NEUROSCIENCE
INTERNATIONAL BRAIN IBRO RESEARCH ORGANIZATION
RESEARCH ARTICLE
C. M. Goeke et al. / Neuroscience xxx (2018) xxx–xxx

Neonatal Ethanol and Choline Treatments Alter the Morphology of Developing Rat Hippocampal Pyramidal Neurons in Opposite Directions

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Abstract—Some of the neurobehavioral deficits identified in children with Fetal Alcohol Spectrum Disorders (FASDs) have been recapitulated in a binge model of gestational third trimester-equivalent ethanol (EtOH) exposure, in which Sprague–Dawley rats are intragastrically intubated between post-natal day (PD) 4 and PD9 with high doses of EtOH. In this model, the ameliorating effects of choline (Chol) administration on hippocampus-dependent behaviors altered by EtOH have also been extensively documented. In the present study, we investigated the effects of EtOH (5 g/kg/day) and/or Chol (100 mg/kg/day) on morphometric parameters of CA1 pyramidal neurons by Golgi–Cox staining followed by Neurolucida tracing and analysis. We found that EtOH increased apical dendrite complexity in male and female pups neonatally exposed to EtOH. EtOH did not significantly affect basal dendrite parameters in female and male rats. Interestingly, Chol treatments decreased basal dendrites' length, number, and maximal terminal distance in male pups. When pups were co-treated with EtOH and Chol, Chol did not rescue the effect of EtOH. In conclusion, EtOH increases while Chol decreases dendritic length and arborization of hippocampal CA1 neurons in PD9 rats. We hypothesize that developmental EtOH exposure induces a premature maturation of neurons, leading to early restriction of neuronal plasticity while Chol treatments delay the normal program of neuronal maturation and therefore prolong the window of maximal plasticity. Chol does not prevent the effects of developmental alcohol exposure on hippocampal pyramidal neurons' morphology characterized in the present study, although whether prolonged Chol administration after developmental EtOH exposure rectifies EtOH damage remains to be assessed. Published by Elsevier Ltd on behalf of IBRO.

Key words: Fetal Alcohol Spectrum Disorders, pyramidal neurons, hippocampus, apical dendrites, basal dendrites, Golgi–Cox staining.

INTRODUCTION

Ethanol (EtOH) abuse during pregnancy may lead to Fetal Alcohol Spectrum Disorders (FASDs) characterized by structural brain abnormalities and compromised cognitive and behavioral functions (Hellemans et al., 2010; Riley et al., 2011). Clinical and preclinical studies indicate that neuronal plasticity and connectivity are affected by *in utero* alcohol exposure; these alterations may play a major role in central nervous system (CNS) dysfunction present in individuals with FASD (Medina, 2011; Lebel et al., 2012; Wozniak et al., 2013).

EtOH affects the development of the CNS throughout gestation (Rice and Barone, 2000). The third trimester of human gestation is characterized by functional maturation of several brain regions, including the hippocampus; this developmental stage in rats occurs mostly during the first nine post-natal days. Major events during this period include a massive increase in brain size (brain growth spurt), proliferation of astrocytes and oligodendrocytes, and dendritic arborization (Rice and Barone, 2000). EtOH exposure during this developmental stage induces microcephaly, cerebellar and hippocampal abnormalities, severe apoptotic neuronal death in the hippocampus and cerebral cortex, and behavioral dysfunctions (Bonthius and West, 1990, 1991; Ikonomidou et al., 2000; Patten et al., 2014). Of particular relevance to the present study is the fact that EtOH alters hippocampus-dependent behaviors in several rodent models of FASD, including models of gestational third trimester-equivalent EtOH exposure (Kelly et al., 1988; Gianoulakis, 1990; Goodlett and Peterson, 1995; Berman and Hannigan, 2000; Johnson and Goodlett, 2002; Christie et al., 2005;

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Abbreviations: BEC, blood ethanol concentration; Chol, choline; CNS, central nervous system; EtOH, ethanol; FASD, Fetal Alcohol Spectrum Disorders; FDR, False Discovery Rate; GD, Gestational Day; PD, post-natal day.

https://doi.org/10.1016/j.neuroscience.2018.01.031
0306-4522/Published by Elsevier Ltd on behalf of IBRO.

Please cite this article in press as: Goeke CM et al. Neonatal Ethanol and Choline Treatments Alter the Morphology of Developing Rat Hippocampal Pyramidal Neurons in Opposite Directions. Neuroscience (2018), https://doi.org/10.1016/j.neuroscience.2018.01.031

Popovic et al., 2006; Thomas et al., 2008, 2010; Patten et al., 2014).

A substantial body of evidence derived from behavioral and neurochemical studies in rats indicate that choline (Chol) improves hippocampal functions in the adult and aging brain and that Chol supplementation during gestation as well as during the early postnatal period improves memory performance throughout life (Zeisel and Niculescu, 2006). More relevant to the present study, Chol has been consistently shown to ameliorate hippocampus-associated behaviors in rats exposed to EtOH during brain development (Thomas et al., 2000, 2004, 2007, 2009, 2010). Additionally, a few studies explored how Chol may ameliorate some of the effects of EtOH (Otero et al., 2012; Tang et al., 2014; Balaraman et al., 2017). For these reasons, Chol is currently being tested clinically for its effectiveness in treating FASD (Wozniak et al., 2015; Nguyen et al., 2016).

EtOH causes long-lasting changes in dendritic arborization and/or number of dendritic spines in different populations of neurons after prenatal and/or neonatal exposure. Neonatal EtOH exposure decreased spine density and dendritic complexity of basal dendrites as well as dendritic spine density in apical dendrites of layer II/III pyramidal neurons of the medial prefrontal cortex (mPFC) in juvenile rats, an effect that was reversed by voluntary exercise (Whitcher and Klintsova, 2008; Hamilton et al., 2010, 2015). In addition, EtOH alters neuronal development, measured as neurite outgrowth, in hippocampal pyramidal neurons *in vitro* (Yanni and Lindsley, 2000; Lindsley et al., 2002, 2003; Yanni et al., 2002; Lindsley and Clarke, 2004; VanDemark et al., 2009; Guizzetti et al., 2010; Giordano et al., 2011; Zhang et al., 2014). Together, this published literature supports the hypothesis that EtOH alters the proper development of neurons leading to altered brain connectivity.

We undertook the present study to investigate the effect of binge EtOH exposure and of the co-treatment with Chol during the third trimester of gestation equivalent, between post-natal day (PD) 4 and PD9, on dendritic arborization of CA1 pyramidal neurons in pups euthanized two hours after the last alcohol exposure on PD9. Our rationale for exploring alterations in neuronal morphology occurring in developing neurons is that appropriate brain development requires developmental events to occur in a synchronized manner, so a delay or acceleration of any given event may have profound functional consequences that may persist throughout life.

EXPERIMENTAL PROCEDURES

Animals

Timed-pregnant Gestational Day (GD) 15 Sprague–Dawley rats were purchased from Charles River (Wilmington, MA) and maintained at the Portland VAMC Veterinary Medical Unit under a 12-h light/dark cycle (lights on from 6:00 to 18:00) at 22 ± 1 °C. Pregnant animals had *ad libitum* access to water and food (chow diet). All animal procedures were approved by the Portland VA Health Care System Institutional Animal Care and Use Committee and followed US National Institutes of Health animal welfare guidelines.

In vivo neonatal EtOH and Chol treatments

On PD4, animals were counted and sexes were determined. When possible, the litters were culled to ten pups, five of each sex and one animal/sex/litter was randomly assigned to one of the following conditions: (1) sham intubation and saline injection control (IC; four female and four male pups), (2) sham intubation and Chol injection (Chol; three female and four male pups), (3) EtOH intubation and saline injection (EtOH; four female and four male pups); (4) EtOH intubation and Chol injection (EtOH + Chol; four female and four male pups); (5) untouched animals that remained with the dam all the time (three female and four male pups; the results from these animals were not presented in this study). In total, data presented in this study were obtained from the analysis of 31 pups derived from four different litters: four females and four males for conditions 1, 3, and 4; three females and four males for condition 2 (one of the litters had only three females). Before the beginning of the treatments, pups were tattooed with subcutaneous injections of India Ink in their paws for identification. Between PD4 and PD9 pups were weighted and injected subcutaneously with saline or 100 mg/kg Chol each day, followed by two EtOH or sham intragastric intubations. Pups that were given EtOH were also given two intubations of milk formula without EtOH at two–hour intervals starting two hours after the last EtOH intubation, to compensate for lack of suckling caused by inebriation; pups not receiving EtOH were sham-intubated at the same intervals (Fig. 1). Intragastric intubation was done by inserting flexible tubing that was dipped into corn oil for lubrication into the esophagus of the neonatal rat. Animals in the EtOH and EtOH + Chol groups received 5 g/kg/day EtOH in milk formula (Similac Advance Early Shield with iron) delivered in two separate feedings two hours apart, at a concentration of 11.9% EtOH in formula, and an intubation volume of 0.0278 ml/g. Rat pups were weighed daily. During the intubation process, rat pups were removed from their dam and placed on a heating pad. On PD9, two hours after the last EtOH intubation, animals were anesthetized by an intraperitoneal injection with a cocktail of Ketamine (500 mg/10 mL, 100 mg/kg), Xylazine (50 mg/10 mL, 10 mg/kg) and Acepromazine (10 mg/10 mL, 1 mg/kg) in 0.9% saline and decapitated. Trunk blood was collected to determine EtOH concentration and the brains were collected for Golgi–Cox staining. Four litters were used in these experiments; all the animals survived throughout the treatments.

Blood EtOH concentration (BEC) determination

Following euthanasia, 20 μl of trunk blood was collected from the animals and mixed into 500 μl of a matrix consisting of 4 mM n-propanol in distilled water. BECs were determined by head-space gas chromatography as previously described (Finn et al., 2007).

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