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Thyroxine administration prevents matrilineal intergenerational consequences of *in utero* ethanol exposure in rats

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ABSTRACT

The neurodevelopmental fetal alcohol spectrum disorder (FASD) is characterized by cognitive and behavioral deficits in the offspring. Conferring the deficits to the next generation would increase overall FASD disease burden and prevention of this transmission could be highly significant. Prior studies showed the reversal of these behavioral deficits by low dose thyroxine (T4) supplementation to the ethanol-consuming mothers. Here we aim to identify whether prenatal ethanol (PE) exposure impairs hippocampus-dependent learning and memory in the second-generation (F2) progeny, and whether T4 administration to the ethanol-consuming dam can prevent it. Sprague-Dawley (S) dams received control diets (*ad libitum* and nutritional control) or ethanol containing liquid diet with and without simultaneous T4 (0.3 mg/L diet) administration. Their offspring (SS F1) were mated with naive Brown Norway (B) males and females generating the SB F2 and BS F2 progeny. Hippocampus-dependent contextual fear memory and hippocampal expression of the thyroid hormone-regulated type 3 deiodinase, (*Dio3*) and neurogranin (*Nrgn*) were assessed. SS F1 PE-exposed females and their SB F2 progeny exhibited fear memory deficits. T4 administration to the mothers of F1 females reversed these deficits. Although SS F1 PE-exposed males also experienced fear memory deficit, this was neither transmitted to their BS F2 offspring nor reversed by prenatal T4 treatment. Hippocampal Dio3 and Nrgn expression showed similar pattern of changes. Grandmaternal ethanol consumption during pregnancy affects fear memory of the matrilineal second-generation progeny. Low dose T4 supplementation prevents this process likely *via* altering allele-specific and total expression of *Dio3* in the hippocampus.

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Introduction

The consumption of alcohol during pregnancy has been linked to a compendium of disabilities collectively referred to as fetal alcohol spectrum disorder (FASD) (Manning and Eugene Hoyme, 2007). These disabilities range from dysmorphology and mental retardation as seen in Fetal Alcohol Syndrome, to cognitive and behavioral deficits that characterize Alcohol-Related Neurodevelopmental Disorder (ARND). FASD affects approximately 2–5% of young children in the United States (May et al., 2014) and puts a significant strain on the health care system, while the prevalence of ARND is not really known, as it does not yet have a defined diagnostic category. Despite the high prevalence, we have limited knowledge to the mechanisms through which ethanol produces these effects. Even less is known about whether and how ethanol might affect future generations.

The deficits and severity of symptoms that arise as a result of prenatal ethanol (PE) exposure vary greatly from one individual to another, even when controlling for the time, and level of ethanol exposure (Guerri et al., 2009). Some of the potential causes of this variability are explored by looking at the genetic vulnerability of the ethanol-consuming mother or their offspring (Tunc-Ozcan et al., 2014). Additionally, vulnerability can be caused by intergenerational effects of ancestral ethanol exposure as environmental factors such as diet, stress, or exposure to teratogens (*i.e.* ethanol) cause a wide range of physiological and behavioral changes across multiple generations (Brasset and Chambeyron, 2013; Gluckman and Hanson, 2004; Govorko et al., 2012; Harper et al., 2014b; Miller et al., 2014). This form of intergenerational effect could result in behavioral or cognitive deficits in individuals not directly exposed to the teratogen. The mechanism of the intergenerational effect is not known, but is likely to be epigenetic in its nature, including changes in the allele-specific expression of imprinted genes across generations (Downing et al., 2011; Haycock, 2009; Mead and Sarkar, 2014; Ramsay, 2010; Sittig et al., 2011b; Tunc-Ozcan et al., 2014).

Hippocampal development is impaired in human FASD (Willoughby et al., 2008) that is paralleled by animal models (Gil-Mohapel et al., 2010; Gil-Mohapel et al., 2011). Consequently, some of the most debilitating effects of ARND are on hippocampus-based learning and memory (Dudek et al., 2014). Animal models employed by most laboratories model ARND, and PE-exposure in rats results in impaired hippocampus-dependent spatial learning and memory as measured

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by the Morris Water Maze (Sittig et al., 2011b; Wilcoxon et al., 2005) or a contextual fear conditioning paradigm (Weeber et al., 2001) that uses the entire experimental environment as the conditioned stimulus and requires the hippocampal formation (Kim et al., 1992; Maren et al., 1998; Phillips and LeDoux, 1992).

The cause of the hippocampus-specific vulnerability in FASD is not known. One of the possible mechanisms is related to PE-induced abnormal thyroid hormone levels during development (Scott et al., 1998; Wilcoxon and Redei, 2004), and the abundance of thyroid hormone receptors (TR) and thyroid hormone regulated genes in the hippocampus (Bastian et al., 2012; Bernal, 2007; Desouza et al., 2005). It is well known that thyroid hormone is essential for normal brain development (Heindel and Zoeller, 2003). Clinical or subclinical hypothyroidism of the mother negatively affects the neuropsychological development of the child (Haddow et al., 1999; Zoeller and Rovet, 2004), and experimental hypothyroidism in developing rats results in impaired learning (Taylor et al., 2014). Decreased serum TSH and thyroxine (T4) has been found in alcohol-consuming pregnant women (Herbstman et al., 2008), and in newborns exposed to alcohol *in utero* (Hernandez et al., 1992). Similar findings in animal models are reported with decreased peripheral free T4, fT3, and TSH in ethanol-consuming pregnant dams (Wilcoxon and Redei, 2004).

We have shown previously that supplementation with T4 during pregnancy can alleviate behavioral and cognitive deficits caused by PE exposure (Gottesfeld and Silverman, 1990; Tunc-Ozcan et al., 2013; Wilcoxon et al., 2005; Wilcoxon and Redei, 2004). One of the mechanisms by which abnormal thyroid homeostasis of the developing brain could result in long term cognitive deficit is alteration in the hippocampal expression of deiodinase 3 (*Dio3*) that metabolizes the biologically active thyroid hormone triiodothyronine (T3) into an inactive metabolite (Gereben et al., 2008). *Dio3* is a preferentially paternally imprinted gene that show allele-specific expression in the adult rat brain as well (Sittig et al., 2011a). PE exposure affects the allele-specific expression of *Dio3* in the hippocampus together with total expression changes (Sittig et al., 2011a; Sittig et al., 2011b). Another thyroid hormone-mediated gene is neurogranin (*Nrgn*), which encodes a neuron-specific postsynaptic protein and plays an important role in synaptic plasticity, learning, and memory (Miyakawa et al., 2001; Wilcoxon et al., 2007).

The work presented here continues the examination of the effects of PE on context-dependent fear memory, activity and anxiety in the first generation offspring directly exposed to alcohol (SS F1), as well as in the second-generation matrilinear (SB F2) and patrilinear (BS F2) progeny. We chose to generate these reciprocal crosses to measure allele-specific expression of the imprinted *Dio3* as we have done previously (Sittig et al., 2011b), but now in the second generation. The main goal of this study was to investigate the intergenerational consequences of PE and attempt to prevent them by simultaneous T4 administration with ethanol during *in utero* development of the SS F1 offspring.

Materials and methods

Animals

The Institutional Animal Care and Use Committee of Northwestern University have approved all animal procedures. Sprague-Dawley (S, Harlan—Indianapolis, IN) and Brown Norway (B, Charles River—Wilmington, MA) rats were housed in a climate-controlled environment with a 14:10 h light/dark cycle (lights on at 6 am) with water provided *ad libitum* throughout the duration of the study. We chose these strains with the knowledge that B is the most phylogenetically divergent inbred rat strain from all others, and S is the most commonly utilized outbred strain (Swerdlow et al., 2008) that has also been employed in all our previous studies (Sittig and Redei, 2010; Wilcoxon et al., 2005). The B and S genomes have been sequenced by the Rat Genome Project and Celera respectively, and therefore, allele-specific expression in imprinted genes can be explored in these crosses as we have done previously (Sittig et al., 2011b).

Female S rats were mated with male S rats and the day of finding sperm in vaginal smears was considered gestational day 1 (GD1). The dams were divided into 4 separate feeding groups: control (C), pair-fed (PF), ethanol (E), and ethanol + thyroxine (E + T4). Control dams received laboratory rat chow diet *ad libitum* throughout gestation while the remaining 3 groups received a liquid diet (Lieber-DeCarli '82; Bio-Serv. Frenchtown, NJ) beginning on GD4 and assigned diet was started at GD8. For E dams, the ethanol percentage in the diet was increased until a final concentration of 5% (w/v) from GD 8–10 and kept constant until G21. The E + T4 group received 0.3 mg/L-thyroxine (Sigma-Aldrich Co, St Louis, MO, USA) in the E-containing liquid diet, which, based on the daily diet consumption, is equivalent to approximately 8μg/100 gBW/day of T4 (Tunc-Ozcan et al., 2013). Each PF dam received liquid diet without E, the volume was matched to the amount of E diet consumed by an E dam. Since we found in our previous study that the low levels of T4 only reversed the PE-induced changes and normalized the thyroid homeostasis, no control groups with added T4 were employed (Tunc-Ozcan et al., 2013).

After GD21 all rats received standard laboratory chow *ad libitum* for the remainder of the study. Around postnatal day (PND) 70, separate cohorts of one-two male and female rats from each litter were used for open field test (OFT), fear conditioning (FC), or brain sample collection to avoid potential litter effects. The morphological parameters of the different groups have been described previously (Harper et al., 2014a).

Upon reaching adulthood (~PND 70), experimentally naive SS F1 male and female offspring of all treatment groups were mated with naive B female and male rats, respectively, thereby generating BS F2 and SB F2 progeny (maternal strain first). All F1 mating pairs received standard laboratory chow and water, *ad libitum*, throughout the experiment. All offspring were weaned at PND 24. Beginning at PND 70, F2 progeny were used either for behavioral testing or for hippocampal expression analyses as was done in the F1 generation.

Experimentally naive adult rat male and female offspring from all generations and treatment groups were sacrificed by decapitation between 10:00 and 12:00 h. Trunk blood was collected into EDTA-coated tubes on ice, and plasma was obtained by centrifugation. Whole hippocampus were immediately dissected and collected directly into RNAlater reagent (Ambion, Austin, TX) and stored at −80 °C.

Behavioral tests

Context dependent fear conditioning (FC) studies were performed on adult animals (PND 70–90), using an automated fear conditioning apparatus (TSE, Bad Homburg, Germany). On the first day of the test, rats were placed in the fear-conditioning chamber for 3 min to habituate to the novel environment. This period was followed by a series of three mild shocks (0.8 mA, 1 s each, 60 s between each shock) administered through an electrified floor grid. Twenty-four hours later the rats were placed in the same chamber for 3 min, and examined for contextual fear memory as measured by freezing duration, velocity, distance traveled and total locomotion through the use of an infrared beam system (detection rate 100 Hz). Any rats that did not respond to the initial shock were excluded from the study.

To test activity and anxiety, the open field test (OFT) was carried out on a separate cohort of adult rats (PND 70–90). The rats were placed in a circular arena (diameter 82 cm) surrounded by a 30 cm high wall and lit to a brightness of approximately 60 lx by indirect overhead lighting. The arena contained an inner concentric circle with a diameter of 50 cm designated as the inner zone. Rats were placed in the center of the arena and allowed to move freely for 10 min with the activity being recorded and tracked by TSE Videomot software (version 5.75, Bad Homburg, Germany). The software recorded and analyzed total distance traveled and time spent in the inner and outer areas of the arena. Between

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