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# Adolescent Intermittent Ethanol (AIE) Increases the Sensitivity to the Reinforcing Properties of Ethanol and the Expression of Select Cholinergic and Dopaminergic Genes within the Posterior Ventral Tegmental Area

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Running Head: EtOH in Adolescence Adult Mesolimbic Dopamine

**Abstract**

Background: Although not legally allowed to consume alcohol, adolescents account for 11% of all alcohol use in the US and approximately 90% of adolescent intake is in the form of an alcohol binge. The adolescent intermittent ethanol (AIE) model developed by the NADIA consortium produces binge-like EtOH exposure episodes. The current experiment examined the effects of AIE on the reinforcing properties of EtOH and genetic expression of cholinergic and dopaminergic factors within the posterior ventral tegmental area (pVTA) in Wistar male and female rats, and in male alcohol-preferring (P) rats.

Methods: Rats were exposed to the AIE or water during adolescence and all testing occurred during adulthood. Wistar control and AIE rats were randomly assigned to groups that self-administered 0- 200 mg% EtOH. Male P rats self-administered 0-100 mg%.

Results: The data indicated that exposure to AIE in both Wistar male and female rats (and male P rats) resulted in a significant leftward shift in dose response curve for EtOH self-administration into the pVTA. Taqman array indicated that AIE exposure had divergent effects on the expression of nicotinic receptors (increased a7, reduction in a4 and a5). There were also sex specific effects of AIE on gene expression; male only reduction in D3 and muscarinic 1 receptors.

Conclusion: Binge-like EtOH exposure during adolescence enhances the sensitivity to the reinforcing properties of EtOH during adulthood which could be part of biological sequelae that are the basis for the deleterious effects of adolescent alcohol consumption on the rate of alcoholism during adulthood.

## Introduction

In US adolescents, the most commonly utilized drug of abuse is alcohol (Johnston et al., 2004). Despite a legal drinking age of 21, 11% of all alcohol consumed in the US is by individuals aged 12 to 20 (OJJDP, 2005). The lack of control of alcohol intake in US adolescents is reflected by the fact that over 90% of US adolescent alcohol consumption occurs in the form of binge drinking (OJJDP, 2005). The consequence of adolescent binge drinking is noted in the more than 4,300 annual deaths in people aged 12 to 20 attributed to excessive alcohol consumption (Sacks et al., 2010). Further evidence of the impact of US adolescent binge drinking is the approximately 119,000 emergency room visits annually for alcohol-related injuries and events (Naeger, 2017). Overall excessive alcohol consumption by US youths results in an annual economic cost of $24 billion (Sacks et al., 2010). Past reports have indicated that 22 and 28% of $10^{th}$ and $12^{th}$ grade students, respectively, reported binge-drinking within the past two weeks (Johnston et al., 2004). Binge-drinking in high school is associated with an increased rate of binge-drinking in college and a propensity for frequent binge-drinking (19-25% report more than 3 episodes of binge drinking per week; Wechsler et al., 1995, 2000; SAMHSA, 2006).

Binge-drinking during adolescence is associated with a greater number of deleterious consequences during adulthood compared to individuals who drank socially or abstained from drinking during adolescence. Age of first drink and the propensity to have binge EtOH drinking episodes during adolescence is associated with increased alcohol involvement, heavier drinking bouts, arrests for driving with ability impaired, and an increased rate of alcohol dependence during adulthood (Hingson et al., 2008; Chou and Pickering, 1992). Epidemiological studies have indicated a 1.3 to 1.6 times increased rate of alcohol dependence in individuals who initiate alcohol use prior to the age of 15 (Dawson et al., 2008).

Despite the mounting evidence of the frequency and importance of adolescent binge alcohol drinking, the vast majority of preclinical research employs adolescent alcohol exposure protocols that do not surpass pharmacologically relevant blood alcohol levels (> 30 mg%) let alone approach the established criteria for binge-like alcohol consumption (> 80 mg%). The Neurobiology of Adolescent Drinking in Adulthood (NADIA) consortium has established an Alcohol Intermittent Exposure (AIE) protocol that results in equivalent exposure to EtOH in subjects and blood alcohol levels that exceed the binge-drinking threshold (c.f., Vetreno et al., 2014).

The adolescent brain is in neuro-developmental flux and is characterized by rapid growth, reorganization, and pruning of neurons throughout adolescence (c.f., Geidd, 2004). Adolescent alcohol exposure can disrupt this normal remodeling of cortical and limbic regions (Spear, 2000). Replicable effects of adolescent alcohol consumption on the adult brain include a hyper-dopaminergic response to stimuli, reduction in choline acetyltransferase (ChAT), alterations in neuroimmune function, and non-specific modulation of epigenetic factors (Spear and Swartzwelder, 2014; Mullholland et al., 2018; Crews et al., 2019).

The hyper-responsive dopaminergic system in adult brains following adolescent alcohol exposure is indicated in numerous reports. Adolescent EtOH consumption (Sahr et al., 2004), as well as peripheral EtOH injections (Bandanich et al., 2007) can increase basal dopamine levels or dopamine reuptake in the AcbSh during adulthood. Similar results are not produced in adult rats with comparable EtOH exposure paradigms (Pascual et al., 2007, 2009). Voluntary adolescent alcohol consumption in male alcohol-preferring (P) rats (BECs approximately 60 mg%) results in increased sensitivity to the reinforcing properties of EtOH (leftward shift in dose response curve) in the posterior ventral tegmental area (pVTA; Toalston et al., 2014). In addition, voluntary adolescent EtOH consumption in P rats reduces the concentration of EtOH required to be microinjected into the pVTA to stimulate dopamine

(DA) release in the nucleus accumbens shell (AcbSh; Toalston et al., 2014). The hyper-responsive adult DA system produced by adolescent EtOH exposure was also indicated by the finding that the magnitude of DA release in the AcbSh induced by microinjections of EtOH into the pVTA was increased in P rats that voluntarily consumed EtOH during adolescence (Toalston et al., 2014). Similarly, consumption of EtOH during adolescence in rats results in greater DA release in the AcbSh following 'risky' choice responding compared to adolescent naïve controls (Nasrallah et al., 2011). Behaviorally, voluntary adolescent EtOH consumption in P rats enhances operant self-administration of EtOH and EtOH reward valance, but not saccharin, during adulthood (Toalston et al., 2015).

The alteration in mesolimbic DA responsivity produced by adolescent EtOH exposure could be the result of neuroadaptations in the inputs into the pVTA and/or the DA neurons within the pVTA. Cholinergic innervation of the VTA stimulates and/or regulates the activation of DA neurons within the pVTA (Mansvelder et al., 2003; Mansvelder and McGehee, 2000). The consistent findings that AIE exposure results in persistent reduction in ChAT in multiple brain regions (c.f., Crews et al., 2019) would suggest an upregulation in cholinergic receptors to maintain functional homeostasis. Upregulation of cholinergic receptors would enhance the propensity of DA neurons to respond to EtOH and other drugs of abuse that act on those receptors. DA receptors in the pVTA are primarily D2/D3 autoreceptors (Le Foll et al., 2005). Alterations in DA autoreceptors or other regulator factors would influence the neuronal activity of pVTA DA neurons (loss of inhibitory influence), and chronic EtOH drinking has been shown to alter D2 autoreceptors in the pVTA (Engleman et al., 2011). Alterations in the inputs into the pVTA or pVTA DA neurons could be the biological basis for the increase in reward sensitivity produced by adolescent EtOH exposure (Toalston et al. 2014, 2015). The pVTA could be considered a neuronal nexus where cholinergic and dopaminergic related factors regulate the effects of adolescent EtOH

exposure on the response to drugs of abuse during adulthood. The current experiments were designed to determine the effects of binge-like exposure to EtOH during adolescence in Wistar male and female rats on the reinforcing properties of EtOH within the pVTA. In addition, the utility of the AIE protocol was assessed by determining if binge-like EtOH exposure produced comparable alterations in the reinforcing properties of EtOH in P rats as that previously observed following voluntary adolescent EtOH consumption (Toalston et al., 2014). The last experiment determined if adolescent binge-like exposure to EtOH resulted in significant alterations in the expression of dopaminergic and acetylcholine (Ach) receptor systems genes in the pVTA of Wistar male and female rats. The overall hypothesis was that adolescent EtOH consumption should enhance the reinforcing properties of EtOH within the pVTA and result in targeted alterations in DA and Ach gene expression in the pVTA.

## Methods

### *Subjects*

As part of the NADIA consortium, a single generation colony of Wistar rats has been established at Indiana University School of Medicine (IUSM; Indianapolis, IN). Breeding is done within the facilities in order to maintain chain of control of subjects and to prevent stress from shipping adolescents. The P rats used in the current experiments were from a substrain of P rats termed Taconic P (tP) rats. Taconic P rats were separated from the IUSM stock of P rats in 2004 and maintained at Taconic Bioscience (Rensselaer, NY) until the rats were returned to IUSM in 2013. The tP rats were maintained for over 30 generations at Taconic Bioscience and represent a highly functional substrain of the P rat. The tP rats have been maintained isolated from the standard P rat since their return to IUSM.

Animals used in this study 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 were in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, the NIH, and the Guide for the Care and Use of Laboratory Animals (2011).

*Adolescent Intermittent EtOH (AIE) Protocol*.

The rats were treated with the standard NADIA AIE protocol. On post-natal day (PND) 21 rats were randomly assigned to either binge (AIE) EtOH exposure or water (Control Intermittent Exposure, CON) only. AIE rats were treated with 4 g/kg EtOH (25% v/v) every other day during adolescence (PND 28-48). The CON group received comparable treatment with water. EtOH and water was administered through oral gavage. All rats were group housed during adolescent treatment and until the onset of experiments PND 90.

*Intracranial Self-Administration (ICSA) Apparatus*

The ICSA test chambers have been extensively described in past (Rodd-Henricks et al., 2000, Rodd et al., 2004). Briefly, an electrolytic microinfusion transducer (EMIT) system controlled the delivery of assigned infusate into the subject via calibrated pulses of current. Depression of the active lever initiated a 5-sec infusion current of 200 μA, resulting in rapid generation of hydrogen gas, increasing the pressure inside the airtight cylinder, pressing a 100 nl bolus of infusate out through the injection tip. Between infusions, current returned to the low quiescent state.

During each infusion, there was a 5-sec time-out period where bar-press responses were recorded yet resulted in no further infusion. In this time-out, the house light and a red cue light were extinguished, while the green cue light over the active lever flashed in 0.5-sec intervals. The assignment of active and inactive lever with respect to the left or right position

was counterbalanced among subjects, and remained the same throughout the experiment for each respective subject.

*ICSA Procedure*

Adolescent EtOH treatment occurred as described above. Food and water were available *ad lib* at all times, except during ICSA testing. ICSA was performed as previously described (Rodd-Henricks et al., 2000, Rodd et al., 2004, 2005). After postnatal day 75, the rats were implanted under isoflurane anesthesia with a guide cannula (22 gauge, Plastics One, Roanoke, VA) stereotaxically aimed 1.0 mm above the pVTA. Coordinates were 5.8 to 6.1 mm posterior to bregma, 2.1 mm lateral, and 8.5 mm ventral from the surface of the skull at a 10 degree angle from the vertical (Paxinos and Watson, 1986). A place-holding stylet (28 gauge, Plastics One) extending 0.5 mm beyond the tip of the guide cannula was inserted at all times, except during test sessions. Subjects were single-housed post surgery, and allowed to recover for 7 days. Three days prior to testing, subjects were handled 5 min per day.

All infusates were prepared freshly on the day of the experiment. Artificial cerebrospinal fluid (aCSF) was used as the vehicle for ICSA infusions. This injection vehicle consisted of (in mM) 120.0 NaCl, 4.8 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, and 10.0 D-glucose. The solution was then sterilized with a syringe filter (pore size 0.2 μM) as previously described (Rodd-Henricks et al., 2000, Rodd et al., 2004b). Ethyl alcohol (190 proof; McCormick Distilling Co., Weston, MO) was dissolved in the vehicle solution to the correct concentration. When necessary, 0.5 N HCl was added to adjust the pH to 7.4 (±0.1).

ICSA was conducted similar to procedures previously described (Rodd-Henricks et al., 2000, Rodd et al., 2004). Briefly, subjects were brought to the testing room, the stylet was removed, and an injection cannula/infusate cylinder was affixed in place. The injection cannula extended 1.0 mm beyond the tip of the guide, into the pVTA. A single,

noncontingent administration of infusate was given at the beginning of the session during this insertion procedure in order to prime the system. Test sessions occurred every other day. No operant shaping techniques were used. Active lever and inactive lever sides were counterbalanced between subjects, remaining the same for each individual rat. Within each 4 hr session, responses on the active lever resulted in 5 sec infusions on a fixed ratio 1 schedule of reinforcement. During infusion and time-out, responses on the active lever were recorded, but did not produce further infusions. Responses on the inactive lever were recorded but did not result in infusions at any time; these responses were used to index non-specific bar-pressing activity. During ICSA sessions 1 through 4 (acquisition), subjects received their respective dose of either the aCSF vehicle or EtOH. During ICSA sessions 5 and 6 (extinction), all subjects received aCSF vehicle only, and in session 7 (reinstatement), the original EtOH concentration was made available.

**Experiment 1: Wistar Male and Female AIE ICSA EtOH Dose Response**

Overall, there were 95 (54 AIE and 41 CON) Wistar males and 74 (37 AIE and 37 CON) Wistar females used in this experiment. Rats were randomly assigned to self-administer 0, 25, 50, 75, 100, 150, or 200 mg% EtOH for the initial 4 ICSA sessions. Group sizes were 6-9/group for AIE males, 5-6/group for AIE females, 5-7/group for CON males, and 5-6/group for CON females.

**Experiment 2: tP Male AIE ICSA EtOH Dose Response**

Overall, there were 50 (25 AIE and 25 CON) tP males used in this experiment. Rats were randomly assigned to self-administer 0, 25, 50, 75, or 100 mg% EtOH for the initial 4 ICSA sessions. All group sizes were n = 5.

**Experiment 3: Taqman Array Assessment of DA and Acetylcholine Receptors in the pVTA Following AIE Treatment**

Conceptually, the pVTA does not have cholinergic neurons, but pre- and post-synaptic cholinergic receptors. Therefore, expression of genes regulating the production of acetylcholine should not be apparent when assaying the pVTA (only genes coding proteins of cholinergic receptors). In contrast, the pVTA DA neurons express the building blocks of DA production (e.g. tyrosine hydroxylase) and primarily DA autoreceptors (D2/D3).

A total of 23 Wistar rats were used in this experiment. Rats were exposed to the AIE (6 males/females) or CON (6 males/5 females) during PND 28-48. Rats were not exposed to any other manipulations prior to brains being harvested on PND 90. Samples were pooled from bilateral micropunches of the pVTA. The protocol for this procedure is detailed in published studies (Truitt et al., 2014). The TaqMan® Low-Density Array (TMLDA) is a multiple RT-PCR assay that quantifiably examines mRNA levels. We have previously used the TMLDA assay to assess the effects of EtOH and NIC microinjected into the pVTA on gene expression in the AcbSh (Truitt et al., 2014) and innate difference in gene expression within the pVTA in genetic models of alcoholism (Deehan et al., 2018). The current TMLDA (Applied Biosystems, Foster City, CA) was a 48 gene array. The selected genes included 6 house-keeping genes and 37 genes associated with DA and Acetylcholine receptor systems (AchR: nAchR – nicotinic acetylcholine receptor). We also included other selected gene targets in the TLMDA (5 genes). The a7 NAchR is part of a cys-loop receptor family that includes the $5HT_3$ (*Hrt3b*a and *Hrt3b*) and Impact (Ancient and Conserved/Imprinted) receptor (Jones and Wonnacott, 2004). Originally, the $5HT_3$ receptor was classified as a NAchR because of a low affinity for acetylcholine (Jackson and Yakel, 1995). Nicotine is not specific for NAchRs. In fact, nicotine binds at lower affinity to the $5HT_3$ receptor

(Impact receptor has a similar binding profile) than any NAchR (c.f., Jackson and Yakel, 1995; Breitinger, Geetha, and Hess, 2001; Gurley and Lanthorn, 1998).

*Histologies*

Upon termination of ICSA experiments, a solution of 1% bromophenol blue dye was injected into the infusion site and the animals sacrificed. Brains were removed and immediately frozen at -80 °C, for slicing into 40-µm sections with a cryostat microtome. Slides were stained with cresyl violet and examined for infusion site verification using the atlas of Paxinos and Watson (1986).

*Statistical Analysis*

ICSA data were analyzed with an Adolescent Exposure x ICSA Dose x Sex x Session mixed ANOVA, with repeated measures on Session performed on the number of active lever responses and the number of infusions separately. For each individual group, lever discrimination was determined by "lever (active vs. inactive) x session" mixed ANOVA with repeated measures on session. Post hoc Tukey's b tests were employed when a significant main effect was found, $p < 0.05$. Extinction was determined by comparing the responses on the active lever during sessions 4, 5, and 6, while reinstatement was determined by comparing the responses on the active lever during sessions 5, 6, and 7.

Results

*Wistar Male and Female AIE ICSA EtOH Dose Response*

A multifactorial ANOVA was performed on the average number of infusions self-administered from sessions 1-4 (Fig. 1). The analysis indicated a significant Adolescent Exposure x ICSA Dose x Sex interaction ($F_{6,141} = 2.46$; $p = 0.027$). This interaction term was

reduced by examining the analysis in each sex. In Wistar males and females, there were significant Adolescent Exposure x ICSA Dose interactions ($F_{6,81} = 20.12$; $p < 0.0001$ and $F_{6,60} = 4.23$; $p = 0.001$ – males and females respectively). The significant 3-way interaction term was also decomposed by holding ICSA Dose constant. Wistar rats given aCSF, 25, or 50 mg% EtOH to self-administer showed no significant Adolescent Exposure x Sex interactions (F values < 1.2; *p* values > 0.285). In contrast, rats self-administering 75, 100, 150, or 200 mg% EtOH had significant Adolescent Exposure x Sex interactions (F values > 12.96; *p* values < 0.002).

The significant 2-way interactions allowed for direct contrasts in each sex across the concentrations of EtOH employed in the ICSA procedure. In AIE Wistar males, there was a significant effect of ICSA Dose on the average number of infusions ($F_{6,47} = 56.13$; $p < 0.0001$). Post-hoc comparisons (Tukey's b) revealed that rats self-administered 75, 100, 150 and 200 mg% EtOH more than rats self-administering aCSF, 25 or 50 mg% EtOH, and that the 150 mg% group infused more than the 75 mg% group. In CON Wistar males, there was also a significant effect of ICSA Dose on the average number of infusions ($F_{6,34} = 21.5$; $p < 0.0001$). Post-hoc comparisons revealed that rats self-administering 150 and 200 mg% EtOH self-infused more often than aCSF, 25, 50, 75, and 100 mg% rats. Directly contrasting the effects of AIE in Wistar male rats revealed that AIE rats self-infused more often than CON rats for 75, 100, 150 and 200 mg% (F values > 33.5; *p* values < 0.0001).

In AIE and CON Wistar females there was also a significant effect of ICSA Dose on the average number of infusions ($F_{6,30} = 14.13$; $p < 0.001$ – $F_{6,30} = 10.94$; $p < 0.001$, respectively). In AIE Wistar female rats, post-hoc comparisons revealed that rats self-administering 75, 100, 150, and 200 mg% infused more often than aCSF, 25, and 50 mg% rats. Post-hoc comparisons in CON Wistar females revealed that rats self-administering 150 and 200 mg% EtOH infused more often than aCSF, 25, 50, 75, and 100 mg% rats. Directly

contrasting the effects of AIE in Wistar female rats revealed that AIE rats self-infused more often than CON rats for 75 and 100 mg% (F values > 44.4; *p* values < 0.0001). Additional analyses were performed to identify the basis for the significant Adolescent Exposure x ICSA Dose x Sex interaction term. The only other significant finding was that in CON rats, Wistar females self-infused 150 mg% more often than Wistar males ($F_{1,15}$ = 41.8; $p < 0.003$).

A mixed factor ANOVA examining lever responses revealed a significant Adolescent Exposure X ICSA Dose x Sex x Session interaction ($F_{36,840,}$ = 3.05; $p < 0.001$). Succinctly, responding on the active lever observed in AIE Wistar male and female rats self-administering 75, 100, 150, and 200 mg% EtOH into the pVTA was significantly higher than aCSF controls during sessions 1-4 and session 7 (left panels - Figs. 2 and 3; all *p* values < 0.004). These rats also displayed discrimination between active and inactive lever during sessions 1-4 and session 7 (all *p* values < 0.002). In CON Wistar males and females, increased active lever responding and lever discrimination was observed only in rats self-administering 150 and 200 mg% EtOH (right panels – Figs. 2 and 3; *p* values < 0.009). All rats displaying significantly higher levels of self-administration compared to aCSF controls reduced responding on the active lever during sessions 5 and 6 (extinction) and reinstated responding during reinstatement (return of original assigned infusate; all *p* values < 0.021).

*P Male AIE ICSA EtOH Dose Response*

The dependent measure average infusions (Sessions 1-4), was analyzed through a multifactor ANOVA with Adolescent Exposure and ICSA Dose as between subject factors (Fig. 4). The overall analysis revealed a significant Adolescent Exposure x ICSA Dose interaction ($F_{4,40}$ = 10.18; $p < 0.0001$). The interaction term was decomposed by holding Adolescent Exposure constant. In AIE P male rats there was a significant effect of ICSA Dose on the average number of infusions ($F_{4,20}$ = 22.3; $p < 0.001$). Post-hoc comparisons

indicated (Tukey's b) that AIE male P rats self-administering 50, 75, and 100 mg% EtOH directly into the pVTA self-infused more than aCSF controls and the 25 mg% EtOH group. In CON male P rats, there was also a significant effect of ICSA Dose ($F_{4,20} = 19.14$; $p < 0.001$) and post-hoc comparisons revealed that rats self-administering only 100 mg% EtOH self-infused more than all other groups. Directly comparing AIE and CON rats by holding ICSA Dose constant indicated that AIE rats self-infused more than CON rats at concentrations of 50 and 75 mg% EtOH ($F_{1,8}$ values > 22.9; $p$ values < 0.001).

The number of lever responses was analyzed through a Mixed Factor Repeated Measure ANOVA with between subject factors of Adolescent Exposure and ICSA Dose and within subject factors of Session and Lever (Fig. 5). The overall analysis revealed a significant Adolescent Exposure x ICSA Dose x Session x Lever interaction ($F_{24,240} = 2.484$; $p < 0.002$). For brevity sake, the analyses revealed that AIE male P rats self-administering 50, 75, and 100 mg% EtOH directly into the pVTA responded more than aCSF and 25 mg% EtOH rats, displayed lever discrimination (active > inactive), reduced responding during aCSF substitution (extinction sessions 5 and 6), and reinstated responding when the original infusate was returned during session 7 ($p$ values < 0.001). Conversely, in CON male P rats only those rats self-administering 100 mg% directly into the pVTA resulted in comparable self-administration behaviors ($p$ values < 0.005).

*Taqman Array Assessment of DA and Acetylcholine Receptors in the pVTA Following AIE Treatment*

Genetic expression was normalized to the expression of housekeeping genes *18S, Gapdh, Ubs, and Ywhaz*. Of the 42 DA and AchR related genes for analysis selected, 13 were flagged by initial Taqman procedure indicating insufficient genetic expression for reliable detection. These genes included *Cdnf, Chat, Chrna1, Chrna2, Chrna10, Chrna9,*

*Chrnd, Chrne, Chrng, Dbh, Drd4, Hrt3b, and Slc22a3*. The $\log^{10}$ values for all validated genes are included in Table 1. Individual ANOVAs were performed on the expression of individual genes with between subject factors of Sex and Adolescent Exposure (Fig. 6). For the expression of 3 genes (*Chrm1, Chrna7, and Drd3*) there was a significant Sex x Adolescent Exposure interaction (*p* values < 0.021). Post-hoc comparisons (Tukey's b) revealed that for expression of the *Chrna7* gene the significant interaction term was produced by the expression of these genes being altered in AIE males in an enhanced function. The significant interaction term for *Chrm1* was produced by CON females having an innately lower level of expression than male CON rats, and AIE resulting in a decrease in expression in males and an increase in expression in females. The significant interaction term for the *Drd3* was based upon only AIE males displaying a reduction in gene expression. There was a significant effect of Adolescent Exposure for the expression of *Chrna4* and *Chrna5* in the pVTA ($F_{3, 16}$ values > 5.3; *p* values < 0.014). Post-hoc analysis revealed that the expression of these genes in both male and female AIE rats were correspondingly reduced compared to CON controls.

## DISCUSSION

The results of the current experiments indicate that binge-like exposure to EtOH during adolescence in unselected male and female Wistar rats results in persistent neuroadaptations in the mesolimbic dopamine system (pVTA) and produces subjects that are more susceptible to the reinforcing/stimulatory actions of EtOH during adulthood. The parallel results in male and female Wistar rats indicated that in the pVTA there is no sex difference in the reinforcing properties of EtOH in non-manipulated subjects (Figs. 1-3). Moreover, exposure to binge-like EtOH during adolescence results in a leftward shift

(increase in sensitivity) in the EtOH dose response curve in the pVTA and in a genetic model of alcoholism (tP rats), exposure to binge-like EtOH in adolescence produces a similar leftward shift in the EtOH dose response curve (increase sensitivity) in the pVTA (Figs. 4 and 5). Lastly, exposure to binge-like EtOH exposure during adolescence results in a persistent upregulation in the expression of α7 nicotinic receptors in the pVTA in Wistar male and female rats as well as other select genes (Fig. 6).

Previous research indicated that voluntary EtOH consumption in P rats results in an increased sensitivity to the reinforcing effects of EtOH in the pVTA and an increased responsivity (leftward and upward shift in dose response curve) in the ability of EtOH microinjected into the pVTA to stimulate DA release in the AcbSh (Toalston et al., 2014). The current data extend the published findings by indicating that experimenter administered binge-like EtOH exposure during adolescence produces comparable results (Figs. 1-7) and thus provide further validation of the AIE model. The consistent finding that adolescent EtOH exposure results in a hyper-dopaminergic state during adulthood is indicated by the findings that systemic administration of EtOH during adolescence results in higher basal DA levels in the Acb (Badanich et al., 2007; Pascual et al., 2009) or DA turnover (increase DA neurotransmission) in the dorsal striatum (Boutros et al., 2018). An increase in DA neurotransmission was also observed in adult P rats following adolescent EtOH consumption (increased DA clearance) within the Acb (Sahr et al., 2004). The AcbSh is associated with reinforcing/motivational properties of drugs of abuse, while the nucleus accumbens core (AcbC) is associated with movement behaviors (c.f., Engleman et al. 2009; Hauser et al., 2015). AIE treatment in Sprague-Dawley rats prevented the reduction in DA levels produced by systemic administration of EtOH during adulthood that was observed in CON and non-manipulated rats (Shnitko et al., 2016).

Adolescent exposure to binge-like EtOH has been shown to reduce the expression in ChAT in multiple brain regions during adulthood (Vetreno et al., 2014, 2019; Vetreno and Crews, 2018). Reduction in ChAT levels in adulthood is also observed following adolescent exposure to other drugs of abuse (Wenk et al., 1993). Conceptually, the reduction in ChAT levels produced by adolescent binge-like EtOH exposure should be associated with an increase in acetylcholine receptors.

EtOH has direct actions on GABA, a7 nAchR, 5HT3a (closely associated with nAchRs), and glutamate (Doyon et al., 2013). Exposure to binge-like levels of EtOH during adolescence resulted in an upregulation in the expression of α7 and a downregulation of α4 and α5 (Fig. 6). Within the VTA activation of the α4, α5 and α6 results in rapid desensitization and internalization of the receptors (Mansvelder et al., 2003; Mansvelder and McGehee, 2000). In contrast, activation of the α7 receptor has both fast and sustained actions within the VTA (Mansvelder et al., 2003). Specifically, the α7 receptor is both a fast action/quickly desensitized ligand gated ion channel (increase $Ca^{2+}$ influx) and a long acting G-protein coupled receptor (Gαq which promotes the sustained release of $Ca^{2+}$ in the neuron; Kabbani and Nichols, 2018). Therefore, alterations in the α7 receptor are more likely to explain the prolonged behavioral and neurochemical effects produced by binge-like EtOH exposure during adolescence than the alterations in the expression of the α4 and α5 genes. Additional recent data that indicates that voluntary EtOH consumption in adolescent P rats reduced the concentration of nicotine microinjected into the pVTA required to stimulate DA release in the AcbSh (Waeiss et al., in press). Furthermore, adolescent voluntary binge-like EtOH drinking in P rats is associated with an increase in α7 labeling and protein levels in the pVTA (Waeiss et al., in press).

In the VTA, α7 nAchR are expressed on presynaptic glutamate neurons, DA neurons, GABA interneurons, and on DA neurons that co-express glutamate, but not on astrocytes or other glia (Jones and Wonnacott, 2004). Each α7 nAchR site of action mediates mesolimbic DA activity, and thus the reward circuit (Keath et al., 2007). The ability of systemically administered nicotine to stimulate DA release in the AcbSh is mediated directly by activating α7 nAchR in the VTA (Schilstrom et al., 1998a, b). Selective activation of α7 nAchR results in the release of glutamate in the VTA, which if paired with postsynaptic depolarization results in long-term potentiation in VTA DA neurons (Mansvelder and McGehee, 2000). In mice, adolescent alcohol consumption increases the sensitivity in select VTA DA neurons to become activated by low concentrations of EtOH (Avengo et al., 2016). Indirect evidence suggests that these hypersensitive DA neurons produced by adolescent EtOH consumption contain α7 nAchRs and may be the subset of VTA neurons that co-express DA and glutamate (Avengo et al., 2016). Thus, the alteration in the sensitivity to the reinforcing properties of EtOH in the pVTA following AIE exposure could be mediated, in part, by the α7 nAchR modulated hypersensitive DA neurons.

The current data indicate that adolescent EtOH exposure results in a persistent reduction in the a4 and a5 NAchR in the pVTA (Fig. 5). The a5 NAchR has been linked to numerous alcohol and drug self-administration behaviors. Human genetic association studies have linked the a3, β4, a5 NAchR locus (A5/A3/B4) with alcohol dependence (Joslyn et al., 2008). Single nucleotide polymorphism assessment has indicated that minor variations within the A5/A3/B4 gene cluster is linked to early alcohol and tobacco initiation in adolescence (Schlaepfer et al., 2008). In human stem cells, exposure to low EtOH concentrations reduces *Chrna5* expression during early human development (Krishnamoorthy et al., 2010). Systemic over-expression of the a5 NAchR results in a reduction in alcohol self-administration (Gallego et al., 2012). Targeted deletion of the a4 NAchR in the VTA

increases nicotine self-administration (no assay of EtOH intake; Peng et al., 2017). In the VTA, a4 NAchR interacts with a5 NAchR in regulating inhibitory GABA interneurons (Hsu et al., 2013). The published literature would suggest that the reduction in the expression of a4 and a5 NAchR in the pVTA during adulthood following EtOH exposure during adolescence would result in a hyper-responsive DA system that would promote EtOH and/or drug self-administration.

The single persistent alteration in gene expression of dopaminergic related genes in the pVTA following adolescent EtOH exposure was the sex specific reduction in *Drd3* (Fig. 6). The D3 receptor in the pVTA is an autoreceptor that when activated reduces the likelihood of DA neuronal activation (Fiorentini et al., 2015). In mice lacking the *Drd3* gene, there is a hyperdominergic state that enhances drug self-administration behaviors (Le Foll et al., 2005; Zhan et al., 2018). In humans, systemic administration of a D3 antagonist enhanced reward seeking in alcohol use disorder subjects (Murphy et al., 2017). Systemic antagonism of the D3 receptor has been shown to reduce self-administration of opioids, cocaine, and other drugs of abuse (c.f., Ashby et. al., 2015). The current data suggests that in Male rats the hyperdopaminergic DA response in the AcbSh following microinjections of EtOH into the pVTA could be the result of the reduction in the inhibitory effects of the D3 receptor (Toalston et al., 2014). Overall, the data indicate that adolescent EtOH consumption in a rodent model of alcoholism produced persistent alterations in the drug reward pathway that is indicative of increased sensitivity to EtOH and a potentiated/prolonged response to EtOH. A recent hypothesis is that adolescent EtOH exposure results in a 'locked-in' phenotype where the brain maintains critical characteristics of adolescence throughout adulthood. Adolescent rats have an enhanced dopaminergic response to EtOH compared to adult rats (Pascual et al., 2009). The current data and past reports (Toalston et al., 2014; Avengo et al., 2016) indicate that the hyper-sensitive DA response in adult rats exposed to

EtOH during adolescence could be another example of a 'locked-in' adult consequence.In addition, the data have indicated a potential target for future analysis. The expression of α7 nAchRs in the pVTA is enhanced by adolescent binge-like EtOH exposure (Fig. 6 and Waeiss et al., in press). The increase in reward sensitivity during adulthood following adolescent EtOH exposure, possibly mediated by alterations in nAchRs in the pVTA, could be the biological basis for the deleterious effect adolescent alcohol consumption has on adult alcoholism. Elucidating specific neuroadaptations within the mesolimbic DA system produced by adolescent EtOH consumption could lead to interventions or treatments to counter the consequences of this common human behavior.

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**Figure Legends**

Figure 1. Depicts the mean (± SEM) average number of infusions self-administered directly into the pVTA during sessions 1-4 in Wistar males (top panel) and females (bottom panel) exposed to AIE or CON. * indicates significantly more infusions than aCSF controls. + indicates significantly more infusions than aCSF controls and AIE > CON. ^ indicates significantly more infusions than aCSF controls and CON females > males.

Figure 2. Depicts the mean (± SEM) number of lever responses in Wistar **male** and **female** AIE (left panels) and CON (right panels) rats given aCSF (top panels), 75 mg% (middle panels) or 100 mg% EtOH (bottom panels) to self-administer directly into the posterior VTA. # indicates significantly more responding on the active lever than that observed in aCSF controls, AIE > CON and discrimination between levers. * indicates significantly more responding on the active lever than that observed in aCSF controls, AIE > CON males and discrimination between levers.

Figure 3. Depicts the mean (± SEM) number of lever responses in Wistar **male** and **female** AIE (left panels) and CON (right panels) rats given 150 or 200 mg% EtOH to self-administer directly into the posterior VTA. * indicates significantly more responding on the active lever than that observed in aCSF controls and discrimination between levers. . + indicates significantly more infusions than aCSF controls and CON females responded more than CON males.

Figure 4. Depicts the mean (± SEM) average number of infusions self-administered directly into the pVTA during sessions 1-4 in Taconic alcohol-preferring (tP) rats exposed to AIE or CON. * indicates significantly more infusions than aCSF.

Figure 5. Depicts the mean (± SEM) number of lever responses in tP **male** AIE (left panels) and CON (right panels) rats given aCSF (top panels), 75 mg% (middle panels) or 100 mg% EtOH (bottom panels) to self-administer directly into the posterior VTA. * indicates significantly more responding on the active lever than that observed in aCSF controls and discrimination between levers.

Figure 6. Depicts the mean (± SEM) change in gene expression in male and female Wistar AIE and CON rats ($log^{10}$ transformation). * indicates statistical difference from CON male controls. + indicates statistical difference from CON male controls and sex differences in AIE male and female rats. ^ indicates innate sex difference in gene expression CON male > CON female.

| Genes of Interest | Control Wistar Male | | Control Wistar Female | | AIE Wistar Male | | AIE Wistar Female | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| *Ache* | 0 | 0.041 | 0.021 | 0.031 | -0.065 | 0.037 | 0.008 | 0.034 |
| *Chrm1* | 0 | 0.116 | ***-0.237*** | **0.031** | ***-0.271*** | ***0.029*** | -0.067 | 0.064 |
| *Chrm2* | 0 | 0.05 | 0.135 | 0.112 | 0.171 | 0.137 | 0.07 | 0.103 |
| *Chrm3* | 0 | 0.028 | 0.106 | 0.03 | 0.133 | 0.105 | 0.019 | 0.095 |
| *Chrm4* | 0 | 0.097 | -0.06 | 0.037 | -0.063 | 0.048 | -0.056 | 0.072 |
| *Chrm5* | 0 | 0.054 | 0.042 | 0.035 | 0.034 | 0.06 | -0.062 | 0.105 |
| *Chrna2* | 0 | 0.328 | -0.133 | 0.223 | 0.143 | 0.164 | -0.187 | 0.135 |
| *Chrna3* | 0 | 0.06 | 0.031 | 0.042 | -0.015 | 0.068 | -0.102 | 0.123 |
| *Chrna4* | 0 | 0.02 | 0.024 | 0.01 | ***-0.088*** | ***0.03*** | ***-0.085*** | ***0.01*** |
| *Chrna5* | 0 | 0.01 | 0.043 | 0.01 | ***-0.29*** | ***0.06*** | ***-0.141*** | ***0.01*** |
| *Chrna6* | 0 | 0.066 | 0.04 | 0.028 | 0 | 0.095 | -0.093 | 0.153 |
| *Chrna7* | 0 | 0.02 | 0.017 | 0.01 | ***0.195*** | ***0.02*** | ***0.0767*** | ***0.01*** |
| *Chrnb1* | 0 | 0.056 | 0.056 | 0.034 | -0.056 | 0.042 | -0.036 | 0.048 |
| *Chrnb2* | 0 | 0.029 | 0.021 | 0.016 | -0.05 | 0.046 | 0.002 | 0.019 |
| *Chrnb3* | 0 | 0.063 | 0.044 | 0.034 | -0.063 | 0.1 | -0.066 | 0.141 |
| *Chrnb4* | 0 | 0.101 | 0.016 | 0.079 | 0.269 | 0.082 | -0.002 | 0.105 |
| *Comt* | 0 | 0.032 | -0.061 | 0.014 | -0.088 | 0.029 | -0.064 | 0.016 |
| *Ddc* | 0 | 0.054 | 0.049 | 0.028 | -0.013 | 0.068 | -0.073 | 0.125 |
| *Drd1* | 0 | 0.16 | -0.041 | 0.038 | -0.164 | 0.157 | 0.019 | 0.045 |
| *Drd2* | 0 | 0.038 | 0.007 | 0.027 | -0.088 | 0.066 | -0.095 | 0.099 |
| *Drd3* | 0 | 0.13 | 0.034 | 0.053 | ***-0.522*** | ***0.217*** | 0.059 | 0.085 |
| *Drd5* | 0 | 0.053 | 0.004 | 0.079 | -0.027 | 0.029 | 0.075 | 0.078 |
| *Htr3a* | 0 | 0.051 | -0.007 | 0.032 | -0.157 | 0.083 | 0.033 | 0.068 |
| *Impact* | 0 | 0.039 | 0.026 | 0.019 | -0.035 | 0.031 | -0.024 | 0.041 |
| *Maob* | 0 | 0.018 | 0 | 0.029 | 0.063 | 0.045 | 0.02 | 0.029 |
| *Oprm1* | 0 | 0.066 | 0.064 | 0.049 | 0.043 | 0.154 | 0.056 | 0.085 |
| *Slc22a3* | 0 | 0.069 | -0.092 | 0.058 | 0.15 | 0.184 | -0.001 | 0.139 |
| *Slc5a7* | 0 | 0.12 | -0.172 | 0.075 | -0.006 | 0.083 | 0.074 | 0.044 |
| *Slc6a3* | 0 | 0.054 | 0.024 | 0.035 | 0.053 | 0.096 | -0.126 | 0.162 |
| *Tbp* | 0 | 0.022 | -0.051 | 0.019 | 0.02 | 0.017 | -0.043 | 0.041 |
| *Th* | 0 | 0.062 | 0.003 | 0.032 | -0.018 | 0.081 | -0.116 | 0.139 |

Table Legend

Table 1. Depicts the mean (± SEM) change in gene expression in male and female Wistar AIE and CON rats ($log^{10}$ transformation) for all detected genes in the pVTA. **Bold** indicates statistical significance (see Figure 6 for details)..