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Long-term Reductions in the Population of GABAergic Interneurons in the Mouse Hippocampus following Developmental Ethanol Exposure

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Abstract—Developmental exposure to ethanol leads to a constellation of cognitive and behavioral abnormalities known as Fetal Alcohol Spectrum Disorders (FASDs). Many cell types throughout the central nervous system are negatively impacted by gestational alcohol exposure, including inhibitory, GABAergic interneurons. Little evidence exists, however, describing the long-term impact of fetal alcohol exposure on survival of interneurons within the hippocampal formation, which is critical for learning and memory processes that are impaired in individuals with FASDs. Mice expressing Venus yellow fluorescent protein in inhibitory interneurons were exposed to vaporized ethanol during the third trimester equivalent of human gestation (postnatal days 2–9), and the long-term effects on interneuron numbers were measured using unbiased stereology at P90. In adulthood, interneuron populations were reduced in every hippocampal region examined. Moreover, we found that a single exposure to ethanol at P7 caused robust activation of apoptotic neurodegeneration of interneurons in the hilus, granule cell layer, CA1 and CA3 regions of the hippocampus. These studies demonstrate that developmental ethanol exposure has a long-term impact on hippocampal interneuron survivability, and may provide a mechanism partially explaining deficits in hippocampal function and hippocampus-dependent behaviors in those afflicted with FASDs.

Key words: interneuron, hippocampus, apoptosis, fetal, alcohol, ethanol, development.

INTRODUCTION

Gestational exposure to ethanol (EtOH) is one of the leading preventable causes of cognitive, physiological, and behavioral deficiencies in children around the world (Autti-Rämö and Granström, 1991; Mattson and Riley, 1999; Hamilton et al., 2003; Kodituwakku, 2007; Simmons et al., 2010; Riley et al., 2011; Mattson et al., 2013). Collectively, deficits induced by EtOH exposure during development are categorized under the umbrella diagnosis of Fetal Alcohol Spectrum Disorders (FASDs), which includes Fetal Alcohol Syndrome, partial FAS, and alcohol-related neurodevelopmental disorders (Chasnoff et al., 2010; May et al., 2014). Despite educational outreach efforts to inform the public on the dangers of exposing the developing fetus to EtOH, a relatively high percentage (2–5%) of children in the United States are exposed to some amount of alcohol during gestation (May et al., 2014), and in some populations up to 63% of children are exposed to EtOH in the womb (Miguez et al., 2009). Children afflicted with FASDs present with an assortment of problems in learning and memory processes, which result, at least in part, from dysfunction within the hippocampal region of the brain (Berman and Hannigan, 2000; Hamilton et al., 2003; Mattson et al., 1996; Uecker and Nadel, 1996, 1998). The hippocampus receives input via the perforant path from superficial layers of the entorhinal cortex, which synapse onto dendrites from granule cells of the dentate gyrus. Granule cells then project mossy fibers to CA3 pyramidal cells, which in turn synapse onto CA1 pyramidal neurons via the Schaffer collaterals (Ribak and Shapiro, 2007). This well-characterized tri-synaptic circuit is intricately regulated by inhibitory, gamma-aminobutyric acid (GABA)-expressing interneurons (Kullmann, 2011). Interneurons represent a minority of neurons in the hippocampus (10–20%) (Olbrich and Braak, 1985; Freedman et al., 1993), but because of their dense axonal arborization, they can innervate hundreds of postsynaptic target dendrites (Lubke et al., 1998; Muller and Remy, 2014; Savanthrapadian et al., 2014), providing tight regulation of circuit-level signaling within the hippocampus (Cobb et al., 1995).

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Abbreviations: BECs, blood EtOH concentrations; EtOH, exposure to ethanol; FASDs, Fetal Alcohol Spectrum Disorders; GABA, gamma-aminobutyric acid; GCL, granule cell layer; PBS, phosphate-buffered saline; PV, parvalbumin; Sst, somatostatin.

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GABAergic interneurons are particularly vulnerable to a variety of insults, including excitotoxicity (Shetty and Turner, 2001; Shetty et al., 2009), ischemic events (Johansen, 1993; Bering et al., 1997), and traumatic brain injury (Lowenstein et al., 1992; Schiavone et al., 2017). Previous research has also demonstrated that interneurons in a variety of brain regions are susceptible to damage by both developmental and postnatal EtOH exposure (Andrade et al., 1992; Moore et al., 1998). Developmental exposure to EtOH reduces interneuron populations in cortical areas (Moore et al., 1998; Miller, 2006; Smiley et al., 2015), and in the cerebellum (Nirgudkar et al., 2016). Relatively little information exists, however, on the impact of developmental EtOH exposure on hippocampal interneuron populations. (Miki et al., 2000) demonstrated that exposure to EtOH during the third trimester equivalent of human gestation in the rat reduced the number of neurons in the hilus of the dentate gyrus, which is an area that contains an abundance of interneurons, but did not specifically identify interneurons in their study. Another set of experiments demonstrated that a single postnatal EtOH exposure led to long-term reductions in parvalbumin (PV)-expressing interneurons in the CA1 region of the mouse brain (Sadrian et al., 2014).

Developmental EtOH exposure causes robust activation of programmed cell death via apoptotic signaling pathways in a variety of cells throughout the brain (Ikonomidou et al., 2000; Olney et al., 2002), including the hippocampus (Camargo Moreno et al., 2017). Until recently, it has remained unknown whether reductions in interneurons caused by developmental EtOH exposure are a result of apoptosis. A recent short report demonstrated that a single dose of EtOH during the early postnatal period increases the expression of cleaved caspase-3, a marker of apoptosis, in interneurons of the hippocampus (Ogievetsky et al., 2017). In the present study, we sought to further understand the effects of early postnatal EtOH exposure on hippocampal interneurons. Using a transgenic mouse model that expresses Venus fluorescent protein in GABAergic interneurons throughout the brain (Wang et al., 2009), mice were exposed to EtOH during the third trimester equivalent of human gestation, which mimics an exposure pattern observed in humans (Ethen et al., 2009). This period of development is also critical for refinement of neuronal circuits impacted by GABAergic signaling (Cellot and Cherubini, 2013; Le Magueresse and Monyer, 2013). We measured the density of interneurons expressing a marker of activated apoptosis in different hippocampal regions, and assessed the number of surviving interneurons in aged mice to understand the long-term trajectory of interneuron numbers in the hippocampus following developmental EtOH exposure.

EXPERIMENTAL PROCEDURES

All experimental procedures described adhered to the U.S. Public Health Service policy on humane care and use of laboratory animals and were approved by the Institutional Care and Use committee of the University of New Mexico Health Sciences Center. For all the experiments described below, experimenters were blinded to treatment group assignment.

Subjects

Venus-VGAT mice were generated as described (Wang et al., 2009). These mice express Venus fluorescent protein (a yellow fluorescent protein variant developed by Dr. Atsushi Miyawaki at RIKEN in Wako, Japan) under control of the vesicular GABA transporter. This leads to Venus expression in virtually every GABAergic and glycinergic neuron throughout the brain (Wang et al., 2009). A breeding colony was established at the University of New Mexico Health Sciences Center Animal Resource Facility. Mice were maintained as heterozygous for the Venus-VGAT transgene (hereafter referred to as Venus-VGAT$^+$), and offspring were group-housed with littermates of the same sex at 22 °C on a reverse 12-h light/dark cycle (lights on at 2000 h) with standard chow and water available *ad libitum*.

Breeding

60- to 180-day-old wild-type C57BL/6 or Venus-VGAT$^+$ female mice were paired with a Venus-VGAT$^+$ male or a wild-type C57BL/6 male breeder, respectively. After pregnancy was evident, the male mice were removed from the cage. Following parturition, postnatal day (P) 1-P2 pups were screened for the presence of the Venus-VGAT transgene by exposing them to 460–495 nm wavelength light and observing yellow fluorescence emitted by the brain with a 520- to 550-nm filter using a "miner's lamp" (Biological Laboratory Equipment Maintenance and Service LTD, Budapest, Hungary).

Tissue collection

To collect tissue, mice were anesthetized with ketamine (250 mg/kg i.p.) and perfused transcardially with 32 °C phosphate-buffered saline (PBS) containing procaine hydrochloride (1g/L; Sigma–Aldrich, St. Louis, MO) and heparin (1USP unit/mL; Sagent Pharmaceuticals, Schaumburg, IL) for 2 min, followed by room-temperature (~21 °C) 4% paraformaldehyde (PFA; Sigma–Aldrich) in PBS for 2 min, then with ice cold 4% PFA in PBS for 5 min. Extracted brains were incubated in 4% PFA in PBS for 48 h at 4 °C with gentle shaking, then cryoprotected in 30% sucrose in PBS for 48 h. Brains were embedded in Optimal Cutting Temperature compound (Fisher Healthcare, Houston, TX, USA) and frozen in isopentane (Avantor Performance Materials, Center Valley, PA, USA) cooled with a bath of 95% EtOH and dry ice. Brains were kept frozen at −80 °C until sectioned in the parasagittal plane on a cryostat (Microm Model HM 505E, Waldorf, Germany) at 50 μm. Floating sections were kept at -20 °C in freezing medium (0.05 M phosphate buffer pH 7.4, 25% glycerol and 25% ethylene glycol).

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