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Peri-adolescent alcohol consumption increases sensitivity and dopaminergic response to nicotine during adulthood in female alcohol-preferring (P) rats: alterations to α7 nicotinic acetylcholine receptor expression

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Abstract

Adolescent alcohol drinking has been linked to increased risk for drug abuse during adulthood. Nicotine microinjected directly into the posterior ventral tegmental area (pVTA) stimulates dopamine (DA) release in the nucleus accumbens (NAc) shell. The α7 nicotinic acetylcholine receptor (nAChR) is a potent regulator of dopaminergic activity in the pVTA. The current experiments examined the effects of peri-adolescent ethanol (EtOH) drinking on the ability of intra-pVTA nicotine to stimulate DA release during adulthood and alterations in α7 nAChR expression within the pVTA. Alcohol-preferring (P) female rats consumed EtOH and/or water during adolescence (post-natal day [PND] 30–60) or adulthood (PND 90–120). Thirty days following removal of EtOH, subjects received microinjections of 1 μM, 10 μM, or 50 μM nicotine into the pVTA concurrently with microdialysis for extracellular DA in the NAc shell. Brains were harvested from an additional cohort after PND 90 for quantification of α7 nAChR within the pVTA. The results indicated that only adolescent EtOH consumption produced a leftward and upward shift in the dose response curve for nicotine to stimulate DA release in the NAc shell. Investigation of α7 nAChR expression within the pVTA revealed a significant increase in animals

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Authors Contribution

ZAR, WJM, and RAW were responsible for the study concept and design. RAW, CPK, and GBC acquired the data. ZAR and RAW performed the data analysis. RAW drafted the manuscript. ZAR, RAW, SRH, EAE, and RLB assisted with interpretation of findings and provided critical revisions for important intellectual content. All authors reviewed the content and approved the final version for publication.

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that consumed EtOH during adolescence compared to naïve animals. The data suggests that peri-adolescent EtOH consumption produced cross-sensitization to the effects of nicotine during adulthood. The interaction between adolescent EtOH consumption and inflated adult risk for drug dependency could be predicated, at least in part, upon alterations in α7 nAChR expression within the mesolimbic reward pathway.

Keywords

α7 nicotinic acetylcholine receptor; adolescent alcohol; ventral tegmental area; dopamine; mesolimbic; nicotine

INTRODUCTION

The prevalence of alcohol (EtOH) drinking in adolescence is high in the United States with 80-90% having consumed EtOH before graduating high school (Johnston et al. 2004). This includes 58% of 12th graders reporting the use of EtOH within the past year and 28% of which engaged in binge drinking within the previous 2 weeks (i.e. > 5 consecutive drinks per drinking episode; Miech et al. 2016; Johnston et al. 2004). Epidemiological data suggests that early initiation of EtOH consumption is associated with a greater risk of developing an alcohol use disorder (AUD) during adulthood (Dawson et al. 2008). Additionally, adolescent EtOH drinking has been linked to increased adulthood use of opioids, cannabis, and other drugs of abuse (Anthony & Petronis 1995). Specifically for nicotine, adolescent binge drinking enhances the likelihood of smoking during adolescence by 88% as well as during adulthood (Best et al. 2000). Conversely, individuals who do not engage in binge drinking have lower rates of smoking during adolescence and adulthood (Bobo & Husten 2000).

Adolescence is a gradual period of transition characterized by widespread neuronal remodeling eventually resulting in the mature adult brain (Spear 2000). Studies of the adolescent brain have consistently demonstrated that numerous regions continue to undergo significant development including the refinement of several neurotransmitter systems. Maturation of cortical and limbic regions during adolescence is marked by excess production of neuronal networks later followed by extensive pruning (Spear 2000; Brenhouse & Andersen 2011).

Dopamine (DA) neurons of the ventral tegmental area (VTA) project to the dorsal striatum, nucleus accumbens (NAc; ventral striatum), amygdala, and prefrontal cortex (PFC). The number of connections, activation of these projections, and firing rate of DA neurons in the VTA reaches its peak during peri-adolescence (McCutcheon et al. 2012; Marinelli & McCutcheon 2014). Moreover, studies in both humans and animals have found mesolimbic and mesocortical DA projections have a heightened sensitivity to reward-related events in the peri-adolescent brain (Hoogendam et al. 2013). Thus, the reorganization and subsequent pruning of connections within this system during adolescence is associated with maturation of the reward pathway with altered excitatory afferents into the VTA. Glutamatergic inputs from the PFC to the VTA are pruned during adolescence, which is associated with a reduction in glutamate receptors (Brenhouse & Andersen 2011; Spear & Swartzwelder 2014). Relevant to the current study, α7 nicotinic acetylcholine receptors (nAChR) are

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located presynaptically on glutamatergic inputs into the VTA, on GABA interneurons in the VTA, and on VTA DA neurons (Jones & Wonnacot 2004).

Alcohol drinking during adolescence affects development through alterations to ongoing neuronal remodeling processes (Spear & Swartzwelder 2014). Lasting alterations found within the mesolimbic system of the adult brain resulting from adolescent EtOH exposure involves (i) reduced cholinergic regulation, (ii) elevated DA stimulation and release, as well as (iii) epigenetic reprogramming (Vetreno et al. 2014; Sahr et al. 2004; Toalston et al. 2014; Kyzar et al. 2019). A number of studies have investigated how these changes affect the response to ethanol (EtOH) in adulthood. For example, animals given voluntary access to EtOH throughout adolescence were able to acquire operant self-administration of EtOH faster and resisted extinction of self-administration compared to animals without prior EtOH exposure (Gass et al. 2014; Rodd-Henricks et al. 2002a, Rodd-Henricks et al. 2002b; Toalston at el. 2015). Adolescent EtOH drinking was also shown to enhance sensitivity to EtOH reward within the posterior VTA (Toalston et al. 2014). This was in addition to increased stimulated DA release within the NAc shell by either peripheral EtOH administration or direct VTA infusions of EtOH during adulthood (Sahr et al. 2004; Toalston et al. 2014).

Research continues to provide evidence demonstrating the influence of adolescent EtOH drinking on long-term EtOH abuse susceptibility. However, there is little preclinical research examining the consequences of adolescent EtOH consumption on responses to other drugs of abuse during adulthood. Specifically, a single study was found to have examined the effect of adolescent EtOH exposure with intragastric administration of up to 5 g/kg three times per day followed by nicotine self-administration in adulthood (Boutros et al. 2016). The limited research in this area provides the opportunity to investigate the impact of voluntary adolescent EtOH exposure on the response to nicotine within the mesolimbic system during adulthood. Additionally, lasting expression changes and the potential involvement of interaction sites for EtOH and nicotine can be explored.

Previous research has established the activation of neuronal nAChRs in the VTA is one site of action behind the effects of EtOH as well as the principal mechanism behind the psychoactive effects of nicotine (De Biasi & Dani 2011; Doyon et al. 2013). These receptors are part of the Cys-loop ligand-gated ion channel superfamily and composed of five subunits. The subunits assemble in various combinations to make up specific nAChR subtypes with unique channel properties, desensitization, and agonist affinity. The various subunits can assemble into hetero- or homopentamers that are expressed on DA, glutamate, and GABA neurons within the VTA (Morel et al. 2018). The α4β2* nAChR (asterisk indicates other potential nAChR subunits) is the most abundant within the VTA, is highly sensitive to ACh and nicotine, but is rapidly desensitized. The α7-homomeric nAChR has been found to desensitize at a slower rate due to a decreased affinity for ACh and is predominately localized to glutamatergic terminals from the PFC that synapse on DA neurons. Previous work has demonstrated both of these nAChR subtypes have significantly altered expression levels following adolescent EtOH exposure and contribute to increased DA release by alcohol and nicotine (Morel et al. 2018; Hauser et al. 2019). Overall, evidence demonstrates significant perturbations in both the dopaminergic and cholinergic systems

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following adolescent EtOH exposure (Vetreno et al. 2018). These long-lasting changes within the mesolimbic circuitry likely alter reward processing and response providing some biological explanation for an increased adult risk for nicotine use.

The present study aimed to determine the long-term effects of voluntary peri-adolescent EtOH consumption on the action of nicotine within the mesolimbic DA system during adulthood. The first experiment determined whether a history of adolescent EtOH drinking produced persistent neuroadaptations in VTA DA neurons. This was tested by examining the ability of intra-VTA nicotine to stimulate DA release in the NAc shell. This was followed by an examination of α7 nAChR levels within the posterior VTA as an indicator of significant and lasting changes to the cholinergic system, following adolescent EtOH consumption.

MATERIALS & METHODS

Subjects.

Female adolescent alcohol-preferring (P) rats from the 79th—85th generations at Indiana University School of Medicine (IUSM; Indianapolis, IN) were utilized in the current study. In order to build on previously published work, as well as the ability to maintain body weight throughout the duration of the study, the following experiments were carried out only in female rats (Ding et al. 2009; Toalston et al. 2014, 2015; Hauser et al. 2019; Waeiss et al. 2019). The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All research protocols were approved by the IUSM Institutional Animal Care and Use Committee and followed the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (NRCC. 2011).

Peri-adolescent & adult EtOH consumption procedure.

The peri-adolescent EtOH drinking model utilized in the present study was carried out in a similar fashion to those published previously (Toalston et al. 2014, 2015; Waeiss et al. 2019). Beginning on PND 28, P female pups were double-housed in plastic shoebox cages. Rats were maintained on a 12-hour reverse light cycle with lights off at 0900. Food and water were available *ad libitum* to all animals throughout the experiment. Ethyl alcohol (95%; McCormick Distilling Co., Weston, MO) was diluted to 15% and 30% (v/v). Rats were provided 24-hour access to water and both EtOH concentrations in the 3-bottle choice (3BC) paradigm beginning on PND 30 until PND 60. A second cohort of P rats, housed under identical conditions, did not receive access to EtOH during this period. Fluid weights were recorded daily while body weights were measured every other day. After PND 60, all animals remained pair housed in plastic shoebox cages without access to EtOH through at least PND 90.

A third cohort of adult female P rats underwent the same drinking procedure. Briefly, beginning at PND 90 the animals were double-housed in plastic shoebox cages. At this time, animals were provided continuous access to water, 15%, and 30% EtOH (v/v) through PND 120. All adult animals were then maintained in plastic shoebox cages while pair housed until at least PND 150. It would be redundant to have both naïve adolescent and adult water

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drinking control groups since the animals would have the same experience. Therefore, the total number of animals in the experiments was reduced by utilizing a single water control group.

Microinjection-Microdialysis Protocol.

The microinjection-microdialysis (micro-micro) procedure was carried out as described previously (Ding et al. 2009; Toalston et al. 2014; Deehan et al. 2018). Given the global effects of adolescent EtOH drinking, the micro-micro procedure allows specific investigations of whether EtOH induced neuroadaptations in the pVTA modulate the activity and DA response to nicotine within the mesolimbic system. Following PND 90 for the adolescent cohorts or PND 150 for the adult cohort, rats were stereotaxically implanted with two ipsilateral guide cannulas in the right hemisphere. While under isoflurane anesthesia, a 22-guage microinjection cannula (Plastics One, Inc., Roanoke, VA) was aimed 1.0 mm above the posterior ventral tegmental area (pVTA). An 18-guage microdialysis cannula was also implanted 3.0 mm above the NAc shell. The coordinates for the pVTA were AP −5.6 mm, ML +2.1 mm, DV −8.0 mm and those for the NAc shell were +1.5 mm, ML +2.0 mm, DV −5.3 mm (Paxinos & Watson 1998). Both cannulas were implanted at a 10° angle and protected with stylets until the micro-micro stages of these experiments. Following surgery, animals were single housed in shoebox cages and allowed at least one week of recovery. During this time, the animals were habituated to the experimental housing and handled daily.

Loop-style microdialysis probes were constructed as previously described (Engleman et al. 2000; Ding et al. 2009; Toalston et al. 2014). Probes were manufactured with an active length of 2.0 mm from regenerated cellulose Spectra/Por (Spectrum Laboratories, Inc., Rancho Dominguez, CA) hollow fiber tubing with an inner diameter of 200 μm and molecular weight cut-off of 13 kDa. One day prior to testing, animals were placed under isoflurane anesthesia and the microdialysis probes were inserted into the NAc shell by extending 3.0 mm below the guide cannula. The micro-micro procedure was carried out the following day.

Experiments were performed in awake freely moving animals. Subjects were placed in the experimental chambers and the microdialysis probes were connected to a Harvard pump (Harvard Apparatus, Holliston, MA) used to continuously perfuse the NAc shell with artificial cerebrospinal fluid (aCSF) at a rate of 1 μL/minute throughout the experiment. Microdialysis aCSF was composed of 140.0 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 2.0 mM Na_2HPO_4 • 7H2O, and 1.0 mM $MgCl_2$ with a pH 7.2 to 7.4. Following a 90-minute washout period, samples were collected in 20-minute intervals beginning with five baseline samples.

Next, rats received a single microinjection challenge directly into the pVTA of either aCSF, 1 μM nicotine, 10 μM nicotine, or 50 μM nicotine solutions. Nicotine HCl (Sigma-Aldrich, St. Louis, MO) concentrations were calculated based on the salt-form. Nicotine concentrations known to be reinforcing and stimulate DA release were selected based on previous intracranial self-administration (ICSA) and micro-micro studies within the pVTA (Hauser et al. 2014a, b; Truitt et al. 2015; Deehan et al. 2018). Importantly, the *in vivo* concentration of nicotine within the pVTA following microinjection is not known but is

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expected to be lower than that emitted due to local metabolism and diffusion during the session. Although intra-pVTA injections produce distinct spatial and temporal gradients not experienced in smokers, nicotine concentrations likely approach what was observed in previous experiments investigating peripheral treatment or self-administration in P rats (27 ng/ml; Hauser et al. 2012; Katner et al. 2015). Passive microinjections were carried out with an electrolytic microinfusion transducer (EMIT) system as described previously (Gatto et al. 1994; Rodd-Henricks et al. 2000; Ding et al. 2009). Briefly, subjects received 30 pulse injections over a 10-minute period designed to simulate average intracranial self-administration (ICSA) levels (Rodd-Henricks et al. 2000; Toalston et al 2014). Each infusion injected 100 nl of solution over 5 seconds and was followed by a 15-second timeout period for a total of 3 μl over the 10-minute infusion session. After the microinjection challenge, six additional 20-minute samples of dialysate samples were collected into tubes containing 5 μl of 0.1 N perchloric acid. All samples were immediately frozen on dry ice and stored at −80 °C until analysis for DA content with high performance liquid chromatography (HPLC).

The present microdialysis study utilized 85 female P rats. Subjects were randomly assigned to one of four microinjection conditions with n = 8–9/group of naïve, n = 7–9/group adolescent EtOH drinkers, and n = 4–6/group of adult EtOH drinkers.

Dialysate Analysis.

DA was analyzed using a reversed phase HPLC system with electrochemical detection (Engleman et al. 2000; Ding et al. 2009; Toalston et al. 2014). Samples were loaded into a 10 μl loop and injected onto an analytical column (BDS Hypersil C18, 3 μm, 150 mm × 2.1 mm, Thermo Fisher Scientific, Waltham, MA). Mobile phase consisted of 0.1 mM EDTA, 8 mM KCl, 50 mM phosphoric acid, 100 mg/L OSA, and 10% MeOH with a pH of 6.0. DA detection occurred with a glassy-carbon electrode and an amperometric detector with the oxidation potential set at 350 mV and sensitivity of 100 pA/V (Decade II EC Detector, Antec Scientific, Netherlands). Signal analysis was carried out with a ChromePerfect (Justice Innovations, Inc., Palo Alto, CA) data station. DA content was then determined through comparison to a standard curve generated from 1 nM, 5 nM, and 10 nM DA solutions.

Histology.

Once the experiments were completed, subjects were euthanized and a solution of 1% bromophenol blue was infused into each microdialysis probe and microinjection cannula. Brains were removed, immediately frozen on dry ice, and stored at −80 °C. Forty-micron sections were then collected with a cryostat microtome for verification of microdialysis probe and microinjection placements.

Immunohistochemistry.

On PND 90, naïve rats (n = 6) and adolescent EtOH drinkers (n = 7) were deeply anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) in PBS. Perfused brains were then removed and post-fixed overnight in 4% PFA at 4 °C. Next, brains were transferred to 30% sucrose in PBS and stored at 4 °C until processed. Serial coronal sections were collected at a

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thickness of 30 μm using a freezing microtome, immediately placed in a cryoprotectant solution, and stored at −20 °C until processing. Based on results from the micro-micro experiment demonstrating a similar response between adult EtOH drinkers and naïve rats, adult EtOH drinkers were not included in the immunohistochemical analysis to reduce the total number of animals used in the present study.

Immunohistochemistry was carried out as described previously (Johnson et al. 2015). Free-floating sections containing the VTA were initially washed with PBS for 30 minutes at room temperature prior to incubation in 1% H_2O_2 in PBS for 20 minutes. Sections were then washed in PBS for 10 minutes and PBS with 0.3% Triton X-100 (PBST) for an additional 20 minutes. Sections were then incubated overnight for 12-16 hours in PBST containing mouse anti-α7 nAChR subunit monoclonal antibody (1:100; Catalog # 836701, BioLegend, San Diego, CA) at room temperature. Following a 30-minute wash in PBST, sections were incubated in biotinylated horse anti-mouse IgG secondary antibody (1:500; Catalog # BA-2000, Vector Laboratories, Burlinghame, CA) for 2 hours. Sections were again washed in PBST for 30 minutes and incubated for 1.5 hours with an avidin–biotin–horseradish peroxidase complex provided in a standard Vector Elite kit (1:1000; Catalog # PK-6100, Vector Laboratories). The resulting peroxidase complexes were visualized by exposure for 10 minutes to a chromogen solution containing 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO) to produce a dark brown reaction product for α7 nAChR subunit staining. Repeated washing in 0.1 M PB was used to terminate the reaction. Sections were then mounted on clean glass slides, dried overnight, dehydrated, and coverslipped with DPX (Electron Microscopy Sciences, Fort Washington, PA).

Due to known concerns with antibodies directed toward α7 nAChRs, steps were taken to determine specificity (Garg & Loring 2017). An experiment to determine non-specific binding was carried out on separate tissue sections by applying the above-mentioned procedure and substituting the primary antibody with an isotype control (IgG1). Another round of immunohistochemistry was conducted with the omission of the primary antibody to determine the contribution of other components to background staining. These experiments (data not shown), as well as those conducted by other research groups, have determined there was relatively little non-specific binding and likely would not influence the analysis (Dominguez del Toro et al. 1994; Mielke & Mealing 2009; Garg & Loring 2017). Additionally, Western blotting was conducted with a different α7 nAChR antibody as a complementary technique to increase confidence in the immunohistochemical findings. Finally, orthogonal data recently published by our group indicate similar alterations to α7 nAChRs following EtOH exposure with TaqMan low-density array (TLDA) cards for real-time PCR (Hauser et al. 2019).

Sections were analyzed by an observer blind to the treatment conditions and the number of α7 nAChR subunit-immunoreactive (α7-IR) cells were counted. Only tissue sections containing the pVTA, located between −5.2 mm bregma and −6.2 mm bregma, were included in the analysis (Paxinos and Watson, 1998). Overall, four to seven sections per brain were analyzed. The total number of cells counted per section was divided by the total volume of each section. Total volumes from the sections counted for the pVTA were

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calculated by multiplying the sum of area, determined with ImageJ analysis software (NIH, Bethesda, MD), by a depth of 19 μm. Data are presented as number of cells per mm^3.

Protein quantification, SDS-PAGE, and Western blotting.

On PND 90, naïve rats (n = 6) and adolescent EtOH drinkers (n = 6) were deeply anesthetized with isoflurane and rapidly decapitated. Brains were quickly removed, flash frozen in isopentane on dry ice, and stored at −80 °C until processed. Serial coronal sections were then collected at a thickness of 300 μm with a freezing microtome. Micro-punches containing the pVTA were obtained using a 1 mm diameter Harris micro-punch (Electron Microscopy Sciences, Hatfield, PA) as described previously (McBride et al. 2009). The tissue was immediately homogenized in 150 μl of ice-cold N-PER lysis buffer supplemented with Halt Protease inhibitor cocktail (Thermo Scientific, Waltham, MA). At 4 °C, samples were incubated in the lysis buffer on a nutator for 2 hours followed by centrifugation at 10,000 × g for 20 minutes. Supernatants were removed and protein concentrations were measured by the bicinchoninic acid assay (Pierce, Rockford, IL). Protein concentrations were determined with 10 μl of lysate and 200 μl of working reagent at an absorbance of 570 nM with a microplate reader (Bio-Rad, Hercules, CA). All samples were analyzed in duplicates and absorbance values averaged. Adult EtOH drinkers were not included based on neurochemical results from the micro-micro experiment.

Approximately 10 μg of processed tissue protein was loaded per lane and separated on 7.5% polyacrylamide denaturing gels (Bio-Rad, Hercules, CA). This was followed by transfer to nitrocellulose membranes (Amersham, Pittsburgh, PA). Membranes were blocked with 5% milk in TBST buffer (50 mM Tris-Cl, pH 7.6; 150 mM NaCl; 0.1% Tween 20) overnight at 4 °C. Next, membranes were incubated for 2 hours at room temperature with rabbit anti-α7 nAChR subunit (1:10,000; ab10096, Abcam, Cambridge, UK) and mouse anti-GAPDH (1:100,000; Catalog # MA5-15738, Thermo Scientific, Waltham, MA). Secondary antibodies were HRP conjugated goat anti-mouse (1:20,000; Catalog # 31430, Invitrogen, Carlsbad, CA) or HRP-conjugated goat anti-rabbit (1:20,000; ab205718, Abcam, Cambridge, UK). Protein band signals were visualized by adding chemiluminescent HRP substrate reagent (Millipore, Billerica, MA). Films were scanned and densitometry was performed with ImageJ software (NIH, Bethesda, MD).

The second α7 nAChR antibody, ab10096, was also scrutinized for specificity. First, ab10096 was preincubated with a synthetic blocking peptide (ab101467, Abcam, Cambridge, UK) corresponding to a region of the human α7 nAChR subunit, and the ab10096 antigen, in a ratio of 1:5 overnight at 4 °C prior to immunoblotting. Next, in order to exclude the possibility of non-specific interactions with other rat antigens, a comparison was made using liver tissue, which has been determined to express negligible levels of α7 nAChR (Mielke & Mealing 2009; Fagerberg et al. 2014). Finally, omitting ab10096 from the Western blotting procedure was also carried out to substantiate specificity. In each of the three control experiments described above, bands at the expected molecular weight were faint or not detected.

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Statistical Analysis.

The average EtOH intake of adolescent drinkers and adult drinkers was calculated based on the last 10 sessions of the 30-day drinking procedure. Contrasting differences between adolescent and adult EtOH consumption was examined with an overall mixed factor ANOVA with between subject factor of 'Age' and a within subject factor of 'Day'. Individual "Day' differences were determined with an unpaired Student's *t*-test ($p < 0.05$). Microdialysis data are expressed as a percentage of basal DA values to correct for subject-to-subject variability (Engleman et al. 2000). Basal dialysate DA values were calculated as the mean of three baseline samples collected prior to the challenge microinjection. The effects of pVTA nicotine microinjections on extracellular DA were analyzed using a Time × Drinking Condition × Nicotine mixed analysis of variance (ANOVA). Post-hoc comparisons used to determine significance were Tukey's b for between group differences or Student's two-tailed *t* for within group differences. Immunohistochemical data were analyzed with the mean number of α7-IR cells per mm^3 calculated from the naïve treatment group and adolescent EtOH drinkers. These values were then compared using a univariate ANOVA ($p < 0.05$). Western blot densitometric signals were normalized to GAPDH. Analysis of signals were then also compared using a univariate ANOVA ($p < 0.05$).

RESULTS

Average daily EtOH intake over the final 10 drinking days were 10.2 ± 0.6 g/kg/day for adolescent drinkers and 10.6 ± 1.2 g/kg/day for adult drinkers (Fig. 1). Analysis revealed that the average EtOH consumption was not significantly different between adolescent drinkers and adult drinkers ($F_{1,53} = 0.47$, $p = 0.50$).

The mean DA levels in dialysate samples from the NAc shell were 1.22 ± 0.12 nM for the naïve group, 1.28 ± 0.11 nM for adolescent drinkers, and 1.17 ± 0.18 nM for adult drinkers. The basal DA levels in dialysis samples are strongly influenced by the rate of recovery through the microdialysis probe. An accurate assessment of extracellular DA levels with microdialysis can only be determined by a quantitative method such as with the No-Net-Flux protocol.

An initial area under the curve (AUC) analysis of DA levels determined whether there were general effects of Drinking History or a dose-response to nicotine over the duration of the experiment. AUC is a summary analysis that provides a definite integral of a value across time to generate a quantitative value. The analysis indicated a significant Drinking History × Nicotine interaction term ($F_{6,73} = 7.11$; $p < 0.001$; Fig. 2). Reducing the significant 2-way interaction by holding Nicotine constant indicated there were no significant group differences following microinjection of aCSF into the pVTA ($F_{2,18} = 0.56$; $p = 0.945$). However, there were significant group differences in animals treated with 1 μM, 10 μM, or 50 μM nicotine ($F_{2,18}$ values > 15.77; p values < 0.001; Fig. 2). Post-hoc comparisons with Tukey's b revealed that at all nicotine concentrations, adolescent EtOH consuming rats had a significantly greater DA response than adult EtOH drinkers or naïve rats.

A more thorough analysis that included individual time points revealed an overall significant Time × Drinking History × Nicotine interaction term ($F_{48,584} = 1.65$; $p = 0.005$; Fig. 3). The

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3-way interaction term was initially decomposed by holding Time constant. A significant Drinking History × Nicotine interaction was found for both the first ($F_{6,73} = 2.84$; $p = 0.015$) and second ($F_{6,73} = 4.00$; $p = 0.002$) samples collected following nicotine microinjection. To determine the effect of Drinking History on the ability of nicotine microinjected into the pVTA to stimulate DA release in the NAc shell at each time point was derived by reducing the significant 2-way interaction term by holding Nicotine constant. There were no significant group differences during the first and second sample period following treatment with aCSF ($F_{2,18} < 1.69$; p values > 0.21). In animals treated with 1 μM, 10 μM, or 50 μM nicotine there were significant group differences during the first and second sample period following microinjection ($F_{2,18}$ values > 7.24; p values < 0.01). Tukey's b post-hoc comparisons revealed that all nicotine concentrations and both sample time points, adolescent EtOH consuming rats had significantly higher levels of DA than adult EtOH drinkers or naïve rats.

The overall 3-way interaction term was also reduced by holding Drinking History constant. For the naïve animals, individual ANOVAs revealed a significant effect of nicotine concentration during the first three samples following microinjection ($F_{3,28}$ values > 2.99; p values < 0.05; Fig. 3). During the first and second samples following pVTA microinjection, Tukey's b post-hoc indicated DA levels in the NAc shell were significantly greater in naïve rats administered 10 and 50 μM nicotine compared to aCSF. During the third sample, DA levels remained significantly elevated in the NAc shell following administration of 50 μM nicotine compared to animals receiving 1 μM nicotine. Within-subject examination of individual nicotine concentrations revealed a significant effect of 50 μM nicotine ($F_{8,56} = 6.19$; $p < 0.001$). Post hoc comparisons indicated DA levels of the first post microinjection sample were significantly higher $t_7 = 5.71$, $p = 0.001$) at 162% of baseline.

Adult EtOH drinkers also exhibited a significant effect of nicotine concentration during the first sample following microinjection ($F_{3,17} = 5.96$; $p = 0.006$; Fig. 3). Adult drinkers receiving 50 μM nicotine into the pVTA produced significantly elevated DA levels in the NAc shell compared to those receiving 1 μM nicotine or aCSF. Within-subject analysis of individual nicotine concentrations revealed a significant effect of 10 μM ($F_{8,24} = 2.52$; $p = 0.038$) and 50 μM nicotine ($F_{8,40} = 2.23$; $p = 0.046$). Post hoc comparisons indicated DA levels of the first sample following 10 μM nicotine microinjection were significantly higher than baseline ($t_3 = 3.35$, $p = 0.044$). Additionally, microinjection of 50 μM nicotine resulted in significantly increased extracellular DA during the first sample to 157% compared to baseline ($t_5 = 3.01$, $p = 0.027$).

Analyses of adolescent EtOH drinkers revealed a significant effect of nicotine concentration during all six samples collected following nicotine treatment ($F_{3,28}$ values > 3.24; p values < 0.05; Fig. 3). Specifically, 1 μM, 10 μM, and 50 μM nicotine produced significantly higher DA levels during the first and second samples post microinjection compared to aCSF. The third post microinjection sample revealed NAc shell DA following 50 μM nicotine treatment was significantly higher than aCSF controls. During the fourth sample, both 10 μM and 50 μM nicotine treated animals exhibited significantly greater DA levels compared to aCSF controls. Additionally, samples five and six indicated elevated extracellular DA in adolescent drinkers that received 1 μM nicotine over aCSF microinjections. Analyses of individual

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nicotine concentrations within-subjects revealed a significant effect of 1 μM, 10 μM, and 50 μM nicotine ($F_{8,56}$ values > 6.98; $p < 0.001$). Post hoc comparisons demonstrated DA levels of samples 1 - 3 and 5 following 1 μM nicotine microinjection were significantly higher than baseline (t_8 values > 2.31, p values < 0.05). Microinjection of 10 μM nicotine resulted in significantly increased extracellular DA during the first four samples compared to baseline (t_7 values > 7.72, p values < 0.05). Lastly, adolescent drinkers that received pVTA microinjections of 50 μM nicotine had significantly greater extracellular DA during the first five samples compared to baseline levels (t_7 values > 3.80, p values < 0.01). Microinjections of 1 μM, 10 μM, and 50 μM nicotine produced maximal DA response during the second sample reaching 197%, 223%, and 232% compared to baseline levels, respectively.

Immunohistochemistry was used to assess α7-IR cells in the pVTA of adolescent EtOH drinkers and naïve rats in adulthood (>PND 90). In naïve rats and adolescent drinkers, examination of α7-IR cells revealed darkly stained cell bodies and processes (Fig. 4). Within the pVTA, α7-IR cells were significantly increased in adolescent drinkers compared to naïve animals ($F_{1,11} = 16.44$, $p = 0.002$). Adolescent EtOH drinking resulted in an increase of α7-IR cells/mm^3 to 193% relative to naïve rats (Fig. 4). Western blot analysis was then utilized to assess α7 nAChR expression in the pVTA. Relative expression levels of α7 nAChR in the pVTA are presented in Fig. 5. Adolescent EtOH consumption significantly increased α7 nAChR expression levels 33% within the pVTA compared to naïve rats ($F_{1,10} = 6.54$, $p = 0.003$).

DISCUSSION

Overall, the findings of the current study indicate that voluntary adolescent, but not equivalent adult, EtOH consumption resulted in long-lasting functional and neurochemical alterations to the mesolimbic DA system. Specifically, adolescent EtOH drinkers developed a cross-sensitization to nicotine during adulthood. Adolescent EtOH consumption produced a leftward and upward shift in the ability of nicotine microinjected into the pVTA to stimulate DA release in the NAc shell that was not present in naïve animals or adult EtOH drinkers (Figs. 2 and 3). Additionally, rats allowed to consume EtOH during adolescence had significantly elevated α7 nAChR expression in the pVTA compared to naïve rats (Figs. 4 and 5).

Overwhelmingly convergent data have indicated that adolescent EtOH exposure results in persistent alterations to the adult brain that mediates future drug responsivity and self-administration. The rewarding and reinforcing properties of EtOH and nicotine are well known to be associated with increased DA release within the NAc (Di Chiara and Imperato 1988; Wise and Rompre 1989). Furthermore, the associated reinforcing effects of pharmacologically relevant levels of EtOH and nicotine within the pVTA has also been established (Exley et al. 2011; Hauser et al. 2014; Rodd-Henricks et al. 2000; Truitt et al. 2014). The pVTA acts as a neural substrate where EtOH and nicotine are self-administered and interact to enhance DA release in the NAc shell (Tizabi et al. 2002, 2007; Rodd et al. 2010; Corrigall et al. 1994). It has been suggested that the hyperdopaminergic state induced by adolescent EtOH exposure could occur through both independent actions as well as through shared neurotransmitter systems of EtOH and nicotine. Repeated observations have

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identified neuroadaptations in the dopaminergic and cholinergic systems in adulthood following adolescent EtOH exposure (Crews et al. 2016). Alterations in DA function in adulthood is observed following voluntary consumption or experimenter administered EtOH during adolescence (Toalston et al., 2014; Sahr et al. 2004; Pascual et al. 2009).

A critical question of adolescent research is whether any observed consequence of EtOH exposure is specific to adolescence. Multiple reports now indicate that adolescent EtOH exposure produces unique adaptations in the dopaminergic system that are not observed following comparable adult consumption. Rats exposed to EtOH during adolescence expressed alterations in DRD1 and DRD2 during adulthood but adult rats treated with EtOH did not display comparable effects (Pascual et al. 2009; Coleman et al. 2011). In addition, an increased sensitivity of neuronal activity within a subset of VTA DA neurons to EtOH is observed specifically following adolescent EtOH self-administration (Avegno et al. 2016).

Importantly, it has also been demonstrated that after almost complete denervation of the NAc and the mesolimbic DA system, voluntary responding for EtOH failed to change (Rassnick et al. 1993). This undoubtedly suggests there are DA-independent neurochemical systems that contribute critically to mediating the reinforcing actions of EtOH and nicotine. The cholinergic system has been shown to be especially susceptible to the effects of adolescent binge-like EtOH exposure. Numerous studies demonstrated long-lasting and global reductions in neurons expressing choline acetyltransferase (ChAT), a marker for cholinergic neurons and enzyme responsible for acetylcholine (ACh) biosynthesis, following an adolescent intermittent EtOH (AIE) binge protocol (Coleman et al. 2011; Ehlers et al. 2011; Vetreno et al. 2014). In addition to an overall reduction in neurons expressing ChAT, chronic EtOH reduced the endogenous function of ChAT, ACh uptake, and expression of acetylcholinesterase (Arendt et al. 1989; Floyd et al. 1997). Alterations in the expression of nAChRs in multiple brain regions have also been reported (Figs. 4 and 5; McClintick et al. 2015, 2016; Hauser et al. 2019). These data add to the current findings indicating that the persistent reduction of ChAT produced by adolescent EtOH exposure results in a compensatory upregulation of nAChRs, specifically α7, in adult brains.

Voluntary EtOH consumption in adolescence, but not adulthood, produced lasting upregulation of α7 nAChRs in the pVTA as well as alterations to the actions of EtOH and nicotine at the α7 nAChR (Doyon et al. 2013; Hauser et al. 2019). The homomeric α7 nAChRs are strongly expressed presynaptically on glutamatergic afferents to the VTA and have been found to contribute to N-methyl-D-aspartate (NMDA) receptor dependent long-term potentiation (LTP) through a 20-fold greater permeability for calcium than the other nAChRs (Mansvelder & McGehee 2000; Mansvelder et al. 2002; Gao et al. 2010). The involvement of glutamate and plasticity have been implicated in several drugs of abuse including EtOH and nicotine (Gipson et al. 2013; Berglind et al. 2009; Campbell et al. 2009; Wang et al. 2008). Specifically, glutamate signaling from the medial prefrontal cortex (mPFC) has been shown to play a significant role in both EtOH and nicotine addiction. Several studies have demonstrated that experience with EtOH increases α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor mediated synaptic transmission and can induce LTP of glutamatergic synaptic afferents onto VTA DA neurons (Stuber et al. 2008; Oliva and Wanat 2016). This has also been associated with increased

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NMDA receptor expression as well as elevated phosphorylation of GluN2B receptor subtype (Pascual et al. 2009). Additional research found increased levels of NR1 and GluR1 subunits, indicating higher levels of both NMDA and AMPA receptors, following long-term exposure to EtOH (Ortiz et al. 1995). Activation of nAChRs by nicotine induces rapid desensitization and internalization, of which α4β2* nAChRs are particularly susceptible (Fenster et al. 1997; Pidoplichko et al. 1997). Despite the rapid transition of α7 nAChRs to a desensitized state, signaling can be spatially and temporally extended through interaction with heterotrimeric GTP-binding proteins (G proteins) via a G protein-binding cluster (GPBC) located on the receptor (Kabbani & Nichols 2018). Specifically, evidence suggests a nicotine induced α7 nAChR interaction with Gαq at the GPBC activates a signaling pathway resulting in prolonged calcium transients and increased neurotransmitter release (Kabbani & Nichols 2018). This unique feature of α7 nAChRs could explain the enhanced dopaminergic response to nicotine by the associated increase in expression levels and subsequent glutamatergic signaling onto VTA DA neurons (Figs. 2-5) following voluntary adolescent EtOH consumption. Collectively, enhanced DA release by nicotine during adulthood may be partly driven by increased levels of α7 nAChRs and subsequent prolonged excitatory signaling of glutamatergic inputs within the VTA. Future experiments hope to establish a causal link between α7 nAChR expression and altered responsivity to nicotine through pharmacological manipulations.

The ability of EtOH and nicotine to cross sensitize is bidirectional. Adolescent, but not adult rats, administered nicotine consumed more EtOH during adulthood than saline treated controls as well as an alteration in GABAA signaling (Thomas et al. 2018). Similarly, exposure to cigarette smoke enhances EtOH consumption during adolescence and adulthood (Burns & Procter 2013). The current data are first to indicate a persistent alteration in the neurochemical response to nicotine within the mesolimbic DA reward system following voluntary adolescent EtOH consumption. The research described above also suggests EtOH and nicotine actions can interact at specific sites, in addition to independent actions, to increase DA release within the NAc. Thus, for both nicotine and EtOH, adolescent exposure to drugs of abuse produce unique neuroadaptations that cross-sensitizes to others during adulthood. The upregulation of α7 nAChRs in the pVTA may be part of the biological basis for reports in humans that heavy adolescent EtOH use greatly enhances adult nicotine use (Dierker et al. 2013).

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Highlights

- Adolescent EtOH drinking increased the sensitivity to nicotine during adulthood.
- Adolescent drinking enhanced the dopaminergic response to nicotine during adulthood.
- VTA α7 nicotinic receptors were elevated in adolescent drinkers compared to controls.
- Adolescent, not adult, drinking produced nicotine cross-sensitization in adulthood.

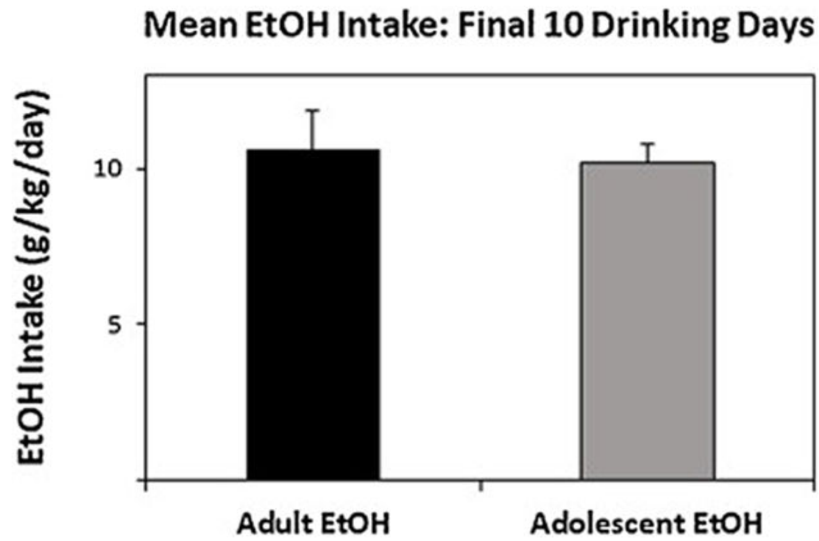

Figure 1.

Average daily EtOH consumption for all animals given free-choice access to 15% and 30% EtOH during adolescence (PND 30–60; n = 32) or adulthood (PND 90–120; n = 24). Intake values are calculated as grams per kilogram per day and averaged into a block of the final 10 days. All data shown are mean +SEM.

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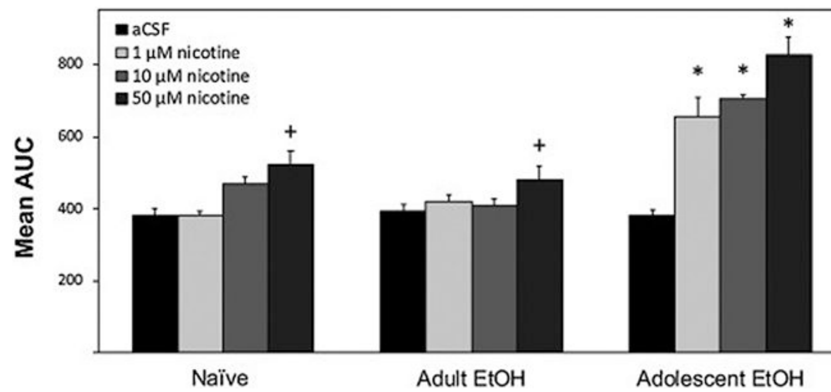

Figure 2.

Mean (+SEM) area under the curve (AUC) values to illustrate the general effects of drinking history and dose-response to nicotine. DA response was determined following microinjections of 0 (aCSF), 1, 10, or 50 μM nicotine administered to adult rats previously allowed to consume EtOH during adolescence (Adolescent EtOH; n = 7–9/group), during adulthood (Adult EtOH; n = 4–6/group), or water only (Naïve; n = 8–9/group). Plus sign (+) indicates significantly greater AUC in rats treated with 50 μM nicotine than respective aCSF treatment. Asterisk (*) indicates significantly greater AUC in Adolescent EtOH rats treated with 1, 10, or 50 μM nicotine compared to respective Naïve, Adult EtOH, and aCSF treatment groups.

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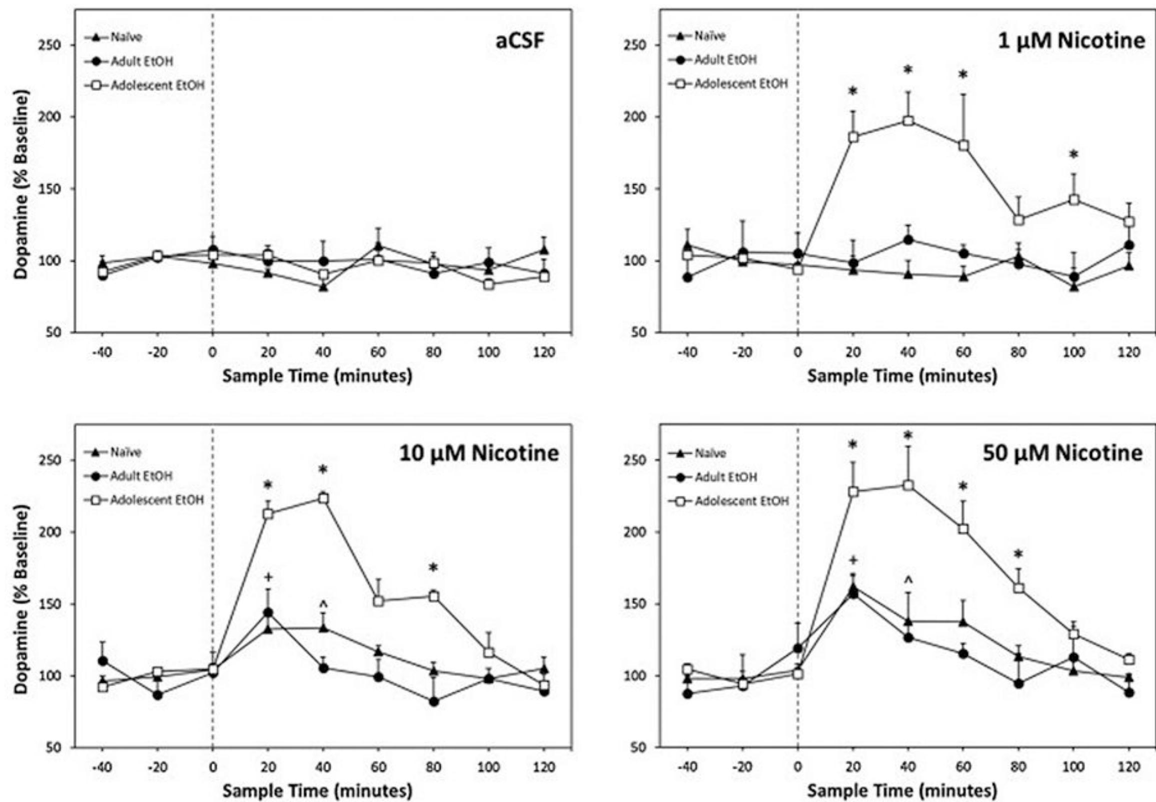

Figure 3.

Mean (+SEM) percentage change in extracellular DA in the NAc shell of adult rats microinjected with 0, 1, 10, or 50 μM nicotine directly into the pVTA previously allowed to consume EtOH during adolescence (Adolescent EtOH; n = 7–9/group), during adulthood (Adult EtOH; n = 4–6/group), or water only (Naïve; n = 8–9/group). Asterisk (*) indicates significantly greater DA levels in Adolescent EtOH rats compared to Naïve, Adult EtOH, and respective baseline DA at the specified time points. Plus sign (+) indicates Naïve and Adult EtOH extracellular DA was significantly higher than respective baseline levels. Carrot (^) indicates Naïve DA levels were significanly increased over baseline.

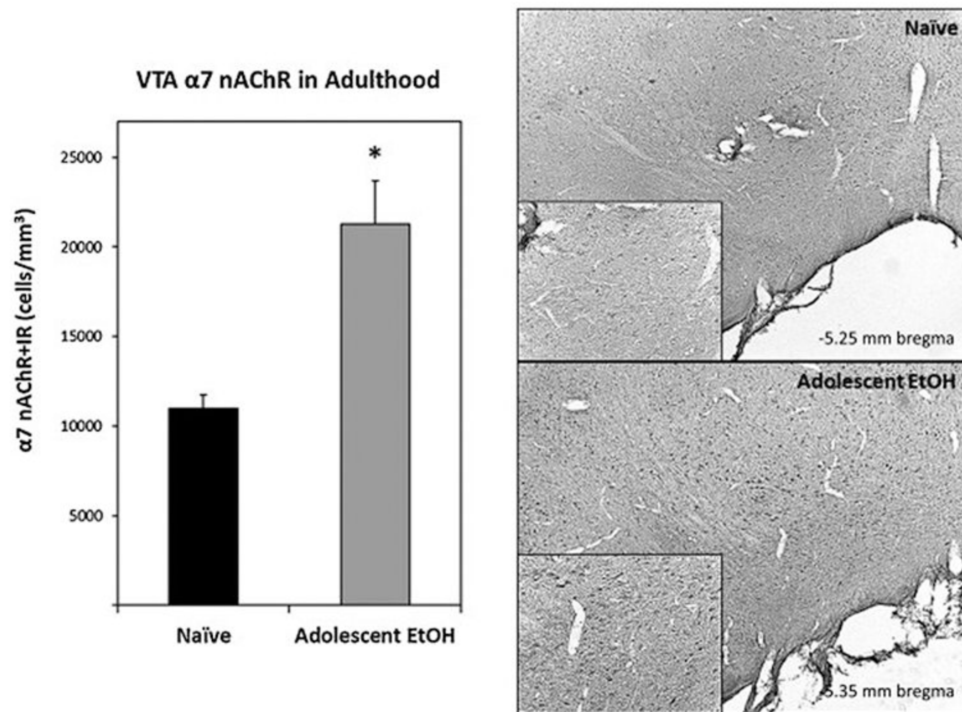

Figure 4.

Left: Mean (+SEM) α7-IR cell counts within the pVTA of adult rats allowed to consume water only (Naïve; n = 6) or EtOH during adolescence (Adolescent EtOH; n = 7). Asterisk (*) indicates significantly greater α7-IR cells compared to Naïve rats. Right: Representative photomicrographs (5x magnification, 20x inset) of α7-IR cells in the pVTA of Naïve and Adolescent EtOH during adulthood.

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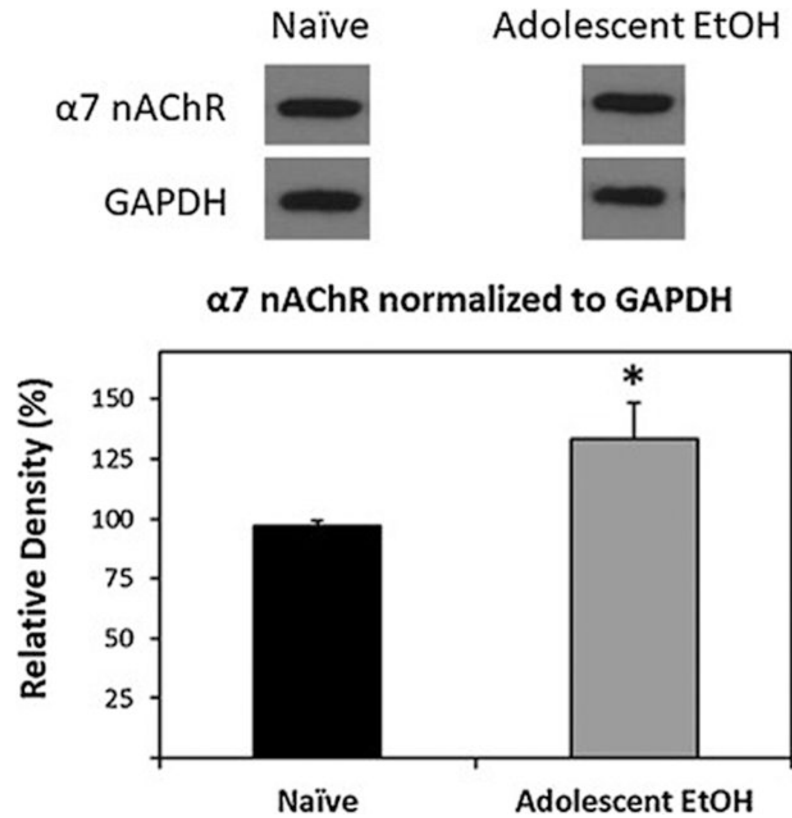

Figure 5.

Representative western blots and group data, normalized to GAPDH, illustrating increased α7 nAChR protein levels in the pVTA of rats allowed to consume EtOH during adolescence (Adolescent EtOH; n = 6) compared to water only controls (Naïve; n = 6) in adulthood. Asterisk (*) indicates significantly greater than controls.