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Donepezil reverses dendritic spine morphology adaptations and *Fmr1* epigenetic modifications in hippocampus of adult rats after adolescent alcohol exposure

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Abstract

Background—Adolescent intermittent ethanol (AIE) exposure produces persistent impairments in cholinergic and epigenetic signaling and alters markers of synapses in the hippocampal formation, effects that are thought to drive hippocampal dysfunction in adult rodents. Donepezil (Aricept), a cholinesterase inhibitor, is used clinically to ameliorate memory-related cognitive deficits. Given that donepezil also prevents morphological impairment in preclinical models of neuropsychiatric disorders, we investigated the ability of donepezil to reverse morphological and epigenetic adaptations in the hippocampus of adult rats exposed to AIE. Because of the known relationship between dendritic spine density and morphology with the fragile X mental retardation 1 (*Fmr1*) gene, we also assessed *Fmr1* expression and its epigenetic regulation in hippocampus after AIE and donepezil pre-treatment.

Methods—Adolescent rats were administered intermittent ethanol for 16 days starting on postnatal day 30. Rats were treated with donepezil (2.5mg/kg) once a day for four days starting 20 days after the completion of AIE exposure. Brains were dissected out after the 4th donepezil dose and spine analysis was completed in dentate gyrus granule neurons. A separate cohort of rats, treated identically, was used for molecular studies.

Results—AIE exposure significantly reduced dendritic spine density and altered morphological characteristics of subclasses of dendritic spines. AIE exposure also increased mRNA levels and H3-K27 acetylation occupancy of the *Fmr1* gene in hippocampus. Treatment of AIE-exposed adult rats with donepezil reversed both the dendritic spine adaptations and epigenetic modifications and expression of *Fmr1*.

Conclusions—These findings indicate that AIE produces long-lasting decreases in dendritic spine density and changes in *Fmr1* gene expression in the hippocampal formation, suggesting

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morphological and epigenetic mechanisms underlying previously reported behavioral deficits after AIE. The reversal of these effects by sub-chronic, post-AIE donepezil treatment indicates that these AIE effects can be reversed by upregulating cholinergic function.

Keywords

Adolescent alcohol; Donepezil; Dendritic Spines; Hippocampus; *Fmr1*; Epigenetics; Histone Acetylation

INTRODUCTION

Alcohol is the world's most widely used recreational drug, and most people in the U.S. begin use during adolescence or young adulthood. U.S. surveys show that 29% of 12th graders and 42% of college students report having had five or more drinks in a row during the last two weeks (NIAAA, 2006). This prevalence of heavy drinking occurs during a period when the brain is undergoing rapid changes in structure and function that make it vulnerable to negative consequences of alcohol exposure (Dahl, 2004, Monti et al., 2005, Pfefferbaum et al., 2016). Adolescence and young adulthood are distinct developmental periods that involve significant behavioral and neurological changes. This period of late brain development is critically important for an individual's ability to function effectively in the adult world, extends from the late teens into the mid-twenties, and has become the subject of intense investigation in both humans and animal models (Spear and Swartzwelder, 2014, Brown et al., 2015, Spear, 2000b). In humans and rodents, adolescents and young adults manifest differential responsiveness to acute ethanol (Acheson et al., 1998, Little et al., 1996, Markwiese et al., 1998, Spear, 2000a, White et al., 2002) and are more sensitive to enduring negative effects of repeated ethanol exposure that persist into adulthood (Spear and Swartzwelder, 2014, Brown et al., 2015). It is well known that drinking onset at young ages is strongly associated with alcohol abuse and compromises of cognitive function in adulthood (Dawson et al., 2008, Hanson et al., 2011, Mota et al., 2013, Nguyen-Louie et al., 2016, Sher and Gotham, 1999, Squeglia et al., 2015).

In order to assess the neural mechanisms underlying adolescent intermittent ethanol (AIE) exposure, and possible preventive or ameliorative treatments, rodent AIE models have evolved that approximate the use patterns and blood ethanol levels experienced by adolescent human drinkers (see Spear, 2016, Spear and Swartzwelder, 2014). One of the most robust and reproduced effects of AIE exposure in animal models is a marked reduction of choline acetyltransferase (ChAT) immunoreactivity in the medial septum and vertical limb of the diagonal band of Broca (Ch1–2), which are rich in cholinergic neurons. These reductions persist well into adulthood (Swartzwelder et al., 2015, Boutros et al., 2014, Vetreno et al., 2014), do not appear to occur after comparable ethanol exposure in adulthood (Vetreno et al., 2014), and are associated with specific memory deficits (Swartzwelder et al., 2015). Thus, it appears that AIE results in a chronic deprivation of hippocampal circuits of cholinergic input. Cholinergic neurons in Ch1–2 project diffusely into the hippocampal formation and play an important regulatory and facilitating role in memory-related neuronal function and plasticity (Madison et al., 1987, Mesulam et al., 1983, Smith and Pang, 2005), circuit activity (Dudar, 1977), and synaptic plasticity (Auerbach and Segal, 1994, Fernandez

de Sevilla and Buno, 2010, Drever et al., 2011). Importantly, their hippocampally projecting axons increase the expression of acetylcholinesterase during adolescence (Armstrong et al., 1987, Gould et al., 1991), and hippocampal ChAT levels peak during early adolescence and remain stable until early adulthood. In addition, the activity of high-affinity choline transporter increases during adolescence before returning to baseline levels in young adulthood (Zahalka et al., 1993). Thus, the developmental trajectory of basal forebrain cholinergic neurons, which begins prenatally, continues through adolescence, and appears particularly susceptible to disruption by adolescent alcohol exposure.

Donepezil (Aricept) is a selective, reversible cholinesterase inhibitor that is used clinically to ameliorate memory-related cognitive deficits. In animal models, it has been shown to protect against olfactory bulbectomy-induced lesions of cholinergic neurons in Ch1–2 and their behavioral sequelae (Yamamoto et al., 2010), which are similar to the pattern of ChAT immunoreactivity reductions we have observed after AIE (Swartzwelder et al., 2015). Donepezil has also been shown to protect against working memory deficits after saporin injections into Ch1–2 (Cutuli et al., 2013), inflammation-induced, hippocampally-mediated working memory deficits after basal forebrain cholinergic lesions (Field et al., 2012), and hippocampal synaptic and neuronal degeneration in a transgenic mouse model of Alzheimer's disease (Cavallucci et al., 2013). Interestingly, donepezil has also been shown to reverse aging-related dendritic spine loss in the hippocampus and prefrontal cortex (Alcantara-Gonzalez et al., 2012, Alcantara-Gonzalez et al., 2010). This is a critical finding given that AIE exposure impairs the cholinergic system and alters dendritic spine density and morphology in cortical and subcortical regions (Pandey et al., 2015, Trantham-Davidson et al., 2017, Jury et al., 2017, Risher et al., 2015a, Boutros et al., 2014, Vetreno et al., 2014, Swartzwelder et al., 2015). Taken together, these findings suggest that pharmacological up-regulation of cholinergic function, in animals with deficient cholinergic input to the hippocampus, can reverse or prevent hippocampal deficits associated with diminished cholinergic input. The present experiments were, therefore, designed to assess the effects of AIE on hippocampal dendritic spine density and morphology and the possible ameliorative effects of donepezil on those AIE-induced changes.

In addition to the translational focus of assessing the possible ameliorative effects of donepezil, we also sought to assess a possible mechanism driving the possible effects of AIE on dendritic spine density. Histone acetylation serves as an active epigenetic mark of genomic activity, and changes in the acetylation status regulate gene expression (Pandey et al., 2017, Krishnan et al., 2014). It has been shown that the fragile X mental retardation 1 (*Fmr1*) gene that encodes fragile X mental retardation protein (FMRP), regulates the morphology of dendritic spines (Grossman et al., 2006a, Grossman et al., 2006b). In addition, deletion of the *Fmr1* gene is associated with increased dendritic spine density and cholinergic deficits in hippocampus (Comery et al., 1997, Grossman et al., 2010, Ivanko and Greenough, 2002, Scharkowski et al., 2017, D'Antuono et al., 2003, Scremin et al., 2015). We reported previously that AIE exposure decreases global H3K9 acetylation in the hippocampus due to increased activity of histone deacetylases (HDAC) and decreases neurogenesis markers in adulthood (Sakharkar et al., 2016), and, as noted above, there is evidence of decreased cholinergic input to the hippocampal formation after AIE (Swartzwelder et al., 2015, Boutros et al., 2014, Vetreno et al., 2014). Thus, it is possible

that enduring alterations of hippocampal synaptic function after AIE (Risher et al., 2015b, Li et al., 2013, Fleming et al., 2013) could be the result of alterations in dendritic spine density, driven by cholinergic alterations linked to *Fmr1* gene expression. To test this hypothesis, we assessed dendritic spine density and *Fmr1* gene expression and histone acetylation in the hippocampal formation after AIE, and determined if post-AIE treatment with donepezil can reverse any observed effects.

MATERIALS AND METHODS

Animals and Ethanol Exposure

The procedures in this study were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and the National Research Council's Guide for Care and Use of Laboratory Animals. In addition, they were approved by the Durham VA Medical Center and the Duke University Animal Care and Use Committees.

For the dendritic spine studies, twenty-four male Sprague-Dawley rats (Charles River, USA) were housed in pairs in a temperature- and humidity-controlled room. They had ad libitum access to food and water. The rats were delivered to the vivarium on postnatal day (PND)-25 and allowed to acclimatize to the vivarium and handling by technicians for five days on a reverse 12:12-hr light:dark cycle (lights off at 9:00 am) prior to beginning AIE or distilled water exposure on PND-30. Exposure consisted of 10 doses of 5 g/kg ethanol (35% v/v in dH₂O at 18.12 mL/kg, VWR, Suwanee, GA, USA) or isovolumetric dH₂O administered by intragastric gavage using a 2 days on, 1 day off, 2 days on, 2 days off schedule for 16 days. The range of gavage volumes across the exposure period was 1.1–5.0ml. Exposure was followed by a 20-day period of no treatment. Twenty-one days following exposure, half of the animals in the AIE pretreatment group and half in the control animals were dosed with 2.5 mg/kg of donepezil (1.88 mL/kg in dH₂O) and the other half in each pretreatment group received dH₂O (1.88 mL/kg) by intragastric gavage once per day for four days. This resulted in four treatment groups. One received water during adolescence and post-treatment (Water/Water), one received AIE during adolescence followed by water post-treatment (AIE/Water), one received water during adolescence followed by donepezil post-treatment (Water/DZ), and one received AIE during adolescence followed by donepezil post-treatment (AIE/DZ).

The ethanol dose was selected in order to produce blood ethanol concentrations (BECs) consistent with adolescent human BECs during binge drinking episodes (Squeglia et al., 2011). We have found that rats of this age, sex, and strain, receiving 5 g/kg ethanol (i.g.), achieved mean blood ethanol concentrations of 199.7 ± 19.9 mg/dl 60 minutes after the first dose, and 172.8 ± 13.3 mg/dl 60 minutes after the last dose (Risher et al., 2015a). A separate group of 36 rats was treated identically for use in the experiments on mRNA levels and epigenetic modifications of *Fmr1*, except that after sacrifice hippocampal tissue was flash frozen.

Neuron Labeling Methods

Because the dorsal - but not ventral - dentate gyrus shows a reduction c-Fos activity in adult mice exposed to ethanol during adolescence (Beaudet et al., 2016), analysis of AIE-induced changes in morphology of dendrites and dendritic spines was limited to granule neurons in the dorsal dentate gyrus. DiIolistic labeling of slices obtained from fixed brains was used to assess the effects of AIE exposure and donepezil treatment on dendritic spine density and morphology in dorsal dentate gyrus granule neurons (Fig. 1A). Brains were prepared for labeling as described previously (McGuier et al., 2015, Uys et al., 2016). Adult rats (PND-70, $n = 6$ rats/group) were anesthetized with urethane (1.2 – 1.5 g/kg, IP) three h after the last donepezil dose and were perfused with 0.1 M phosphate buffer (PB) followed by 1.5% paraformaldehyde (PFA) in PB. Brains were blocked and post-fixed in 1.5% PFA for 1 h at room temperature. Brains were washed and shipped to the Medical University of South Carolina overnight in PB. Coronal sections (150 μm thick) were prepared using a vibratome. DiI-coated tungsten particles (1.3 μm diameter) were delivered to the slices using a modified Helio Gene Gun (Bio-Rad; Hercules, CA) fitted with a polycarbonate filter (3.0 μm pore size; BD Biosciences; San Jose, CA). Slices were left overnight at 4°C in PB to allow the DiI to completely diffuse through labeled neurons and sections were post-fixed in 4% PFA for 1 h at room temperature. After mounting with Prolong Gold Antifade mounting media (Life Technologies; Carlsbad, CA), images were acquired ($n = 5\text{--}6$ rats/group; 3 – 6 dendritic sections from each rat; 25–30 sections/treatment group; 111 total sections) on a Zeiss LSM 510 confocal microscope using a 63X oil objective (NA = 1.4). Images of granule neuron dendrites (~60 μm length sections) were collected at a 3X zoom (voxel size: $47 \times 47 \times 100$ nm) and a 4 frame average and were then deconvolved using AutoQuant X (Media Cybernetics; Rockville, MD). Distal sections of granule neuron dendrites (75 microns from the soma) that receive entorhinal cortex inputs (Witter, 2007) were collected for analysis.

Chronic ethanol exposure during development and adulthood can alter density and morphology of subpopulations of spines (Risher et al., 2015a, McGuier et al., 2015, Uys et al., 2016, Pandey et al., 2015, Trantham-Davidson et al., 2017, Jury et al., 2017, Kroener et al., 2012). Imaris XT (version 8.3.1, Bitplane; Zurich, Switzerland) was used to generate a filament of the dendritic shaft and spines following our routine procedures (Fig. 1B). Dendritic spines (2336 ± 210 spines/treatment group; 389.3 ± 19.0 spines/rat) were classified into 4 categories (stubby, long, filopodia, and mushroom) based on the spine length and the width of the spine head and neck, where L is spine length, H_D is spine head diameter, and N_D is spine neck diameter. Long spines were identified as having a $L > 0.75$ μm and < 3 μm , mushroom spines had a $L < 3.5$ μm , $H_D > 0.35$ μm and a $H_D > N_D$, stubby spines had a $L < 0.75$ μm , and filopodia were identified as having a $L < 3$ μm . Images were acquired and spines were identified using Imaris software by an experimenter that was blind to the experimental groups. Data on dendritic spine parameters were averaged for each dendritic section and were collated from the Imaris output via a custom script written in Python. Although visual inspection of the classification algorithm clearly identifies spines based upon distinctive morphological characteristics (Uys et al., 2016), some studies have reported a continuum of spine head diameters along the same dendrite (Konur et al., 2003, Wallace and Bear, 2004). Dendritic spine head diameters were parsed into 0.15 μm internals

(head diameters 0.2 μ m, 0.21–0.35, 0.36–0.5, etc.) following previously reported methods (Shen et al., 2009, Uys et al., 2016).

Expression of the *Fmr1* Gene

Total RNA was extracted from whole hippocampal tissue of Control and AIE exposed animals with or without donepezil treatment using the Qiagen miRNAeasy and DNase kits (Qiagen, Inc., Valencia, CA) as we have described previously (Centanni et al., 2014; Kyzar et al., 2017). Total RNA was then reverse-transcribed at 37°C for 2 hours using the high capacity cDNA RT kit (Life Technologies, Carlsbad, CA). The reaction was stopped by a 5-minute incubation at 85°C. Expression of fragile X mental retardation 1 (*Fmr1*) (Forward, 5'-AAA GTC CAG AGG GGG ATG GT-3', Reverse, 5'-TCT CTC CAA ACG CAA CTG GT-3') was quantified by RT-PCR with SYBR Green mastermix (BioRad, Berkeley, CA, USA). Hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) (Forward, 5'-TCC TCA GAC CGC TTT TCC CGC-3', Reverse, 5'-TCA TCA TCA CTA ATC ACG ACG CTG G-3') was used as the internal control. The Ct value of the *Fmr1* gene was corrected with the Ct value of the respective *Hprt1* internal standard gene. The Ct values were calculated for each sample by subtracting the mean Ct value of the Water/Water group and the respective fold changes were calculated using the Ct method (Schmittgen and Livak, 2008, Centanni et al., 2014).

Histone Acetylation of the *Fmr1* Gene

Histone H3-K9/14 acetylation (H3-K9/14ac) and H3-K27 acetylation (H3-K27ac) of the *Fmr1* gene was measured in the whole hippocampus of control and AIE exposed animals with or without donepezil treatment using chromatin immunoprecipitation (ChIP) assay. The procedure is similar to the methods as described by us earlier (Kyzar et al., 2017). Briefly, hippocampal brain tissue was fixed in methanol-free formaldehyde and DNA sheared by sonication. The resulting DNA-chromatin complex was immunoprecipitated with antibody directed to either H3-K9/14ac (Millipore, 06-599) or H3-K27ac (Cell Signaling, 4353S), and the precipitated DNA was quantified using qPCR with SYBR Green mastermix (BioRad, Berkeley, CA, USA). Primers were designed for the promoter region of the *Fmr1* gene (Forward, 5'-CCTCGCTTCCTCCTGTACAA-3', Reverse, 5'-GCGCAAAGGGTAGTAACAGG-3') as well as towards a predicted site for the binding of cAMP responsive binding element (CREB) protein in the *Fmr1* gene body (Forward, 5'-GCCATTCTGCTTTATTCTAGTTATACTC-3', Reverse, 5'-AGTCAGAATTTGTGGAAATCTGTTG-3'). The input DNA Ct value was subtracted from the Ct value of each respective sample and the Ct method (Kyzar et al., 2017, Schmittgen and Livak, 2008, Pandey et al., 2015) was used to determine the fold change of H3-K9/14ac and H3-K27ac levels in the *Fmr1* gene. Results are presented as fold change relative to the Water/Water control group.

Statistical Analysis

Dendritic spine data were analyzed as mixed linear modeling (SAS Proc Mixed) that has been used previously in studies of this type (McGuier et al., 2015, Uys et al., 2016), and was selected because of the capacity to handle unbalanced and complex repeated-measures data and the ability to model the variance and correlation structure of repeated-measures

experimental designs (Littell et al., 1998). The dendritic spine variables were nested within animal and were further nested across the sequential slices within rat. All gene expression and epigenetic modification data were analyzed by two-way ANOVAs using GraphPad Prism software (version 6.07; GraphPad Software, Inc., La Jolla, CA). Tukey's test was used for all post-hoc analyses.

RESULTS

Dendrites and Dendritic Spine Density

AIE-induced changes in morphology of dendrites and dendritic spines was assessed in granule neurons in the dorsal dentate gyrus ($n = 6$ rats/group, $n = 3 - 6$ dendritic sections/rat, 25–30 sections/treatment group; and $n = 111$ total dendritic sections; Fig. 1A,B). Data from one control rat were excluded due to a high degree of variability across section that met statistical significance as an outlier. Consistent with other reports of 'reactive' dendrites induced by chronic ethanol exposure (Zhou et al., 2007, Uys et al., 2016), AIE exposure significantly increased dendrite diameter of dentate gyrus granule neurons in adult rats (main effect of AIE: $F(1,20) = 35.77$, $p < 0.0001$; Fig. 1C). Donepezil treatment did not affect dendrite diameter in water-treated rats nor did it reverse the increase in dendrite diameter in AIE-treated rats ($F(1,20) = 1.26$, $p = 0.27$). A significant AIE X donepezil interaction was observed for total dendritic spine density in AIE exposed and donepezil treated rats ($F(1,19) = 5.56$, $p = 0.029$; Fig. 1D). Post-hoc analysis revealed that total spine density on dentate gyrus granule neurons was significantly reduced in AIE exposed rats ($p = 0.0004$). While treatment with donepezil alone did not affect total spine density in water-treated controls ($p = 0.982$), donepezil treatment significantly reversed the effects of AIE exposure on total spine density ($p = 0.008$), thus supporting our general hypothesis that donepezil would reverse the effects of AIE on dendritic spine density.

Spine Subclass Density

We next determined if AIE exposure affected spine subclasses and if treatment with donepezil was able to reverse those morphological adaptations. Spines were classified into four subclasses (i.e., filopodia, stubby, mushroom, and long) according to their length and head/neck diameters (see Fig. 2A). In addition to analysis of these spine parameters, total spine volume and spine head and neck volume were calculated and analyzed. The results for these analyses are shown in Table 1. AIE exposure significantly reduced the density of long and mushroom shaped spines, and there was a main effect of donepezil on stubby spine density (Fig. 2B).

Spine Morphology

There was a significant reduction in dendritic spine volume and diameter in long and stubby spines and filopodia after AIE, and treatment with donepezil completely reversed this adaptation in long and stubby spines (Fig. 3A,B). The length of dendritic spines within specific subclasses was largely unaffected by AIE or donepezil exposure, with the exception of a minor, but significant decrease in the length of long spines in rats treated with donepezil (Fig. 3C). The head diameter and volume of long and stubby spines were significantly reduced in AIE exposed rats, and these adaptations in the morphology of spine terminal

points were reversed by donepezil (Fig. 4A,B). Spine neck volume and diameter were also significantly reduced in long and stubby spines in AIE exposed rats (Fig. 5A,B). Accordingly, spine neck length was increased by AIE in long and stubby spines, and donepezil treatment completely reversed this change in long spines (Fig. 5C). In filopodia, spine head diameter and neck volume and diameter were significantly reduced in AIE-exposed adult rats, but unlike in long spines, donepezil did not reverse these adaptations (Fig. 4 and Fig. 5). Consistent with other studies reporting effects of donepezil on dendritic spines in hippocampus and prefrontal cortex (Alcantara-Gonzalez et al., 2012, Alcantara-Gonzalez et al., 2010), analysis showed main effects of donepezil on stubby spine density (Fig. 2B), filopodia spine length (Fig 3B), and the neck length of mushroom spines and filopodia (Fig. 5C).

As a second level analysis, data were parsed into 0.15 μm intervals according to their spine head diameters following previously reported methods (Uys et al., 2016). AIE exposure significantly increased the number of spines with head diameters $\leq 0.2 \mu\text{m}$ (interaction: $F(1,20) = 11.50$, $p = 0.0029$; post-hoc, $p < 0.0001$) and reduced the prevalence of spines with head diameters in the 0.36 – 0.5 μm interval (interaction: $F(1,20) = 18.73$, $p = 0.0003$; post-hoc, $p < 0.0001$; Fig. 6). In both intervals, post-AIE treatment with donepezil significantly reversed these effects ($\leq 0.2 \mu\text{m}$, post-hoc, $p = 0.0078$; 0.36 – 0.5 μm interval; post-hoc, $p = 0.0093$). We also observed a small but significant reduction in the prevalence of spine head diameters in the 0.51 – 0.65 μm interval in adult rats exposed to AIE (main effect of AIE: $F(1,20) = 15.67$, $p = 0.0008$).

AIE and Epigenetic Regulation of *Fmr1* expression

The next set of studies examined if AIE altered *Fmr1* expression in hippocampus, and if AIE-induced changes were reversed by donepezil treatment. We further examined if the expression of *Fmr1* gene is regulated by histone acetylation mechanisms in the hippocampus in adulthood, after AIE. We found that AIE exposure significantly increased the *Fmr1* expression in the hippocampus and this effect was completely normalized by donepezil treatment (interaction: $F(1,23) = 6.813$, $p = 0.0156$; $n = 5\text{--}8/\text{group}$; Fig. 7A). These results support our hypothesis that AIE may modulate *Fmr1* expression via cholinergic mechanisms. Next, we determined the occupancy of H3K9/14ac and H3K27ac of the *Fmr1* promoter and CREB binding site of the *Fmr1* gene body using ChIP assay. H3K9/14ac levels at the promoter of the *Fmr1* gene were significantly decreased in adulthood after AIE, an effect that was not modulated by donepezil treatment (main effect of AIE: $F(1,23) = 28.46$, $p < 0.0001$; $n = 6\text{--}8/\text{group}$; Fig. 7B). These epigenetic modifications were not changed at the CREB binding site of *Fmr1* gene body either by AIE or donepezil treatment (main effect of AIE: ($F(1,24) = 1.152$, $p = 0.294$); main effect of DZ: ($F(1,24) = 1.671$, $p = 0.208$); $n = 6\text{--}8/\text{group}$; Fig. 7B). Interestingly, H3K27ac occupancy at the promoter of *Fmr1* gene is not changed by AIE or donepezil treatment (main effect of AIE: ($F(1,24) = 0.157$, $p = 0.695$); main effect of DZ: ($F(1,24) = 0.189$, $p = 0.668$); $n = 6\text{--}8/\text{group}$; Fig. 7C), whereas its occupancy at the CREB binding site of the *Fmr1* gene was significantly increased by AIE, which was normalized by donepezil treatment in adulthood (interaction: $F(1,24) = 12.77$, $p = 0.0015$; $n = 6\text{--}8/\text{group}$; Fig. 7C). These findings suggest a novel regulatory site within the *Fmr1* gene that is modulated by H3K27 active mark during AIE exposure as well

as donepezil treatment. Furthermore, these novel data suggest the possibility that AIE-induced increased expression of *Fmr1* may be regulated by H3K27 acetylation of the CREB binding site within the *Fmr1* gene body but not by changes in H3K9/14 acetylation of promoter of the gene.

DISCUSSION

The major finding in this manuscript is that donepezil treatment reversed several adaptations in dendritic spines and genetic and epigenetic modifications of *Fmr1* in hippocampus of adult rats exposed to alcohol during adolescence. To our knowledge, this is the first report to show pharmacological reversal of morphological and molecular adaptations produced by AIE. Previous studies have shown that AIE reduces ChAT immunoreactivity in areas Ch1–2 that send cholinergic projections to the hippocampal formation (Swartzwelder et al., 2015, Boutros et al., 2014, Vetreno et al., 2014). Thus, the present findings indicate that targeting this impaired cholinergic system with a clinically used cholinesterase inhibitor is a viable treatment approach to ameliorate hippocampal deficits induced by alcohol exposure during adolescence. In addition, we found that AIE alters *Fmr1* transcript levels and histone acetylation at the promoter region and CREB binding site of the gene, in whole hippocampal tissue. We previously reported that FMRP regulates chronic alcohol-induced homeostatic plasticity in hippocampus (Spencer et al., 2016), and a recent study demonstrated that synthesis of new GABA_B receptors, which modulate hippocampal local circuit function (Mott et al., 1990), requires FMRP (Wolfe et al., 2016). Thus, the present findings further indicate that FMRP is a critical protein mediating alcohol actions, and add to a growing literature that AIE induces epigenetic modifications in cortical and subcortical regions (Kokare et al., 2017, Sakharkar et al., 2016, Pandey et al., 2017, Pandey et al., 2015, Trantham-Davidson et al., 2017).

The effects of adolescent alcohol exposure on dendritic spines are complex and differ depending on brain region and time since last exposure (Pandey et al., 2015, Trantham-Davidson et al., 2017, Jury et al., 2017). In a previous study, we reported that while AIE exposure does not alter total spine density in CA1 pyramidal neurons, though the prevalence of long spines and filopodia were significantly increased (Risher et al., 2015a). Here, we found that AIE exposure enlarged dendritic shafts, which is consistent with reactive dendrites (Zhou et al., 2007, Uys et al., 2016), and decreased total spine density in dentate gyrus granule neurons. The latter finding is similar to a previous publication that reported reductions in granule neuron spine density when alcohol exposure began in late adolescence (Golub et al., 2015). The present analysis of spine subclasses showed that AIE exposure reduced the density of long and mushroom-shaped spines, and detailed analysis of the morphological characteristics of spine subclasses revealed reductions in overall spine size and spine head size, as measured by both volume and diameter. Moreover, AIE exposure altered characteristics of dendritic spine necks. This is an intriguing finding because spine necks act as diffusion barriers that isolate spino-somatic voltage signals and biochemically compartmentalize spine terminal points from dendritic shafts (Tonnesen et al., 2014, Jayant et al., 2017). Although density was reduced only among long and mushroom spines, AIE exposure impacted the morphological characteristics of all four spine subclasses in the dentate gyrus. This detailed morphological analysis of dendritic spines adds to the growing

evidence that AIE exposure has an enduring detrimental impact on hippocampal structure and function (Broadwater et al., 2014, Swartzwelder et al., 2015, Li et al., 2013, Risher et al., 2013, Fleming et al., 2013).

The present findings suggest that donepezil, which is used clinically to enhance the hypo-functioning cholinergic system in Alzheimer's disease patients, represents a possible pharmacotherapy to reverse AIE-induced deficits in cholinergic signaling. Moreover, donepezil treatment increases dendritic spine density and dendritic arborization in hippocampal neurons (Alcantara-Gonzalez et al., 2012, Alcantara-Gonzalez et al., 2010), underscoring the possibility that cholinergic modulation of spine density may be an important component of functional hippocampal activity. Donepezil also enhances vesicular acetylcholine transporter immunoreactive boutons in a rat model of cholinergic degeneration in nucleus basalis (Ch-4) projection neurons (Ginestet et al., 2007), a region that has also been shown to have reduced ChAT immunoreactivity after AIE (Swartzwelder et al., 2015). Here, donepezil reversed AIE-induced changes in total spine density in dentate granule neurons, while also reversing morphological changes in spine subclass volume and size. In control rats, donepezil had few and modest effects on spine subclass density and morphological characteristics, likely due to the relatively short treatment period. In contrast, its ability to reverse AIE-induced morphological adaptations was quite robust, as a complete reversal of deficits was observed in many of the spine variables that were measured, including parameters of spine heads and necks. Thus, these findings demonstrate that targeting the cholinergic system can quickly reverse morphological abnormalities emerging from adolescent alcohol exposure, while leaving uncompromised spine features intact.

In addition to the spine changes, another major finding reported in this manuscript is that AIE exposure increased *Fmr1* transcript levels in hippocampus. Similar to many of the morphological changes, the enhanced expression of *Fmr1* levels observed in AIE-exposed rats was reversed by donepezil treatment. *Fmr1* knockout mice have higher dendritic spine density in dentate gyrus granule neurons with a higher proportion of long, thin spines (Grossman et al., 2010). Spine density gradually increases in granule neurons before reaching adult levels between PND-30 and PND-60 (Grossman et al., 2010), and recent evidence has shown that FMRP controls spine stabilization during development (Cruz-Martin et al., 2010). It is noteworthy that the increase in *Fmr1* expression and parallel reduction in spine density (and long spines, in particular) in the AIE-exposed rats is opposite of what is observed when *Fmr1* is deleted (Grossman et al., 2010). Although speculative, these data suggest that the spine changes observed in rats with a history of AIE may be mediated through elevated FMRP signaling. Since spines in granule neurons are still reaching maturation during AIE exposure, *Fmr1* expression adaptations represent a possible mechanism that regulates morphological abnormalities. This hypothesis is supported by evidence that donepezil reverses both the spine changes and the elevation in *Fmr1* expression in AIE-exposed rats. It is important, however, that these data be interpreted cautiously because the *Fmr1* measurements were made in whole hippocampal tissue whereas spines were assessed only on dentate granule cells. Still, we believe that the data on *Fmr1* gene expression are valuable in this context because they lead suggest the intriguing hypothesis that the spine changes may be related to changes in *Fmr1* expression. Thus, they

lay the groundwork for future studies that can address the specific hippocampal nuclei hypothesis.

In addition, while these findings implicate FMRP signaling as a possible molecular mechanism for regulation of the spine adaptations in AIE-treated rats, we also provide evidence that this may occur via changes in histone acetylation of the *Fmr1* gene specifically at the CREB binding site. A recent study has reported that AIE exposure reduced markers of neurogenesis and increased cellular toxicity of immature neurons in dentate gyrus (Broadwater et al., 2014). Moreover, markers of neurogenesis and spine density were reduced in newborn granule neurons in a late adolescent alcohol exposure model (Golub et al., 2015). Interestingly, donepezil treatment in adult rats elevated hippocampal neurogenesis and enhanced CREB phosphorylation (Kotani et al., 2008), providing evidence that activating cholinergic systems enhances neurogenesis via CREB signaling. In the present study, we observed elevated H3-K27 acetylation at the CREB binding site of *Fmr1* in AIE-exposed rats, an effect that was completely reversed by donepezil treatment. These results show that the CREB binding site of the *Fmr1* gene is sensitive to AIE-induced H3-K27 acetylation. These interactions are consistent with previous evidence linking functional coupling of CREB and FMRP proteins (Hwu et al., 1997, Wang et al., 2012, Smith et al., 2006). Furthermore, increased binding of H3-K27 acetylated proteins at the CREB binding site may change the conformation of the gene and allow gene expression to occur. We observed that the AIE-induced increase in *Fmr1* expression paralleled the increase in H3-K27 acetylation of CREB binding site of *Fmr1* gene in the hippocampus after AIE in adulthood. We previously reported that global H3-K9 acetylation is decreased by AIE in the hippocampus in adulthood (Sakharkar et al., 2016). Here, we observed that H3-K9/14 acetylation is decreased at the promoter but not at the CREB binding site of *Fmr1* in the hippocampus after AIE in adulthood. Interestingly, this histone modification is not sensitive to donepezil treatment and appears to not be regulating the *Fmr1* expression. Together, these data provide evidence for the interesting mechanism that the CREB binding site of the *Fmr1* gene is susceptible to epigenetic modifications that possibly regulate its expression after AIE in adulthood and this is modulated by donepezil treatment.

In recent years it has become clear that in both humans (see Brown et al., 2015) and animal models (Spear, 2016, Spear and Swartzwelder, 2014), adolescence is a period during which individuals are highly sensitive to enduring negative effects of repeated ethanol exposure, which have been described across multiple domains of brain and behavioral function. The present findings add to this timely and important literature in both translational and mechanistic ways. The reductions of dendritic spine density and *Fmr1* expression in the hippocampal formation indicate enduring AIE effects on both morphological and molecular endpoints in this structure, which is involved in both cognitive and affective functioning. That both of these effects were ameliorated by sub-chronic donepezil well after the AIE exposure suggests 1) that certain AIE effects are pharmacologically reversible and 2) that these specific effects may be driven, in part, by the depletion of cholinergic input to the hippocampal formation (Swartzwelder et al., 2015, Boutros et al., 2014, Vetreno et al., 2014). Thus, the present findings identify both neuromorphological and gene expression changes that suggest specific mechanisms whereby AIE may alter hippocampal function, and an anti-cholinesterase agent in clinical use (donepezil) that reverses those effects in

adulthood, long after the last ethanol dose. These findings also point toward specific epigenetic mechanisms that may underlie AIE-induced pathophysiology and a specific class of therapeutic agents that may be capable of reversing it. Importantly, AIE has been shown to reduce ChAT immunoreactivity not only in regions that project to the hippocampal formation, but also to the amygdala and frontal cortex, both of which have been implicated in mediating the enduring effects of AIE. Thus, further studies of those regions may reveal similar possible mechanisms and therapeutic opportunities.

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Fig. 1. Effects of adolescent intermittent ethanol (AIE) exposure and donepezil (DZ) post-treatment on dendrites and total dendritic spine density in adult dentate gyrus granule neurons. **(A)** Representative 3D image of a DiI labeled granule neuron in the dorsal dentate gyrus. Yellow arrow denotes axon stemming from the soma and yellow box represents a typical section of dendrite used for analysis. **(B)** Representative labeling of a section of dendrite showing the Imaris filament recreation of the dendritic shaft (shown in yellow) and spines (shown in blue). Quantitation of **(C)** dendrite diameter (main effect of AIE: $F(1,20) = 35.77$, $***p < 0.0001$) and **(D)** total dendritic spine density (interaction: $F(1,19) = 5.56$, $p = 0.0293$; $***p < 0.001$ vs Water/Water; $**p < 0.01$ vs AIE/Water) in granule neurons from adult rats exposed to AIE and DZ. For all dendrite and dendritic spine analysis, there were $n = 5-6$ rats/group, $n = 3-6$ dendritic sections/rat, and $n = 111$ total dendritic sections.

Fig. 2.

Adaptations in dendritic spine subclasses in adult rats exposed to adolescent intermittent ethanol (AIE) and donepezil (DZ) post-treatment. **(A)** Schematic illustrating parameters used to classify spines based on their morphological characteristics (H_D = head diameter; L = length; N_D = neck diameter; N_L = neck length). **(B)** Changes in density of dendritic spine subclasses in AIE and DZ treated rats (Long spines: * $p = 0.0131$, Water vs AIE; Stubby spines: ** $p = 0.0056$, Water vs DZ; Mushroom spines: *** $p < 0.001$, Water vs AIE).

Fig. 3.

Morphological adaptations in spine subclass (A) volume (Filopodia: $*p = 0.0196$, Water/Water vs AIE/Water; Long spines: $***p < 0.0001$, Water/Water vs AIE/Water, $*p = 0.0313$, AIE/Water vs AIE/DZ; Stubby spines: $***p < 0.0001$, Water/Water vs AIE/Water, $*p = 0.046$, AIE/Water vs AIE/DZ), (B) diameter (Filopodia: $*p = 0.032$, Water/Water vs AIE/Water; Long spines: $***p < 0.001$, Water/Water vs AIE/Water; $**p < 0.01$, AIE/Water vs AIE/DZ; Stubby spines: $**p < 0.01$, Water/Water vs AIE/Water, $*p = 0.0176$, AIE/Water vs AIE/DZ), and (C) length in adult rats exposed to AIE and DZ (Filopodia: $*p = 0.0279$, Water vs DZ).

Fig. 4.

Donepezil reverses AIE-induced morphological adaptations in dendritic spine terminal point

(**A**) diameter (Filopodia: $*p = 0.0215$, Water/Water vs AIE/Water; Long spines: $***p < 0.0001$, Water/Water vs AIE/Water; $**p = 0.0029$, AIE/Water vs AIE/DZ; Stubby spines: $***p < 0.0001$, Water/Water vs AIE/Water, $*p = 0.0336$, AIE/Water vs AIE/DZ, $*p = 0.0456$, Water/Water vs AIE/DZ) and (**B**) volume (Long spines: $***p < 0.0001$, Water/Water vs AIE/Water, $**p = 0.0088$, AIE/Water vs AIE/DZ; Stubby spines: $***p < 0.0001$, Water/Water vs AIE/Water, $*p = 0.0362$, AIE/Water vs AIE/DZ).

Fig. 5.

Morphological characteristics of dendritic spine necks after AIE exposure and donepezil post-treatment. Changes in spine neck (A) volume (Filopodia: $*p = 0.0221$, Water/Water vs AIE/Water; Long spines: $**p = 0.0017$, Water vs AIE; Stubby spines: $*p = 0.073$, Water vs AIE), (B) diameter (Filopodia: $*p = 0.0161$, Water/Water vs AIE/Water; Long spines: $***p = 0.0001$, Water/Water vs AIE/Water; $**p = 0.0036$, AIE/Water vs AIE/DZ; Stubby spines: $*p = 0.031$, Water vs AIE), and (C) length (Filopodia: $*p = 0.0455$, Water vs DZ; Long spines: $**p = 0.0025$, Water/Water vs AIE/Water; $**p = 0.0027$, AIE/Water vs AIE/DZ; Stubby spines: $*p = 0.0203$, Water/Water vs AIE/Water; Mushroom: $*p = 0.047$, Water vs DZ) in dentate gyrus granule neurons.

Fig. 6.

The spine head diameter frequency distribution was significantly changed by AIE exposure and donepezil post-treatment (0.2 μm : *** $p < 0.0001$, Water/Water vs AIE/Water, ** $p = 0.0078$, AIE/Water vs AIE/DZ; 0.36–0.5 μm : *** $p < 0.0001$, Water/Water vs AIE/Water, ** $p = 0.0093$, AIE/Water vs AIE/DZ; 0.51–0.65 μm : *** $p = 0.0008$, Water vs AIE).

Fig. 7.

Effects of AIE and donepezil treatment on *Fmr1* gene expression and its regulation by histone modifications (H3K9/14 and H3K27 acetylation) in the hippocampus in adulthood. (A) *Fmr1* gene expression data is represented as fold changes in mRNA levels. (* $p = 0.012$, Water/Water vs AIE/Water; ** $p = 0.0047$, AIE/Water vs AIE/DZ). (B,C) ChIP data is represented as fold changes in the occupancy of H3K9/14 and H3K27 acetylation of both promoter and gene body of *Fmr1* (CREB binding site: * $p = 0.027$, Water/Water vs AIE/Water; ** $p = 0.0004$, AIE/Water vs AIE/DZ). All values mean $\pm$ SEM and derived from $n = 5-8$ /group.

Table 1

Spine subclass statistics (DF = (1,20) for all tests).

	Test	Filopodia		Long		Stubby		Mushroom	
		F value	P value	F value	P value	F value	P value	F value	P value
Density	Group	0.52	0.4778	7.42	0.0131*	1.00	0.3302	14.83	0.001*
	Drug	0.93	0.3466	3.86	0.0633	9.62	0.0056*	0.86	0.3643
	Interaction	0.35	0.5610	0.74	0.4006	0.92	0.3489	4.28	0.0517
Length	Group	0.25	0.6216	2.32	0.1432	0.36	0.5558	0.10	0.7549
	Drug	0.5.62	0.0279	4.18	0.0542	0.49	0.4914	3.41	0.0795
	Interaction	0.30	0.5904	3.75	0.0670	0.37	0.5502	.20	0.6563
Diameter	Group	3.15	0.0911	11.69	0.0027	11.23	0.0032	0.50	0.4889
	Drug	1.77	0.1978	8.23	0.0095	4.83	0.0399	3.42	0.0793
	Interaction	5.59	0.0283	10.04	0.0048	6.79	0.0169	1.60	0.2200
Head Diameter	Group	3.45	0.0782	27.15	<.0001	28.01	<.0001	0.56	0.4629
	Drug	0.32	0.5786	3.52	0.0755	1.55	0.2272	1.36	0.2574
	Interaction	6.45	0.0195	16.81	<.0006	9.7	0.0055	4.25	0.0524
Volume	Group	5	0.0369	16.47	0.0006	21.8	<.0001	1.53	0.2301
	Drug	0.4	0.5329	4.02	0.0586	3.98	0.0599	0.23	0.6348
	Interaction	4.92	0.0382	5.78	0.0260	4.61	0.0443	2.35	0.1412
Head Volume	Group	3.31	0.0839	25.16	<.0001	23.24	<.0001	0.4	0.5339
	Drug	0.9	0.3542	2.54	0.1270	2.3	0.1452	2.14	0.1592
	Interaction	2.29	0.1457	13.43	0.0015	7.81	0.0112	3.52	0.0752
Neck Length	Group	0.34	0.5674	5.85	0.0252	3.79	0.0659	0.05	0.8281
	Drug	4.55	0.0455*	6.94	0.0159	0.42	0.5235	4.48	0.0470*
	Interaction	0.48	0.49060	11.48	0.0029	6.75	0.0172	0.05	0.8270
Neck Diameter	Group	4.54	0.0458	17.68	0.0004	4.78	0.0408*	1.16	0.2941
	Drug	1.78	0.1973	6.24	0.0214	1.85	0.1884	0.07	0.7892
	Interaction	5.99	0.0237	11.18	0.0032	2.01	0.1712	1.69	0.2086
Neck Volume	Group	4.75	0.0414	13.2	0.0017*	6.74	0.0173*	1.46	0.2411
	Drug	0.62	0.4399	3.95	0.0609	2.33	0.1430	0.63	0.4369
	Interaction	4.83	0.0400	4.01	0.0588	0.67	0.4232	1.66	0.2121

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Bold font denotes significant interactions.

* denotes significant main effects when interactions did not reach $p < 0.05$.

Simple effect d values from Tukey's post-hoc tests are reported in the figure legends, when appropriate.