

ORIGINAL ARTICLE

Hippocampus-dependent memory and allele-specific gene expression in adult offspring of alcohol-consuming dams after neonatal treatment with thyroxine or metformin

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Fetal alcohol spectrum disorder (FASD), the result of fetal alcohol exposure (FAE), affects 2–11% of children worldwide, with no effective treatments. Hippocampus-based learning and memory deficits are key symptoms of FASD. Our previous studies show hypothyroxinemia and hyperglycemia of the alcohol-consuming pregnant rat, which likely affects fetal neurodevelopment. We administered vehicle, thyroxine (T4) or metformin to neonatal rats post FAE and rats were tested in the hippocampus-dependent contextual fear-conditioning paradigm in adulthood. Both T4 and metformin alleviated contextual fear memory deficit induced by FAE, and reversed the hippocampal expression changes in the thyroid hormone-inactivating enzyme, deiodinase-III (*Dio3*) and insulin-like growth factor 2 (*Igf2*), genes that are known to modulate memory processes. Neonatal T4 restored maternal allelic expressions of the imprinted *Dio3* and *Igf2* in the adult male hippocampus, while metformin restored FAE-caused changes in *Igf2* expression only. The decreased hippocampal expression of DNA methyltransferase 1 (*Dnmt1*) that maintains the imprinting of *Dio3* and *Igf2* during development was normalized by both treatments. Administering *Dnmt1* inhibitor to control neonates resulted in FAE-like deficits in fear memory and hippocampal allele-specific expression of *Igf2*, which were reversed by metformin. We propose that neonatal administration of T4 and metformin post FAE affect memory via elevating *Dnmt1* and consequently normalizing hippocampal *Dio3* and *Igf2* expressions in the adult offspring. The present results indicate that T4 and metformin, administered during the neonatal period that is equivalent to the third trimester of human pregnancy, are potential treatments for FASD and conceivably for other neurodevelopmental disorders with cognitive deficits.

Molecular Psychiatry advance online publication, 20 July 2017; doi:10.1038/mp.2017.129

INTRODUCTION

Despite efforts in awareness and prevention, 1 in 10 pregnant women still reports alcohol consumption.¹ As a result, fetal alcohol spectrum disorder (FASD) affects 2–11% of children worldwide, with increasing prevalence and presumably even more unreported and undiagnosed cases.² Despite its significance, there are still no validated biological treatments for FASD.^{3,4} Some preclinical studies suggest that choline administration is beneficial,^{5,6} while others found it without effect for FASD.⁷ Current treatments, such as stimulants, antidepressants, neuroleptics and anti-anxiety drugs, alleviate those FASD symptoms common to many psychiatric disorders but are not specific for FASD.⁸ Therefore, specific treatments are needed to prevent or reverse fetal alcohol-induced defects.

Hippocampal development is impaired in human FASD,⁹ consequently some of the most debilitating effects of FASD are on hippocampus-based learning and memory¹⁰ that is mirrored in animal models of fetal alcohol exposure (FAE).^{11,12} The cause of this cognitive vulnerability is not yet known, but one possible mechanism is via abnormal thyroid hormone levels during development of the alcohol-exposed fetus.^{13,14} Excessive alcohol consumption decreases thyroxine (T4) levels,^{15–17} and alcohol use during pregnancy has been reported with significant changes in thyroid function of neonates.^{18,19} Preclinical studies demonstrate

that maternal alcohol consumption during pregnancy interferes with thyroid hormone availability or function.^{11,13,14,20–22} Furthermore, clinical or subclinical hypothyroidism of the mother negatively affects neuropsychological development of the child,^{23,24} and experimental hypothyroidism in developing rats results in impaired learning.^{12,21}

Sufficient levels of thyroid hormones are essential for normal brain development, and fetus is dependent on maternal T4 before the adequate functioning of its own thyroid glands.²⁵ Maternal T4 reaching the fetal brain is being deiodinated to the biologically active form of thyroid hormone (triiodothyronine, T3) in the glia and then transported to the neuron.²⁶ In the neuron, right amount of T3 can regulate the transcription of thyroid hormone-dependent genes. Thus, ethanol-induced maternal hypothyroxinemia can limit the availability of T3 to the fetal brain and affect the regulation of neurodevelopmental genes.

Alternatively, even if necessary amount of T3 reaches the fetal neurons, it can be excessively metabolized by elevated levels of thyroid hormone-inactivating enzyme, deiodinase-III (*Dio3*) to its inactive form.²⁷ Indeed, increased hippocampal *Dio3* expression leads to decreased local T3 levels with subsequent changes in target gene transcription.²⁷ Administration of T4 during gestation normalizes the increased transcript levels of *Dio3* in the hippocampus of *in utero* ethanol-exposed adult offspring.²⁸ Therefore, administering T4 to the ethanol-consuming dams can be effective

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Received 9 January 2017; revised 8 May 2017; accepted 9 May 2017

via reversing the maternal hypothyroxinemia¹⁴ and/or by reducing the expression of *Dio3* in the fetal and subsequently the adult hippocampus. Both mechanisms can result in alleviation of FAE-caused hippocampus-based cognitive deficits of the adult offspring, as observed.^{11,21,28}

Abnormal thyroid function is often concomitant with glucose metabolic dysfunction.²⁹ Both the ethanol-consuming dams and their adult offspring are indeed hyperglycemic without any changes in their insulin levels.^{30,31} This phenomenon suggests insulin resistance, namely an increase in release of insulin from the pancreas is required to maintain normal plasma glucose levels. As insulin pathway genes, including insulin-like growth factor 2 (*Igf2*), are associated with hippocampus-based learning and memory processes,^{32–35} peripheral and central insulin resistance may also contribute to FAE-induced learning and memory deficits.³⁶ Decreased hippocampal levels of *Igf2* is detrimental to cognition.³⁷ Given that FAE leads to decreased *Igf2* expression during development,^{38,39} normalizing *Igf2* expression could reverse FAE-induced cognitive deficits.⁴⁰ Metformin, the most widely used insulin-sensitizing drug, is known to affect *Igf2* expression,⁴¹ provide neuroprotection against ethanol-induced neurodegeneration,⁴² enhance short-term memory⁴³ and spatial memory formation.⁴⁴ Thus, metformin is a logical choice to explore as a potential treatment to reverse FAE-induced memory deficits.

Both *Dio3* and *Igf2* are imprinted genes, known to be preferentially expressed from the paternal allele in the placenta.^{45,46} However, both of these genes show a preferential maternal expression in the adult hippocampus.^{12,47} The reason for this 'switch' from paternal to maternal expression is not known, but imprinting in general is regulated by differential DNA methylation of the maternal and paternal allele.⁴⁸ Because DNA methylation is altered by FAE,^{39,49–51} the FAE-engendered changes in allelic expression of *Dio3* (ref. 21) and *Igf2* (ref. 38) likely occur via modifications of DNA methylation in these imprinted loci.⁵² Methylation maintenance during development by the DNA methyltransferase 1 (*Dnmt1*)⁴⁸ and expression regulation by the enhancer-blocker CCCTC-binding factor (*Ctcf*)-binding sites are highly analogous in the *Dio3* and *Igf2* imprinted regions.⁵³ If FAE affects DNA methylation and imprinting processes of these genes similarly, the potential beneficial effects of neonatal T4 or metformin treatment could be via reversing the effects of FAE on these imprinted genes and their common regulators.

The primary objective of this study was to identify the effects of neonatal administration of T4 or metformin, respectively, on adult FAE offspring with the goal of finding potential treatment for FAE-induced cognitive deficits. The desirable time frame of treatment is the period post FAE, and in a neurodevelopmental stage that is equivalent to the third trimester of human pregnancy. Successful treatment at this period increases our study's translational value, as some women reduce alcohol consumption toward the end of pregnancy,⁵⁴ neurodevelopment can still be affected, and administration of treatments to the women is more feasible than treating the newborns. The secondary objective was to determine whether neonatal treatment with T4 or metformin would normalize hippocampal expressions of *Dio3* and *Igf2*. Therefore, we administered T4 and metformin to neonatal rats during postnatal days 1–10 after the maternal consumption of alcohol ceased, when the offspring have already been exposed to the detrimental effects of alcohol. Both male and female offspring were investigated, as sex differences in the effects of FAE have been reported both in human⁵⁵ and animal studies.^{56,57} We hypothesized that altered maternal thyroid and glucose functions by alcohol consumption generates an adverse intrauterine environment for the development of the offspring. We further hypothesized that these adverse effects can be reversed by neonatal T4 and metformin administration within a specific developmental window.

MATERIALS AND METHODS

Please see Supplementary Materials and Methods for a more detailed description of the methods.

Animals

All animal procedures were approved by the Northwestern University Animal Care and Use Committee. All rats were housed in controlled environment and received water *ad libitum*. For the *ex vivo* and *in vivo* treatment studies, maternal diets and animal procedures were performed as described previously.⁵⁸ Briefly, adult female Sprague–Dawley (S) rats (Harlan, Indianapolis, IN, USA) were mated with adult Brown Norway (B) males (Charles River, Wilmington, MA, USA). We chose to study the S by B (SB) offspring because of their vulnerability to FAE.^{12,27} This cross also allows studying allele-specific expression of imprinted genes. Pregnant females received control (C, *ad libitum* standard lab chow) or pair-fed (PF) and ethanol (E) liquid diets (Lieber-DeCarli '82; Bio-Serv, Frenchtown, NJ, USA) during gestation days (G) 8–21. The E diet contained 5% ethanol (w/v, 35% ethanol-derived calories). PF dams received the same amount of isocaloric liquid diet as the paired E dams. Regular laboratory chow was provided *ad libitum* to all pregnant rats on G21 and their offspring.

Neonates from each prenatal diet group (C, PF and E) and each litter received T4 (0.05 $\mu\text{g g}^{-1}$ per day; Sigma, St. Louis, MO, USA)⁵⁹ or metformin (200 $\mu\text{g g}^{-1}$ per day; Sigma),⁶⁰ or distilled water as vehicle by intraperitoneal injection in a volume of 10 $\mu\text{l g}^{-1}$ for 10 days, postnatal days 1–10. Two different cohorts of animals were used in the T4 and metformin studies with their own matched vehicle littermates. The two sets of vehicle cohorts were statistically the same and therefore combined throughout the study.

The thyroxin dose administered to neonates restored the attenuated thyroid-stimulating hormone levels in the adult FAE offspring without altering thyroid-stimulating hormone levels of the control animals (Supplementary Figure 1). The dose of metformin has been chosen from published data,⁶⁰ and the human equivalent of this dose is 2400 mg per 75 kg per day,⁶¹ which is within the recommended dose range.

In two additional studies, postnatal day 1 pups of C animals received vehicle, 5-aza-2'-deoxycytidine (5-Aza; 1 $\mu\text{g g}^{-1}$ per day; Sigma)⁶² or 5-Aza and metformin (5-Aza+Met; 1 and 200 $\mu\text{g g}^{-1}$ per day, respectively) by intraperitoneal injection for 10 days.

The number of animals in each experimental condition is provided in the figure legends.

Primary hippocampal culture

Primary hippocampal cultures were prepared from embryonic day 18 fetuses of dams on C, PF or E diets ($n=3$ dams per diet) as described previously.⁵⁸ Hippocampal neurons were plated at a density of 8×10^5 cells per 60 mm dish and cultured for 10 days. Metformin (final concentration of 0.4 mM in sterile water) was administered every 48 h.⁶³ All cultures were incubated at 37 °C with 5% CO₂ and processed for RNA isolation.

Behavioral testing

Context-dependent fear conditioning. One to two male and female adult offspring per litter were tested as described previously.²¹ Rats were placed into the automated fear-conditioning apparatus (TSE, Bad Homburg, Germany) for 3 min followed by three mild shocks (0.8 mA, 1 s each, 60 s between each shock). Twenty-four hours later, the animals were placed into the same chamber for 3 min without shock exposure, and their freezing behavior was measured. Rats that did not respond to the initial shock were excluded from the study. Two weeks later, adult offspring were killed between 10:00 and 12:00 h.

RNA isolation and quantitative real-time PCR

Whole hippocampi were dissected as described⁶⁴ and collected directly into RNAlater reagent (Ambion, Austin, TX, USA). RNA was isolated by using Direct-zol RNA MiniPrep (Zymo Research, Orange, CA, USA) and reverse transcription was performed using SuperScript VIL0 Master Mix (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was conducted as described previously⁵⁸ with 5 ng cDNA, specific primer pairs (Supplementary Table 1) and SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using the ABI 7900HT cyclor.

Pyrosequencing

We used the identified single-nucleotide polymorphisms in the *Dio3* exon¹² and in the 3'-untranslated region of *Igf2* (ref. 47) between S and B strains to track allele-specific expression. PCR was conducted using a forward primer and biotinylated reverse primer flanking the single-nucleotide polymorphisms. Purification of biotinylated PCR products and pyrosequencing were performed by EpigenDx (Hopkinton, MA, USA) using a sequencing forward primer.

Statistical analysis

Ex vivo data were analyzed by two-way analysis of variance (ANOVA; prenatal diet and *ex vivo* treatment). All *in vivo* data containing prenatal diet were analyzed by three-way ANOVA (prenatal diet, neonatal drug treatment and sex). Significant ANOVA results were followed by Bonferroni *post hoc* tests corrected for multiple comparisons. In the 5-Aza studies, data were analyzed by two-way ANOVA (neonatal drug treatment and sex). As there were no differences in sex effects, male and female data of the 5-Aza studies were combined and analyzed by unpaired two-tailed Student's *t*-test. All statistical analyses were performed using Systat (Chicago, IL, USA) and GraphPad Prism 7 (La Jolla, CA, USA) programs.

RESULTS

Please see Table 1 for the summary of the different neonatal treatments' effects on the measured consequences of FAE.

Neonatal T4 treatment restores FAE-induced hippocampal fear memory deficit and *Dio3* expression

FAE resulted in a fear memory deficit in male and female adults, measured as decreased freezing duration in the contextual fear-

conditioning test ($F(2,110) = 5.75$, $P < 0.01$, sex \times diet, $F(2,110) = 2.78$, not significant; Figure 1a). Neonatal T4 treatment, administered after FAE, alleviated this deficit without affecting the memory of C and PF offspring (diet \times drug, $F(2,110) = 3.53$, $P < 0.05$).

Expression of hippocampal *Dio3* was increased in the hippocampus of male and female E offspring (diet, $F(2,57) = 10.89$, $P < 0.01$; Figure 1b). Neonatal T4 administration reduced *Dio3* expression at large, even more so in the E offspring to the levels of T4-treated controls (drug, $F(1,57) = 117.37$, $P < 0.01$; diet \times drug, $F(2,57) = 4.92$, $P < 0.01$).

Body weights of all groups of animals at weaning and in adulthood are shown in Supplementary Tables 2 and 3.

Metformin affects FAE-induced gene expression changes in primary hippocampal culture

As metformin has never been used in FAE studies, its efficacy was evaluated first in our *ex vivo* primary culture model.⁵³ Metformin is known to affect insulin pathway genes and its effect on the FAE-induced changes of these genes were examined.⁵⁸ The *ex vivo* model consisted of our routine FAE ethanol administration *in vivo* and subsequent evaluation of gene expression after 10 days of metformin or vehicle treatment of the fetal hippocampal culture.⁵⁸ Among the previously validated insulin pathway genes, FAE effects persisted after 10 days in culture for transcript levels of insulin receptor (*Insr*; $F(2,33) = 3.74$, $P < 0.05$), *Igf2* ($F(2,35) = 7.07$, $P < 0.01$) and growth factor receptor bound protein 10 (*Grb10*; $F(2,37) = 3.58$, $P < 0.05$) (Figures 2a–c). Metformin treatment

Table 1. Summary of changes as a result of neonatal treatments

	Weaning weight		Adult weight		Fear memory		Allelic expression				Total expression							
							<i>Dio3</i>		<i>Igf2</i>		<i>Dio3</i>		<i>Igf2</i>		<i>Dnmt1</i>		<i>Ctcf</i>	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
FAE+T4	↔	↔	↔	↑	↑	↑	↑	NC	↑	NC	↓	↓	↑	↑	↑	↑	↔	↔
FAE+metformin	↑	↑	↑	↑	↑	↑	↔	NC	↑	NC	↓	↓	↑	↑	↑	↑	↓	↓
5-Aza+metformin	↑	↑	↑	↑	↑	↑	↔	↔	↑	NC	↓	↓	↑	↑	↑	↑	↓	↓

Abbreviations: FAE, fetal alcohol exposure; NC, no FAE-related changes. ↔, did not differ from FAE. ↓ or ↑, reversed by treatment.

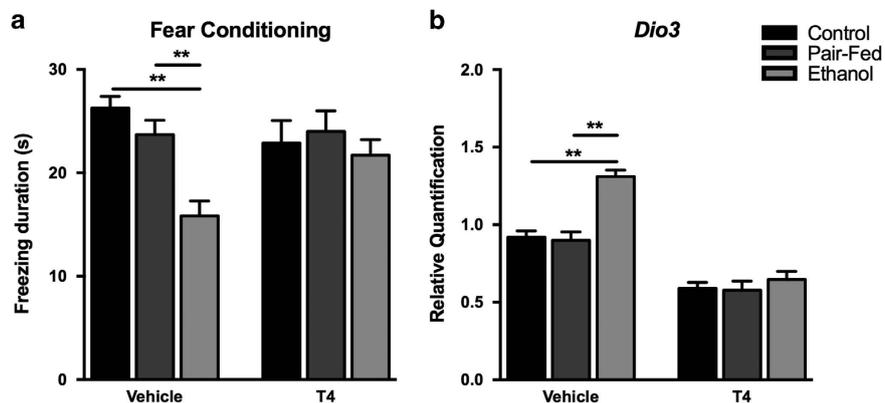


Figure 1. Hippocampal contextual fear memory deficit and increased *Dio3* expression in adult fetal alcohol exposure offspring are alleviated by neonatal T4 treatment. (a) Memory deficit was measured as decreased time (in seconds) spent freezing in the second day of contextual fear-conditioning test. Male and female neonates of dams on different diet from gestation day 8 to 21 received either T4 ($0.05 \mu\text{g g}^{-1}$ per day) or vehicle treatment between postnatal days 1 and 10, and were tested in adulthood ($N = 16\text{--}24$ per prenatal diet/neonatal treatment; sex combined). (b) Hippocampal transcript levels of *Dio3* were measured by quantitative real-time PCR. $N = 8\text{--}15$ per prenatal diet/neonatal treatment. Data represented as mean \pm s.e.m. $**P < 0.01$, Bonferroni *post hoc* test within neonatal treatments.

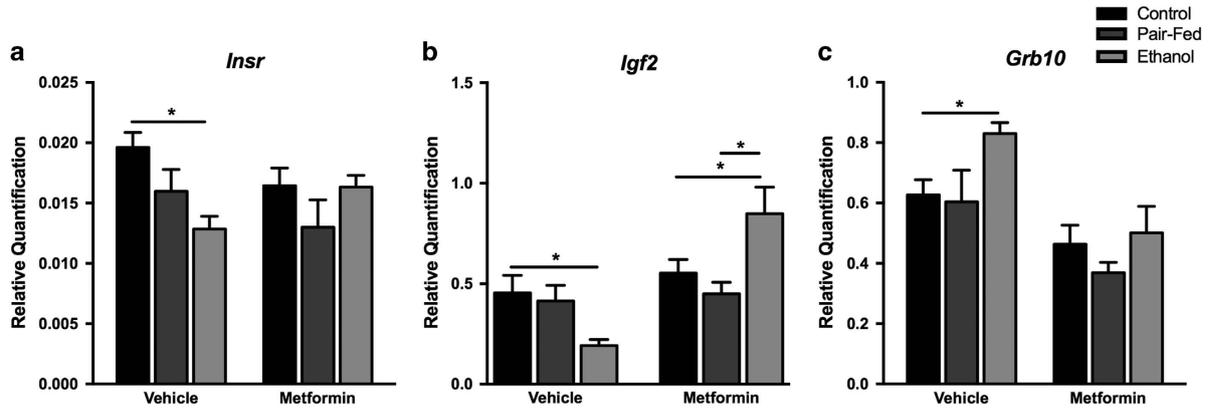


Figure 2. Administration of metformin reverses fetal alcohol exposure-induced changes in gene expression in the *ex vivo* primary hippocampal culture. Metformin (0.4 mM, every 48 h, for 10 days) was administered into primary hippocampal culture obtained from fetuses of the three prenatal diet groups (males and females combined). Transcript levels of (a) *Insr*, $N=5-9$ per prenatal diet/treatment, (b) *Igf2*, $N=6-8$ per prenatal diet/treatment and (c) *Grb10*, $N=6-9$ per prenatal diet/treatment. Details are as described in Figure 1. $*P < 0.05$, Bonferroni *post hoc* test within prenatal treatment groups after *in vitro* treatment.

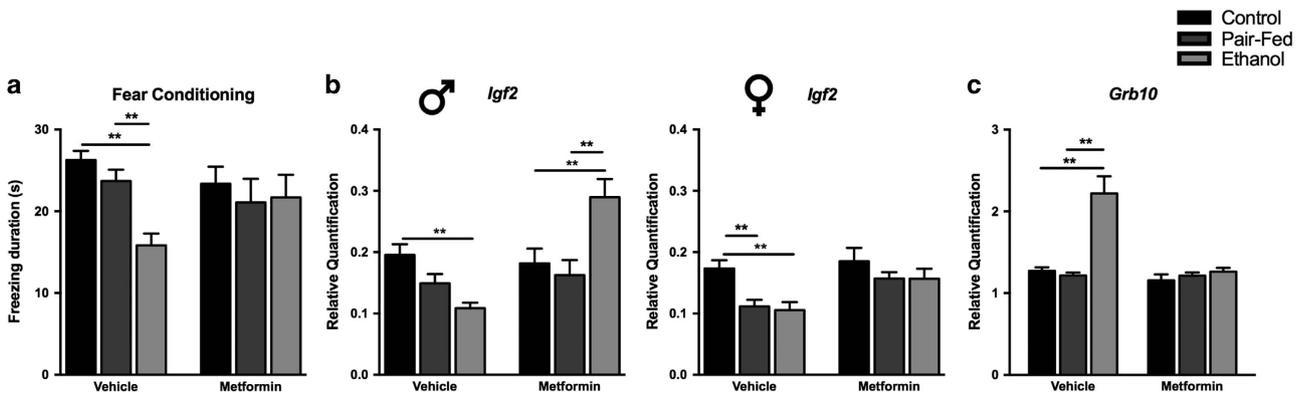


Figure 3. Neonatal metformin treatment reverses fetal alcohol exposure-induced fear memory deficit and hippocampal *Igf2* and *Grb10* expression in adult offspring. (a) Metformin administration ($200 \mu\text{g g}^{-1}$ per day, from postnatal day 1 to 10) reversed the fear memory deficit of adult ethanol offspring. $N=11-24$ per prenatal diet/neonatal treatment; sex combined. Transcript levels of (b) *Igf2*, male ($N=4-11$ per prenatal diet/neonatal treatment) and female ($N=5-8$ per prenatal diet/neonatal treatment), and (c) *Grb10* ($N=8-13$ per prenatal diet/neonatal treatment, sex combined) in the hippocampus of adult offspring. Details are as described in Figure 1. $**P < 0.01$, Bonferroni *post hoc* test within prenatal treatment groups.

reversed the FAE-induced decrease in the expression of *Insr* and *Igf2* (*Insr*: diet \times drug, $F(2,33)=3.08$, $P=0.05$; *Igf2*: drug $F(1,35)=16.5$, $P < 0.01$; diet \times drug $F(2,35)=9.33$, $P < 0.01$) (Figures 2a and b). Metformin also normalized the FAE-induced increase in *Grb10* levels (drug, $F(1,37)=19.76$, $P < 0.01$; Figure 2c).

Effects of neonatal metformin treatment on FAE-induced fear memory deficit and hippocampal *Igf2* expression

Neonatal metformin treatment tended to alleviate the FAE-induced fear memory deficit in both male and female adult offspring (diet, $F(2,93)=6.37$, $P < 0.01$; sex \times diet, $F(2,93)=1.09$, not significant; drug, $F(1,93)=3.56$, $P=0.06$; Figure 3a).

Hippocampal transcript levels of *Insr* were not different between the prenatal diet groups (data not shown). However, metformin not only reversed the FAE-induced decrease but also enhanced the expression of *Igf2* in the adult male hippocampus (diet, $F(2,71)=5.04$, $P < 0.01$; sex, $F(1,71)=10.41$, $P < 0.01$; drug, $F(1,71)=22.14$, $P < 0.01$; diet \times drug, $F(2,71)=11.32$, $P < 0.01$; sex \times diet \times drug, $F(2,71)=6.36$, $P < 0.01$). In females, the FAE- and PF-induced decrease in *Igf2* expression were normalized, but not enhanced by metformin (Figure 3b). Hippocampal *Grb10* transcript levels of E male and female adult offspring were normalized by

neonatal metformin (diet, $F(2,55)=9.78$, $P < 0.01$; sex, $F(1,55)=2.15$, not significant; drug, $F(1,55)=16.81$, $P < 0.01$; diet \times drug, $F(2,55)=7.63$, $P < 0.01$; Figure 3c).

Body weights of all groups of animals at weaning and in adulthood are shown in Supplementary Tables 4 and 5.

Treatment specificity in hippocampal allele-specific and total expression of *Dio3* and *Igf2*

Determining the parental contribution to the expression of the imprinted *Dio3* and *Igf2* genes was possible due to single-nucleotide polymorphisms previously identified between the S and the B strains.^{12,47} We were not able to track allele-specific expression for *Grb10* because there are no single-nucleotide polymorphisms between the S and B cDNAs.

Allele-specific expression of hippocampal *Dio3* was affected by FAE and neonatal T4 or metformin treatments in a sex- and treatment-specific manner. Decreased maternal allelic expression of hippocampal *Dio3* tended to be normalized by T4 in the adult FAE male (diet, $F(2,65)=4.07$, $P < 0.05$; drug, $F(1,65)=3.08$, $P=0.08$; diet \times sex \times drug, $F(2,65)=2.63$, $P=0.08$; Figure 4a). In contrast, the decreased maternal allelic expression of hippocampal *Dio3* was not normalized by metformin in the adult FAE male

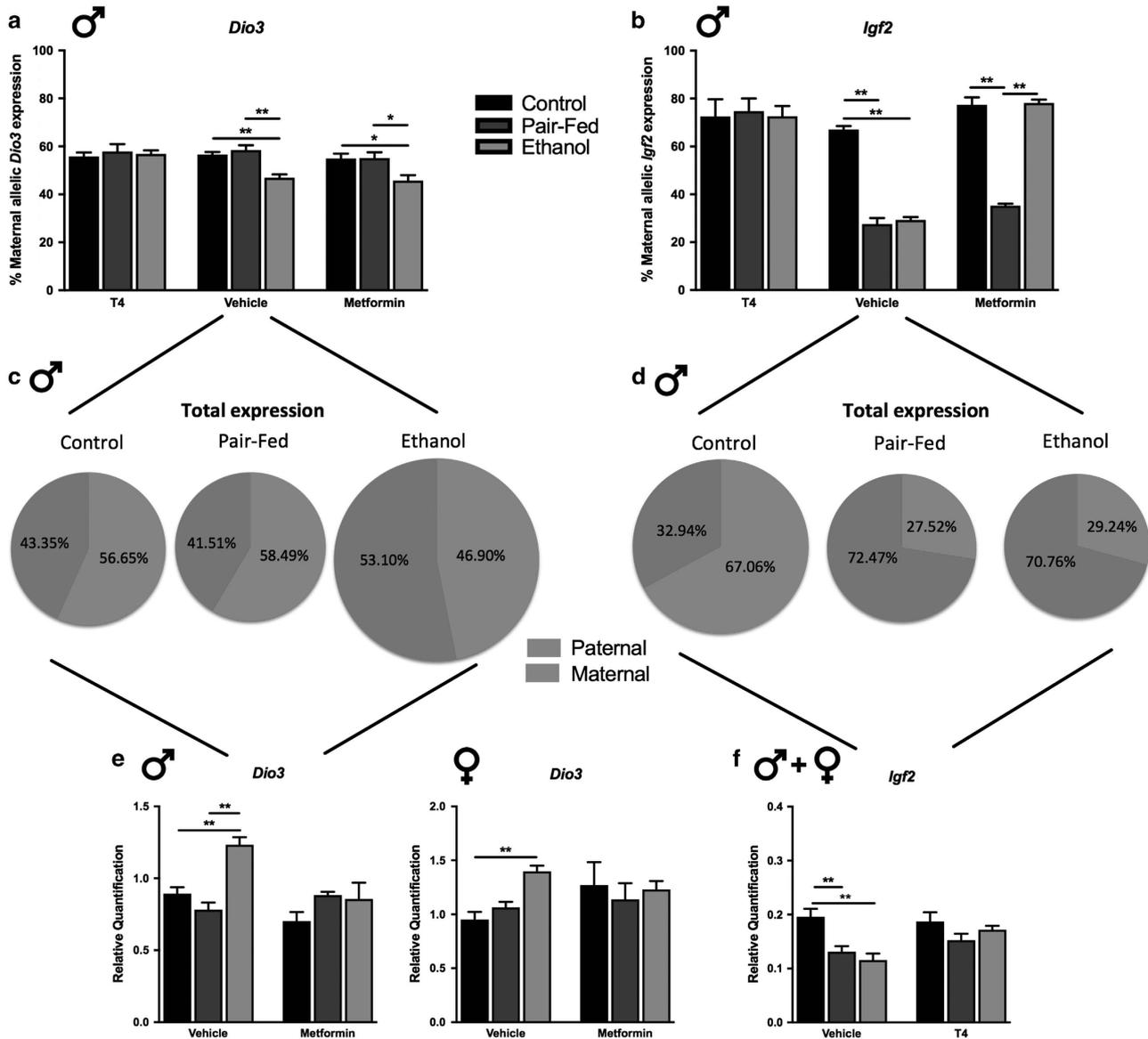


Figure 4. Changes in allele-specific and total expression of hippocampal *Dio3* and *Igf2* are sex- and treatment-dependent. Allele-specific expression in adult male hippocampus was determined by pyrosequencing using the already-verified single-nucleotide polymorphisms (a) *Dio3* ($N=4-10$ per prenatal diet/neonatal treatment) and (b) *Igf2* ($N=3-11$ per prenatal diet/neonatal treatment). Schematic representation of the maternal and the paternal allelic contributions to the total hippocampal expression of (c) *Dio3* and (d) *Igf2* (drawn not to the scale). (e) Effects of neonatal metformin treatment on fetal alcohol exposure-altered transcript levels of hippocampal *Dio3* in adult offspring ($N=4-8$ per sex/prenatal diet/neonatal treatment). (f) Total transcript levels of *Igf2* in the hippocampus of adult offspring in response to FAE and neonatal T4 treatment ($N=8-20$ per prenatal diet/neonatal treatment, sex combined). Details are as described in Figure 1. * $P < 0.05$, ** $P < 0.01$, Bonferroni *post hoc* test within prenatal treatments.

hippocampus (Figure 4a). There were no effects of FAE, T4 or metformin on *Dio3* expression in the females (Supplementary Figure 2a).

Allelic expression of hippocampal *Igf2* also showed sex differences in response to FAE and was affected by both treatments. Maternal contribution to *Igf2* expression was significantly lower in the adult PF and FAE male offspring compared to controls (diet, $F(2,69) = 23.76$, $P < 0.01$; sex \times diet, $F(2,69) = 49.40$, $P < 0.01$; Figure 4b). Both T4 and metformin treatment reversed the FAE-induced decrease (T4: drug, $F(1,67) = 133.68$, $P < 0.01$; sex \times drug, $F(1,67) = 4.85$, $P < 0.05$; metformin: drug, $F(1,69) = 32.44$, $P < 0.01$; sex \times drug, $F(1,69) = 30.45$, $P < 0.01$). However, only the neonatal T4 treatment restored the decrease in maternal allelic *Igf2* expression in the adult male PF hippocampus (Figure 4b). There

were no effects of FAE or treatments on *Igf2* expression in the females (Supplementary Figure 2b).

To interpret the parental dosage effects (allelic expression) in the light of total expression, a schematic representation of the maternal and the paternal allelic contributions to the total hippocampal expression of *Dio3* and *Igf2* is shown in Figures 4c and d, respectively (drawn not to the scale).

The effects of T4 and metformin on the FAE-induced changes in the hippocampal total expression of *Dio3* and *Igf2* have been shown on Figures 1b and 3b, respectively. If an overlapping mechanism is responsible for these treatment effects, T4 and metformin would also alter FAE-induced changes in *Igf2* and *Dio3*, respectively. Indeed, the FAE-induced increase in the total expression of *Dio3* was normalized by metformin in the

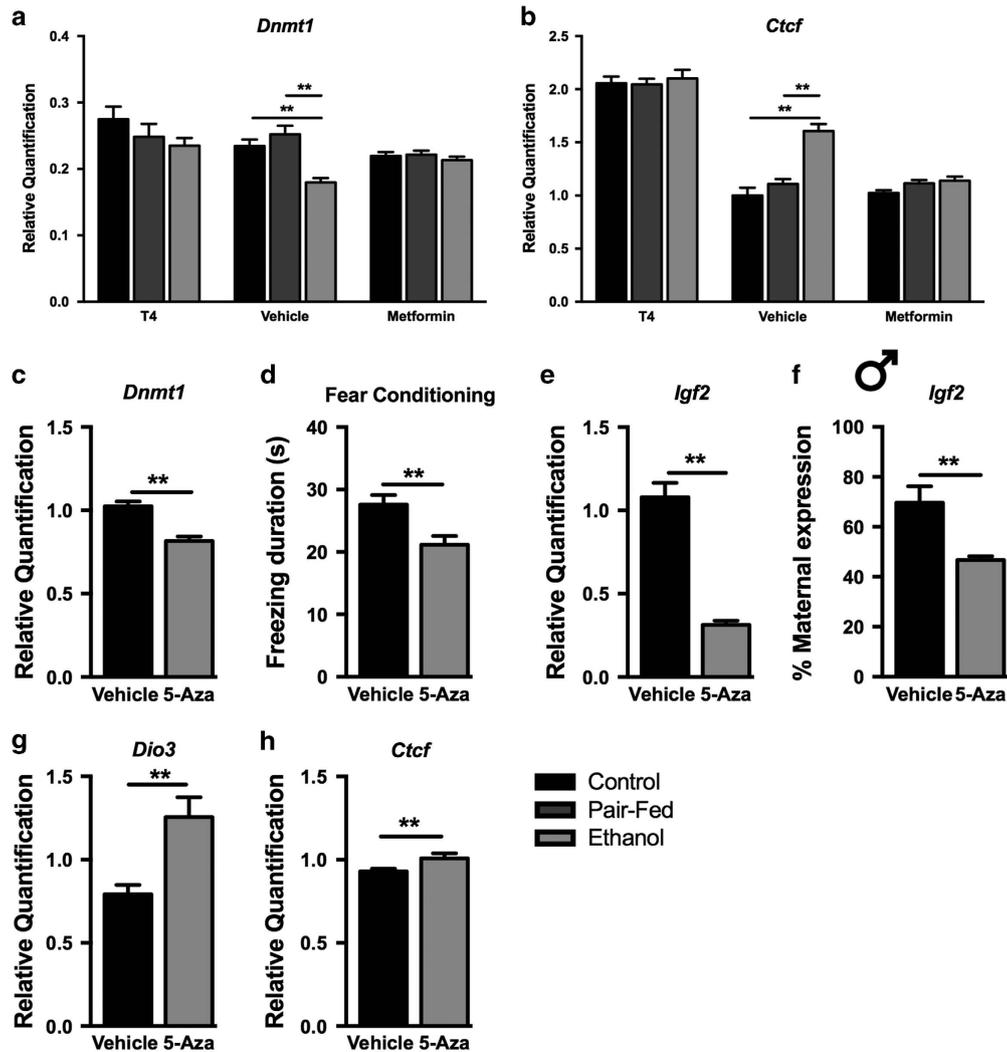


Figure 5. The role of *Dnmt1* in fetal alcohol exposure (FAE)-induced deficits. (a) Hippocampal *Dnmt1* expression was affected by FAE and alleviated by neonatal treatments ($N=7-20$ per prenatal diet/neonatal treatment, sex combined). (b) Treatment-specific changes in adult hippocampal *Ctcf* expression (sex combined T4: $N=9-10$ per prenatal diet/neonatal treatment; metformin: $N=7-13$ per prenatal diet/neonatal treatment). Data are normalized to vehicle control animals. (c-h) Administration of *Dnmt1* inhibitor 5-aza-2'-deoxycytidine (5-Aza, $1 \mu\text{g g}^{-1}$ per day, from postnatal day 1 to 10) to control neonates mimicked the effects of FAE in fear memory deficit and hippocampal gene expression changes in the adult offspring. $N=13-14$ per neonatal treatment, sex combined, except (f) $N=4-5$ males per neonatal treatment. Details are as described in Figure 1. $**P < 0.01$ between treatment groups.

hippocampus of adult male, while the correction by metformin was not complete in the female (sex, $F(1,62)=27.53$, $P < 0.01$; diet \times drug, $F(2,62)=4.32$, $P < 0.05$; sex \times drug, $F(1,62)=9.61$, $P < 0.01$; diet \times sex \times drug, $F(2,62)=3.00$, $P=0.05$; E vehicle vs E metformin $P < 0.01$; Figure 4e). Decreased transcript levels of *Igf2* in the E and PF hippocampus were restored to control levels by neonatal T4 (drug, $F(1,63)=9.20$, $P < 0.01$; diet \times drug, $F(2,63)=3.42$, $P < 0.05$) in both sexes equally (Figure 4f). These latter effects parallel those of T4 on *Dio3* expression (Figure 1b).

Dnmt1 as potential mediator of FAE effects

Dnmt1 is involved in learning and memory processes,⁶⁵ known to be affected by FAE⁶⁶ and it is the primary regulator of DNA methylation maintenance relevant to allele-specific imprinting processes.⁴⁸ Transcript levels of *Dnmt1* were decreased in the adult hippocampus by FAE (diet, $F(2,69)=15.21$, $P < 0.01$) and normalized by both treatments with no sex effects (T4: drug, $F(1,63)=13.22$, $P < 0.01$; diet \times drug, $F(2,63)=3.13$, $P < 0.05$;

metformin: diet \times drug, $F(2,69)=9.37$, $P < 0.01$; Figure 5a). *Ctcf*, one of the main regulator of allele-specific *Igf2* expression,⁶⁷ showed increased hippocampal expression after FAE (diet, $F(2,52)=20.92$, $P < 0.01$). Neonatal T4 did not reverse this effect, but rather it increased *Ctcf* expression equally in all prenatal treatment groups (T4: drug, $F(1,45)=197.39$, $P < 0.01$; diet \times drug, $F(2,45)=31.49$, $P < 0.01$; Figure 5b). In contrast, neonatal metformin reversed the FAE-induced increase in *Ctcf* expression (metformin: drug, $F(1,52)=4.35$, $P < 0.05$; diet \times drug, $F(2,52)=5.54$, $P < 0.01$; Figure 5b).

As a proof of concept for the role of *Dnmt1* in FAE-induced memory deficit, 5-Aza, a *Dnmt1* inhibitor that preferentially inhibits *Dnmt1* (refs 66,68) was administered to control neonates between postnatal days 1 and 10. As expected, 5-Aza treatment reduced *Dnmt1* expression in the adult hippocampus (drug, $F(1,25)=24.46$, $P < 0.01$; Figure 5c). Neonatal 5-Aza treatment mimicked the effects of FAE on fear memory in both male and female adults (drug, $F(1,25)=7.19$, $P < 0.05$; Figure 5d). Total and maternal allelic expression of hippocampal *Igf2* were decreased by 5-Aza, the

latter with a male-specificity similar to the FAE effects (allelic expression in males, $F(1,13)=12.59$, $P < 0.01$; total expression in males and females, $F(1,24)=72.26$, $P < 0.01$; Figures 5e and f). No effect of 5-Aza treatment has been seen in the allelic expression of *Igf2* in the female hippocampus, and in *Dio3* allelic expression in either sex (Supplementary Figures 3 and 4). However, total hippocampal expression of both *Dio3* and *Ctcf* were increased by the 5-Aza treatment, again confirming the similarity to FAE effects (*Dio3*, $F(1,24)=61.02$, $P < 0.01$; *Ctcf*, $F(1,25)=4.52$, $P < 0.05$; Figures 5g and h). Metformin administered together with 5-Aza eliminated all of the 5-Aza effects (Supplementary Figure 5).

Body weights at weaning and in adulthood are shown in Supplementary Table 6.

DISCUSSION

The major finding of the present study reveals the therapeutic potential of neonatal T4 and metformin treatments, administered after FAE, for reversing hippocampus-dependent cognitive deficits in adult offspring. The mechanism by which these treatments are effective is via restoring the FAE-induced decrease in hippocampal *Dnmt1* expression. Indeed, both FAE and direct inhibition of *Dnmt1* caused a memory deficit, while reversing the decreased *Dnmt1* expression by neonatal T4 and metformin treatments normalized fear memory. These treatments also restored FAE-induced changes in hippocampal total expression of *Dio3* and *Igf2*, two imprinted genes known to affect hippocampal memory processes. However, only the male hippocampus showed allelic expression changes of *Dio3* and *Igf2* post FAE, of which only *Igf2* were normalized by both of these treatments.

Dnmt1 is the major maintenance DNA methyltransferase that preserves DNA methylation patterns.⁴⁸ Recently, *Dnmt1* has been associated with the etiology of autism,⁶⁹ neurodegenerative diseases and other central nervous system disorders.⁶⁵ Decreased transcript levels of *Dnmt1* impairs learning and memory processes.^{70,71} Correspondingly, administering the *Dnmt* inhibitor 5-Aza impairs the formation of contextual fear memory.⁷² Our results are in agreement with these findings as both FAE and neonatal 5-Aza administration decreased hippocampal expression of *Dnmt1*, in parallel with contextual fear memory. Conversely, the FAE-induced deficits in *Dnmt1* expression and memory were both reversed by neonatal T4 and metformin treatments. Similarly, choline deficiency and supplementation modulate *Dnmt1* expression in parallel with learning and memory performance.^{73,74} The effect of choline on *Dnmt1* is relevant to this study, because preclinical studies suggest that choline administration is also beneficial for FAE-induced learning and memory deficits.^{5,6}

Dio3 and *Igf2* are two imprinted genes with allele-specific methylation patterns conserved by *Dnmt1*.⁴⁸ *Dnmt1* expression is inversely correlated with the expression of *Dio3* (ref. 75) and linearly with *Igf2*.⁷⁶ These patterns are confirmed in this study, where after FAE adult hippocampus showed increased *Dio3* and decreased *Igf2* expression concurrently with decreased *Dnmt1* expression. Treatments with T4 and metformin reversed these effects and also normalized fear memory. Indeed, decreasing hippocampal levels of *Dio3* and increasing that of *Igf2* are known to improve memory.^{21,28,77} As the FAE-induced deficit in *Dnmt1* expression is also normalized by the treatments, and *Dnmt1* is the major regulator of imprinting maintenance, the possible involvement of other imprinted genes in reversing the memory deficit after FAE still exist.

Sex specificity of the FAE effects and of the treatments was also observed in the allelic expression of *Dio3* and *Igf2* in the hippocampus, as neither FAE nor the treatments altered allelic expression of these genes in adult females. This sex-specific effect of FAE on the allelic expression of two imprinted genes with different imprinting regulators could be caused either by sex differences in the imprinting processes or by peripubertal

estrogen-induced compensatory mechanisms. Other developmental studies have shown sex-specific consequences of *in utero* treatments in the expression of *Dio3* (ref. 78) and male-specific FAE effects have also been observed for *Dio3*.^{12,28} Interestingly, structural sex differences have also been found in human FASD subjects, although the small sample size in that study precluded to identify sex differences in memory performance.¹⁰ Nevertheless, smaller size of anterior hippocampus is found in FASD males, but not females, compared to controls. It appears that in general males demonstrate greater *in utero* vulnerability.⁷⁹ FASD also shows higher prevalence in boys,⁵⁵ confirming to this general finding. Male-specific vulnerability may relate to the sequence of sex-specific timing of re-methylation and imprint establishment and the period of alcohol exposure. As a result, sex differences in the effect of FAE on allele-specific expression of imprinted genes could occur.

The sex-specific effects that emerge after puberty could be the consequence of estrogen-induced compensatory regulation of *Dio3* and *Igf2* expression,^{80,81} or the sex specificity of their epigenetic regulation during this period.^{82,83} The divergence of the total expression from the sex-specific allelic expression further suggests hormonal regulation of either the allele-specific or the total expression of *Dio3* and *Igf2*. Interestingly, opposite to the current findings, female specificity in the total expression of *H19*, a long noncoding RNA reciprocally imprinted with *Igf2*, has been found without sex differences in allelic expression.⁸⁴ As estrogen responsive elements are present in both the *Dio3* and *Igf2* imprinted regions (Supplementary Figure 6), estrogen-induced increases in maternal allelic expressions of these genes could compensate for the FAE effects in the female hippocampus.

Limitations of the current study include the timing of the treatments, although we have changed it from maternal²¹ to neonatal T4 administration to reverse the detrimental effects post FAE. Ideally, the time frame of treatment should be expanded all the way to puberty, as it is argued that neurodevelopmental changes still occur during puberty.⁸³ Furthermore, different alcohol exposure regimens and species could be investigated for determining the general efficacy of these treatments. These future explorations will provide further confirmation for the translational value of T4 and metformin in the treatment of FASD. Another interesting finding, although not a limitation, in this study is the pair-feeding effects on the hippocampal expression of *Igf2*. Recently described consequences of restricted feeding indicate major changes in adult hippocampal total and allele-specific expression of *Igf2* similarly to that found in the present study for pair-feeding.⁴⁷ The significance that metformin reversed the allele-specific expression changes caused by FAE, but not by pair-feeding, suggests that FAE effects are reversible (at least in this time frame), while those of PF are not. This latter suggestion is in agreement with the conclusions of the Dutch famine study,⁸⁵ where altered methylation of the *IGF2* imprinting control region and *IGF2* expression persist way into adulthood.

In the current study, both neonatal T4 and metformin administration mitigated FAE-induced hippocampal fear memory deficits, even when administered after the termination of *in utero* ethanol exposure. The convergent effect of neonatal T4 and metformin treatments in reversing this memory deficit in parallel with increasing hippocampal *Dnmt1* expression suggests methylation maintenance as a common mechanism of these treatments. As neonatal treatments restored hippocampal *Dio3* and *Igf2* expressions in addition to those of *Dnmt1*, it is possible that both of these treatments affect memory via elevating *Dnmt1* and consequently *Dio3* and *Igf2* expressions in the *in utero* ethanol-exposed adult offspring. We believe these results are the first to show that post-FAE T4 or metformin are potential therapeutic agents for FASD when administered in a time frame equivalent to the third trimester in human pregnancy. The translational value of this study is further increased by the fact that T4 and metformin

are commonly and safely used in pregnant women and, therefore, they could be explored in clinical trials for the treatment of FASD.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Sarah Chung, Wendy Luo, Kathryn M Harper, Jeanie K Meckes and Laura J Sittig for their contributions to this manuscript. This work was supported by NIH AA017978 to EER.

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