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Selective reduction of cerebral cortex GABA neurons in a late gestation model of fetal alcohol spectrum disorder

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ABSTRACT

Fetal alcohol spectrum disorders (FASD) are associated with cognitive and behavioral deficits, and decreased volume of the whole brain and cerebral cortex. Rodent models have shown that early postnatal treatments, which mimic ethanol toxicity in the third trimester of human pregnancy, acutely induce widespread apoptotic neuronal degeneration and permanent behavioral deficits. However, the lasting cellular and anatomical effects of early ethanol treatments are still incompletely understood. This study examined changes in neocortex volume, thickness, and cellular organization that persist in adult mice after postnatal day 7 (P7) ethanol treatment. Post mortem brain volumes, measured by both MRI within the skull and by fluid displacement of isolated brains, were reduced 10–13% by ethanol treatment. The cerebral cortex showed a similar reduction (12%) caused mainly by lower surface area (9%). In spite of these large changes, several features of cortical organization showed little evidence of change, including cortical thickness, overall neuron size, and laminar organization. Estimates of total neuron number showed a trend level reduction of about 8%, due mainly to reduced cortical volume but unchanged neuron density. However, counts of calretinin (CR) and parvalbumin (PV) subtypes of GABAergic neurons showed a striking >30% reduction of neuron number. Similar ethanol effects were found in male and female mice, and in C57BL/6By and BALB/cJ mouse strains. Our findings indicate that the cortex has substantial capacity to develop normal cytoarchitectonic organization after early postnatal ethanol toxicity, but there is a selective and persistent reduction of GABA cells that may contribute to the lasting cognitive and behavioral deficits in FASD.

Introduction

Fetal alcohol spectrum disorders (FASD) are one of the most common causes of mental disabilities, and affect as many as 1% of children born in the United States (May et al., 2009). Along with cognitive deficits including memory, attention, and sensory perception, FASD is associated with reduced brain volume that persists into adulthood (Bakoyiannis et al., 2014; Norman, Crocker, Mattson, & Riley, 2009).

Animal models have been instrumental in providing insights into the brain areas and cellular processes that are affected by FASD. They have demonstrated that the effects of ethanol vary depending on the gestational timing of exposure. Treatment during the rodent early postnatal period, approximating the human third trimester, causes both widespread apoptotic cell death in the brain and long-lasting behavioral deficits (Ikonomidou et al., 2000; Sadrian, Wilson, & Saito, 2013; Wilson, Peterson, Basavaraj, & Saito, 2011; Wozniak et al., 2004). This is a period when neurogenesis is largely complete, and the brain is rapidly growing (Miller, 1988). Treatments early in this period, around the time of birth in rats, cause pronounced neurodegeneration in hypothalamic and ventral thalamic regions, compared to treatments around P3 that especially affect the dorsal thalamus and hippocampal regions, or treatments around P7 that especially affect cerebral cortex (Ikonomidou et al., 2000). Earlier treatments that model the first and second trimesters of human development also produce anatomical and behavioral changes, although they are somewhat distinct from the late gestational effects (Guerri, 1998; Sadrian et al., 2013). While these earlier ethanol treatments do not cause such widespread apoptosis, they have been shown to disrupt neurogenesis and cell migration (Cuzon, Yeh, Yanagawa, Obata, & Yeh, 2008).

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Understanding the enduring effects of FASD on the brain is an area of ongoing research. In human adolescents and adults, FASD causes reduced volumes of gray matter and white matter throughout the forebrain. Gray matter volume reductions as large as 10% have been described across regions of the cerebral cortex, with even larger changes in subcortical structures, including hippocampus and striatum (Coles et al., 2011; Lebel et al., 2012; Nardelli, Lebel, Rasmussen, Andrew, & Beaulieu, 2011; Norman et al., 2009). Adult animals that were exposed to fetal alcohol typically have a similar profile of volume reductions (Coleman, Oguz, Lee, Styner, & Crews, 2012; Leigland, Ford, Lerch, & Kroenke, 2013). In some models, histological studies showed that these volume reductions corresponded to reduced neuron numbers (Berman & Hannigan, 2000; Ieraci & Herrera, 2007; Miller, 2006; Olney, 2004). More detailed examinations found evidence of altered dendritic length and spine density (Berman & Hannigan, 2000; Cui et al., 2010; Lawrence, Otero, & Kelly, 2012; Susick, Lowing, Provenzano, Hildebrandt, & Conti, 2014), and found evidence of disrupted organization of cortical sensory areas (Margret et al., 2006; Medina, Krahe, & Ramoa, 2005; Miller & Potempa, 1990). Additionally, in several cortical and subcortical brain regions there are findings of reduced GABAergic cell density, suggesting that inhibitory circuits in the cerebral cortex may be especially vulnerable to ethanol toxicity (Coleman et al., 2012; Sadrian, Lopez-Guzman, Wilson, & Saito, 2014; Sadrian et al., 2013). At present, it is unclear to what extent these changes are the direct result of cell loss induced by ethanol toxicity, or are secondary effects caused by compensatory brain plasticity that occurs in response to ethanol-induced damage. In particular, inhibition of GABA function in early postnatal development is known to disrupt the normal development of cortical circuits (Le Magueresse & Monyer, 2013).

We recently demonstrated that mouse piriform cortex has reduced PV-immunolabeled GABA cells, along with increased neuron excitability, reduced paired-pulse inhibition, and enhanced oscillatory coherence, caused by ethanol treatment at both early and late gestational stages (Sadrian et al., 2013, 2014). The findings suggest that deficits of GABAergic inhibition may cause a characteristic profile of electrophysiological deficits. In the current study, we used a model of late gestational ethanol treatment (mouse P7) to determine whether a similar reduction in GABA cells is present throughout the neocortex, and we measured total neuron number to determine to what extent the neuron reduction was selective for GABAergic neurons. We also evaluated cortical thickness, surface area, and laminar organization, to evaluate the general features of adult cortical disruption caused by perinatal ethanol. Additionally, we included comparisons of C57BL/6By and BALB/cJ strains of mice, as these strains have well-established differences in alcohol preference and are commonly used as genetic animal models in alcohol-related research (Fish et al., 2010; Rodgers & McClearn, 1962; Vadasz, Baker, Joh, Lajtha, & Reis, 1982; Vadasz, Saito, et al., 2007).

Materials and methods

Subjects and ethanol exposure

Twenty four mice were used, comparing 12 ethanol-treated to 12 saline-treated animals. The two treatment groups were matched to include equal numbers of C57BL/6By and BALB/cJ mice, with equal number of males and females from each strain. Mice were originally obtained from The Jackson Laboratory (Bar Harbor, Maine) in 1978, and maintained by brother × sister mating at the Nathan Kline Institute. Dams and their litters were housed individually in standard mouse cages, and maintained on *ad libitum* food and water. P7 pups were injected subcutaneously with saline or ethanol (2.5 g/kg) twice at 0 h and 2 h as described previously (Saito, Mao, Wang, Vadasz, & Saito, 2007) according to the methods originally described for C57BL/6 mice (Olney et al., 2002). This treatment induces a peak blood alcohol level of ~0.5 g/dL when the truncal blood is collected at 0.5, 1, 3, and 6 h following the second ethanol injection, as analyzed using Alcohol Reagent Set (Pointe Scientific, Canton, MI, USA) (Saito et al., 2007). This alcohol level is similar to those reported by others (Wozniak et al., 2004; Young & Olney, 2006). After injections, pups were returned to the litter, and weaning occurred at P25–30. All procedures were approved by the Nathan Kline Institute IACUC and were in accordance with NIH guidelines for the proper treatment of animals.

MRI data acquisition

After survival to 72–89 days of age, animals were anesthetized by intraperitoneal injection with 200 mg/kg ketamine and 10 mg/kg xylazine, and transcardially perfused with heparinized 4% paraformaldehyde in phosphate buffer, pH 7.2. The mouse heads were left in fixative overnight. The skin, lower jaw, ears, and cartilaginous nose tip were removed and the head placed in 50 mL tubes in 0.01% sodium azide in PBS for 3–7 days at 4 °C. The head was then transferred to a 0.3% solution of MultiHance (gadobenate dimeglumine 529 mg/mL, Bracco Diagnostics Inc., Monroe Township, NJ, USA) and 0.01% sodium azide in PBS and rocked for 7 days at 4 °C. One day before the MRI scan, the head was placed in a 15 mL tube with the contrast reagent flombin (Sigma, catalog #317926). One day after scanning, the whole brain was removed and stored in phosphate-buffered saline with 0.03% sodium azide.

Images were acquired on a 7.0 T Agilent (Santa Clara, CA) 40 cm bore system. The gradient coil insert had an internal diameter of 12 cm with a maximum gradient strength of 600 mT/m and minimum rise time of 200 μs with customized second- and third-order shim coils. A Morris Instruments (Ontario, Canada) 1.5 cm inner diameter solenoid transmit coil was used for radiofrequency transmission and reception. The images used in this study were acquired with 150 μm^3 isotropic resolution and were taken from a data set of magnetic resonance diffusion measurements. For image analysis of 24 mouse brains, a mask was created automatically using the Automatic Registration Toolbox software (www.nitrc.org/projects/art). Individual brain extractions were manually corrected by using the ITK-snap (www.itk-snap.org) software and by consulting a mouse brain atlas (Paxinos & Franklin, 2004).

Histological processing and immunolabeling

Whole brain volumes, including cerebellum and brainstem, were measured by a fluid displacement method (Dorph-Petersen et al., 2005; Scherle, 1970). After cryoprotection by 2 day immersion in 20% buffered glycerol, brains were divided into two groups containing either 12 males or 12 females, and each group was embedded in a protein matrix for simultaneous sectioning and tissue processing (Smiley & Bleiwas, 2012). Coronally oriented brains were sectioned at 50 μm thickness. A series including every 6th consecutive section was mounted and dried on chrome-alum coated glass slides, and processed for Nissl staining with thionin before dehydration and coverslipping with Permount™.

For immunolabeling, neurons were visualized with mouse anti-PV diluted to 1:2500 (Sigma, catalog #P3088) rabbit anti-CR diluted to 1:5000 (Swant, catalog #7699/4) or mouse-anti-neuron specific protein (NeuN) diluted to 1:2000 (Millipore, catalog #MAB377). For immunoperoxidase labeling with CR and PV antibodies, series of every 6th consecutive section were exposed to primary antibodies for 3 days in 1% normal serum, 0.03% Triton X-100, and phosphate