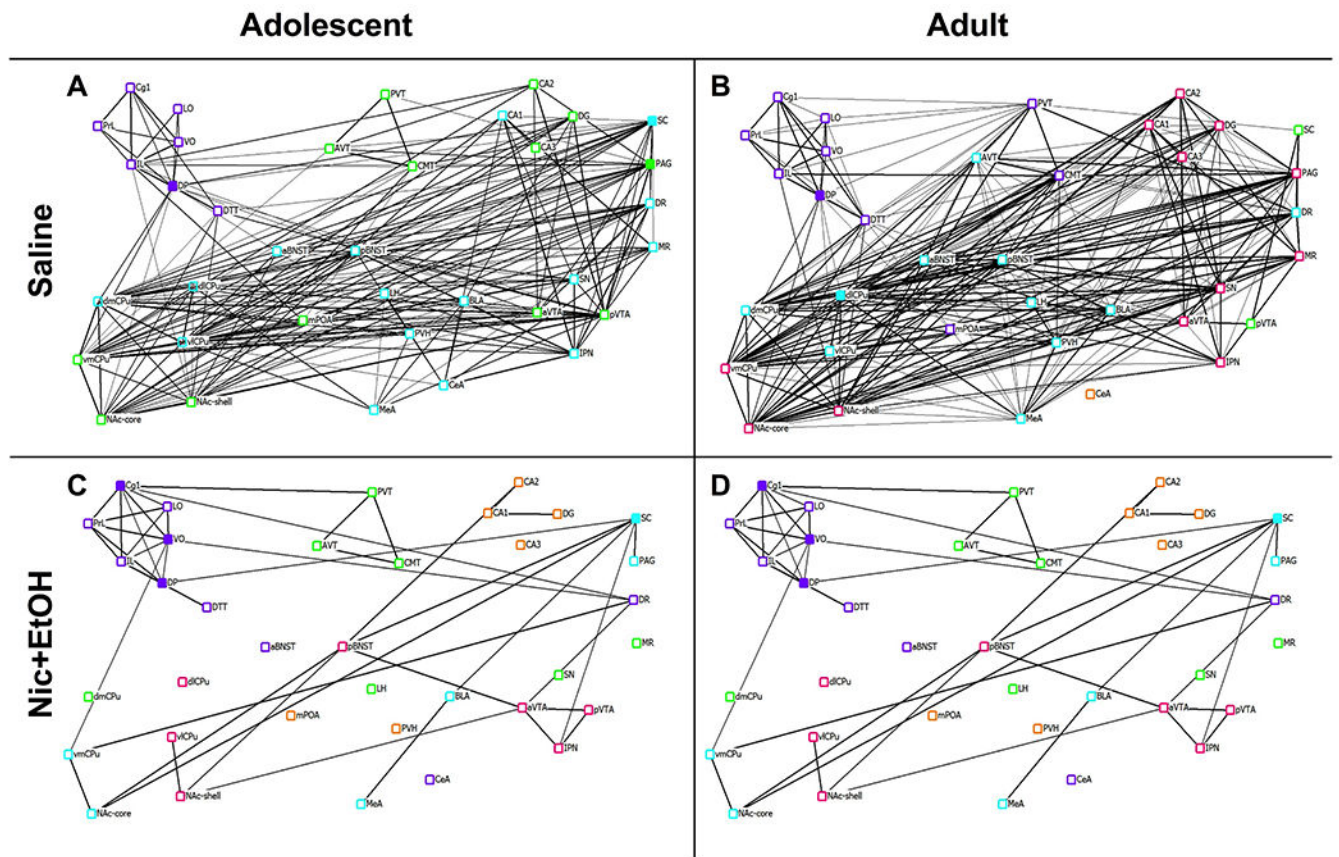
Figure 5. Baseline and Nic+EtOH-mediated functional networks.

Networks of correlated *cFos* mRNA expression in saline-treated (A, B) or Nic+EtOH-treated (C, D) adolescents and adults. Each node represents a brain region presented in pseudoanatomical space. Significant r-values (p<0.05) denoting association between two brain regions (CGE, analogous to functional connectivity) were displayed as lines (edges) connecting two brain regions (nodes). The thickness of the line denotes the strength of the r-value, while color distinguishes between r-values that were negative (red) and positive (black). Functional subcommunities are denoted by node color. Network hubs, designated based on degree and betweenness centrality, are denoted by node size and solid color fill.

Figure 6. Nic+EtOH-induced changes in functional networks.
Difference maps visually represent significant differences (corrected by false discovery rate, q=0.10) between baseline saline networks and Nic+EtOH networks in (A) adolescents and (B) adults. Each node represents a brain region presented in pseudoanatomical space. Lines indicate functional relationships between nodes that are significantly changed by Nic+EtOH treatment, where dashed red lines indicate a loss of positive *cFos* CGE and dashed black lines indicate a gain of positive *cFos* CGE.

Table 1.

Names and abbreviations of brain regions of interest

	Abbreviation	Full name		Abbreviation	Full name
Cortex	VO	Ventral orbital cortex	**Amygdala**	MeA	Medial amygdala
	LO	Lateral orbital cortex		CeA	Central amygdala
	Cg1	Cingulate cortex		BLA	Basolateral amygdala
	PrL	Prelimbic cortex	**Thalamus**	PVT	Paraventricular nucleus of the thalamus
	IL	Infralimbic cortex		AVT	Anteroventral nucleus of the thalamus
	DP	Dorsal peduncular cortex		CMT	Centromedial nucleus of the thalamus
	DTT	Dorsal tenia tecta	**Midbrain**	aVTA	Anterior ventral tegmental area
Striatum	NAc-core	Nucleus accumbens core		pVTA	Posterior ventral tegmental area
	NAc-shell	Nucleus accumbens shell		SN	Substantia nigra
	dmCPu	Dorsomedial caudate putamen		IPN	Interpeduncular nucleus
	dlCPu	Dorsolateral caudate putamen		PAG	Periaqueductal gray
	vmCPu	Ventromedial caudate putamen		SC	Superior colliculus
	vlCPu	Ventrolateral caudate putamen		DR	Dorsal raphe nucleus
Stress nuclei	aBNST	Anterior bed nucleus of the stria terminalis		MR	Median raphe nucleus
	pBNST	Posterior bed nucleus of the stria terminalis	**Hippocampus**	CA1	CA1
	PVH	Paraventricular nucleus of the hypothalamus		CA2	CA2
	LH	Lateral hypothalamus		CA3	CA3
	mPOA	Medial preoptic area		DG	Dentate gyrus

Table 2.

Network hub analysis

Drug	Age	Region	k #SDs	Bc #SDs
Saline	**P32**	PAG		2.72
		SC		2.55
		DP		2.43
	P90	DP		3.17
		dlCPu		2.70
Nic+EtOH	**P32**	SC		3.47
		DP		2.48
		Cg1	2.04	2.04
		VO	2.04	
	P90	VO		3.11
		DP		2.74
		BLA		2.54
		CA2		2.16

Nodes are designated as network hubs if its degree (k) and/or betweenness centrality (Bc) value was at least two standard deviations (SDs) from the network mean.