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

Elif Tunc-Ozcan^a, Kathryn M. Harper^a, Evan N. Graf^a, and Eva E. Redei^{a,*}

^aThe Asher Center, Department of Psychiatry and Behavioral Sciences, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

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 (0.3mg/l diet) administration. Their offspring (SS F1) were mated with naïve 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.

Keywords

prenatal ethanol; fear conditioning; thyroxine administration; *Dio3*; neurogranin; free T3

*Corresponding author: Eva E. Redei, The Asher Center, Department of Psychiatry and Behavioral Sciences, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611., (312) 908-1791 (phone), (312) 503-0466 (fax), e-redei@northwestern.edu.

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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 dismorphology 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 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 hour light/dark cycle (lights on at 6am) 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 8ug/100gBW/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 naïve SS F1 male and female offspring of all treatment groups were mated with naïve 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 naïve 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 RNeasy lysis 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 minutes to habituate to the novel environment. This period was followed by a series of three mild shocks (0.8 mA, 1 sec each, 60 sec between each shock) administered through an electrified floor grid. Twenty-four hours later the rats were placed in the same chamber for 3 minutes, 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 100Hz). 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 lux 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 minutes 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 each test, the arena was cleaned with a 1.25% acetic acid solution to eliminate cues caused by odor.

Radioimmunoassay

Experimentally naïve rats from different prenatal diet groups were sacrificed by decapitation for tissue and blood collection. Plasma was collected from trunk blood was used for measurements of free T3 (fT3). fT3 levels were measured by RIA as described previously (Sittig and Redei, 2010). Assay was manufactured by MP Biomedicals, LLC (Irvine, CA, USA) and the assay sensitivity and coefficient of variation were 0.6 pg/ml and 8%, respectively.

RNA isolation and Quantitative RT-PCR

Total RNA extraction, reverse transcription and quantitative PCR (qPCR) were performed as described previously (Tunc-Ozcan et al., 2013). Briefly, RNA was isolated using Trizol reagent according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). Reverse transcription of 1 µg total RNA was performed by using the TaqMan Reverse Transcription kit (Applied Biosystems, Branchburg, NJ, USA). Real-time PCR was conducted with the ABI 7900 system using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were performed in triplicate and reached threshold amplification within 32 cycles. Levels of transcripts were determined relative to GAPDH and a general calibrator using the 2^{-C_t} method. Primers were designed using the ABI Primer Express 3.0 program (given sequences are 5' to 3') and were constructed to span an exon junction: *Dio3* (F:TGGCTCGAACTGGCAACTTT; R:ATTGCGCTTTGCTGAAATAGC), *Nrgn* (F:CCTGAACTACCAACCCAGCAT; R:ATCTTCTTCCTCGCCATGTG) and *Gapdh* (F:CAACTCCCTCAAGATTGTCAGCAA; R:GGCATGGACTGTGGTCATGA).

Pyrosequencing

To facilitate the identification of allelic *Dio3* expression in SB F2 and BS F2 hybrid offspring, we used the identified SNP in the *Dio3* exon between S and B strains (Sittig et al., 2011a). To measure the allelic ratio, PCR was conducted using forward primer and a biotinylated reverse primer flanking the SNP: F:ATCTGCGTATCCGACGACAAC, R:biotin-TCATGGGCCTGCTTGAAGAA. Purification of biotinylated PCR products and pyrosequencing were performed by EpigenDx, Inc. (Worcester, MA, USA) using a sequencing forward primer.

Statistical analyses

Data are presented as means $\pm$ SEM. The plasma fT3, total *Dio3* and *Nrgn* expression data was normalized to the C offspring value. Data were analyzed within generation first by 2-

way ANOVA (diet, sex), and subsequently subjected to hypothesis testing analyses using one-way ANOVA, or, in some cases, Student's t-test. When there was no significant effect of sex, or sex by diet interaction, male and female data were combined and analyzed by one-way ANOVA (diet). When there was either a sex effect or sex by diet interaction, male and female data were additionally analyzed, separately, by one-way ANOVA (diet). Bonferroni adjusted post hoc tests were used to identify differences between C, PF, E and E+T4 groups, with significance set at $P < 0.05$, as shown in the figures. Cohen's d effect sizes were calculated by using the online effect size calculator at <http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD30.php> for ANOVA as well as pair-wise comparisons.

Results

Behavior

Contextual fear memory of the SS F1 offspring exposed to PE was impaired as measured by decreased freeze duration upon re-exposure to the context on the second day of the FC test (diet: $F(3,81)=4.00$, $p < 0.05$, Cohen's $d=1.50$). Although there were no significant sex differences in this effect, we also analyzed male and female SS F1 offspring separately, to illustrate the intergenerational transmission by lineage. PE-exposed SS F1 male offspring showed contextual fear memory deficit with a significantly decreased freeze duration in the second day of the FC ($F(3,49)=3.39$, $p < 0.05$, $d=-0.60$) (Figure 1A). However, administration of T4 to the ethanol-consuming dam did not reverse this impairment in their SS F1 male offspring. These findings were mirrored by the distance traveled measure; PE-exposure resulted in a significant increase in distance traveled compared to controls, with no recovery as a result of T4 supplementation ($F(3,49)=10.73$, $p < 0.01$, $d=0.73$) (Figure 1A). Examining FC behavior of the SS F1 males' BS F2 progeny revealed no sex differences; therefore, male and female data were combined. There were no significant differences in freeze duration or distance traveled in the FC test between the BS F2 groups whose fathers were exposed to the different prenatal diets *in utero* (Figure 1B).

PE-exposed SS F1 females showed impaired contextual fear memory with a significantly decreased freeze duration ($F(3,35)=7.42$, $p < 0.01$, $d=-1.15$; PF vs E $t(15)=2.29$, $p < 0.05$, $d=1.11$) and increased distance travelled ($F(3,35)=6.02$, $p < 0.01$, $d=1.00$; PF vs E $t(15)=2.18$, $p < 0.05$, $d=1.06$), which were both reversed by T4 supplementation to the E-consuming mothers (Figure 2A). SB F2 progeny of the PE-exposed SS F1 females also exhibited significant decreases in freeze duration ($F(3,58)=4.19$, $p < 0.01$, $d=-0.53$) and increases in distance travelled ($F(3,58)=6.07$, $p < 0.01$, $d=0.51$) compared to controls; the latter effect remained to be reversed by T4 supplementation to their E-consuming grandmothers (Figure 2B). There were no sex differences within groups, therefore, male and female SB F2 data were combined.

Anxiety and activity levels were measured in the OFT to investigate whether they contributed to the fear memory deficit observed in the SSF1 and SB F2 offspring. Neither activity, nor anxiety level findings were commensurate with the fear-memory deficit. Total activity levels were not affected by prenatal diets (Supplemental Figures 1 and 2). PE increased anxiety levels of the SS F1 male offspring, but decreased it in the females, as measured by distance travelled in the inner area of the OFT (diet: $F(3,82)=4.15$, $p < 0.05$; sex:

$F(1,82)=7.17$, $p<0.01$; interaction: $F(3,82)=6.21$, $p<0.01$, $d=-0.46$). T4 supplementation reversed the PE effects in the SS F1 offspring. Since total activity was not altered by PE or T4 supplementation, the increased activity, and decreased freezing observed in the FC (Figures 1 and 2) represented a specific memory deficit induced by PE and reversed by T4 in the SS F1 offspring.

Thyroid hormone status and expression of thyroid hormone-regulated *Dio3* and *Nrgn*

There were no sex differences in the plasma free T3 (fT3) levels, in total expression of *Dio3* and *Nrgn* and allele-specific expressions of *Dio3* for both BS F2 and SB F2 progeny, therefore, male and female data were combined in the following analysis.

Plasma fT3 levels were significantly higher in PE-exposed SS F1 males and females compared to control offspring (diet: $F(3,48)=8.03$, $p<0.01$, $d=0.78$) (Figure 3A). This PE-induced increase was normalized by simultaneous administration of T4 to the E consuming mothers in both SS F1 males and females. BS F2 progeny of the SS F1 males did not show any difference in fT3 levels by diet (Figure 3B), while the SB F2 progeny of the SS F1 females showed elevated levels of fT3 in the E group that was reversed in E+T4 group ($F(3,80)=13.0$, $p<0.01$, $d=0.51$) (Figure 3C).

Both male and female SS F1 offspring of E consuming dams displayed significantly decreased transcript levels of *Nrgn* in the hippocampus compared to both C and PF offspring, which was increased by prenatal T4 supplementation (diet: $F(3,33)=8.52$, $p<0.01$, $d=2.84$) (Figure 4A). Similar to fT3, BS F2 progeny showed no prenatal diet-induced differences in *Nrgn* transcript levels (Figure 4B), while SB F2 progeny mimicked the SS F1 pattern with a significantly decreased *Nrgn* expression in E group that was reversed in the E +T4 group ($F(3,38)=18.31$, $p<0.01$, $d=-1.11$) (Figure 4C).

Hippocampal transcript levels of *Dio3* were significantly higher in PE-exposed SS F1 females compared to either controls, while there was only a trend of increased *Dio3* expression in PE-exposed SS F1 males (diet: $F(3,18)=6.23$, $p<0.01$, sex: $F(1,28)=5.02$, $p<0.05$, $d=2.14$) (Figure 5A). Maternal T4 administration reversed the PE-induced increase in the SS F1 females. BS F2 progeny showed no differences among diet groups in their hippocampal *Dio3* transcript levels (Figure 5B), while SB F2 progeny had increased total expression of hippocampal *Dio3* in the E group compared to both controls that was normalized in the E+T4 group ($F(3,40)=5.25$, $p<0.05$, $d=0.58$) (Figure 5C).

The *Dio3* gene is a partially imprinted gene. Allele-specific expression analyses by pyrosequencing revealed preferentially paternal *Dio3* expression in the hippocampus of the BS F2 progeny with no significant effect of grandmaternal diets (Figure 6A). Hippocampus of the SB F2 offspring of SS F1 females also exhibited preferential paternal expression in all groups except the E group, which had an inverted imprinting pattern such that the maternal *Dio3* allele predominated ($F(3,35)=3.54$, $p<0.05$, $d=-0.34$) (Figure 6B). Grandmaternal T4 administration during pregnancy concomitantly with E reversed this imprinting pattern.

Discussion

This study demonstrated for the first time that hippocampus-associated phenotypes that are affected by prenatal ethanol exposure are transferred to the next generation via matrilineal transmission. Furthermore, as T4 supplementation to the ethanol-consuming dam consistently reversed the affected phenotypes in the SS F1 E females, their offspring showed this normalization as well.

We focused on hippocampus-related phenotypes because this brain region is affected in individuals with FASD (Astley et al., 2009; Berman and Hannigan, 2000; Kodituwakku, 2007; Willoughby et al., 2008). Animals exposed to ethanol *in utero* exhibit specific alterations in hippocampal-dependent spatial learning and memory tests (Sutherland et al., 1997; Wilcoxon et al., 2005). Contextual fear conditioning is also hippocampus-dependent (Maren et al., 2013; Saxe et al., 2006), and it is known to be affected by PE (Weeber et al., 2001). In agreement with these findings, both male and female offspring of ethanol consuming dams showed deficit in fear memory as measured by decreased freezing and inversely increased activity in the fear-provoking context. Simultaneous administration of a low dose T4 to the ethanol-consuming dam did not reverse this deficit in the male SS F1 offspring, but restored fear memory in the PE-exposed SS F1 female to the level of controls. The sex difference in the T4 effect is not in agreement with the reversal of the elevated fT3 levels by maternal T4 administration in both males and females exposed to PE. The cause of this difference in T4 efficacy is not known, but similar sex difference has been observed in T4 efficacy previously (Rumbaugh et al., 1978).

The elevated fT3 levels in the SS F1 offspring of E dams were concomitant with decreased expression of hippocampal *Nrgn* and both were normalized by maternal T4 administration. Neurogranin (or RC3) is a neuron-specific protein that affects cognitive function and is thought to be directly regulated by T3 (Chaalal et al., 2014; Guadano-Ferraz et al., 1997; Huang et al., 2006). However, the decreased expression of hippocampal *Nrgn* in the light of increased plasma fT3 in response to PE suggests that the local thyroid hormone-milieu may differ from the peripheral one and/or it is affected by PE (Shukla et al., 2010; Sittig et al., 2011b). The local thyroid hormone availability is regulated by hippocampal *Dio3*, which metabolizes the biologically active T3 to the inactive T2. The PE-induced increase in the total expression of *Dio3* likely results in local hypothyroidism, as T3 and *Dio3* levels are closely and inversely related in the hippocampus (Sittig et al., 2011b). Should that be the case, local T3 levels would be independent of plasma fT3, similarly as we have shown it previously (Shukla et al., 2010). The increased hippocampal *Dio3* expression, therefore, could result in decreased *Nrgn* levels in the same brain region. The only caveat in this explanation is that SS F1 E females showed a dramatically greater increase in their hippocampal *Dio3* levels compared to their male counterparts, while their suppressed *Nrgn* expression was comparable. As *Dio3* expression is known to be induced by estrogen, the female-specific exaggerated increase of *Dio3* expression in the PE hippocampus is likely a synergistic effect between estrogen and the PE-induced elevated fT3 (Hernandez, 2005). Future studies could address this phenomenon.

The explanation for the decreased paternal allelic expression, but increased total *Dio3* expression in the SB F2 hippocampus is a complex one. *Dio3* expression is known to be positively regulated by thyroid hormones (Tu et al., 1999). However, the elevated total levels of hippocampal *Dio3* in the SB F2 offspring of PE-exposed mothers and the reversal of this elevation by grandmaternal T4 administration is the opposite of the expected effect if it is due to thyroid hormone levels. One potential mechanism by which total *Dio3* expression could increase while paternal contribution to *Dio3* is decreased is increased methylation of the intergenic differentially methylated region (IGDMR) on the maternal allele. This increased methylation could produce results similar to that of maternal deletion of the IGDMR, namely increased *Dio3* total expression (Lin et al., 2003). Alternatively, because total expression of *Dio3* was significantly higher, while the paternal contribution was significantly lower in the SB F2 E hippocampus, the total *Dio3* increase is very likely due to increased maternal expression. Since, in the control offspring, hippocampal expression of *Dio3* is about 60% paternal, maternal expression is mostly silenced. Thus, the increased maternal expression in the SB F2 E hippocampus might be the result of a lack of silencing of maternal *Dio3*. We could only speculate that this lack of silencing of the maternal allele is an unorthodox imprinting mechanism. One mechanism by which this could occur is the reduced expression of non-coding RNAs, such as *Rian* and *Mirg*, in the maternal allele of the SB F2 E progeny (see Figure 7). A somewhat similar phenomenon has been described, where maternal transmission of the *Gtl2LacZ* insertion “paternalized” the maternal allele leading to an increase in the maternal expression of *Dio3* (Charalambous et al., 2012).

Most described phenotypes showed a transmission to the next generation via the matrilineal lineage. The lineage specificity of this transmission could be assigned to maternal genetic- or behavior-specific effects, or to a differential influence of ethanol on female and male germ cells of the SS F1 fetus. Genetic differences between the S and the B mothers could result in the behavioral and hippocampal gene expression differences between the SB F2 and the BS F2 crosses. We found clear behavioral differences between the SB and BS F1 offspring of SS and BB mothers, respectively (Sittig et al., 2011a). These differences could be the results of strain differences in maternal behavior independent of PE effects. Although we and others found no differences in the maternal behavior of the ethanol consuming dams compared to controls (Marino et al., 2002; Matta and Elberger, 2007; McMurray et al., 2008), the increased anxiety and depression-like behaviors (Hellemans et al., 2010; Wilcoxon et al., 2005) of the female PE-exposed offspring could affect their maternal behavior towards their F2 progeny (Meaney, 2001; O'Connor et al., 2002).

When the dam is exposed to ethanol (or other toxicant), both the F1 embryo and the F2 generation germ cells are also directly exposed. Therefore, disease phenotypes in the F1 and F2 generations might still be due to the toxicology of direct exposure to the environmental factor, in this case ethanol. The sex difference in transmission, the observed matrilineal transmission, can occur via a direct effect of E on the SS F1 oocyte leading to a deficit in fear memory in their offspring. This possibility is supported by human studies investigating whether the growth of the fetus of a non-smoking mother Influenced by the smoking of either grandmother while pregnant (Miller et al., 2014). They found, together with three other studies (Hypponen et al., 2003; Misra et al., 2005), that maternal, but not paternal, prenatal exposure to cigarette affects their child's birth characteristics.

The matrilineal transmission could also be the result of differential vulnerability of the male and female germline to E, the sex differences in the establishment of imprinting genes contributing to the phenotypes, or the sex differences in the schedule of reprogramming the germ cells. The first two possibilities need further exploration. Assessing the vulnerability of the F3 generation to ancestral maternal alcohol consumption could in part clarify the validity of these possibilities. However, regarding the sex differences in the schedule of reprogramming the germ cells during embryonic development and gonadal sex determination, genome-wide demethylation does not have a significant time difference between male and female gametes, but remethylation follows sex specific timelines (Reik et al., 2001). Additional information would help to determine if these schedule differences interact with ethanol exposure to lead to the matrilineal transmission.

Many adverse prenatal conditions lead to intergenerational transmission of specific deficits. This has been shown for humans (Chamorro-Garcia and Blumberg, 2014; Veenendaal et al., 2013) and animals (Constantinof et al., 2015; Iqbal et al., 2012). Whether maternal alcohol consumption is specific in its effect on the next generation cannot be ascertained at this date. The matrilineal nature of the intergenerational transmission we described here suggests some level of specificity for alcohol. The fact that the effect sizes for many significant measures decreased from the SS F1 E offspring to the SB F2 E progeny imply the attenuation of these deficits to the second generation. Should this be the case in humans, without further alcohol consumption by the daughters with FASD, these deficits would eventually wash out throughout the generations. However, the increased rate of alcoholism in FASD (Nizhnikov et al., 2016) makes the probability high of this intergenerational transmission to persist into subsequent generations.

In conclusion, the present study signifies the effect of maternal prenatal exposure to ethanol on the cognitive performance of their offspring. If the alterations induced by the maternal prenatal exposure are immediately corrected, such that seems to be the case by the concomitant administration of T4 to the ethanol consuming dam, the offspring of these PE-exposed mothers are cognitively rehabilitated. Should these intergenerational consequences of PE exposure be present in human FASD, such as was shown for nicotine exposure, they would have major socioeconomic and health consequences for the future generations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Prenatal ethanol exposure affects fear memory of males and females
- Deficits from maternal prenatal ethanol exposure are transmitted to progeny
- Maternal T4 with ethanol lessens deficit in female offspring and their progeny

Figure 1. Maternal ethanol consumption leads to deficit in fear memory in male offspring that is not transmitted to their BS F2 progeny. No effect of prenatal T4 treatment in either generation Time (in seconds) spent freezing and distance (in centimeters) travelled in the second day of contextual fear conditioning paradigm by A) SS F1 males, and B) their BS F2 offspring (male and female data combined). Data represented as mean $\pm$ SEM. N=16-24/group. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA followed by Bonferroni post-hoc test.

Figure 2. Maternal ethanol consumption leads to deficit in fear memory in female offspring that is transmitted to their SB F2 progeny. Prenatal T4 treatment of the ethanol consuming mother eliminated the deficit in the SS F1 females and in their SB F2 offspring

Time (in seconds) spent freezing and distance (in centimeters) travelled in the second day of contextual fear conditioning paradigm by A) SS F1 females, and B) their SB F2 offspring (male and female data combined). Data presented as mean $\pm$ SEM. N=12-21/group.

* $p < 0.05$, ** $p < 0.01$ by oneway ANOVA followed by Bonferroni post-hoc test; ^ $p < 0.05$ by Student's t-test.

Figure 3. Prenatal ethanol exposure causes elevated plasma free T3 levels in both male and female SS F1 rats and the latter's SB F2 progeny, which is normalized by administration of T4 in the ethanol containing liquid diet

Plasma free T3 levels of A) SS F1 males and females, B) their BS F2 and C) SB F2 progeny (there were no sex differences, so male and female data were combined for the BS and SB F2 progeny). Free T3 was measured by radioimmunoassay. Data presented as mean $\pm$ SEM. N=12-25/group. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA followed by Bonferroni post-hoc test.

Figure 4. The decrease in hippocampal neurogranin (*Nrgn*) transcript levels of prenatal ethanol-exposed SS F1 rats and their SB F2 progeny is normalized by administration of T4 in the ethanol containing liquid diet to the mother

Nrgn transcript levels of A) SS F1 males and females, B) their BS F2 and C) SB F2 progeny (there were no sex differences, so male and female data were combined for BS and SB F2 progeny). Transcript levels were measured by quantitative real-time RT-PCR and normalized to *Gapdh* and a general calibrator using the 2^{-C_t} method. Data presented as mean $\pm$ SEM. N=6-14/group. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA followed by Bonferroni post-hoc test.

Figure 5. The increase in *Dio3* transcript levels in the hippocampus of prenatal ethanol-exposed SS F1 female rats and their SB F2 progeny is normalized by administration of T4 in the ethanol containing liquid diet

Dio3 transcript levels of A) SS F1 males and females, B) their BS F2 and C) SB F2 progeny (there were no sex differences, so male and female data were combined for the BS F2 and SB F2 progeny). Transcript levels were measured by quantitative real-time RT-PCR and normalized to *Gapdh* and a general calibrator using the 2^{-C_t} method. Data presented as mean $\pm$ SEM. N=6-13/group. *p<0.05, **p<0.01 by one-way ANOVA followed by Bonferroni post-hoc test.

Figure 6. Maternal prenatal ethanol exposure increases maternal expression of hippocampal *Dio3* in the SB F2 progeny that is reversed by the presence of concomitant T4
 Allele-specific expression of *Dio3* in the hippocampus of A) BS F2 and B) SB F2 progeny (there were no sex differences, so male and female data were combined). Data presented as mean $\pm$ SEM. N=5-12/group. * $p < 0.05$ by one-way ANOVA followed by Bonferroni post-hoc test.

Figure 7. Schematic representation of the ~1.01 Mb imprinted *Dlk1-Dio3* region on the rat chromosome 6:134-135 Mb

Genes, thought to be expressed from the paternally allele (*Dlk1*, *Rtl1* and *Dio3*), are marked by dark blue boxes and those expressed from the maternally allele (*Rian* and *Mirg*; *C/D snoRNA* and *miRNA* genes) by red boxes, respectively. Silent alleles are marked with light grey boxes. Differentially methylated regions are indicated by filled and open lollipops (methylated and unmethylated, respectively). The bigger lollipops indicate the location of intergenic differentially methylated region (IGDMR).