HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2023 September 15.

Published in final edited form as:

Neuropharmacology. 2022 September 15; 216: 109170. doi:10.1016/j.neuropharm.2022.109170.

MUTATION OF THE α5 NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNIT INCREASES ETHANOL AND NICOTINE CONSUMPTION IN ADOLESCENCE AND IMPACTS ADULT DRUG CONSUMPTION

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Abstract

Alcohol and nicotine are commonly used during adolescence, establishing long-lasting neuroplastic alterations that influence subsequent drug use and abuse. Drinking- and smoking-related traits have been extensively associated with variation in *CHRNA5* – the gene that encodes the α5 subunit of neuronal nicotinic acetylcholine receptors (nAChRs). The single nucleotide polymorphism (SNP) rs16969968 in *CHRNA5* encodes an amino acid substitution (D398N) that alters the function and pharmacokinetics of α5-containing nAChR. When expressed in rodents,

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CONTRIBUTIONS

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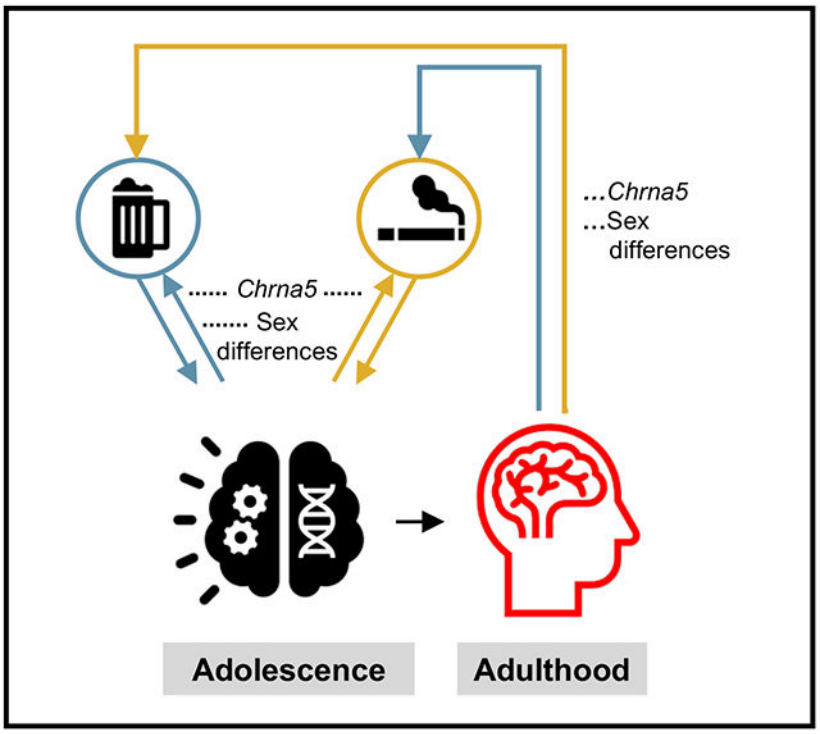
CONFLICT of INTEREST STATEMENT

The other authors declare no competing financial interests in relation to the work described in this manuscript.

this variant results in increased ethanol and nicotine operant self-administration. How disruption of α5-containing nAChRs influences adolescent ethanol and nicotine intake, and how it modulates interactions between these drugs has not been previously explored. In the present study, we examined volitional ethanol and nicotine consumption in adolescent mice (post-natal day 30 – 43) of both sexes with mutated (SNP) or lacking (KO) the α5 nAChR subunit. The effect of adolescent alcohol or nicotine exposure on home cage consumption of the opposite drug in adulthood and its modulation by *Chrna5* mutation and sex were examined. During adolescence, we found that α5 nAChR disruption increases nicotine intake in mice of both sexes, but the effect on alcohol intake was only observed in females. The sex-specific increase in alcohol consumption in α5 SNP and KO was replicated in adulthood. The effect of adolescent alcohol or nicotine exposure on subsequent intake of the opposite drug in adulthood is modulated by sex and *Chrna5* mutation. These observations suggest sex differences in the genetic architecture of alcohol dependence, and modulators of alcohol and nicotine interactions.

Graphical Abstract



Keywords

Chrna5; rs16969968; Alcohol; Nicotine; Voluntary intake; Adolescence; Nicotinic acetylcholine receptors

1. INTRODUCTION

Alcohol and nicotine are commonly used during adolescence, a critical period of neurodevelopment. According to the 2019 National Survey on Drug Use and Health

(NSDUH), 1 out of 5 adolescents ages 12 to 17 reported past-year alcohol use (SAMHSA, 2019a), with alcohol use disorder (AUD) affecting approximately 0.4 million adolescents in this age group in the United States (SAMHSA, 2019b). Moreover, binge drinking – defined as consumption of more than 4 and 5 standard drinks on the same occasion in women and men, respectively (SAMHSA, 2020) – is a risky drinking pattern commonly observed in both adolescents (11% past-month) and adults (26% past-month). Binge drinking has been shown to increase the likelihood of experiencing both short-term and long-term negative consequences, and is prevalent among patients with an AUD diagnosis (Hingson et al., 2017).

Binge drinking levels are also correlated with lifetime smoking (Hingson et al., 2017), which has been reported to be initiated by age 18 in nearly 90% of adults who smoke cigarettes (U.S. Department of Health and Human Services, 2012). In fact, almost 1 in every 4 high school students reported using a tobacco product in 2020 (Gentzke et al., 2020). Multiple longitudinal studies have similarly provided evidence of a strong association between adolescent smoking and alcohol use, dependence, and binge drinking (Brook et al., 2002; Ellickson et al., 2001; Grant, 1998; Lewinsohn et al., 1999; Strong et al., 2016, but also see review by Mathers et al., 2006). There is also substantial evidence for increased tobacco use in individuals who initiated alcohol use or had alcohol-related problems during adolescence (Dierker et al., 2013; Paavola et al., 2004). Altogether, these highly replicated interactions underscore the necessity to identify neural correlates of the harmful adaptations established in response to early alcohol or nicotine use and that serve as gateway for subsequent drug use.

Adolescence is a developmental period during which neuronal network connectivity is structurally and functionally reorganized across regions that govern brain function, processing of stimuli and behavioral output. Many neurotransmitter systems undergo major plasticity during development (see review by Yuan et al., 2015). Of particular interest is the expression of the α5 nicotinic acetylcholine receptor (nAChR) subunit in the ventral tegmental area (VTA), which changes throughout development with transcript levels reaching a post-natal maximum at the beginning of adolescence (post-natal day (PND) 21) and decreasing significantly in adulthood (PND 60) in rats (Azam et al., 2007). The α5 nAChR subunit is also expressed in other brain regions that are highly implicated in alcohol and nicotine addiction, such as the substantia nigra (SN) and the habenulo-interpeduncular nucleus (mHb-IPN) axis (Hsu et al., 2013; Wada et al., 1990), and is integrated in multiple nicotinic receptor subtypes (such as α4β2α5) that have been shown to modulate nicotine-related accumbal dopamine release (Salminen et al., 2004; Zoli et al., 2002).

The α5 nAChR subunit has been consistently and robustly implicated in both alcohol and tobacco use disorders, with strong associations between genetic variants in the gene that encodes it (i.e., *CHRNA5)* and drinking-/smoking- related traits in humans (Bierut et al., 2008; Choquet et al., 2013; Hartz et al., 2012; Wang et al., 2009a, 2009b; Weiss et al., 2008). More specifically, the single nucleotide polymorphism (SNP) rs16969968 in *CHRNA5* (Bierut et al., 2008) that results in an aspartate (D) to asparagine (N) substitution (D398N) and a partial loss of function has been examined in relationship to nicotinic effects. Studies have shown that cells expressing the SNP demonstrate reduced calcium

influx, and VTA dopamine neurons in rats expressing the SNP are less responsive to nicotine (Bierut et al., 2008; Kuryatov et al., 2011; Morel et al., 2014a; Sciaccaluga et al., 2015). In preclinical studies examining levels of drug intake in male rodents, expression of the α5 SNP was shown to produce a rightward shift in the dose-response curve of nicotine intravenous operant self-administration (Morel et al., 2014b), and increase ethanol oral operant self-administration under fixed and progressive ratio schedules (Besson et al., 2019). Altogether, these findings suggest an important modulatory role of α5*nAChRs in sensitivity to drug-related positive reinforcement. As increased reward-seeking behavior is characteristic of adolescence and there is abundant evidence of changes in reward processing during development (for review, see Galván, 2010), it is possible that the α5*nAChRs may also be critical modulators of alcohol and nicotine consumption during adolescence. Thus, the goal of this study was to investigate the potential involvement of the α5*nAChR system on the relationship between adolescent drinking or smoking and the subsequent predisposition to misuse nicotine or alcohol in adulthood. Specifically, we examined the interactions between alcohol, nicotine and genetic variation in the gene encoding the α5 nAChR subunit.

2. MATERIALS AND METHODS

2.1. Experimental Subjects

This study utilized female and male mice with a C57BL/6J genetic background carrying a null mutation in *Chrna5* (α5 knockout, KO), or constitutively expressing the rs16969968 SNP (α5 SNP), and their wild-type (WT) littermates. The generation of the α5 KO mice was originally described in Salas et al., 2003 and the mice are periodically backcrossed to the GSP C57BL/6J strain (# 000664; The Jackson Lab, Bar Harbor, ME) to minimize genetic drift. The α5 SNP line was generated from mouse embryonic stem cells with the C57BL/6 genetic background and genomic constructs derived from a C57BL/6 bacterial artificial chromosome library (Sciaccaluga et al, 2015), which allows the generation of mutant mice without the need to backcross to the C57BL/6 background. Mice were bred in-house and were group-housed with same-sex littermates at weaning (PND 21). All mice were housed in standard ‘shoebox’ cages (7.6 in x 5.1 in x 15 in) with 1 cm corn cob bedding, a cotton nestlet, and *ad libitum* access to fluid and food (Labdiet 5053, PMI, Brentwood, MO) throughout the study. The mice were housed in a temperature- and humidity- controlled room (68 – 75 °F, 30 – 60% relative humidity) with a 12-h light/12-h dark cycle. For consumption studies in which mice were single-housed, a tent-shaped cardboard house (i.e., Shepherd shack®, Shepherd Specialty Papers, Watertown, TN) was added to the cage for additional environmental enrichment. All procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC).

Adolescent mice self-administered ethanol or nicotine for 14 days starting on PND 30. This period of development (PND 30-44) is analogous to human adolescence based on brain maturation, hormonal changes, and behavioral characteristics (Spear, 2000).

2.2. Chemical Reagents

2.2.1. Ethanol—Ethanol solutions were prepared by diluting 190 proof ethanol (Decon Labs, Inc. King of Prussia, PA) in distilled water.

2.2.2. Nicotine and Saccharin—Nicotine solutions were prepared by dissolving (−)-nicotine hydrogen tartrate salt (Sigma-Aldrich®, Milwaukee, WI) and saccharin (Sigma-Aldrich®, Milwaukee, WI) in distilled water. All concentrations are provided as freebase equivalents.

2.3. EXPERIMENT #1: Effect of adolescent binge-like drinking on adult nicotine intake

2.3.1. Adolescent Treatment – Ethanol drinking-in-the-dark (DID) in three-bottle choice (3BC)—WT, α5 SNP and α5 KO mice were single-housed 2-3 days before starting the ethanol DID 3BC. Three bottles containing filtered water were provided during this habituation period. Starting on PND 29-31, mice consumed ethanol in a 3BC for 4 h/day during the dark phase of the light cycle (Figure 1, “Adolescence”). This paradigm leads to moderate consumption of alcohol which has been shown by others (Wolstenholme et al 2020) to increase ethanol consumption in adulthood. Additionally, we chose this “forced”, alcohol-only exposure to model binge-like drinking, which is highly prevalent among adolescents to enhance the translational value of the study. For the 3BC, mice had access to three different concentrations of ethanol (5, 10 and 15% (v/v)) simultaneously. These ethanol concentrations were chosen based on previous observations from 2BC procedures where concentrations higher than 15% result in low preference for an ethanol bottle over a water bottle, potentially suggesting aversion (Quijano Carde’ & De Biasi, 2022). The solutions were provided in 25-mL bottles with straight stainless-steel, open-tip tubes. The bottles were weighed before and after each drinking session and the difference in weight was used to determine ethanol dose and bottle preference. The adolescent mice were weighed daily immediately before the start of each drinking session. Although we did not identify a main effect of genotype on weight (data not shown), ethanol intake was corrected for body weight (g/kg/4h) to account for any potential differences. Control mice were single housed during adolescence but did not have access to ethanol. Empty cages with bottles were monitored for leakage in all experiments.

Blood plasma ethanol concentrations (BECs) were measured in a subset of C57BL/6J mice exposed to the ethanol DID 3BC paradigm during adolescence. For this, trunk blood was collected immediately after the last session (DID day 14, PND 43) and kept on ice until centrifuged at 4 °C for 15 min at 10,000 RPMs. The plasma was stored at −80 °C until analyzed. To determine BECs, samples were diluted in deionized water (1:50) and analyzed in duplicate using a colorimetric ethanol assay from Sigma-Aldrich® (CAT MAK076, St. Louis, MO).

2.3.2. Adult Testing – Intermittent access to 200 mg/L nicotine in a two-bottle choice (2BC)—Following adolescent ethanol or control treatment, nicotine consumption was evaluated using a nicotine 2BC for a total of five weeks (Figure 1, Adulthood). Briefly, WT, α5 SNP and α5 KO mice had access to 200 mg/L nicotine + 0.2% (w/v) saccharin and 0.2% (w/v) saccharin for 4 days/week, followed by 3 days of abstinence. During

abstinence, mice had access to two bottles of filtered water. Four-day nicotine dose was determined using the change in bottle weight, and the level of nicotine consumption for each mouse was calculated by averaging 4-day intake across the five weeks of exposure. Adult, ethanol-treated α5 KO mice weighed significantly more than WT mice at the beginning of nicotine exposure; however, there was no significant difference by the end of the experiment. There were no differences in weight in ethanol-treated α5 SNP mice compared to control at the beginning or end of the exposure, and there were no significant differences by genotype in weight of control-treated mice at any time. To account for potential differences in bodyweight, nicotine intake was expressed as mg/kg.

2.4. EXPERIMENT #2: Effect of adolescent nicotine exposure on adult alcohol intake

2.4.1. Adolescent Treatment – Forced access to nicotine in the drinking water—

For the nicotine treatment, WT and α5 KO mice were group-housed with same-genotype/sex littermates. During the first week of adolescent treatment (days 1-7), mice had access to 100 mg/L nicotine + 0.2% (w/v) saccharin (Figure 4, “Adolescence”). During the second week (days 8-14), the concentration of nicotine was increased to 200 mg/L nicotine + 0.2% (w/v) saccharin (Salas et al., 2009; Perez et al., 2015; O’Neill et al., 2018). Bottles were weighed every 2-3 days to determine dose consumed by each cage, and mice were weighed at this time. Weight varied according to genotype in males—alpha 5 KO male mice weighed more than WT mice—and according to treatment in females—nicotine-treated female mice weighed less than saccharin-treated mice. Therefore, intake was expressed as mg/kg to adjust for differences in weight. Control-mice were given 0.2% saccharin without nicotine during adolescence. Contrary to the 2BC testing we used for adult mice, adolescent mice were exposed to a nicotine solution as the sole source of fluids, based on the observation that mice will not voluntarily consume physiologically relevant levels of nicotine when given a choice prior to establishment of dependence. We therefore opted for the forced nicotine exposure paradigm for this set of experiments to increase the probability of observing effects of the adolescent exposure in adulthood.

Plasma cotinine levels in adolescent mice with forced access to nicotine were examined in a separate cohort. Briefly, trunk blood was collected 6 h into the dark phase of the light cycle on the last day of adolescent treatment (PND 43). Blood samples were centrifuged at 4 °C and 10,000 RPM for 15 min, and plasma was stored at −80 °C until analyzed. Cotinine levels in the blood plasma were determined using the Mouse/rat cotinine ELISA from Calbiotech, Inc. (C0096-100; El Cajon, CA).

2.4.2. Adult Testing – Ethanol DID 2BC—

Following adolescent nicotine or control treatment, WT and α5 KO mice were single-housed on PND 60 to begin habituation to photobeam-based lickometers, described below. Briefly, ethanol intake was examined using a 4-h/day DID protocol in which mice had access to an ethanol-containing solution and water simultaneously (Figure 4, “Adulthood”). To evaluate potential differential responses to different ethanol concentrations, we evaluated three concentrations in increasing order (5%, 10%, 15% (v/v)). Ethanol drinking behavior was evaluated for three days at each concentration and averages for each concentration were used for our analyses. During the rest of the day (i.e., 20 h), mice had access to two bottles of filtered water. As described

above, alpha5 KO mice weighed significantly more than WT mice and therefore, intake was expressed as g/kg to correct for any potential weight differences.

The photobeam-based, home-cage lickometers used in this study were manufactured in-house as described by Godynyuk et al., 2019, with modifications to the housing to account for differences in cage type. Each lickometer was equipped with two bottles that consisted of 15-mL conical tubes fitted with HYDRO-PAC, Inc. valves (Catalog no.: HYP-VALVES; Seaford, Delaware). Housing was 3D printed using Acrylonitrile butadiene styrene (ABS) and cleaned in a commercial dishwasher between animals. The open-source code was modified to time stamp individual beam breaks ("events"), allowing for increased temporal resolution.

2.5. Data and Statistical Analyses

Prism 9 (version 9.2.0) and R (version 1.3.1056) were used to perform statistical analyses, which were conducted in a blind fashion. Specific statistical tests utilized for each experiment are specified in the Results. Briefly, normality and equality of variances were assessed using the Kolmogorov-Smirnov test and Bartlett's test, respectively. Parametric data were analyzed using analyses of variance (ANOVAs) followed by Bonferroni's multiple comparisons test. Welch's ANOVA followed by Dunnett's T3 multiple comparisons test was used for data with unequal variances. Non-parametric data were analyzed using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Statistical association between variables was examined using Pearson's correlation coefficient. Differences in the proportion of "heavier" drinkers was assessed using a two-tailed binomial test. Significance threshold was set to α=0.05 for all analyses. Data are shown as individual subjects, mean ± standard error of the mean (SEM), or median ± 95% confidence interval (CI).

Given the ample evidence of significant effect of sex on voluntary alcohol and nicotine consumption in the home cage in mice (Klein et al., 2004; Patten et al., 2021; Radke et al., 2021), data from female and male mice were analyzed separately when investigating effects of *chrna5* mutation. Given that most previously published studies examining the effect of adolescent exposure to ethanol or nicotine on adult consumption of these drugs used forced exposures (e.g., IP injections, oral gavage, osmotic mini pumps), we also analyzed 'WT' data separately and reported them in separate subsections in the Results.

2.5.1. Lickometers—Lickometer data were processed using custom-written scripts in R. Events lasting more than 10 s were operationally defined as spurious and were excluded. These lickometers allowed for the estimation of home-cage drinking behavior in mice that included (i) total bout duration (bouts operationally defined as events with cumulative duration of ≥250 ms and inter-event interval of <1000 ms), (ii) latency to start drinking (time to first bout), and (iii) number of bouts during the first 30 min of the session, which serves as proxy for consumption during the early phase of the session.

2.5.2. Determination of drinking categories—A common observation in alcohol addiction research is the presence of subpopulations with varying degrees of alcohol use based on quantity-frequency measures, and there is great interest in investigating risk factors for more severe use (i.e., heavier drinking phenotypes). To examine whether *Chrna5*

mutation increases risk of worse drinking behavior during adolescence, mice were divided in severity categories. To do this, we started by determining a threshold for 4-h "heavier" drinking based on the relationship between BEC and ethanol intake. In C57BL/6J mice, a significant correlation between 4-h ethanol intake and plasma ethanol concentration was observed at the end of the DID 3BC session on PND 43 (Pearson $r = 0.7633$, 95% CI: 0.4605 to 0.9069, p=0.0002; Supplemental Figure 1). A simple linear regression of the BEC vs. intake data was used to estimate the level of intake required in adolescent mice to achieve a plasma ethanol level of 20 mg/dL. This plasma ethanol level was chosen because in C57BL/6J mice, adolescent 4-h/day alcohol consumption associated with BECs of ~20 mg/dL is sufficient to significantly increase alcohol consumption in adulthood (Wolstenholme et al., 2020). In addition, in humans, BECs of 20 mg/dL have been reported to induce relaxation, loss of judgement/shyness, slight euphoria and inability to track quickly moving objects (U.S. Department of Transportation, 2021). Runs test did not detect significant deviation from linearity (p=0.4842), and the relationship between the two variables was described by the following equation: y = 9.416x – 23.88, where y is BEC in mg/dL and x is ethanol intake in g/kg/4h. With this equation, the 95% confidence interval of the ethanol intake needed to reach 20 mg/dL ethanol in the plasma was calculated to be 3.862 - 5.125 g/kg/4h. Thus, the upper limit of the 95% CI (i.e., 5.125) – which is also close to the third quartile of the data (i.e., 5.3 g/kg/4h) – was used to identify the heavier drinking days in adolescence. Then, using the number of heavier drinking days and the aggregated ethanol dose, hierarchical clustering with average-linkage metric for distance was used to identify clusters. Animals were divided in two groups, where mice in cluster #1 ('lighter drinkers') consumed an average of 54.0±1.5 g/kg/14d with 2.0±0.2 heavier drinking days, while mice in cluster #2 ('heavier drinkers'), achieved an average total dose of 73.3±0.9 g/kg/14d with 7.8±0.4 heavier drinking days.

3. RESULTS

3.1. EXPERIMENT #1: Effect of adolescent binge-like drinking on adult nicotine intake

3.1.1. Chrna5 *variation alters alcohol drinking behavior during adolescence in female mice*—

Daily alcohol consumption was examined in adolescent mice with access to ethanol in the DID-3BC (Figure 1, "Adolescence"). This paradigm did not result in drinking to intoxication. Overall, mice consumed amounts of ethanol estimated to result in BECs ≥ 20 mg/dL (heavier drinking) approximately 40% of days, and the percentage of mice reaching BECs ≥ 20 mg/dL increased over time (~20% on days 1 to 3, and ~60% on days 12 to 14). Repeated measure (RM) two-way ANOVA of daily 4-h dose in α5 WT, SNP and KO female mice revealed significant main effects of genotype ($F_{genotype}[2,31] = 6.287$, p=0.0051) and time ($F_{time}[13, 403] = 11.87$, p<0.0001), and a trend for genotype-by-time interaction ($F_{interaction}[26,403] = 1.459$, p=0.0699) (Figure 2A). Bonferroni's multiple comparisons test revealed significant differences between WT and *Chrna5* mutated mice between DID days 4 and 11, potentially suggesting accelerated escalation in mice with disrupted α5*nAChRs. The total ethanol exposure (i.e., aggregated ethanol dose; g/kg/14 d) was used to evaluate overall differences between genotypes (Figure 2B). One-way ANOVA detected significant differences between genotypes (F[2,31] = 6.287, p=0.0051), with increased 14-day ethanol intake in α5 SNP (p=0.0058) and α5 KO (p=0.0135) female

mice compared to WT mice. There was also a trend for a higher occurrence of heavier drinking days (i.e. above threshold) in mice with the Chrna5 mutation [one-way ANOVA, $F(2,31)=2.863$, $p=0.0723$] (Figure 2C). These results suggest an effect of α5*nAChR disruption in alcohol consumption during adolescence in female mice. However, when these behaviors were examined in male mice, no significant effect of genotype was detected on 4-h ethanol dose ($F[2,30] = 1.281$, $p=0.2925$), aggregated dose ($F[2,30] = 1.281$, $p=0.2925$), or number of heavier drinking days (Kruskal-Wallis test, $p=0.8846$) (Figure 2E–G).

The proportion of mice classified as heavier (moderate rather than light) drinkers in WT, α5 SNP, and α5 KO male mice was 36, 18, and 27%, respectively (Figure 2H). Interestingly, in females a statistically significant increase in the proportion of heavier drinkers was observed in the α5 SNP (55%, $p=0.0002$) and α5 KO (58%, $p<0.0001$) groups compared to WT females (9%) using two-tailed binomial tests (Figure 2D).

Preference for each ethanol concentration in the ethanol DID 3BC was evaluated using RM two-way ANOVA (Supplemental Figure 2). The effect of *Chrna5* genotype on bottle preference did not reach statistical significance in females ($F_{5\%}[2, 31] = 1.2$, $p=0.3208$; $F_{10\%}[2, 31] = 2.3$, $p=0.2818$; $F_{15\%}[2, 31] = 0.99$, $p=0.3824$) or in males ($F_{5\%}[2, 30] = 0.56$, $p=0.5751$; $F_{10\%}[2, 30] = 3.3$, $p=0.0583$; $F_{15\%}[2, 30] = 0.040$, $p=0.9608$). However, time significantly altered bottle preference. In general, mice of both sexes displayed an increase in the preference for the 15% ethanol bottle over time, which was accompanied by a decrease in the preference for the 5% ethanol bottle. It is possible that this transition to higher preference for more concentrated ethanol solutions is associated with taste habituation and/or the development of tolerance following repeated binge-like drinking. The change in bottle preference contributes to the escalation in ethanol consumption observed in the DID 3BC, as evidenced by significant correlations between ethanol dose and bottle preference (positive between intake and 15% preference, and negative between intake and 5% preference; correlation matrices not shown).

3.1.2 Mice with disrupted α5*nAChRs consume higher doses of nicotine in adulthood—

We observed that *Chrna5* genotype had a significant effect on voluntary nicotine consumption ($F_{females}[2, 14.4] = 13.0$, $p=0.0006$; $F_{males}[2, 16.9] = 13.4$, $p=0.0003$). In both females and males, *Chrna5* SNP ($p_{females}=0.0357$, $p_{males}=0.0006$) and KO mice ($p_{females}=0.0013$, $p_{males}=0.0123$) consumed significantly higher doses of nicotine. This is in accordance with previous reports (Bagdas et al., 2019; Meyers et al., 2021).

3.1.3. Adolescent binge-like drinking increases voluntary nicotine consumption in adult WT mice—

Following the adolescent alcohol treatment (3.1.1) and maturation, mice were allowed to consume nicotine via the drinking water in a 2BC paradigm. We first evaluated the effect of adolescent ethanol exposure on adult nicotine intake in WT mice. Two-way ANOVA of the data from WT mice of both sexes revealed a significant main effect of adolescent alcohol exposure on nicotine intake in adulthood ($F[1, 45] = 14.9$, $p=0.0004$), but no significant main effect of sex ($F[1, 45] = 0.61$, $p=0.4374$) or sex-by-adolescent treatment interaction ($F1, 45] = 0.07$, $p=0.7869$) (Supplemental Figure 3A). Adolescent alcohol consumption via the 14-day DID 3BC increased adult nicotine intake in both male (Bonferroni, $p=0.0325$) and female (Bonferroni, $p=0.0094$) WT mice.

3.1.4. *Adolescent alcohol exposure influences adult nicotine intake in a sex-specific and* Chrna5*-dependent manner*—

We then investigated how *Chrna5* mutation influences the effect of adolescent ethanol drinking on subsequent nicotine consumption in adulthood. In females, there was a significant main effect of genotype ($F[2, 60] = 9.8$, $p=0.002$), as well as a genotype-by-adolescent alcohol treatment interaction ($F[2, 60] = 8.2$, $p=0.0007$) (Figure 3A). There was a pattern for increased nicotine intake in WT mice exposed to alcohol compared to same-genotype control females; however, Bonferroni multiple comparisons were not statistically significant. In contrast, significantly lower levels of nicotine intake were observed in α5 KOs exposed to alcohol in adolescence compared to same-genotype control females ($p=0.0079$). In males, there were significant main effects of genotype ($F[2, 63] = 7.8$, $p=0.0009$) and adolescent alcohol treatment ($F[1, 63] = 4.0$, $p=0.0498$), but no genotype-by-adolescent treatment interaction ($F[2, 63] = 1.1$, $p=0.3462$) (Figure 3B).

To further investigate the apparent protective effect of α5 nAChR subunit deletion from the adverse consequence of adolescent binge drinking on adult nicotine consumption in female mice, correlations between levels of alcohol and nicotine intake were explored for each group using Pearson correlation coefficient (Figure 3C). Interestingly, total aggregated ethanol dose was strongly inversely correlated with average 4-day nicotine intake in adulthood only in α5 KO female mice (Pearson $r = -0.66$, $p=0.04$), with higher levels of alcohol consumption during adolescence resulting in lower nicotine consumption in adulthood. This observation suggests a dose-dependent effect of ethanol on nicotine consumption in this group.

3.2. EXPERIMENT #2: Effect of adolescent nicotine exposure on adult alcohol intake

3.2.1. α5 deletion results in increased nicotine intake during adolescence

—A separate group of mice was exposed to nicotine via the drinking fluid during adolescence (Figure 4, “Adolescence”). In both female and male mice, RM two-way ANOVA revealed significant main effect of genotype ($F_{females}[1,14] = 15.7$, $p=0.0014$; $F_{males}[1,18] = 8.0$, $p=0.0113$) and genotype-by-concentration interaction ($F_{females}[1,14] = 9.9$, $p=0.0071$; $F_{males}[1,18] = 9.1$, $p=0.0074$) on average nicotine consumption per cage (Figure 5A and 5B). Significantly increased nicotine consumption was observed in α5 KO at 200 mg/L (Bonferroni, $p_{females}<0.0001$; $p_{males}=0.0008$), but not at 100 mg/L (Bonferroni, $p_{females}=0.1061$, $p_{males}=0.5252$) compared to WT mice. Trunk blood was collected on PND43 from a subset of mice to compare plasma cotinine levels between WT and α5 KO mice, thereby confirming nicotine exposure. In accordance with their cage-level increased nicotine consumption at the cage level in α5 KO mice compared to WT, both female (Unpaired t-Test: $p=0.0171$) and male (Unpaired t-Test: $p=0.0300$) α5 KO mice had higher levels of cotinine in the plasma compared to same-sex WTs (Supplemental Figure 4). No significant effect of *Chrna5* genotype was observed on saccharin consumption during adolescence in the control groups (Supplemental Figure 5).

3.2.2. α5 *deletion increases adult ethanol consumption in female but not male mice*—

We then wanted to examine whether the effect of *Chrna5* mutation on ethanol intake observed during adolescence – in which female mice with mutated *Chrna5* consumed

more ethanol than WT controls – was also observed in adulthood. In females, RM two-way ANOVA detected a close-to-significant effect of genotype ($F_{genotype}[1, 19] = 4.1$, p=0.0584) in the absence of a significant genotype-by-concentration interaction ($F_{2way}[2, 38] = 0.8$, p=0.4394). When collapsed across concentrations, α5 KO females consumed an average of 3.3±0.3 g/kg/4h, while WT females consumed significantly less (2.5±0.3 g/kg/4h) (t-Test: p=0.0254). Genotype did not have a significant effect ($F_{genotype}[1, 18] = 1.4$, p=0.2556) and did not significantly interact with ethanol concentration ($F_{2way}[2, 36] = 1.2$, p=0.3259) in male mice. Moreover, the average ethanol consumption between WT and α5 KO male mice did not significantly differ (1.8±0.3, and 1.2±0.2 g/kg/4h, respectively; t-Test: p=0.1278). This finding was further replicated in an independent cohort of mice that was tested in the ethanol DID 2BC in adulthood but was not exposed to saccharin during adolescence (Supplemental Figure 6).

3.2.3. Adolescent nicotine exposure increased adult ethanol intake in adult female, but not male, WT mice—

We then investigated the effect of adolescent nicotine treatment on adult ethanol consumption in WT mice of both sexes (Figure 6A and 6B, "WT"). Analysis of WT data using RM three-way ANOVA revealed significant main effects of adolescent nicotine treatment ($F[1, 37] = 5.1$, p=0.0303), sex ($F[1, 37] = 13.7$, p=0.0003), and ethanol concentration ($F[2, 74] = 95.7$, p<0.0001) (Supplemental Figure 3B). However, the only significant interaction was between sex and ethanol concentration ($F_{2way}[2, 74] = 11.7$, p<0.0001). WT data for each sex were evaluated separately using RM two-way ANOVA to further investigate the effect of adolescent nicotine exposure on ethanol consumption when provided at different concentrations. In females, adolescent nicotine treatment had a significant effect on ethanol dose consumed ($F[1, 19] = 8.8$, p=0.0079). Post hoc analysis showed that nicotine intake during adolescence only caused a significant increase in alcohol intake when provided at 5% (p=0.0224), with a trend at 15% (p=0.0600). However, when male WT data was analyzed separately, RM two-way ANOVA did not detect a significant main effect of adolescent nicotine treatment ($F[1, 18] = 0.36$, p=0.5572), nor did it detect a significant ethanol concentration-by-adolescent treatment interaction ($F[2, 36] = 0.4674$). This suggests that the level of exposure to nicotine during adolescence used in this study was sufficient to increase adult ethanol consumption in females, but not males with functional α5*nAChRs.

3.2.4. *Adolescent nicotine consumption affects adult binge-like ethanol drinking in a sex-specific and* Chrna5*-dependent manner—*

We then examined the effect of adolescent nicotine consumption on adult alcohol drinking phenotypes in WT and α5 KO mice of both sexes (Figure 6). Using RM three-way ANOVA, a significant genotype-by-adolescent nicotine treatment interaction was only observed on female ethanol dose (Figure 6A, $F_{2way}[1, 37] = 8.5$, p=0.0060) and preference (Figure 6C, $F_{2way}[1, 37] = 14.3$ p=0.0006). Additional comparisons planned *a priori* were performed in dependent variables collapsed across ethanol concentrations for each group. Bonferroni's multiple comparisons test following two-way ANOVAs were used to compare outcomes from control- and nicotine- treated mice in each genotype-sex group. These analyses showed that nicotine increased ethanol dose and preference in WT females (Figure 6A and 6C, respectively; $p_{WT\text{-}dose}$=0.0187, $p_{WT\text{-}pref}$=0.0002) and α5 KO males (Figure 6B and 6D, respectively;

$p_{KO\text{-}dose}$=0.0086, $p_{KO\text{-}pref}$=0.0943). This suggests a protective effect of α5 nAChR subunit deletion from the effect of adolescent nicotine exposure in females that conversely results in increased susceptibility in male mice. It is possible that the observation in males is an effect of adolescent nicotine dose mediated by α5 deletion (KO males consume more nicotine than WTs), especially considering that α5 KO males consumed levels of nicotine comparable to those observed in α5 WT females (Figure 5) that led to increased adult alcohol consumption (Figure 6A). Although nicotine-exposed α5 KO males consumed significantly more ethanol than saccharin-exposed α5 KO males we did not identify a concomitant difference in body weight, and this together with the *ad libitum* availability of food suggest that it is unlikely that increased alcohol consumption is the result of increased calorie-seeking.

RM three-way ANOVA followed by planned comparisons as described above were used to analyze data from lickometers—specifically the latency to the first bout ("eagerness" to initiate drinking), the number of bouts in the beginning of the session ("loading" behavior), and bout duration (time spent engaging in focused drinking)—as differences in the patterns of ethanol intake can reveal important information on reward and reinforcement (Figure 7). In summary, the changes in vulnerability to consume higher doses of ethanol in response to the adolescent nicotine exposure were not accompanied by statistically significant changes in the parameters of drinking microstructure examined in this study, although non-significant trends for an effect of adolescent treatment were observed in α5 KO males (Figure 7B, D, F).

4. DISCUSSION AND CONCLUSIONS

Genetic variation in the *CHRNA5* gene has been correlated with alcohol and nicotine use disorders (Bierut et al., 2008; Wang et al., 2009a), as well as age of initiation of alcohol and tobacco use in humans (Schlaepfer et al., 2008). Moreover, evidence of a strong effect of adolescent smoking or drinking on increased risk of adult misuse of alcohol or tobacco products is ample and robust. In this study, we investigated the involvement of α5* nAChRs in the reciprocal effects of ethanol and nicotine when the first exposure to one of these drugs occurs during adolescence. For this, the interactive effects of adolescent alcohol/nicotine exposure and the *Chrna5* genetic mutation on adult drug consumption were evaluated in mice of both sexes.

4.1. *Effects of* Chrna5 *mutation on adolescent ethanol and nicotine consumption*

In adolescence, we found that disruption of the α5 nAChR subunit increased nicotine consumption in mice of both sexes (Figure 5), but only increased ethanol consumption in female mice (Figure 2). This sex-specific effect of *Chrna5* mutation on alcohol binge-like drinking was replicated in adulthood (see Supplemental Figure 6 and Results for details). The absence of an effect of α5*nAChR disruption on ethanol intake in home cage DID or two-bottle choice procedures in adult male rodents is in accordance with previous reports (Besson et al., 2019; Dawson et al., 2018; Santos et al., 2013). Notably, these studies had not included females, which seem to be more vulnerable to the effects of *Chrna5* mutation on responses to ethanol. However, one study employing male rats showed that the α5 SNP results in increased ethanol self-administration under fixed ratio (FR) and progressive ratio

(PR) schedules of reinforcement and ethanol seeking during extinction and reinstatement (Besson et al., 2019). Nonetheless, when examining ethanol intake when it was provided at different concentrations under an FR5 schedule, the same group only described a small difference in the ethanol dose-response relationship in the absence of a significant effect of *Chrna5* genotype. The lack of a positive correlation between home cage consumption and operant self-administration of ethanol has been reported before (Files et al., 1998; Koros et al., 1999; Wilson et al., 1997), and partially reflects the ability of operant procedures to assess appetitive behaviors that cannot be readily examined in home-cage, non-operant procedures. Furthermore, post-ingestive effects can modulate subsequent consumption, resulting in undetectable changes in consummatory behavior caused by genetic variation. This could explain why we did not detect an effect of the *Chrna5* SNP on ethanol intake in males.

It is possible that these differences are related to sex hormone-induced changes in α5 nAChR subunit expression (Gangitano et al., 2009) given that progesterone levels start to differ between females and males during peripuberty in rodents and humans (Bell, 2018; Pignatelli et al., 2006). The observation that females, but not males, with disrupted α5*nAChRs displayed increased volitional alcohol consumption in bottle-choice procedures may indicate a sex-modulated effect of *Chrna5* mutation on the reinforcing properties of ethanol, resulting in increased vulnerability to overconsume and misuse alcohol. Other methods to investigate ethanol reinforcement – such as taste and place conditioning – are needed to further investigate this hypothesis in adolescent and adult female mice. For example, it has been shown that male mice lacking the α5 nAChR subunit do not display conditioned place preference (CPP) when the conditioned stimulus is paired with an ethanol dose that produces strong CPP in WT male mice (Dawson et al., 2018) – but other doses have not been examined. Evaluation of the ethanol dose-CPP response relationship in mice of both sexes would allow further investigation of the sex-dependent involvement of the α5*nAChRs in ethanol reward and reinforcement.

4.2. Adult voluntary intake of nicotine or ethanol following adolescent exposure to the opposite drug

In this study, we report a modulatory effect of *Chrna5* mutation on the influence of adolescent exposure to nicotine or ethanol on voluntary consumption of these drugs during adulthood. Previous work in humans has shown that smoking during adolescence impacts interhemispheric connectivity and gray matter volume of the ventromedial prefrontal cortex (PFC), and this effect appears to be exacerbated in carriers of the rs16969968*A allele (Chaarani et al., 2019). Alcohol consumption during adolescence has also been shown to produce disturbances in brain morphometry, to which the PFC seems to be particularly vulnerable as well (De Bellis et al., 2005; Medina et al., 2008; Squeglia et al., 2015). Thus, both alcohol and nicotine exposure can affect midbrain and corticostriatal circuits that are important for salience attribution, reward processing, decision making and self-control (see review by Goldstein and Volkow, 2011). The expression of the α5 nAChR subunit in regions across these circuits suggests a role of the α5*nAChRs in communication and actions driven by these circuits (Azam et al., 2002; Hsu et al., 2013; Picard et al., 2013; Zoli et al., 2002). Moreover, drug-promoted adaptations in corticostriatal circuits have been

extensively implicated in addiction and seem to facilitate the transition to compulsive drug use. For instance, an imaging study showed that the degree of synchronization between the dorsal anterior cingulate cortex (dACC) and the ventral striatum-extended amygdala during nicotine withdrawal is predictive of nicotine addiction severity (Hong et al., 2010). Notably, findings from the same study investigating resting state functional connectivity (rsFC) in corticostriatal circuits found evidence indicating a modulatory effect of *CHRNA5* variation on nicotine-related alterations of top-down inhibitory control. Specifically, the *CHRNA5* rs16969968 SNP was shown to modulate the magnitude of the decrease in connectivity between these structures caused by chronic smoking (Hong et al., 2010). Reduced frontostriatal functional connectivity has also been observed following binge-like ethanol exposure during adolescence in rats (Broadwater et al., 2018), and is predictive of relapse in humans (Camchong et al., 2013). Thus, both ethanol and nicotine exposure during adolescence may impair maturation of corticolimbic connections, perpetuating impulsivity and risk-taking behaviors characteristic of adolescence and that can predispose an individual to misuse addictive substances. Considering that previous work has shown that dysfunction of the α5*nAChR system modulates baseline brain network connectivity and adaptations of these circuits in response to chronic drug use (Hong et al., 2010), our findings suggest that *Chrna5* variation also strongly influences subsequent acute exposures, their motivational valence and potential to expedite the establishment of alcohol/nicotine dependence. Certainly, more research investigating the rs16969968 modulation of aberrant long-term plasticity in response to drug exposure during development and its consequences on later drug self-administration is warranted.

4.3. *Sex-specific influences of* Chrna5 *mutation on the effects of adolescent ethanol or nicotine exposure*

The discrepancy between females and males could be related to basal sex differences in the dopaminergic system. For instance, it has been shown that females and males differ in the number, morphology, function, and cholinergic modulation of VTA DA neurons (reviewed by Zachry et al., 2020), which express the α5 nAChR subunit (Azam et al., 2002; Wada et al., 1990). Previous work has shown sexually dimorphic behavioral and transcriptional effects of *Chrna5* mutation in rodents (Correa et al., 2019; Gangitano et al., 2009). Our observations also suggest that the consequences of *CHRNA5* rs16969968 on adolescent drug use may be different between females and males. Although it is challenging to make inferences based on previously published work that has widely excluded females from their experimental designs, there is some evidence of neuroplastic changes involving the cholinergic system following drug exposure that are modulated by sex. It has been shown that animals undergoing withdrawal from chronic nicotine show heightened levels of acetylcholine (ACh) in the nucleus accumbens (NAc, Carcoba et al., 2018, but see Nestby et al., 1999 for ethanol) and IPN (Correa et al., 2019), with the effect in the latter structure being more robust in females. Elimination of ACh via choline acetyltransferase (ChAT) KO in habenular neurons projecting to the IPN – where *Chrna5* is densely expressed (Grady et al., 2009) – blunts the reinforcing properties of nicotine (i.e., withdrawal and CPP, Frahm et al., 2015) in male rats. A similar behavioral phenotype pertaining to nicotine (Salas et al., 2009) and ethanol reinforcement (Dawson et al., 2018) has been shown in mice lacking the α5 nAChR subunit. Although speculative, this suggests that activation

of IPN neurons via α5*nAChRs contributes to the mechanisms of drug reinforcement, and thus enhancement of cholinergic signaling in this brain region in individuals with disrupted α5*nAChRs could compensate for their baseline deficiencies, resulting in less severe drug-taking phenotypes. Furthermore, Nestby et al., 1999 showed that the effect of ethanol withdrawal on increased ACh release in the striatum was dose-dependent in males. Whether this phenomenon also occurs in ethanol-treated female rodents during withdrawal and extends to other brain regions is unknown. However, it is tempting to speculate that this ethanol dose-dependent ACh enhancement during protracted withdrawal could explain the lower nicotine consumption observed in α5 KO females exposed to alcohol during adolescence, as well as the negative correlation between adolescent aggregated ethanol dose and adult weekly nicotine intake (Figure 3A and C).

Ethanol has been shown to induce transcriptional changes in a sex-dependent manner, with differences in gene ontology category over-representation (Hashimoto and Wiren, 2008) and minimal overlap of differentially expressed genes between male and female C57BL/6J and DBA/2J mice (Hitzemann et al., 2021). Sex differences have also emerged in studies examining the effects of nicotine withdrawal on α5 expression levels in the IPN (Correa et al., 2019). Moreover, the expression of high-affinity nAChRs (e.g., α4β2) is increased in response to ethanol (Eriksson et al., 2000; Tarren et al., 2017) and adolescent nicotine (Doura et al., 2008). Therefore, there is evidence that the α5 subunit might play a regulatory role on drug-related neuroplastic changes in number/type of nAChRs, which could explain some of our behavioral observations on modulation of adolescent treatment by genotype. However, it should be noted that although the α5-containing α4β2 nAChR subtype is a common native receptor variant (Gotti et al., 2006; Zoli et al., 2002), the α4β2α5 nAChRs have been reported to be resistant to nicotine-induced upregulation (Hoegberg et al., 2015; Mao et al., 2008). It is also important to mention that there is evidence that ethanol and nicotine do not always produce identical changes in nAChR number and function in the brain (see reviews by Feduccia et al., 2012; Lajtha and Sershen, 2010). Undoubtedly, more research is needed to unveil the mechanism behind the effect of α5 nAChR subunit disruption on adult alcohol/nicotine self-administration following adolescent consumption of these substances. Understanding how sex, genetic variation and drug exposure interactively modulate communication across brain regions implicated in addiction may be useful to develop novel treatments for alcohol and nicotine dependence.

4.4. Conclusion

This study suggests that α5*nAChR-mediated neurotransmission may modulate long-term neuroplasticity in response to developmental exposure to alcohol or nicotine. Further investigation is needed to identify α5-dependent alterations and if they could be targeted in adulthood to modulate voluntary alcohol or nicotine consumption. Understanding how adolescent alcohol or tobacco use re-wire the brain to promote drug use in adulthood may help in the development of cessation therapies for individuals with early onset of use of these drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

SOURCES OF SUPPORT

This work was supported by the National Institutes of Health grant U01 AA025931 to MDB. VAR is supported by Division of Intramural Clinical and Biological Research (Z1A AA000466), National Institute on Alcohol Abuse and Alcoholism, NIH.

ABBREVIATIONS:

AUD	alcohol use disorder
nAChR	nicotinic acetylcholine receptor
VTA	ventral tegmental area
PND	post-natal day
IPN	interpeduncular nucleus
SNP	single nucleotide polymorphism
WT	wildtype
KO	knockout
DID	drinking-int-the-dark
2BC	two-bottle choice
3BC	three-bottle choice
BEC	blood plasma ethanol concentration
ANOVA	analysis of variance
RM	repeated measures
CI	confidence interval
CPP	conditioned place preference
PFC	prefrontal cortex

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HIGHLIGHTS

- Disrupted α5*nAChRs increase ethanol intake in adolescent and adult female mice
- Adolescent ethanol or nicotine exposure increases drug intake in adulthood
- The effect of adolescent drug exposure is modulated by *Chrna5* mutation and sex

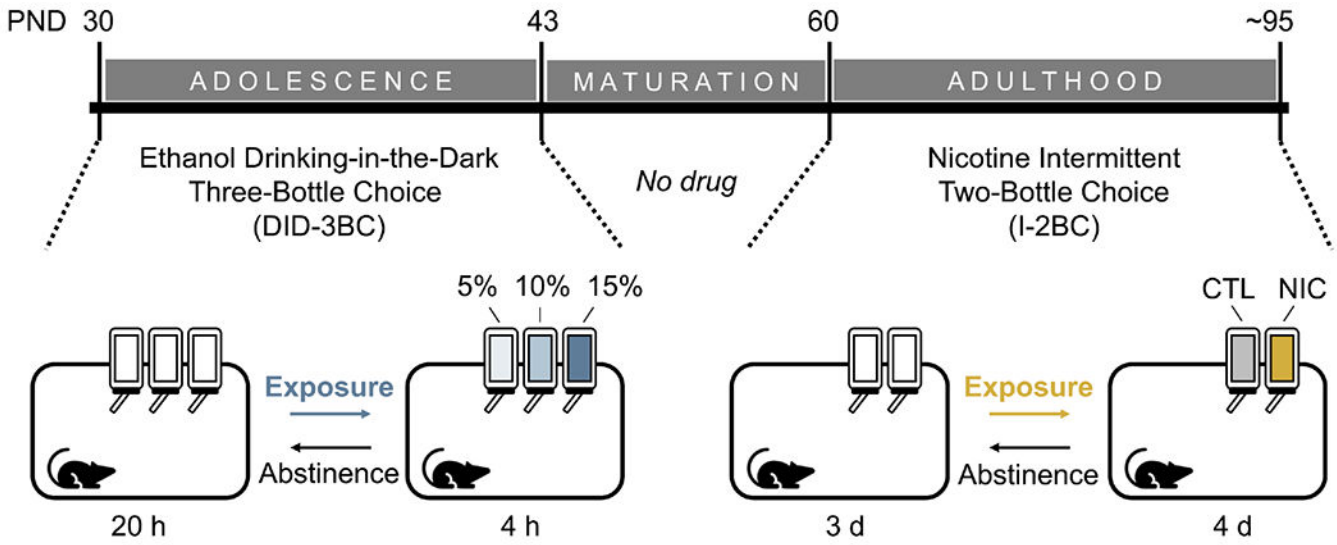

FIGURE 1. Schematic of Experiment #1 - adolescent ethanol drinking-in-the-dark (DID) followed by nicotine intermittent two-bottle choice (I-2BC) in adulthood.

Alcohol consumption during adolescence was evaluated in WT, α5 SNP and α5 KO mice of both sexes using a model of binge-like drinking (DID). Briefly, mice received simultaneous access to three bottles of ethanol-containing solutions (5, 10, 15%) during 4 h every day from post-natal day (PND) 30 to 43. Mice had no access to ethanol or nicotine during maturation (PND 43-60). In adulthood (PND 60), nicotine consumption was administered via the drinking fluid at a concentration of 200 mg/L intermittently for a total of five weeks. Saccharin (0.2% w/v) was added to both the nicotine solution and the control bottle. CTL = control solution, NIC = nicotine solution. A total of 125 mice were utilized in this experiment.

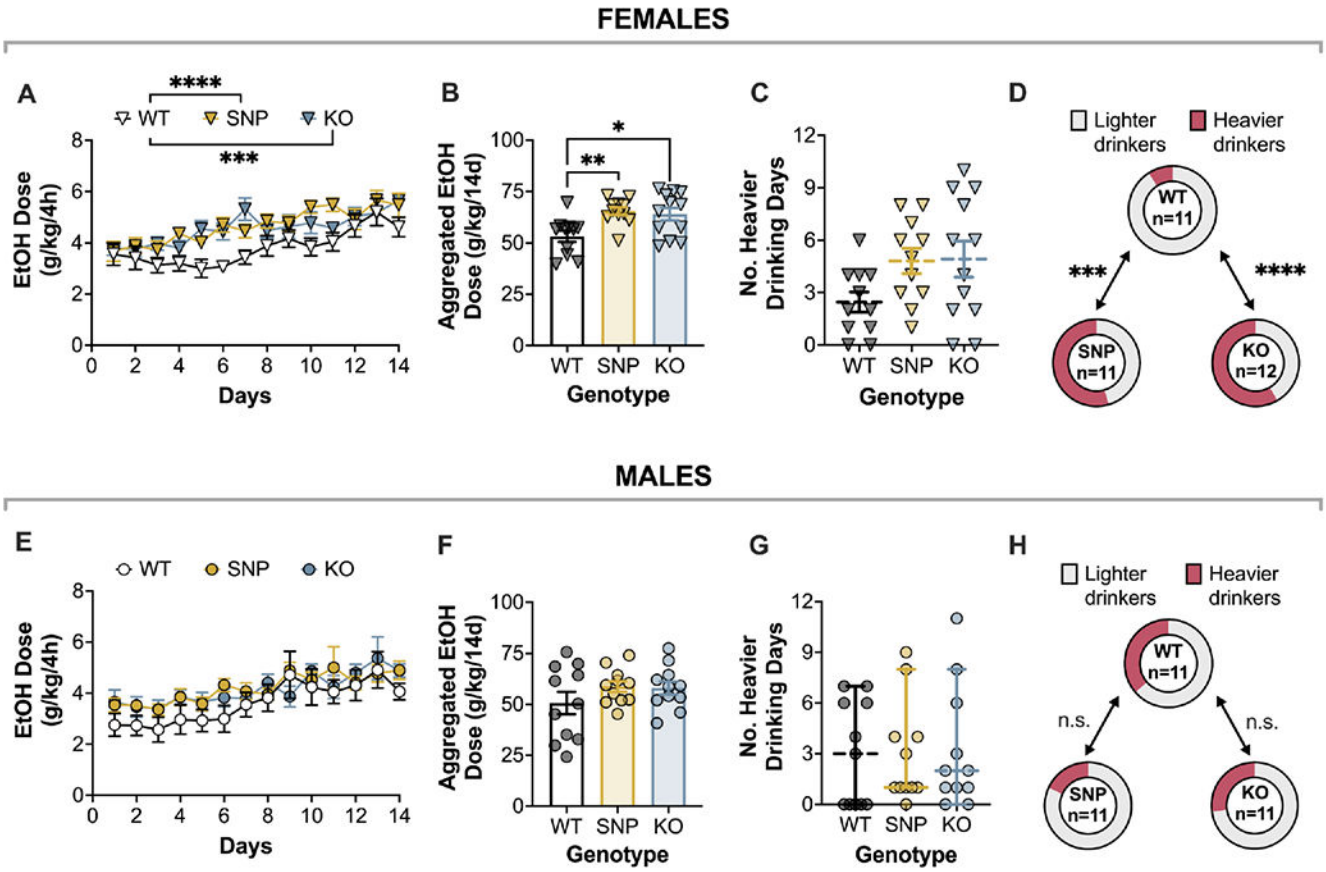

FIGURE 2. Disruption of α5-containing nicotinic acetylcholine receptors affects alcohol consumption during adolescence in female, but not male mice.

WT, α5 SNP and α5 KO mice of both sexes were allowed to self-administer ethanol during adolescence in an in-cage model of binge drinking. Daily 4-h ethanol dose (A, E) and aggregated ethanol dose (B, F) were evaluated. The occurrence of heavier drinking days (>5.125 g/kg/4h predictive of plasma ethanol concentration greater than 20 mg/dL; see Methods for more details) was also assessed during adolescence (C, G). Lastly, hierarchical clustering was utilized to group mice based on total level of consumption and number of heavier drinking days (D, H). Overall, α5 nAChR subunit deletion or dysfunction resulted in worse alcohol drinking behavior during adolescence in female, but not male mice. (A, B, C, E, F) show mean ± SEM, (G) shows median ± 95% CI, (D, H) show proportions. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n = 11-12/group.

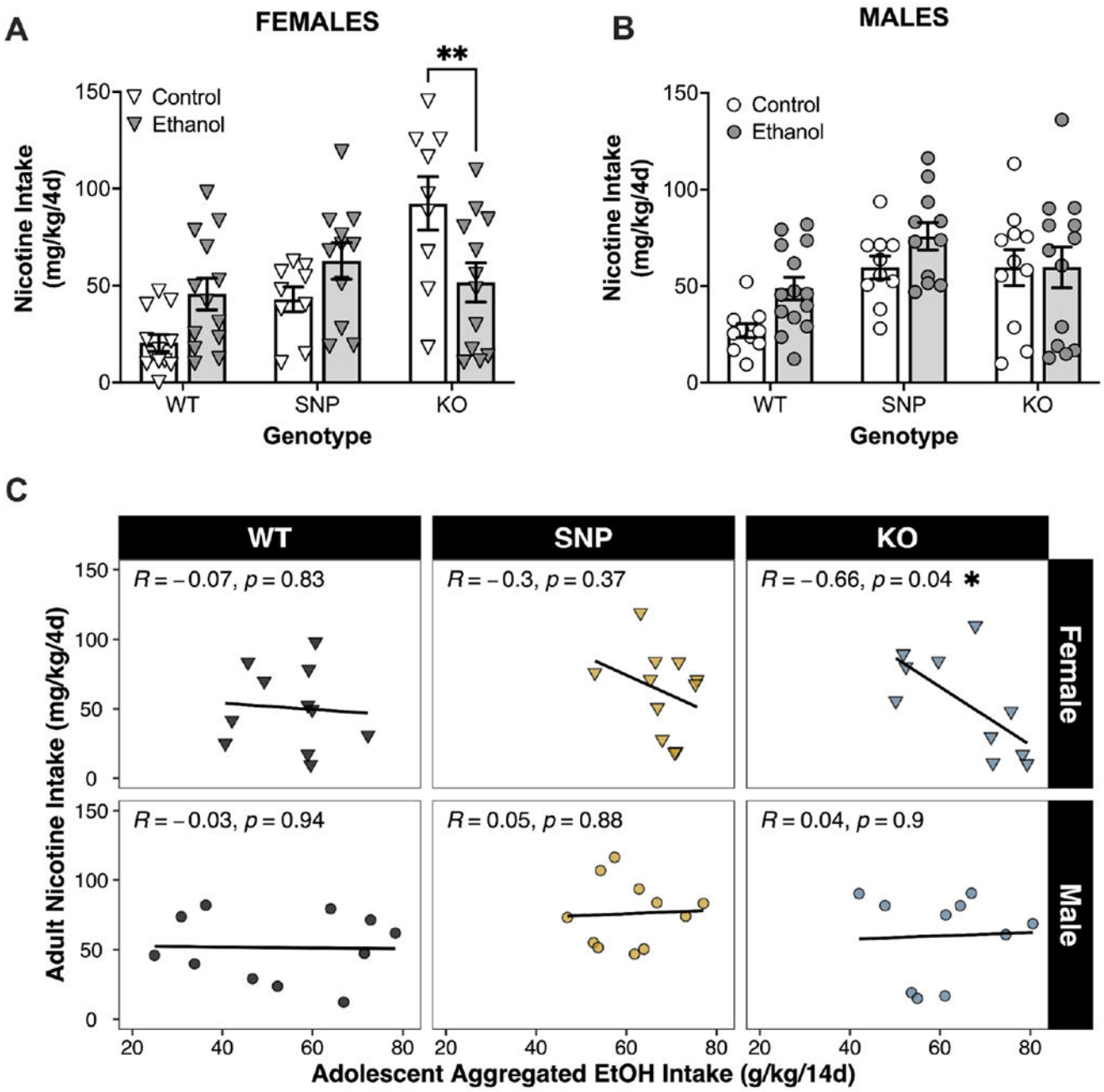

FIGURE 3. α5-dependent and sex specific effect of adolescent ethanol exposure on adult nicotine consumption.

WT, α5 SNP and α5 KO mice of both sexes exposed to ethanol via the drinking-in-the-dark three-bottle choice (DID 3BC) were allowed to self-administer nicotine *per os* in adulthood. Average nicotine intake was examined in females (A) and males (B). Genotype and adolescent alcohol treatment had a significant interactive effect on adult nicotine intake in females (p=0.0007), but not in males (p=0.3462). Interestingly, α5 KO females exposed to alcohol during adolescence consumed significantly less nicotine in adulthood compared to control-treated α5 KO females. This effect was further investigated by

examining correlations between levels of adolescent ethanol and adult nicotine intake (C). A significantly negative correlation was observed in α5 KO females whereas correlations for all the other groups were not statistically significant. (A, B) show mean ± SEM, (C) shows individual values with line of best fit. **p<0.01. n = 9-14/group.

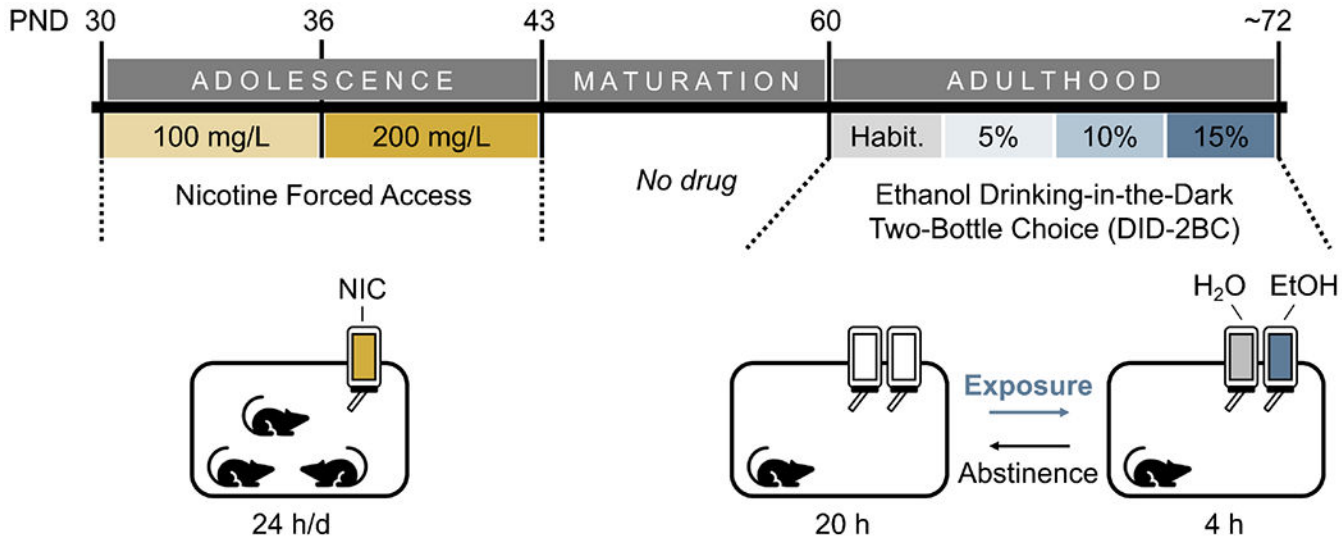

FIGURE 4. Schematic of Experiment #2 - adolescent nicotine exposure followed by ethanol drinking-in-the-dark (DID) in adulthood.

Group-housed WT and α5 KO mice of both sexes had forced access to nicotine or control saccharin solution during adolescence. Nicotine-treated mice had access to two bottles of 100 mg/L nicotine from PND 30 to 36, and to 200 mg/L nicotine from PND 37 to 43. Saccharin (0.2% w/v) was added to nicotine solution to mask its bitter taste. Control-treated animals had access to saccharin solution (0.2% w/v) during the entire adolescent treatment. Mice had no access to ethanol or nicotine during maturation (PND 43-60). On PND 60, mice were single-housed and habituated to lickometers for 3-5 days. Ethanol consumption was then evaluated using a drinking-in-the-dark two-bottle choice (DID-2BC), where mice had access to each ethanol concentration for 3-4 days. NIC = nicotine solution, EtOH = ethanol solution. A total of 120 mice were utilized in this experiment.

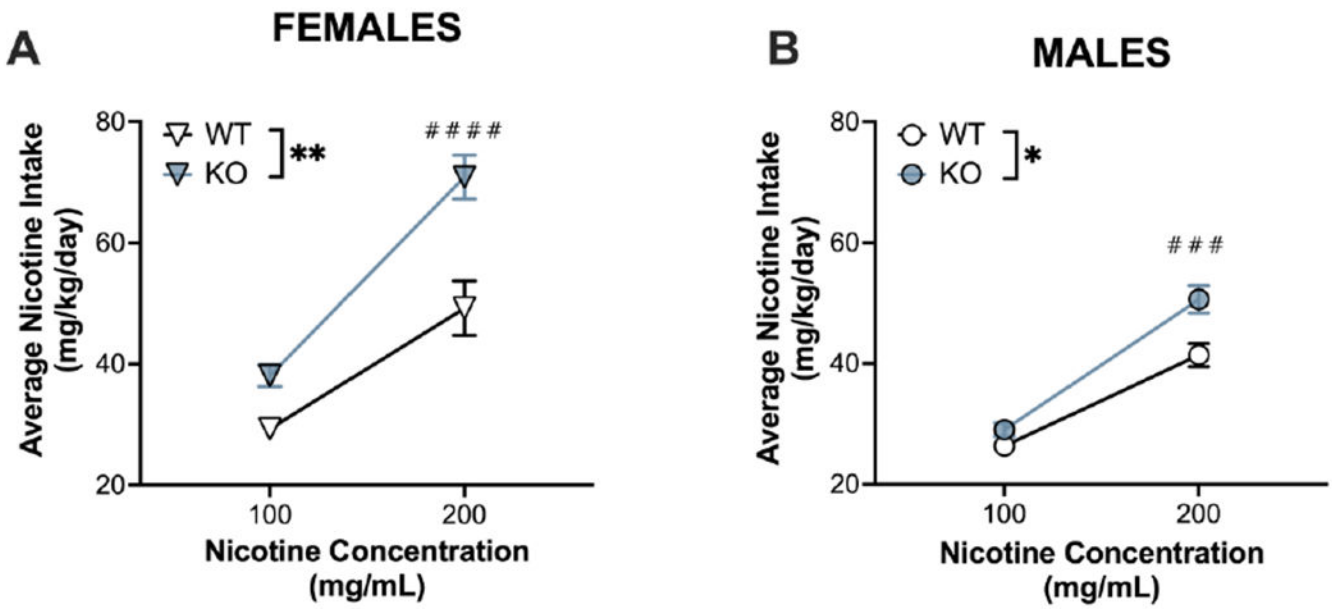

FIGURE 5. Mice with *Chrna5* knockout display increased nicotine intake during adolescence.
Nicotine or control solution was provided via the drinking water to adolescent WT and α5 KO mice of both sexes. Female and male mice with an α5 nAChR subunit deletion showed higher levels of nicotine consumption at the highest concentration of nicotine provided (i.e., 200 mg/mL nicotine in 0.2% saccharin). In a separate group of mice, trunk blood was collected on post-natal day (PND) 43 and cotinine concentration was measure in the plasma. α5 KO mice had increased levels of nicotine compared to WT mice (Supplemental Figure 4). (A, B) show mean ± SEM. *p<0.05, **p<0.01 for main effect of genotype; ###p<0.001, ####p<0.0001 WT vs KO at specified concentration, n = 7-10/group.

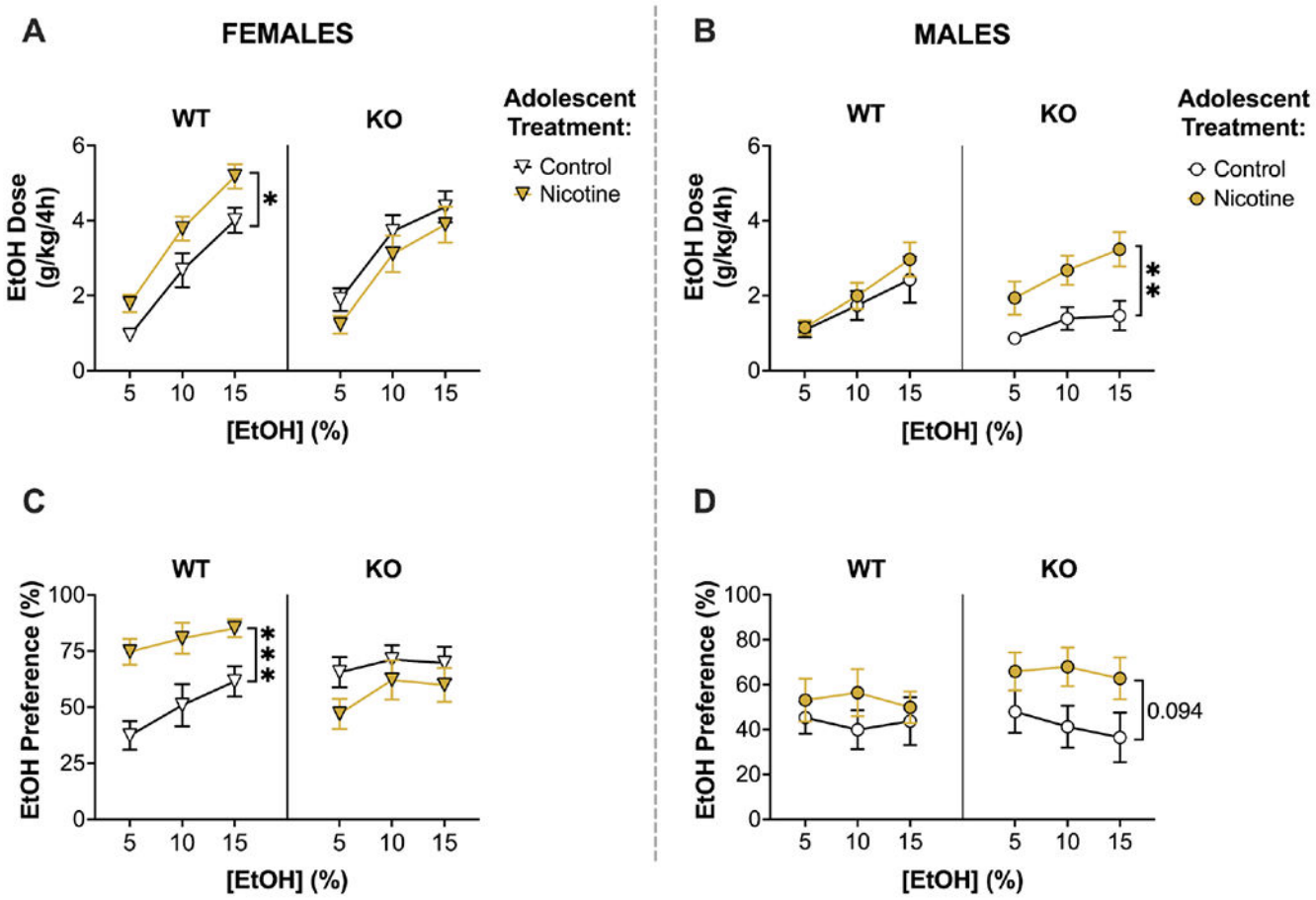

FIGURE 6. Adult alcohol consumption is influenced by adolescent exposure to nicotine in a sex-specific and α5- dependent manner.

Alcohol consumption (A, B) and preference (C, D) in the drinking-in-the-dark two-bottle choice (DID 2BC) model was examined in WT and α5 KO mice of both sexes (Left column: females, Right column: males) treated with saccharin or nicotine during adolescence. RM three-way ANOVA did not reveal significant interactions of ethanol concentration with other variables. Therefore, two-way ANOVAs followed by Bonferroni's multiple comparisons tests were used to further investigate differences in the groups. P-values and significance asterisks indicate results from post hoc comparisons. All data are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 main effect of adolescent treatment in specified genotype group. n = 10/group.

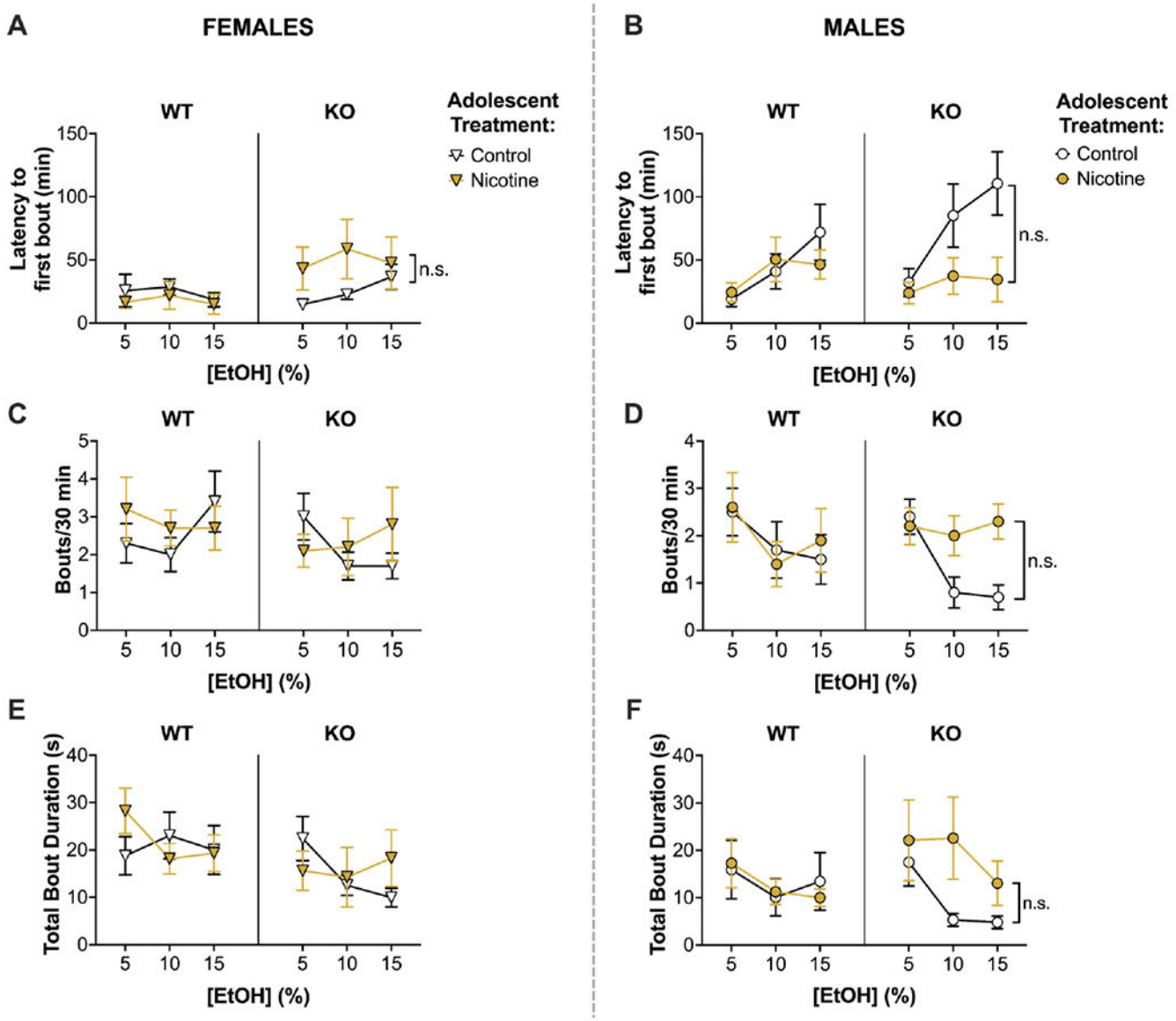

FIGURE 7. Adolescent nicotine exposure produces minor changes in ethanol drinking microstructure in α5 KO male mice.

Photobeam-based lickometers were used to examine the microstructure of the drinking behavior of WT and α5 KO mice exposed to saccharin or nicotine during adolescence. Bouts are operationally defined as events at the ethanol sipper fulfilling duration (cumulative time ≥250 ms) and inter-event interval (≤1 s) criteria. The following descriptors of drinking behaviors were examined: (A, B) time to first bout, (C, D) number of bouts during the first 30 min of the session (proxy for early phase drinking), and (E, F) total time spent drinking in bouts. RM three-way ANOVA did not reveal significant interactions of ethanol concentration with other variables. Therefore, two-way ANOVAs followed by Bonferroni's multiple comparisons tests were used to further investigate differences in the groups. No

significant differences were observed between groups. All data are shown as mean ± SEM. n = 10/group.