Supplemental Materials and Methods

Animals:

All animal procedures were approved by the Northwestern University Animal Care and Use Committee. All rats were housed in a temperature and humidity controlled environment with a 12h light cycle and had water *ad libitum*. For the *ex vivo* and *in vivo* treatment studies, maternal diets and animal procedures were performed as described previously.[1] Shortly, adult (70 days old) female Sprague-Dawley (S) rats (Harlan, Indianapolis, IN, USA) were mated with adult Brown Norway (B) males (Charles River, Wilmington, MA, USA) overnight. We chose to study the S by B (SB) offspring because of their vulnerability to FAE.[2, 3] This cross also allows studying allele-specific expression of imprinted genes. Pregnant females were assigned to one of three diet groups, control (C, *ad libitum* standard lab chow), pair-fed (PF) and ethanol (E). Pregnant rats in the PF and E groups received liquid diet (Lieber-DeCarli ’82; Bio-Serv. Frenchtown, NJ, USA) during gestation day (G) 8-21. The E diet contained 5% ethanol (w/v, 35% ethanol-derived calories). PF dams received an amount of isocaloric liquid diet that matched the paired E dam’s diet consumption the previous day. The regular laboratory chow was provided to all pregnant rats and their offspring *ad libitum* starting on G21*.* Offspring were weighed at weaning (PD24) and adulthood (PD60).

Neonates from each diet group (C, PF, and E) and each litter were randomly assigned to one of two neonatal treatment groups (metformin or T4 and distilled water as vehicle). Metformin and T4 studies were separate, in two different cohorts of animals with their own matched vehicle littermates. Neonates received metformin (200µg/gr/day) (Sigma, St. Louis, MO, USA),[4] T4 (0.05µg/gr/day) (Sigma, St. Louis, MO, USA)[5] and vehicle, respectively, by intraperitoneal injection in a volume of 10µl/gr for 10 days PD1-10. In two additional studies, PD1 pups of C animals were randomly assigned to receive 5-aza-2'-deoxycytidine (5-Aza; 1µg/gr/day) (Sigma, St. Louis, MO, USA)[6] or 5-Aza+metformin (200µg/gr/day) (5-

Aza+Met) with their respective vehicle littermates for 10 days PD1-10. All solutions were prepared immediately prior to use under sterile conditions. During treatments, all pups were separated from the dam for equivalent periods of time. Due to the nature of the experiments and automated TSE behavior systems, no blinding was done throughout the experiments.

Animal numbers in each experimental condition is provided in figure legends.

Primary hippocampal culture:

Dissociated hippocampal cultures were prepared from embryonic day 18 (E18) fetuses[1] of dams that received C, PF, or E diets (n=3 dams/diet) as described above. Pregnant dams were euthanized with CO_2 on E18 and fetal hippocampi were isolated. Individual fetal hippocampi dissected and cleaned of meninges. Hippocampal neurons were dissociated using trypsin (0.25% for 15min at 37°C), followed by trituration with a fire-polished Pasteur pipette. The cells were combined by litters and plated with minimum essential medium (MEM, Gibco-Life Technologies, Grand Island, NY, USA) with 10% horse serum (Gibco-Life Technologies, Grand Island, NY, USA) onto poly-l-lysine-coated (Sigma, St. Louis, MO, USA) dishes at the density of $8x10^5$ cells/60mm dish and cultured for 10 days (day in vitro (DIV) 10). Four hours after plating, the medium was changed to glia-conditioned MEM containing ovalbumin (0.1%), sodium pyruvate (0.1 mM) and N2 supplements.[7, 8] The first metformin and vehicle treatment was given at this time and continued until DIV10. Metformin was dissolved in double distilled water (ddWater) first and used in the culture at final concentration of 0.4mM (every 48h), which was chosen from literature[9] and did not cause cell death as seen by microscopy compared to concentration of 1.6mM in the pilot study (data not shown). ddWater was used as the vehicle. Drug solutions were prepared fresh in a sterile environment and added directly to the culture medium. All cultures were incubated at 37°C with 5% CO_2. At DIV10, all cultures were processed for RNA isolation.

RNA Isolation and Quantitative Real-Time PCR (qPCR):

RNA was isolated by using Direct-zol™ RNA MiniPrep (Zymo Research, Orange, CA, USA) according to manufacturer's protocol, as described previously[1] Subsequently, reverse transcription of 0.5µg total RNA was performed using SuperScript® VILO™ Master Mix (Invitrogen, Carlsbad, CA, USA). qPCR was conducted as described previously [1] with 5 ng cDNA, specific primer pairs and SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) using the ABI Prism 7900HT cycler. Relative quantification (RQ) was determined relative to *18S*, as an endogenous control and a general calibrator using the $2^{-\Delta\Delta Ct}$ method.

Supplemental Table 1: qPCR primer sequences.

Gene	Sequence 5' - 3'
Dio3	F: CTGTTCCCGCGCTTCCTA R: GTCCCTTGTGCGTAGTCGAG
Igf2	F: CCGTACTTCCGGACGACTTC R: CGTCCCGCGGACTGTCT
Insr	F: TCAATGGGCAGTTTGTGGAA R: GGTTGGGCAAACTTTCTGACA
Grb10	F: CAACCAAGAAGCCAACCAG R: TCCACGGATGAGTTAATATCGTT
Dnmt1	F: TCATCTAGTTCGGTGGCTACGA R: TTAGCGGGACCCTGAAGTG
Ctcf	F: TGGCAGAGCATTCAGAACAGTAAC R: TGTGAGGACGAGTACCTGTGTGT
Gapdh	F: CAACTCCCTCAAGATTGTCAGCAA R: GGCATGGACTGTGGTCATGA

Supplemental Results

Weaning and Adult body weights of offspring:

Neonatal T4 treatment:

At weaning (24 days old), male pups weighed more than females ($F(1,88)=4.47$, $p<0.05$) and FAE decreased body weight in both sexes ($F(2,88)=42.60$, $p<0.01$). Neonatal T4 treatment increased weight in general ($F(1,88)=54.97$, $p<0.01$), but did not reverse the FAE-induced decrease at this age (sex*diet*drug, $F(2,88)=2.81$, $p=0.06$) (Supplemental Table 2). Adult FAE females weighed less than controls (sex, $F(1,88)=2428.13$, $p<0.01$; diet, $F(2,88)=10.29$, $p<0.01$). Neonatal T4 abolished the weight difference of E females from their controls, but not of males (sex*diet*drug, $F(2,88)=7.95$, $p<0.01$). Maternal pair-feeding reduced body weight of adult male offspring only (sex*diet, $F(2,88)=4.14$, $p<0.05$) (Supplemental Table 3).

Neonatal metformin treatment:

Weaning weights of E pups were lower than controls' (diet, $F(2,56)=21.27$, $p<0.01$), but metformin reversed this effect (diet*drug, $F(2,56)=6.48$, $p<0.01$) (Supplemental Table 4). Adult weights reflected the same changes by prenatal diet and neonatal metformin treatment with the exception of an effect of pair-feeding (sex, $F(1,54)=2216.16$, $p<0.01$; diet, $F(2,54)=19.73$, $p<0.01$). Specifically, while FAE-induced lower body weights were normalized by metformin treatment, those of PF remained lower (Supplemental Table 5).

Neonatal 5-aza-2'-deoxycytidine (5-Aza) treatment:

The Dnmt inhibitor, 5-Aza, administered to control neonates between PD 1 -10 reduced weaning weight with no significant sex effect (drug: $F(1,31)=94.01$, $p<0.01$). This effect was eliminated by the simultaneous administration of metformin to the 5-Aza-treated neonates (Supplemental Table 6). Adult weights of 5-Aza-treated animals remained lower, which were again reversed after simultaneous administration of metformin (drug: $F(1,28)=55.14$, $p<0.01$; sex, $F(1,28)=1331.00$, $p<0.01$) (Supplemental Table 6).

Supplemental Table 2: Weaning body weights of offspring in different prenatal diet and neonatal T4 treatment groups.

	Male			Female		
	Control	Pair-Fed	Ethanol	Control	Pair-Fed	Ethanol
Vehicle	45.9 ± 1.6	43.7 ± 2.0	34.9 ± 1.5**$^{\#\#}$	39.2 ± 1.7	40.6 ± 1.0	36.1 ± 1.4$^{\#}$
T4	50.1 ± 0.7	47.0 ± 1.3	43.3 ± 1.2**$^{\$\$}$	47.1 ± 0.6$^{\$\$}$	49.0 ± 0.8$^{\$\$}$	40.2 ± 0.7**$^{\#\#\$}$

* p<0.05, ** p<0.01 compared to C; $^{\#}$ p<0.05, $^{\#\#}$ p<0.01 compared to PF. $^{\$}$ p<0.05, $^{\$\$}$ p<0.01 compared to Vehicle. Data is represented as mean ± SEM. N=6-10/group

Supplemental Table 3: Adult body weights of offspring in different prenatal diet and neonatal T4 treatment groups.

	Male			Female		
	Control	Pair-Fed	Ethanol	Control	Pair-Fed	Ethanol
Vehicle	299.4 ± 2.7	268.0 ± 3.5**	288.2 ± 3.5$^{\#\#}$	197.2 ± 3.5	194.9 ± 4.8	182.7 ± 3.1**$^{\#}$
T4	297.2 ± 3.5	289.1 ± 2.4$^{\$\$}$	284.1 ± 2.0*	186.8 ± 3.5	180.0 ± 1.5$^{\$\$}$	181.3 ± 1.6

* p<0.05, ** p<0.01 compared to C; $^{\#}$ p<0.05, $^{\#\#}$ p<0.01 compared to PF; $^{\$\$}$ p<0.01 compared to Vehicle. Data is represented as mean ± SEM. N=6-10/group

Supplemental Table 4: Weaning body weights of offspring in different prenatal diet and neonatal metformin treatment groups.

	Male			Female		
	Control	Pair-Fed	Ethanol	Control	Pair-Fed	Ethanol
Vehicle	42.0 ± 1.9	46.6 ± 1.6	36.2 ± 1.1*$^{\#\#}$	44.0 ± 1.8	43.8 ± 1.7	32.4 ± 0.6**$^{\#\#}$
Metformin	43.3 ± 2.4	45.0 ± 1.4	40.8 ± 1.7	41.4 ± 0.9	42.3 ± 1.0	39.0 ± 0.5$^{\$\$}$

* p<0.05, ** p<0.01 compared to C or vehicle; # p<0.05, ## p<0.01 compared to PF. $^{\$\$}$ p<0.01 compared to Vehicle. Data is represented as mean ± SEM. N=4-8/group

Supplemental Table 5: Adult body weights of offspring in different prenatal diet and neonatal metformin treatment groups.

	Male			Female		
	Control	**Pair-Fed**	**Ethanol**	**Control**	**Pair-Fed**	**Ethanol**
Vehicle	296.2 ± 1.6	272.5 ± 2.2**	290.8 ± 1.0**[#]	190.6 ± 2.3	177.8 ± 3.0**	178.2 ± 1.2**
Metformin	297.3 ± 3.7	281.0 ± 5.3*	292.5 ± 4.3	191.8 ±1.1	175.6 ± 3.1*	185.9 ± 3.3

* p<0.05, ** p<0.01 compared to C or vehicle; # p<0.05 compared to PF.
Data is represented as mean ± SEM. N=4-8/group

Supplemental Table 6: Weaning and adult body weights of offspring in Control group after vehicle and 5-Aza or 5-Aza with metformin (5-Aza+Met) treatments.

WEANING	**Control**	
	Male	**Female**
Vehicle (5-Aza)	42.2 ± 1.5	45.2 ± 1.3
5-Aza	33.4 ± 0.8**	32.6 ± 0.8**
Vehicle (5-Aza+Met)	44.0 ± 2.6	45.4 ± 1.9
5-Aza+Met	42.7 ± 1.7	42.0 ± 1.5

** p<0.01 compared to vehicle
Data is represented as mean ± SEM. N=6-11/group

ADULT	**Control**	
	Male	**Female**
Vehicle (5-Aza)	295.8 ± 1.9	185.4 ± 3.4
5-Aza	271.2 ± 3.7**	166.3 ± 2.6**
Vehicle (5-Aza+Met)	302.8 ± 4.8	192.3 ± 2.1
5-Aza+Met	296.8 ± 4.0	193.2 ± 1.9

** p<0.01 compared to vehicle.
Data is represented as mean ± SEM. N=6-9/group

Supplemental Figure Legends

Supplemental Figure 1. Thyroid function of adult offspring in different prenatal diet groups after neonatal T4 treatment. Plasma thyroid stimulating hormone (TSH) levels of neonatal vehicle or T4-treated male (N= 4-6/diet/treatment group) and female (N= 4-7/diet/treatment group) offspring of dams receiving different prenatal diets. *$p < 0.05$ Bonferroni post hoc test. Data are represented as means ± SEM.

Supplemental Figure 2. FAE or neonatal drug treatments have no effects on hippocampal-allele-specific expression of *Dio3* and *Igf2* in adult female offspring. Allele-specific expression of hippocampal **a)** *Dio3* (N=4-10/diet/treatment group) and **b)** *Igf2* (N=3-11/diet/treatment group) in the adult female offspring was determined by PCR, using forward primer and a biotinylated reverse primer flanking the already verified SNPs. Data represented as mean ± SEM.

Supplemental Figure 3. Neonatal administration of DNA methyltransferase inhibitor has no effect on hippocampal-allele specific expression of *Igf2* in adult female offspring. N=4-6/group. Details are same as Supplemental Figure 1.

Supplemental Figure 4. Neonatal administration of DNA methyltransferase inhibitor has no effect on hippocampal-allele specific expression of *Dio3* in adult offspring of either sex. N=4-6/group. Data represented as mean ± SEM.

Supplemental Figure 5. Neonatal metformin treatment concomitant with 5-Aza alleviates the *Dnmt1* inhibitor-induced fear memory deficits and gene expression changes in adulthood. (a-

f) Neonatal 5-aza-2'-deoxycytidine (5-Aza)-induced fear memory deficit and gene expression changes were reversed by concomitant neonatal metformin treatment with 5-Aza (5-Aza+Met). Data represented as mean ± SEM. Number of subjects: (**a-f)** Vehicle=13, Aza+Met=15, except **d)** N=4 and 5, respectively.

Supplemental Figure 6. Schematic representation of the *Dio3* and *Igf2* imprinted gene clusters. The *Dlk1-Dio3* imprinted region gives rise to paternally expressed *Dlk1*, *Rtl1* and *Dio3* genes and multiple maternally expressed non-coding RNAs, as miRNAs and snoRNAs. The imprinted control region (ICR) is intergenic and active on the maternal allele. The *Igf2-H19* imprinted region includes the paternally expressed *Igf2* (not in the hippocampus) and maternally expressed *H19* genes. The active maternal ICR is intergenic, where CCCTC-binding factor (CTCF) binds and forms an insulator to block the enhancer access to *Igf2*, thus silencing the *Igf2* expression. The clusters and genes are not drawn to scale.

Supplemental References

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