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Full-length Article

Adolescent intermittent ethanol reduces serotonin expression in the adult raphe nucleus and upregulates innate immune expression that is prevented by exercise

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ABSTRACT

Serotonergic neurons of the raphe nucleus regulate sleep, mood, endocrine function, and other processes that mature during adolescence. Alcohol abuse and binge drinking are common during human adolescence. We tested the novel hypothesis that adolescent intermittent ethanol exposure would alter the serotonergic system that would persist into adulthood. Using a Wistar rat model of adolescent intermittent ethanol (AIE; 5.0 g/kg, i.g., 2-day on/2-day off from postnatal day [P]25 to P55), we found a loss of dorsal raphe nucleus (DRN) serotonin (5-HT)-immunoreactive (+IR) neurons that persisted from late adolescence (P56) into adulthood (P220). Hypothalamic and amygdalar DRN serotonergic projections were reduced following AIE. Tryptophan hydroxylase 2, the rate-limiting 5-HT synthesizing enzyme, and vesicular monoamine transporter 2, which packages 5-HT into synaptic vesicles, were also reduced in the young adult midbrain following AIE treatment. Adolescent intermittent ethanol treatment increased expression of phosphorylated (activated) NF-κB p65 as well as markers of microglial activation (i.e., Iba-1 and CD11b) in the adult DRN. Administration of lipopolysaccharide to mimic AIE-induced innate immune activation reduced 5-HT+IR and increased phosphorylated NF-κB p65+IR similar to AIE treatment. Voluntary exercise during adolescence through young adulthood blunted microglial marker and phosphorylated NF-κB p65+IR, and prevented the AIE-induced loss of 5-HT+IR neurons in the DRN. Together, these novel data reveal that AIE reduces 5-HT+IR neurons in the adult DRN, possibly through an innate immune mechanism, which might impact adult cognition, arousal, or reward sensitivity. Further, exercise prevents the deleterious effects of AIE on the serotonergic system.

1. Introduction

Adolescence is a highly conserved neurodevelopmental period that marks the transition from childhood to adulthood, and is characterized behaviorally by increased social interactions and risk-taking (i.e., novelty- and sensation-seeking (Spear, 2011)). In parallel, the brain undergoes significant maturation of neurocircuitry and refinement of several neurotransmitter systems (Coleman et al., 2011; Crews et al., in press; Spear, 2000; Weir et al., 2012), including the serotonergic system (Lidov and Molliver, 1982; Shoval et al., 2014). Serotonin (5-hydroxytryptamine, 5-HT)-producing neurons are primarily located within the brainstem raphe nuclei and are generated prenatally in the brain (Lauder, 1990). Serotonergic neurons innervate multiple brain regions and these projections undergo significant refinement during adolescence (Dori et al., 1996; Xu et al., 2001). Serotonin is a neuromodulatory neurotransmitter involved in synaptic plasticity, learning and memory, mood regulation, sleep, and endocrine processes that mature during adolescence, and dysregulation of this system is linked to several psychiatric disorders, including depression, impulsivity, and alcohol dependence (Michelsen et al., 2007; Muller and Homberg, 2015; Nautiyal et al., 2015). It is currently unknown whether adolescent binge drinking alters populations of 5-HT-immunopositive neurons in the adult raphe nucleus.

In humans, adolescent risk-taking and sensation-seeking behaviors coincide with the onset of alcohol and drug experimentation (Windle et al., 2008). Studies reveal that by age 14, binge drinking is common among youth in the United States, with current statistics reporting heavy episodic binge drinking (i.e., >5 consecutive drinks per binge drinking episode) in approximately 5% of 13–14 year old 8th graders, 22% of 12th graders, and >40%

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of college students (Johnston et al., 2013; White et al., 2006). Since the adolescent rat brain has been found to be more sensitive to alcohol neurotoxicity (Crews et al., 2000), maturational processes occurring in the adolescent brain suggest adolescence may be a particularly vulnerable period of elevated risk for later development of addiction and other disorders (Crews and Boettiger, 2009). Employing the rodent adolescent intermittent ethanol (AIE) model of human adolescent binge drinking, our laboratory and others found evidence of long-term cognitive dysfunction, increased impulsivity and anxiety-like behaviors, increased alcohol preference and drinking, and increased expression of multiple innate immune genes in adulthood (Crews et al., in press; Spear and Swartzwelder, 2014; Vetreno and Crews, 2012, 2015; Vetreno et al., 2013). However, it is unknown if adolescent binge ethanol exposure leads to long lasting changes in the adult serotonergic system.

Multiple studies have found that alcoholism is associated with innate immune gene induction in the brain (see e.g., Cui et al., 2015). There is increased expression of microglial (He and Crews, 2008) and innate immune markers (Crews et al., 2013; Vetreno et al., 2013) in post-mortem human alcoholic brain samples. Adolescent intermittent ethanol treatment in rats also increases innate immune gene expression in the prefrontal cortex (Vetreno and Crews, 2012; Vetreno et al., 2013) and hippocampus (Vetreno and Crews, 2015). In *ex vivo* slice culture, innate immune signaling reduces 5-HT+IR neurons (Hochstrasser et al., 2011). Since adult alcohol use disorders and other drinking problems are associated with an earlier age of drinking onset (Sher and Gotham, 1999) and dysfunction of the serotonergic system is associated with increased alcohol consumption and dependence (LeMarquand et al., 1994a,b), it is imperative to determine the effect of adolescent binge ethanol exposure on the adult serotonergic system. In the current study, we tested the novel hypothesis that AIE treatment would alter serotonergic neurons that would persist into adulthood. To test this hypothesis, 5-HT+IR in the dorsal raphe nucleus was assessed following treatment with our model of adolescent intermittent ethanol (AIE). Lipopolysaccharide (LPS), which is known to increase brain innate immune gene expression, was used to determine if brain innate immune gene induction would mimic the AIE-induced loss of 5-HT+IR neurons in the adult raphe nucleus. Further, previous studies find that voluntary exercise prevents ethanol-induced neuropathology in adult mice (Crews et al., 2004). Thus, we sought to determine whether wheel running would prevent the AIE-induced innate immune response and 5-HT+IR neuronal loss in adulthood. Our findings suggest that voluntary exercise can prevent the loss of 5-HT expression and brain innate immune upregulation by AIE. The novel findings presented are consistent with adolescent binge drinking leading to long-lasting changes in innate immune signaling in the adult raphe nucleus that contribute to reductions in 5-HT+IR neurons.

2. Materials and methods

2.1. Animals

Young time-mated pregnant female Wistar rats (embryonic day 17; Harlan Sprague-Dawley, Indianapolis, IN) were acclimated to our animal facility prior to birthing at the University of North Carolina at Chapel Hill. On postnatal day 1 (P1; 24 h after birth), litters were culled to 10 pups (6 males and 4 females) and housed with their dam in standard clear plastic tubs with shavings until

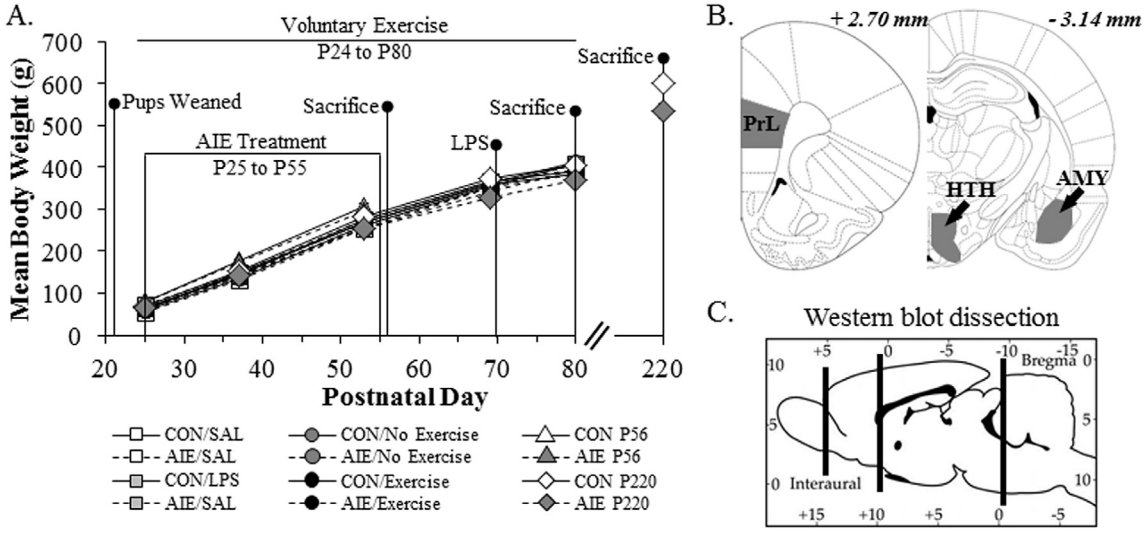

Fig. 1. Graphical representation of the adolescent intermittent ethanol (AIE) exposure paradigm. (A) Rats were treated from postnatal day (P) 25 to P55 with either a single daily dose of ethanol (AIE; 5.0 g/kg 20% ethanol w/v, i.g.) or a comparable volume of water (CON) on a two-day on/two-day off administration schedule. Blood ethanol concentrations (BEC) were measured one hr after ethanol exposure on P38 and P54. Twenty-four hours (P56; CON = 8, AIE = 8), 25 days (P80; CON = 8, AIE = 8), and 165 days (P220; CON = 7, AIE = 7) following the conclusion of AIE, rats were sacrificed for immunohistochemistry and Western blot analysis (P80). A subset of CON- and AIE-treated animals was treated with lipopolysaccharide (LPS; 1.0 mg kg, i.p., CON = 8, AIE = 8) or saline (SAL; CON = 8, AIE = 8) on P70 and sacrificed on P80. An additional subset of CON- and AIE-treated animals was exposed to voluntary wheel running from P24 to P80 and sacrificed on P80 (No Exercise: CON = 8, AIE = 8; Exercise: CON = 9, AIE = 10). Body weights were assessed at the initiation of AIE (P25), the conclusion of AIE (P55), and the conclusion of each experiment, depending on the endpoint. Across experiments, we observed that all subjects evidence dramatic weight gains (main effect of Age: Exercise study, $F_{[2,62]} = 5152.9$, $p < 0.01$; LPS study, $F_{[2,54]} = 4203.5$, $p < 0.01$; Aging study: P56, $F_{[1,14]} = 3926.4$, $p < 0.01$; P80, $F_{[2,28]} = 4434.8$, $p < 0.01$; P220, $F_{[2,24]} = 1553.9$, $p < 0.01$). Further, there were no differences in body weights across treatments and conditions (Exercise study: main effect of Treatment – $F_{[1,31]} = 0.9$, $p > 0.05$; main effect of exercise – $F_{[1,31]} = 1.1$, $p > 0.05$; LPS study: main effect of Treatment – $F_{[1,28]} = 1.8$, $p > 0.05$; main effect of LPS – $F_{[1,28]} = 0.01$, $p > 0.05$; Aging study: P56 – main effect of Treatment: $F_{[1,14]} = 1.4$, $p > 0.05$; P80 – main effect of Treatment: $F_{[1,14]} = 0.03$, $p > 0.05$) with the exception of the P220 study in which we observed that AIE-treated animals weighed approximately 10% less than CON subjects at P220 (CON = 622 ± 18 g, AIE = 553 ± 17 g [one-way ANOVA: $F_{[1,12]} = 7.9$, $p < 0.05$]). (B) Representative photomicrographs based on the atlas of Paxinos and Watson (1998) defining the regions of interest assessed for serotonin terminal field immunoreactivity. PrL: prelimbic cortex; HTH: hypothalamus; AMY: amygdala. (C) Representative photomicrograph depicting the brain regions dissected for Western blot analysis. The frontal cortex and midbrain were dissected and used for Western blot analysis of serotonergic protein expression. (all p's < 0.05) that did not differ as a function of treatment during AIE exposure (repeated measures ANOVAs: all p's > 0.9; see Fig. 1A). However, AIE-treated animals weighed approximately 10% less than CON subjects at P220 (CON = 622 ± 18 g, AIE = 553 ± 17 g [one-way ANOVA: $p < 0.05$]).