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Postnatal choline supplementation selectively attenuates hippocampal microRNA alterations associated with developmental alcohol exposure

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ABSTRACT

Prenatal alcohol exposure can result in a range of physical, neuropathological, and behavioral alterations, collectively termed fetal alcohol spectrum disorders (FASD). We have shown that supplementation with the nutrient choline reduces the severity of developmental alcohol-associated deficits in hippocampal-dependent behaviors and normalizes some aspects of hippocampal cholinergic development and DNA methylation patterns. Alcohol's developmental effects may also be mediated, in part, by altering microRNAs (miRNAs) that serve as negative regulators of gene translation. To determine whether choline supplementation alters ethanol's long-lasting effects on miRNAs, Sprague-Dawley rats were exposed to 5.25 g/kg/day ethanol from postnatal days (PD) 4–9 via intubation; controls received sham intubations. Subjects were treated with choline chloride (100 mg/kg/day) or saline vehicle subcutaneously (s.c.) from PD 4-21. On PD 22, subjects were sacrificed, and RNA was isolated from the hippocampus. MiRNA expression was assessed with TaqMan Human MicroRNA Panel Low-Density Arrays. Ethanol significantly increased miRNA expression variance, an effect that was attenuated with choline supplementation. Cluster analysis of stably expressed miRNAs that exceeded an ANOVA $p < 0.05$ criterion indicated that for both male and female offspring, control and ethanol-exposed groups were most dissimilar from each other, with choline-supplemented groups in between. MiRNAs that expressed an average 2-fold change due to ethanol exposure were further analyzed to identify which ethanol-sensitive miRNAs were protected by choline supplementation. We found that at a false discovery rate (FDR)-adjusted criterion of $p < 0.05$, miR-200c was induced by ethanol exposure and that choline prevented this effect. Collectively, our data show that choline supplementation can normalize disturbances in miRNA expression following developmental alcohol exposure and can protect specific miRNAs from induction by ethanol. These findings have important implications for the mechanisms by which choline may serve as a potential treatment for FASD.

Introduction

Prenatal alcohol exposure can disrupt development of the fetus via multiple mechanisms. Alcohol may alter cell proliferation, migration, differentiation, synaptogenesis, and myelination, depending on the developmental timing and other exposure parameters (Camarillo & Miranda, 2008; Gil-Mohapel, Boehme, Kainer, & Christie, 2010; Kane, Phelan, & Drew, 2012; O'Leary-Moore, Parnell, Lipinski, & Sulik, 2011; Santillano et al., 2005; Tingling et al., 2013; Wilhelm & Guizzetti, 2016). But alcohol may also lead to long-lasting changes in cell function, altering gene expression (Downing et al., 2011, 2012) and compromising synaptic plasticity (Gil-Mohapel et al., 2010; Medina, 2011), all of which contribute to long-lasting pathology in neural structure and function. Such disruptions lead to behavioral and cognitive alterations (Hamilton et al., 2010; Mattson, Crocker, & Nguyen, 2011), effects that have serious consequences on the quality of life of individuals prenatally exposed to alcohol and their families.

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Protection against alcohol's teratogenic effects is critical, as prevention efforts have not effectively reduced alcohol consumption among many pregnant women (Centers for Disease Control and Prevention, 2015; May et al., 2008). In fact, recent estimates suggest that the prevalence of fetal alcohol spectrum disorders (FASD), the range of effects caused by prenatal alcohol exposure on the offspring, is around 34 per 1000 in the United States, with higher prevalence in other areas around the globe, such as South Africa (113 per 1000) (Roozen et al., 2016). Given that 10% of women report some alcohol consumption during pregnancy in the U.S. (Center for Behavioral Health Statistics and Quality, 2015) and that almost half of pregnancies are unplanned (Finer & Zolna, 2011), FASD will continue to be a problem. Elucidation of factors that can reduce the severity of FASD may lead to effective intervention strategies.

Alcohol's teratogenic effects may be modified by a number of factors, including nutritional variables (Abel, 1995; Young, Giesbrecht, Eskin, Aliani, & Suh, 2014). We have shown that administration of choline, an essential nutrient, can reduce the severity of alcohol's teratogenic effects, including physical (Thomas, Abou, & Dominguez, 2009), neuropathological (Monk, Leslie, & Thomas, 2012; Otero, Thomas, Saski, Xia, & Kelly, 2012), and behavioral outcomes (Monk et al., 2012; Ryan, Williams, & Thomas, 2008; Thomas, Garrison, & O'Neill, 2004; Thomas, Idrus, Monk, & Dominguez, 2010; Thomas, La Fiette, Quinn, & Riley, 2000; Thomas & Tran, 2012). Specifically, we have shown that prenatal choline supplementation during prenatal alcohol exposure mitigates ethanol-related reductions in birth and brain weights, delays in incisor emergence, and delays in reflex development, including impairments in hindlimb coordination (Thomas et al., 2009). Choline also mitigates delays in the development of spontaneous alternation and long-lasting deficits on working memory induced by prenatal alcohol exposure (Thomas et al., 2010), both of which depend on the functional integrity of the hippocampus. But importantly, choline can reduce the severity of fetal alcohol effects, even when administered postnatally and after the alcohol exposure has occurred. When administered during the early postnatal period, choline targets ethanol's effects on behaviors associated predominantly with hippocampal function, reducing the severity of open field overactivity (Monk et al., 2012; Thomas, Garrison, et al., 2004), and deficits in reversal learning (Thomas, Garrison, et al., 2004), working memory (Thomas et al., 2000), spatial learning (Ryan et al., 2008; Thomas et al., 2010), and trace classical conditioning (Thomas & Tran, 2012; Wagner & Hunt, 2006). In contrast, when administered during this period of development, choline does not reduce ethanol's effects on behaviors that depend on the functional integrity of the cerebellum (Thomas, O'Neill, & Dominguez, 2004; Thomas & Tran, 2012; Wagner & Hunt, 2006), although one study reported that choline supplementation from postnatal day (PD) 1–20 reduces the severity of motor deficits associated with acute ethanol on PD 5 (Bearer, Wellmann, Tang, He, & Mooney, 2015). These data suggest that when administered after PD 10, choline targets the hippocampus.

However, it is not clear how choline achieves these behavioral effects. Choline acts, in part, as a precursor to the neurotransmitter acetylcholine (Zeisel, 2013; Zeisel & Niculescu, 2006) and we have found that choline supplementation attenuates alcohol-related changes in hippocampal cholinergic receptors (Monk et al., 2012). Choline also acts as a methyl donor and can, therefore, influence gene expression (Niculescu & Zeisel, 2002; Zeisel, 2006). In fact, we have found that choline attenuates alcohol-related changes in hippocampal DNA methylation (Otero et al., 2012). Choline's actions as an epigenetic factor mean that it may affect many target pathways that influence development. In this study, we tested the hypothesis that choline reduces the effects of developmental alcohol exposure, in part, by attenuating the effects of alcohol on a class of small non-protein-coding RNAs termed microRNAs (miRNAs).

MiRNAs regulate cellular function and maturation state by repressing the translation of networks of protein-coding genes (for review, see Miranda, 2014). We previously showed that early developmental exposure to alcohol resulted in alterations in miRNAs (Sathyan, Golden, & Miranda, 2007). Moreover, these alterations could explain ethanol's teratogenic effects on neural and craniofacial development (Pappalardo-Carter et al., 2013; Sathyan et al., 2007; Tsai et al., 2014). Pertinent to the presumptive mechanisms of action of choline, we also previously showed that ethanol-sensitive miRNAs were regulated by epigenetic mechanisms (Pappalardo-Carter et al., 2013) and that the effects of ethanol on miRNAs could be prevented and reversed by ligands that acted at nicotinic acetylcholine receptors (Balaraman, Winzer-Serhan, & Miranda, 2012; Tsai et al., 2014). These data suggested to us that choline supplementation might be a means to reverse the effects of developmental alcohol exposure on miRNA pathways. Therefore, the present study examined whether the nutrient choline would modify ethanol's effects on miRNAs. To examine this, we used a third trimester model of ethanol exposure, which produces hippocampal pathology. Subjects received choline both during and after ethanol exposure to maximize effects.

Materials and methods

Ethanol and choline treatment

Subjects were offspring from the Center for Behavioral Teratology mating colony at San Diego State University. The mating colony is housed in a temperature and humidity-controlled environment, and food and water was available *ad libitum*. Male and female Sprague-Dawley rats were housed overnight and the presence of a seminal plug at the bottom of the cage indicated mating. Pregnant dams were then singly housed. The day after birth, litters were culled to eight, with four males and four females, whenever possible.

On PD 4, pups were randomly assigned to groups in a 2 (ethanol, sham) x 2 (choline, saline) x 2 (male, female) design. No more than one sex pair per litter was assigned to a treatment group to minimize litter effects. A total of 48 subjects (6/group) were generated. Ethanol was administered via oral intubation. Consistent with our previous studies of third trimester alcohol exposure (Monk et al., 2012; Ryan et al., 2008; Schneider & Thomas, 2016; Thomas & Tran, 2012), subjects received 2.625 g/kg ethanol in a nutritionally balanced milk formula (11.9% v/v) twice per day, 2 h apart, for a daily dose of 5.25 g/kg/day from PD 4-9, to mimic exposure during the third trimester-equivalent period of human development (Workman, Charvet, Clancy, Darlington, & Finlay, 2013). Ethanol-exposed subjects were also given two additional intubations of milk formula with no alcohol (2 h apart) to minimize growth differences. Sham controls received intubations, but no formula during the four daily intubations. Intubations took just a few minutes and subjects remained with the dam between intubations. From PD 4-21, subjects were injected subcutaneously (s.c.) with choline chloride (100 mg/kg/day) or saline, as previous studies have shown benefits of choline chloride at this dose during postnatal development (Monk et al., 2012; Otero et al., 2012; Ryan et al., 2008; Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007).

On PD 6, 20 μL of blood was collected via a tail clip, 1.5 h after the second ethanol treatment to determine peak blood alcohol concentration (BACs; mg/dL). Blood samples were centrifuged and plasma samples were analyzed using the Analox Alcohol Analyzer (Model AM1, Analox Instruments; Lunenburg, MA). On PD 7, each