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Modeling Fetal Alcohol Spectrum Disorder: validating an *ex vivo* primary hippocampal cell culture system

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Abstract

Background—Fetal Alcohol Spectrum Disorder (FASD) is the leading non-genetic cause of mental retardation. There are no treatments for FASD to date. Preclinical *in vivo* and *in vitro* studies could help in identifying novel drug targets as for other diseases. Here, we describe an *ex vivo* model that combines the physiological advantages of prenatal ethanol (E) exposure *in vivo* with the uniformity of primary fetal hippocampal culture to characterize the effects of prenatal E. The insulin signaling pathways are known to be involved in hippocampal functions. Therefore, we compared the expression of insulin signaling pathway genes between fetal hippocampi (*in vivo*) and primary hippocampal culture (*ex vivo*). The similarity of prenatal E effects in these two paradigms would deem the *ex vivo* culture acceptable to screen possible treatments for FASD.

Methods—Pregnant Sprague-Dawley rats received one of three diets: *ad libitum* standard lab chow (control-C), isocaloric pair-fed (PF, nutritional control), and E containing liquid diets from gestational day (GD) 8. Fetal male and female hippocampi were collected either on GD21 (*in vivo*) or on GD18 for primary culture (*ex vivo*). Transcript levels of *Igf2*, *Igf2r*, *Insr*, *Grb10*, *Rasgrf1* and *Zac1* were measured by RT-qPCR.

Results—Hippocampal transcript levels differed by prenatal treatment in both males and females with sex differences observed in the expression of *Igf2* and *Insr*. The effect of prenatal E on the hippocampal expression of the insulin pathway genes was parallel in the *in vivo* and the *ex vivo* conditions.

Conclusions—The similarity of gene expression changes in response to prenatal E between the *in vivo* and the *ex vivo* conditions ascertain that these effects are already set in the fetal hippocampus at GD18. This strengthens the feasibility of the *ex vivo* primary hippocampal culture as a tool to test and screen candidate drug targets for FASD.

Keywords

Prenatal ethanol; primary culture; hippocampus; fetal; insulin pathway genes

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Introduction

Fetal Alcohol Spectrum Disorder (FASD) is manifested in a variety of behavioral, cognitive, and physical abnormalities in the offspring of alcohol consuming pregnant mothers. In the United States, FASD may affect as many as two to five percent of school age children (May et al., 2009). This makes FASD the leading non-genetic cause of mental retardation in the United States (Mizejewski, 2010). Despite educational efforts, 7.6% of pregnant women still consume alcohol (Centers for Disease Control and Prevention, 2012) and there are no treatments for FASD to date; therefore, treatments are needed to alleviate or reverse the consequences of FASD.

Animal models provide a simple and reliable method to study the effects of ethanol on the developing brain and to screen potential therapeutics (Patten et al., 2014). Primary neuronal cultures are shown to be useful to study the neurodevelopmental effects of prenatal ethanol (E) exposure (Yanni and Lindsley, 2000). Direct *in vitro* administration of E can capture the teratogenic toxic effects (Yanni and Lindsley, 2000), but it lacks the ability to recapitulate physiological and indirect consequences of gestational E exposure. This drawback of the *in vitro* FASD models is resolved in a recent *ex vivo* study, where E administration occurs *in vivo* and the evaluation of gene expression occurs *ex vivo* (Tyler and Allan, 2014). The authors show that moderate prenatal E exposure alters the expression of several genes involved in neurogenesis.

In the present study, we used a similar approach by combining our established animal model of prenatal E exposure with culturing the fetal hippocampus to assess expression of genes related to learning and memory deficits. The advantage of this *ex vivo* model is that E administration occurs *in vivo* via the pregnant mother, just like in human FASD, so that it recapitulates aspects of second trimester gestational E exposure. The subsequent primary hippocampal culture makes it possible to screen drug targets aiming to reverse molecular deficits that might represent potential treatments for hippocampal-based cognitive deficits caused by prenatal E exposure.

We targeted the hippocampus for our studies, because functional and morphological changes of the hippocampus exposed to E by maternal drinking are well known (Willoughby et al., 2008, Gil-Mohapel et al., 2010). Additionally, impaired hippocampus-dependent spatial and social learning/memory is shown both in animal models of FASD (Wilcoxon et al., 2005, Sittig et al., 2011b, Weeber et al., 2001, Tunc-Ozcan et al., 2013) and FASD patients (Hamilton et al., 2003). It appears likely that many of the cognitive and behavioral deficits of FASD may involve the compromised neurodevelopment of the hippocampus.

Since E acts on and beyond the plasma membrane without having a predominant specific receptor-binding site, prenatal E exposure can affect transcriptional regulation of different signaling pathways (Thibault et al., 2005). One of these pathways is the insulin and insulin-like growth factor signaling pathway that is involved in learning and memory processes of the hippocampus (Zhao et al., 2004, McNay and Recknagel, 2011). Prenatal E leads to insulin resistance in rat neonates (Chen et al., 2004) as well as adults (Yao and Nyomba,

2008). Additionally, prenatal E-caused alterations of the insulin pathway during early neurodevelopment persist into adulthood in the brain (de la Monte et al., 2005). Furthermore, delivery of insulin directly into hippocampus enhances learning and memory in rats, while blockade of endogenous hippocampal insulin markedly impairs spatial learning and memory (McNay and Recknagel, 2011). Several genes in the insulin pathway are known to be involved in learning and memory; such as the insulin receptor (*Insr*) (Zhao et al., 2004), the insulin-like growth factor 2 (*Igf2*) (Chen et al., 2011), the Igf2 receptor (*Igf2r*) (Chen et al., 2011, Lee et al., 2015), the ras-guanine nucleotide releasing factor 1 (*Rasgrf1*) (Fernandez-Medarde et al., 2007) and the growth factor receptor-bound protein 10 (*Grb10*) (Ma et al., 2013). Interestingly, the pleomorphic adenoma gene-like 1 (*Plagl1/Zac1*) gene regulates a gene network involving *Igf2*, *Igf2r*, *Grb10* and *Rasgrf1* (Varrault et al., 2006, Hoffmann and Spengler, 2012, Charalambous et al., 2007). *Zac1* also plays a major role in the coordinated expression of these genes (Charalambous et al., 2007). Overall, prenatal E exposure disrupts regulation of insulin homeostasis, which may directly or indirectly lead to hippocampus-based cognitive deficit of the offspring.

Here we compared the effect of prenatal E exposure on the expression of insulin pathway genes between the gestational day (GD) 21 fetal hippocampus and the *ex vivo* primary hippocampal culture established on GD18 and cultured for three days. The similarity of prenatal E effects between the *in vivo* and the *ex vivo* models allows us to use the *ex vivo* model as a practical tool for screening potential treatments for alleviating the molecular effects of prenatal E in the future.

Methods

Animals

All animal procedures were approved by the Northwestern University Animal Care and Use Committee. Maternal diet and animal procedures were performed as described previously (Tunc-Ozcan et al., 2013, Sittig et al., 2011b). We chose to generate a Sprague-Dawley (SD) and Brown Norway (BN) cross, because their offspring have shown vulnerability to prenatal E (Sittig et al., 2011b). Shortly, adult female SD rats (Harlan, Indianapolis, IN, USA) were mated with adult BN males (Charles River, Wilmington, MA, USA) overnight. Gestational day 1 (GD1) was assigned by the presence of sperm in vaginal smears. Pregnant females were assigned to one of three diet groups, control (C, *ad libitum* standard lab chow), pair-fed (PF) and ethanol (E). All pregnant rats, except those assigned to the control group, received *ad libitum* isocaloric liquid diet (Lieber-DeCarli '82; Bio-Serv. Frenchtown, NJ, USA) from GD4 to GD8 for habituation. On GD8, rats started their assigned diets. The E diet was introduced in stages starting on GD8 and by GD10 E rats received a full 5% ethanol (w/v, 35% ethanol-derived calories) diet. PF dams received an amount of isocaloric liquid diet that matched the paired E dam's diet consumption on the previous day. All rats were housed in a temperature and humidity controlled environment with a 12h light cycle (lights on at 6am) and had water *ad libitum*.

Sample Collection

In vivo study—Pregnant dams were sacrificed by decapitation on GD21 between 0900h and 1100h as previously described (Sittig and Redei, 2010). The uterine horns were quickly removed and placed on ice. Fetal sex was determined based on anogenital distance. One male and one female offspring from each litter were used to avoid litter effect. Fetal heads were collected directly into RNAlater® reagent (Ambion, Austin, TX, USA) and kept at room temperature for 24h before hippocampi were dissected on ice using a dissection microscope according to the Atlas of Prenatal Rat Brain Development (Altman & Bayer, CRC Press, 1995). Then, tissues were transferred into fresh RNAlater® and stored at -80°C.

Ex vivo study—Dissociated hippocampal cultures were prepared from GD18 fetuses (Ferreira et al., 1995) of dams that received C, PF, or E diets as described above. Briefly, pregnant dams on GD18 were euthanized with CO₂, fetuses isolated and individual fetal hippocampi dissected and cleaned of meninges. Hippocampal neurons were dissociated using trypsin (0.25% for 15 min at 37°C), followed by trituration with a fire-polished Pasteur pipette. The cells were plated with minimum essential medium (MEM) with 10% horse serum onto poly-l-lysine-coated dishes at the density of 800,000 cells/60mm dish or onto poly-l-lysine-coated coverslips. After 4h, the medium was changed to glia-conditioned MEM containing ovalbumin (0.1%), sodium pyruvate (0.1 mM) and N2 supplements (N2 medium). All cultures were incubated at 37°C with 5% CO₂ and processed 72h after plating [days *in vitro* 3 (DIV3)].

The sex of each fetus was determined first by the anogenital distance. Confirmation of sex was carried out by the presence or absence of the sex-determining region Y (*Sry*) gene. *Sry* was amplified using Taqman PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA) with 100ng of genomic DNA and primers (Forward primer sequence: GCAAGTTGGCTCAACAGA, and reverse primer sequence: GTTTCTGCTGTAGTGGTA). PCR products were run on a gel and visualized; a band corresponding to the size of the *Sry* PCR product (450bp) was observed in male fetuses, while female fetuses showed no products.

Quantitative RT-PCR (qPCR)

RNA was isolated by using Direct-zol™ RNA MiniPrep (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Reverse transcription and qPCR were performed as described previously (Tunc-Ozcan et al., 2013). Briefly, reverse transcription of 1µg total RNA was performed by using the TaqMan Reverse Transcription kit (Applied Biosystems, Branchburg, NJ, USA). qPCR was conducted with the ABI Prism 7900HT system using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Reactions were performed in triplicate from 5ng cDNA and reached threshold amplification within 32 PCR cycles. Relative quantification (RQ) was determined relative to *Gapdh*, as the endogenous control and to a general calibrator, using the 2^{-Ct} method. We confirmed that *Gapdh* expression was not affected by any of the prenatal treatments by measuring the cycle threshold (CT) values of *Gapdh* and *18S* across the different diet groups (Supplemental Table 1). Therefore, *Gapdh* was kept as the endogenous control for all qPCR analyses.

Immunocytochemistry (ICC)

Hippocampal neurons cultured on coverslips were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) containing 0.12mmol/L sucrose for 15min on DIV3. Fixed cells were stored in 4°C in PBS until ICC. They were then permeabilized in 0.3% Triton X-100 in PBS for 4min at room temperature (RT) and rinsed for 5min, three times in PBS. Next, the cells were incubated in 10% bovine serum albumin (BSA) in PBS for 1h at RT to block non-specific binding. After blocking, the following primary antibodies in 1% BSA were used overnight at 4°C for double immunofluorescence labeling: anti-neuron-specific Class III β -tubulin (clone TuJ1, 1:200; Sigma, St Louis, MO) and anti- α -tubulin (1:200; Sigma). The next day, coverslips were rinsed 5min, three times in PBS and incubated with the following secondary antibodies in 1% BSA for 1 hour at 37°C: Alexa Fluor 488 anti-mouse and Alexa Fluor 568 anti-rabbit IgG (1:200; Molecular Probes, Eugene, OR). Photometrics Cool Snap HQ2 camera coupled with a fluorescent microscope (Nikon Diaphot, Melville, NY, USA) was used for taking the images.

Statistical analysis

Data is shown as mean $\pm$ standard error of the mean. Sample numbers are indicated in the figure and table legends. Sample numbers varied in the analyses of the different transcripts because different outliers, defined as two standard deviations away from the mean, were removed.

Data was normalized to the control mean to allow ready comparison between the *in vivo* and the *ex vivo* data with the obvious differences in neuronal numbers. Data was first analyzed using two-way ANOVA with prenatal treatment and sex as factors, when there were no significant sex differences, male and female data were combined and analyzed by one-way ANOVA. Student's t-test was used for hypothesis testing. Bonferroni corrections for post-hoc comparisons were used. ANOVA results are described in the results section, while post-hoc comparisons are indicated on the figures and in the figure legends. Significance was considered $p < 0.05$. Statistical analyses were done by GraphPad Prism 6 (La Jolla, CA, USA).

Results

Hippocampal culture characterization

To analyze the presence of glia and the morphological differences by prenatal treatment, we fixed and stained C, PF and E hippocampal cultures using a neuron-specific tubulin antibody (TUJ1). Hippocampal cultures usually show a well-defined sequence of morphological changes (Paganoni and Ferreira, 2005). These are defined by stages. Upon plating, hippocampal neurons are surrounded by a lamellipodial veil (stage I), which is followed by extension of several undifferentiated minor processes within the first 12 hours (stage II). Then, the cells are polarized within 1-2 days by showing one long process as the axon, which exceeds the length of one of the minor processes (stage III). The minor processes differentiate into dendrites 4 days later (stage IV) (Paganoni and Ferreira, 2005). There were no significant differences in terms of developmental stage of hippocampal neurons between the different diet groups. They all shared a common morphology that identifies the stage II

and III across diet groups (Figure 1). In each diet group, 3 random regions were selected and all showed the same morphology as the represented panels in Figure 1. No sex differences were observed.

Double immunofluorescence labelling using an anti- α -tubulin antibody and the neuron-specific TUJ1 antibody showed that these cultures contained mainly pyramidal neurons. Very few glial cells (less than 1%) were present in all prenatal diet groups, as no cells stained only with the anti- α -tubulin antibody were found. To confirm that similar number of neurons were present across the prenatal treatment groups, we measured transcript levels of the neuronal marker *Fox3* (NeUN) by qPCR. There were no significant sex differences in the transcript levels, so male and female data was combined. One-way ANOVA followed by Bonferroni post-hoc test and/or Student's t-test showed no significant differences between prenatal diet groups in transcript levels of *Fox3* (Supplemental Figure 1).

Sex and prenatal ethanol effects on hippocampal gene expression in the *in vivo* and the *ex vivo* studies

Sex differences were present in transcript levels in both the *in vivo* fetal hippocampi and the *ex vivo* hippocampal culture obtained from the control dams (Table 1). These sex differences were more numerous in the *in vivo* fetal hippocampi, where only *Grb10* expression showed no sex differences. In contrast, only *Igf2* and *Insr* showed sex differences in their expression in the *ex vivo* culture, which directionally agreed with the *in vivo* data.

Prenatal diet altered the expression of all investigated transcripts significantly in the *in vivo* fetal hippocampus (Table 2). In addition, *in vivo* expression of *Igf2*, *Insr*, *Grb10* and *Rasgrf1* differed by sex, and by an interaction effect of prenatal diet and sex (Table 2). Prenatal diet had a sex-dependent effect on hippocampal *Igf2r* expression, while *Zac1* levels were not affected by the sex of the fetus (Table 2 and Supplemental Figure 2).

The major differences between the effects of prenatal diet and sex on *in vivo* fetal hippocampal transcript levels and *ex vivo* hippocampal neuronal expression were notable in the lack of significant sex or interaction effects for *Igf2r* and *Rasgrf1* expression in the hippocampal culture (Table 3). *Zac1* expression was not affected by sex neither in the *ex vivo* hippocampal culture either (Table 3 and Supplemental Figure 3).

Since the aim of the study was to validate the *ex vivo* primary hippocampal culture against the *in vivo* fetal hippocampus, these comparisons were carried out for the sexes separately.

***In vivo* vs. *ex vivo* of male hippocampus**—Hippocampal transcript levels of *Igf2* were decreased in the E compared to C and PF males in both *in vivo* ($F(2,9)=18.38$, $p<0.01$) and *ex vivo* ($F(2,12)=15.08$, $p<0.01$) (Figure 2A). *Igf2r* and *Insr* expressions decreased in PF compared to C, but were higher in the E than the PF group both in *in vivo* and *ex vivo* (*in vivo* *Igf2r*: $F(2,10)=44.42$, $p<0.01$; *Insr*: $F(2,10)=4.81$, $p<0.05$ and *ex vivo* (*Igf2r*: $F(2,11)=15.34$, $p<0.01$; *Insr*: $F(2,11)=20.72$, $p<0.01$) (Figures 2B and 2C). Both *in vivo* and *ex vivo* studies showed significant increases in hippocampal *Grb10* transcript levels by E compared to both C and PF groups ($F(2,9)=6.68$, $p<0.05$ and ($F(2,12)=13.48$, $p<0.01$, respectively) (Figure 2D). There were no significant differences in transcript levels of

Rasgrf1 between the different prenatal diet groups in the *in vivo* hippocampus, although there was a trend toward an increase of transcript levels ($p<0.09$) in the E group compared to C and PF groups (Figure 2E). The *ex vivo* culture showed a similar profile with significantly increased *Rasgrf1* expression in the E culture ($F(2,12)=5.38$, $p<0.05$) (Figure 2E). Lastly, transcript levels of *Zac1* were significantly lower in both PF and E groups than those of C in both *in vivo* ($F(2,11)=13.51$, $p<0.01$) and *ex vivo* ($F(2,11)=18.11$, $p<0.01$) (Figure 2F).

***In vivo* vs. *ex vivo* of female hippocampus**—Transcript levels of *Igf2* were significantly lower in the PF compared to C and E groups *in vivo* ($F(2,11)=22.14$, $p<0.01$) (Figure 3A). In contrast, in the *ex vivo* study, *Igf2* expression was significantly greater in the E compared to both C and PF groups ($F(2,10)=12.12$, $p<0.01$) (Figure 3A). Similarly to males, *Igf2r* transcript levels in the *in vivo* and the *ex vivo* female hippocampus showed a significant decrease in the PF group compared to C and an increase by E compared to the PF group (*in vivo*: $F(2,11)=10.94$, $p<0.01$; *ex vivo*: $F(2,10)=8.11$, $p<0.05$) (Figure 3B). Transcript levels of *Insr* in both PF and E groups compared to C in both *in vivo* ($F(2,11)=14.93$, $p<0.01$) and *ex vivo* ($F(2,10)=11.31$, $p<0.01$) (Figure 3C). Significantly higher levels of *Grb10* transcript was observed in E compared to both C and PF groups in both *in vivo* ($F(2,10)=30.38$, $p<0.01$) and *ex vivo* ($F(2,10)=7.85$, $p<0.01$) (Figure 3D) in female hippocampus as seen in the males. *Rasgrf1* transcript levels were significantly higher in the E group compared to C and PF in the *in vivo* study ($F(2,10)=16.53$, $p<0.01$) (Figure 3E). In the *ex vivo* culture, *Rasgrf1* expression profile showed the same trend with increased levels of *Rasgrf1* transcript in the E and PF groups compared to C ($F(2,10)=6.66$, $p<0.05$) (Figure 3E). Similarly to males, female *Zac1* expression was significantly decreased by E compared to C in both *in vivo* ($F(2,11)=9.59$, $p<0.01$) and *ex vivo* ($F(2,9)=5.13$, $p<0.05$) (Figure 3F).

Discussion

The major conclusion of the present study, based on our current findings, is that the effects of prenatal E are already set in the fetal hippocampus at gestational day (GD) 18, as hippocampal expression changes of neuronal genes show little or no deviation from those that were exposed to E up to gestational day (GD) 21. This finding strengthens the feasibility of the *ex vivo* primary hippocampal culture as a tool to test and screen candidate drug targets for FASD. Additionally, the similarities and differences in the sex-specific expression of insulin signaling pathway genes in the control fetal hippocampi vs. cultured hippocampal neurons can identify which of these genes are under sex chromosomal regulation and which ones are regulated by sex hormones. The sex-specific effects of prenatal E on the expression of these genes are further highlighted by the differences between the sex-related developmental stages of the fetal hippocampi on GD21 and hippocampal neurons cultured on GD18. Finally, some of the transcripts show changes only by prenatal E, while others are clearly affected by the caloric restriction of the pair-feeding as well. This differential vulnerability to E or PF may illuminate the role of these genes in regulation of hippocampal metabolism with direct or indirect effects on hippocampus-specific learning and memory-related functions.

Comparison of the gene expression profiles of selected insulin pathway genes in the *in vivo* fetal hippocampus and the *ex vivo* primary hippocampal culture showed persistent and similar effects. This similarity exists despite the seeming differences in the presence of auxiliary insulin in the *ex vivo* culture medium as well as the differences in developmental stages of the fetal brain in the two different time points, GD18 vs. GD21. Insulin is present in the brain of humans and animals (Havrankova et al., 1978, Dorn et al., 1983). Insulin is already present in the fetal brain (Unterman et al., 1993) and in our hippocampal culture medium in the *ex vivo* experiment. Thus, the effects of prenatal E on the expression of insulin pathway genes are modified similarly by the presence of insulin *in vivo* and *ex vivo*. The other important issue is the developmental difference between GD18 and GD21. The *ex vivo* hippocampus was cultured at GD18, which is an early developmental time point for the hippocampus, although, the DIV3 hippocampal neurons show even more advanced developmental stage than GD21 (Paganoni and Ferreira, 2005, Pressler and Auvin, 2013). Thus, the developmental difference is less of an issue than the three more days of exposure to E of the *in vivo* fetal hippocampus. Indeed, the prenatal E-induced effects on the cultured hippocampal neurons are qualitatively the same on GD18 as the E effects on the *in vivo* hippocampus on GD21, which suggests that these effects are programmed earlier and even before the development of hippocampus is largely completed. Nevertheless, the longer exposure to prenatal E heightened the E effects observed *in vivo* in the expression of *Igf2* in the male hippocampus, *Igf2r* and *Grb10* in both the male and the female hippocampus, and *Rasgrf1* in the female hippocampus compared to those in the *ex vivo* culture.

Differences between the *in vivo* and *ex vivo* sex-effects in the expression of insulin signaling-related genes in the control hippocampi, and the timing of the tissue collection in the two methods suggest the origin of these sex differences. Only the expressions of *Igf2* and *Insr* show sex differences in cultured hippocampal neurons, while more extensive sex differences were found in the fetal hippocampi harvested after the prenatal testosterone surge on GD18-19 (Welsh et al., 2008). Expression levels of several imprinted genes are variable between sexes in the developing brain, even before the presence of any sex hormones in the body (Faisal et al., 2014). Differential genomic imprinting in the brain according to the sex of an individual (Gregg et al., 2010) would be the reason for this expression differences, since changes in imprinting status can alter transcript levels of imprinted genes (Sittig et al., 2011a). Sex differences in the expression of *Igf2* and *Insr* are likely regulated by sex chromosome-related mechanisms. Indeed sex-specific regulation of *Igf2* expression has been proposed: murine female cells are hypomethylated at the imprinted *Igf2* gene, and the *Igf2* differentially methylated region (DMR) 2 is more heavily methylated in the male than in the female cells (Durcova-Hills et al., 2004). It has been proposed that re-methylation of the DMR within the *Igf2* gene is primarily regulated by the embryo's sex chromosomes. Sex differences in the expression of hippocampal *Insr* has been found as being higher in male than in female rats on postnatal day one (Hami et al., 2012), but no sex differences in fetal *Insr* expression has been reported to date prior to the prenatal testosterone surge.

In the male rat, testosterone surges markedly during embryonic days 18–19 and again during the first few hours following parturition. Both testosterone and estrogen levels are higher in the GD19 male compared to the female hippocampus (Konkle and McCarthy, 2011). The *in*

vivo, but not *ex vivo*, sex differences in the expression of *Igf2r*, *Rasgrf1* and *Zac1* indicate the potential significance of the prenatal testosterone surge affecting the expression of these genes. The simplistic explanation for the sex differences in the expression of these genes in the fetal GD21 hippocampus is that they are regulated by estrogen, the metabolic consequence of the testosterone surge in the brain. *Igf2r*, for example, is negatively regulated by estrogen in breast cancer cells (Mathieu et al., 1991). If this regulation occurs in the hippocampus, the lower levels of *Igf2r* we found in the male fetal hippocampus could be the result of increased estrogen metabolized after the prenatal testosterone surge. Regarding *Rasgrf1* and *Zac1*, no clear evidence of transcriptional regulation by estrogen or testosterone has been found.

Sex-differential effects of prenatal E could be seen in the expression profile of *Igf2* both *in vivo* and *ex vivo* hippocampus. Males showed a significantly decreased *Igf2* expression after E compared to both PF and C. In contrast, female fetal hippocampi showed a more profound *Igf2* expression difference in response to PF than that of E. The interesting finding of the present study is that the same environmental insult had different effects on the gene expression depending on the sex of the offspring. The male-specific effect of E on the expression of fetal hippocampal *Igf2* parallels the male specificity of cognitive and social behavior deficits in the adult prenatal E-exposed offspring (Sittig et al., 2011b). Since incidence of FASD is higher among males than females (Thanh et al., 2014) and animal models supports this sex bias (Tunc-Ozcan et al., 2013), these sex differences in the effect of prenatal E are of significance.

Igf2 is an imprinted gene that is thought to be expressed from the paternal allele. In contrast, in the hippocampus it is preferentially maternally expressed (Harper et al., 2014), due to epigenetically regulated differential methylation marks. Sex-specific regulation of these epigenetic marks has been shown. For example, the influence of *in utero* exposure to cigarette smoking on methylation of the *IGF2* DMR is sex dependent: the maternal smoking-related increase in methylation is more pronounced in the male offspring (Murphy et al., 2012). Sex-specific epigenetic consequences of prenatal exposures to environmental insults that affect the whole organism, therefore, could be a general mechanism of sex-biased prevalence of neurodevelopmental disorders. Considering the sex-specific effects of E on *Igf2* expression, and that *Igf2* has cognition-enhancing abilities (Chen et al., 2011), it is possible that early restoration of hippocampal *Igf2* levels could attenuate the behavioral and cognitive consequences of FASD.

The effects of prenatal E are often difficult to be separated from prenatal nutritional effects, since the alcohol consuming pregnant mother has nutritional deficiencies. The average weight and body mass index of FASD mothers is lower than control subjects (May et al., 2011), and nutrition is a major contributing factor to the consequences of FASD (Shankar et al., 2006). This finding is mirrored by animal studies as well, including the present one, despite our efforts to control for nutritional effects. It is shown that prenatal malnutrition influences the expression levels of *Igf2* in a sex-specific manner as females demonstrate a more pronounced decrease in transcript levels (Radford et al., 2012). The present study also show these prominently lower levels of *Igf2* in the PF group, compared to C, in the *in vivo* female hippocampus, which is mirrored by decreased hippocampal *Igf2* expression induced

by calorie restriction in the adult offspring (Harper et al., 2014). Similarly, *in vivo* and *ex vivo* hippocampal expressions of *Igf2r* and *Insr* were lower in the PF group of both sexes. Pair-feeding of the mother lowered the *Zac1* expression in both *in vivo* and *ex vivo* male hippocampus. *Ex vivo* transcript levels of *Rasgrf1* were, in contrast, increased by pair-feeding compared to controls in both male and female hippocampus. In a recent study, expressions of several genes were similarly altered in the hippocampus by E and PF compared to controls (Lussier et al., 2015). The authors suggest, that these changes might be the results of reduced caloric availability, which is the common effect of E exposure and pair-feeding. PF dams receive the same amount of diet as E dams, but they consume it fast, leaving them in a fasting state with its metabolic consequences until the next feeding. It is, therefore, a feasible and testable hypothesis that pair feeding and E affect metabolic states of the periphery and of the brain in a partially overlapping, but also separate manner.

Long-lasting outcomes of prenatal E exposure include aberrant fetal programming with their effect on critical, and time-sensitive molecular processes during neurodevelopment, which coexist in time with the exposure to E (Kleiber et al., 2013, Zhou, 2012). Supplementation of thyroxin (Tunc-Ozcan et al., 2013) or choline (Thomas et al., 2010) to the ethanol-consuming dams has shown to be effective in reversing some of the cognitive deficits seen in FASD models. However, exploration of specific treatment paradigms post-E exposure would benefit from the very efficient *in vitro* methods used for screening drug targets in other areas of research (Astashkina et al., 2012). Direct *in vitro* models are not ideal, because of the disruptive effects of E on cell composition (Dolganiuc and Szabo, 2009) and the lack of physiologically significant indirect effects of E via the pregnant dam. Therefore, it is likely that the *in vitro* hits of potential drug targets will lack efficacy *in vivo* as it has been seen before (Medina-Franco et al., 2013). The advantage of the *ex vivo* model is that the E administration occurs *in vivo*, via the pregnant mother, just like in human FASD, but the primary hippocampal neurons-based screening and manipulations occur *in vitro*. An advanced application of this advantageous procedure has been explored in other fields of drug discovery (Yu et al., 2014). In the present study, we confirmed the persistent and parallel influence of *in vivo* prenatal E exposure on the gene expression profiles of selected insulin pathway genes in the *in vivo* fetal hippocampus and the *ex vivo* primary hippocampal culture on DIV3. Thus, the *ex vivo* primary hippocampal culture could be useful to develop and characterize drug-based interventions, which then can be investigated *in vivo*, to reverse E-induced hippocampal dysregulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Examples of cultured hippocampal neurons of Control (a and b), Paired (c and d) and Ethanol (e and f) fetuses obtained from male GD18 hippocampi and cultured for three days Neurons were fixed and immunostained using the neuron-specific tubulin (TUJ1) (a, c and e) and α -tubulin (b, d and f) antibodies. Developmental stage of hippocampal neurons indicates stage II (extension of several undifferentiated minor processes) and III (one long process as the axon, which exceeds the length of one of the minor processes) in all prenatal diet groups. Note the absence of glial cells (TUJ1 (-) cells) in panels b, d and f. Scale bar = 20 μ m.

Figure 2. Gene expression profiles of A) *Igf2*, B) *Igf2r*, C) *Insr*, D) *Grb10*, E) *Rasgrf1*, and F) *Zac1* in the fetal GD21 male hippocampus (*in vivo*) and in the primary hippocampal culture (*ex vivo*) obtained from male GD18 hippocampi and cultured for three days

Transcript levels were measured by quantitative RT-PCR and normalized to *Gapdh* and a general calibrator using the 2^{-Ct} method. For comparison purposes, all PF and E measures were normalized to their respective C values, and the relative quantification (RQ) values of C are listed in Table 1. *p<0.05, **p<0.01 by one-way ANOVA followed by Bonferroni post-hoc test. ^ p<0.05 by Student's t-test (C vs PF: *Insr* t(7)=3.16; *Rasgrf1* t(8)=2.75). Data presented as mean $\pm$ SEM. *In vivo* N=4-5/group, *ex vivo* N=4-5/group.

Figure 3. Gene expression profiles of A) *Igf2*, B) *Igf2r*, C) *Insr*, D) *Grb10*, E) *Rasgrf1*, and F) *Zac1* in the fetal GD21 female hippocampus (*in vivo*) and in the primary hippocampal culture (*ex vivo*) obtained from female GD18 hippocampi and cultured for three days

Transcript levels were measured by quantitative real-time RT-PCR and normalized to *Gapdh* and a general calibrator using the 2^{-Ct} method. For comparison purposes, all PF and E measures were normalized to their respective C values, and the relative quantification (RQ) values of C are listed in Table 1. * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA followed by Bonferroni post-hoc test. ^ $p < 0.05$, ^^ $p < 0.01$ by Student's t-test (*Igf2r*: C vs PF $t(6) = 2.55$

and C vs E $t(7)=2.44$; *Rasgrfl*: C vs PF $t(7)=3.74$). Data presented as mean $\pm$ SEM. *In vivo* N=4-5/group, *ex vivo* N=3-5/group.

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Table 1
Sex differences in transcript levels measured in control *in vivo* fetal hippocampi and control *ex vivo* primary hippocampal cultures

Genes	<i>In vivo</i> (RQ)		<i>Ex vivo</i> (RQ)	
	Male	Female	Male	Female
<i>Igf2</i>	2.517 ± 0.33	0.519 ± 0.04 **	0.023 ± 0.01	0.012 ± 0.01 **
<i>Igf2r</i>	1.190 ± 0.12	3.823 ± 0.31 **	1.763 ± 0.14	1.890 ± 0.23
<i>Insr</i>	2.053 ± 0.12	4.197 ± 0.18 **	0.911 ± 0.03	1.321 ± 0.14 *
<i>Grb10</i>	0.442 ± 0.13	0.301 ± 0.03	0.194 ± 0.02	0.152 ± 0.02
<i>Rasgrf1</i>	2.564 ± 0.13	0.189 ± 0.05 **	0.239 ± 0.03	0.276 ± 0.02
<i>Zac1</i>	1.049 ± 0.09	2.240 ± 0.20 **	2.777 ± 0.23	2.228 ± 0.22

Data presented as mean ± SEM. N=4-6/group.

**
p<0.01,

*
p<0.05 by Student's t-test.

RQ: Relative quantification in qPCR.

Table 2
Results of statistical analyses of the *in vivo* fetal hippocampal transcript levels by prenatal diet and fetal sex

Gene	Two-way ANOVA results		
	Interaction	Diet	Sex
<i>Igf2</i>	F(2,16)=27.02, p<0.01	F(2,16)=10.68, p<0.01	F(1,16)=116.2, p<0.01
<i>Igf2r</i>	F(2,17)=6.93, p<0.01	F(2,17)=50.64, p<0.01	X
<i>Insr</i>	F(2,19)=7.74, p<0.01	F(2,19)=6.29, p<0.01	F(1,19)=9.04, p<0.01
<i>Grb10</i>	F(2,17)=7.35, p<0.01	F(2,17)=26.42, p<0.01	F(1,17)=4.77, p<0.05
<i>Rasgrf1</i>	F(2,15)=5.39, p<0.05	F(2,15)=17.14, p<0.01	F(1,15)=11.72, p<0.01
<i>Zac1</i>	X	F(2,20)=22.59, p<0.01	X

Table 3
Results of statistical analyses of the transcript levels in the *ex vivo* primary hippocampal cell culture by prenatal diet and fetal sex

Gene	Two-way ANOVA results		
	Interaction	Diet	Sex
<i>Igf2</i>	F(2,22)=26.78, p<0.01	X	F(1,22)=52.33, p<0.01
<i>Igf2r</i>	X	F(2,19)=22.11, p<0.01	X
<i>Insr</i>	F(2,21)=7.20, p<0.01	F(2,21)=18.62, p<0.01	F(1,21)=15.60, p<0.01
<i>Grb10</i>	X	F(2,22)=16.22, p<0.01	F(1,22)=10.71, p<0.01
<i>Rasgrf1</i>	X	F(2,22)=11.25, p<0.01	X
<i>Zac1</i>	X	F(2,20)=18.86, p<0.01	X