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Intergenerational and parent of origin effects of maternal calorie restriction on *Igf2* expression in the adult rat hippocampus

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Abstract

Insulin-like growth factor 2 (*Igf2*) regulates development, memory and adult neurogenesis in the hippocampus. Calorie restriction (CR) is known to modulate non-neuronal *Igf2* expression intergenerationally, but its effect has not been evaluated on brain *Igf2*. Here, Sprague-Dawley (S) dams underwent moderate CR between gestational days 8–21. To identify parent of origin expression pattern of the imprinted *Igf2* gene, their offspring (SS F1) were mated with naïve male or female Brown Norway (B) rats to obtain the second generation (BS and SB F2) progeny. CR did not affect adult hippocampal *Igf2* transcript levels in SS F1 males or their BS F2 progeny, but increased it in SS F1 females and their SB F2 offspring. The preferentially maternal *Igf2* expression in the SB F2 control male hippocampus relaxed to biallelic with CR, with no effect of grandmaternal diet in any other groups. Thus, allele-specific and total expression of hippocampal *Igf2* is affected by maternal, grandmaternal CR in a strain and sex-specific manner.

Keywords

allele-specific expression; imprinting; Sprague-Dawley rat; Brown Norway rat; sex-specific; matrilineal; grandmaternal calorie restriction

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Conflict of Interest
The authors have no conflict of interest.

Contributors
Conceived and designed the experiments: EER. Identified and confirmed SNP: LBKH. Performed the experiments: KMH, ETO, ENG. Analyzed the data: KMH. Wrote the manuscript: KMH. Edited and revised manuscript: KMH, ETO, ENG, EER, LBKH. Approved final version of the manuscript: KMH, ETO, ENG, EER, LBKH

Introduction

Insulin-like growth factor 2 (*Igf2*) is involved in growth and development (for review see Bergman et al. (2013)). Among its effects on the CNS, *Igf2* increases the survival of 17–19 days old neurons in the hippocampus while also promoting neural stem cell proliferation (Agis-Balboa et al., 2011; Bracko et al., 2012). Additionally, the effects of *Igf2* on adult hippocampal neurogenesis is connected to hippocampus based learning and memory (Agis-Balboa et al., 2011). Upregulation of hippocampal *Igf2* plays an important role in extinction of contextual fear memory and increases memory retention in an inhibitory avoidance task (Agis-Balboa et al., 2011; Chen et al., 2011).

Igf2 is an imprinted gene; expressed from the paternal allele under the control of a differentially methylated region (Bergman et al., 2013). Interestingly, though *Igf2* is maternally imprinted in the embryonic brain, it becomes paternally imprinted in specific regions of the adult human and mouse brain, i.e. the globus pallidus and hypothalamus (Gregg et al., 2010; Pham et al., 1998). Since changes in imprinting status can alter transcript levels of imprinted genes (Sittig et al., 2011), conditions that change the imprinting status of *Igf2* could have lasting effects on learning and memory by affecting the transcript levels. Gestational nutrition might be one such condition, since rodents born of diabetic mothers show altered non-neuronal *Igf2* levels with concomitant increased methylation (Ding et al., 2012), while decreased methylation alters non-neuronal *IGF2* transcript levels in humans born during famine (Heijmans et al., 2008). Moreover, gestational methylation changes at the *Igf2* locus has been shown to be transferrable to the second generation (Ding et al., 2012; Stouder et al., 2011), evoking the possibility of intergenerational inheritance of *Igf2*-related neurodevelopmental deficits.

Our goal is to determine the intergenerational effect of calorie restriction (CR) on total and allele-specific expression of *Igf2* in the rat hippocampus. To achieve this, offspring of Sprague-Dawley (S) dams, with and without CR, were mated with naïve Brown Norway (B) male and female rats (Figure 1) to distinguish the maternal or paternal transmission of grandmaternal CR on hippocampal *Igf2* expression in the second generation. Additionally, allele-specific expression of hippocampal *Igf2* could be measured on the SB and BS F2 progeny using this mating paradigm. Moderate maternal CR during pregnancy increased hippocampal total expression of *Igf2* in the female SS F1 offspring, which was transferred to their SB F2 progeny. Furthermore, the preferentially maternal expression of hippocampal *Igf2* relaxed to biallelic expression in SB F2 control male offspring with grandmaternal CR, with no other group showing this effect. Therefore, allele-specific and total expression of hippocampal *Igf2* are affected by maternal and grandmaternal CR in a sex-specific manner.

Materials and Methods

The Northwestern University Animal Care and Use Committee approved all procedures. After mating male and female S rats overnight (Harlan, Indianapolis, IN, USA), sperm positive vaginal smear marked gestational day one (GD1). Pregnant females were divided between control (C), laboratory rat chow and water *ad libitum*, and CR groups. From GD4-8, CR rats were provided with water and a liquid diet (Lieber-DeCarli ’82; Bio-Serv.

Frenchtown, NJ, USA) *ad libitum* to acclimatize them to the diet as described previously (Harper et al., 2014). From GD8-21, CR rats consumed an average of 21.8 to 26.8 kcal per 100 g^{-1} of body weight per day. This represents approximately 83–89% of the daily caloric intake of C dams, which is a mild CR with no significant body weight difference between the F1 C vs CR pups (Harper et al., 2014). At all other times regular laboratory chow and water were available *ad libitum*. One to two rats of each sex/litter/prenatal treatment of SS F1 offspring were mated with naïve B rats (Charles River, Wilmington, MA, USA) to generate SB and BS F2 progeny. B is the most phylogenetically divergent inbred rat strain (Swerdlow et al., 2008), and the B and S genomes have been sequenced by the Rat Genome Project and Celera, respectively. All of which increased the chances that we would be able to identify a single nucleotide polymorphism (SNP) between the B and S cDNAs at the *Igf2* locus that we could use to measure allele-specific contribution to the expression of hippocampal *Igf2*.

Sample preparation and qPCR

At approximately 70 days of age, one-two rats of each sex/litter (N=9–12/group) were sacrificed for tissue collection. Brains were directly placed into RNAlater© (Life technologies, Grand Island, NY). Whole hippocampus was dissected as previously described (Sittig et al., 2011). RNA was isolated by the Trizol© reagent (Life technologies, Grand Island, NY), then converted to cDNA by ABI Reverse Transcription kit (Foster City, CA). For qPCR, SYBR Green PCR Master Mix (ABI, Foster City, CA) was used on the ABI Prism 7300. Reactions were performed in triplicate and ΔΔCt method was employed using 18S (primers from Ambion, Grand Island, NY) as endogenous control. *Igf2* primers were; forward CCGTACTTCCGGACGACTTC and reverse CGTCCCGCGGACTGTCT.

Pyrosequencing

A SNP of A/G at Chromosome 1:222725126 bp in the 3′ untranslated region of *Igf2* was identified between the B (A) and S (G) strains (rs8143502) by sequencing, and the SNP was confirmed in the SB and BS F2 offspring. Both forward and biotinylated reverse primers, that flank the SNP, were designed by EpigenDx (Worchester, MA, USA). After PCR, the purification and pyrosequencing of the PCR product were carried out by EpigenDx, which gave the percentage of the A vs G allele in the *Igf2* (N=3–4/sex/cross/prenatal treatment). In the reciprocal F2 crosses, the maternal contribution to *Igf2*, “A” in BS F2 and “G” in SB F2, is shown.

Statistical analysis

Data were analyzed by two-way ANOVA (sex and prenatal treatment) for the F1 generation and three-way ANOVA (cross, sex and prenatal treatment) for the F2 generation. The F2 generation was also analyzed by two-way ANOVA for hypothesis testing. When appropriate, Bonferroni post-hoc comparisons were made. $p<.05$ was considered statistically significant. Statistical analyses were carried out using Systat 11 (Chicago, IL, USA).

Results

Female SS F1 offspring had significantly greater hippocampal *Igf2* levels than males (sex: $F_{1, 34}$=12.25, p<.01; Figures 2A & B), and CR caused further increases in these levels in females (prenatal treatment: $F_{1,34}$=2.85, p=.10; sex X prenatal treatment: $F_{1,34}$=3.57, p=.068; female C vs CR p<.05). There was no effect of CR on hippocampal *Igf2* levels in SS F1 male progeny.

In the second generation, a significant difference in *Igf2* transcript levels was observed between SB vs BS (cross: $F_{1,37}$=84.83, p<.01), due to higher *Igf2* expression in the BS F2 progeny (Figure 2C & D). Grandmaternal treatment affected only the SB F2 progeny (prenatal treatment: $F_{1,37}$=3.09, p=.09; cross X prenatal treatment: $F_{1,37}$=1.05, p=.31; sex: $F_{1,37}$=2.45, p=.13; sex X prenatal treatment: $F_{1,37}$=2.123, p=.15; sex X cross: $F_{1,37}$=.001, p=.97; sex X prenatal treatment X cross: $F_{1,37}$=.50, p=.49). A within cross two-way ANOVAs revealed that grandmaternal CR had no effect on the BS F2 progeny (prenatal treatment: $F_{1,15}$=.13, p=.73), while SB F2 CR offspring have significantly increased hippocampal *Igf2* levels compared to C (prenatal treatment: $F_{1,20}$=16.17, p<.01; Figure 2D).

By using the A/G SNP, a significant difference in allele-specific expression between the crosses (cross: $F_{1,17}$=45.74, p<.01) and a trend towards a cross-specific prenatal treatment effect (cross X prenatal treatment X sex $F_{1,17}$=3.63, p=.074; Figures 2E & F) on hippocampal *Igf2* were found. In general, *Igf2* showed similar, preferentially maternal expression in both male and female, C and CR BS F2 hippocampi (prenatal treatment: $F_{1,7}$=2.26e-005, p=1.0; Figure 2E). In contrast, although SB F2 C male hippocampus also exhibited preferentially maternal *Igf2* expression, this maternal expression became biallelic in the male SB grandoffspring of CR grandmothers. Moreover, female SB F2 progeny of both C and CR grandmothers showed biallelic hippocampal expression of *Igf2* (prenatal treatment X sex: $F_{1,10}$=5.15, p<.05; Figure 2F).

Discussion

Here we describe for the first time a sex and lineage-specific effect of calorie restriction on hippocampal *Igf2* expression. Specifically, only females and their progeny responded by increased expression of hippocampal *Igf2* to maternal or grandmaternal CR. Furthermore, the matrilineal male offspring of grandmothers on calorie restricted diet during pregnancy show biallelic hippocampal *Igf2* expression in contrast to the preferential maternal expression of control male grandoffspring.

Maternal diet affects fetal growth directly, by determining the amount of nutrients available, and epigenetically modulating gene activity in the fetus. Nutritional changes during critical periods of gestation may have long-lasting effects on progeny. *Igf2* is a very important growth-regulatory imprinted gene, and its alteration by maternal nutrition has been studied thoroughly. In humans, famine during pregnancy causes persistent hypomethylation of *Igf2*, leading to biallelic expression in plasma (Heijmans et al., 2008), which is still present 60 years after famine exposure. In contrast, a high sugar/fat diet during pregnancy increases placental *Igf2* expression (Sferruzzi-Perri et al., 2013). These results indicate that various

nutritional changes during pregnancy can alter imprinting of *Igf2*, and consequentially *Igf2* transcript levels in various tissues in the offspring.

To our knowledge, this is the first report of adult hippocampal *Igf2* imprinting, which is consistent with the 80% preferential maternal expression found in the adult mouse hypothalamic preoptic area and medial prefrontal cortex (Gregg et al., 2010). In the present study, however, we found that both cross and sex affects allelic expression of hippocampal *Igf2*, such that only SB females exhibited biallelic expression. Such sex and cross-specific parent of origin effect is found for hippocampal *Dio3* gene in the BS and SB (F1) offspring (Sittig et al., 2011). In that case, the cross and sex specific changes in the imprinting of *Dio3* led, in the adults, to behavioral vulnerabilities to adverse prenatal conditions, which might occur in response to changes in *Igf2* imprinting status as well.

Our results have implications for nutritional effects on hippocampus based learning and memory. Increases in hippocampal *Igf2* expression immediately after training in both the avoidance learning task and contextual fear memory lead to increased memory retention (Chen et al., 2011), but during extinction training in the contextual fear memory task it allows for normal extinction (Agis-Balboa et al., 2011). Additional behavioral changes, due to increased hippocampal *Igf2* expression, are possible. For example, a placental knockout mouse model of *Igf2* with limited nutrition *in utero* shows increased anxiety in adulthood (Mikaelsson et al., 2013). However, a disconnect between decreased hippocampal *Igf2* expression and anxiety-like behavior has also been shown in a study using a mouse model of a schizophrenia-associated disorder. Still, deficits in adult neurogenesis and working memory in this model are restored by Igf2 administration (Ouchi et al., 2013). Thus, higher basal *Igf2* expression could be beneficial for the retention of short-term memory, and thus female offspring, and their progeny, of CR dams might show superior performance on hippocampal based learning and memory tasks, such as contextual fear conditioning. Interestingly, moderate CR in baboons during gestation and lactation has been shown to improve cognitive performance in female offspring (Rodriguez et al., 2012), which is in agreement with our predictions based on the *Igf2* expression changes in female offspring. It is important to emphasize that both the baboon study and the present one employed CR and not a nutritional restriction. In CR, animals are maintained on the proper nutrients and vitamins needed for a healthy diet, but have a reduced daily calorie consumption.

The prevalence of metabolic disorders, including obesity and hyperglycemia, in women of childbearing years is on the rise in the US (Ramos and Olden, 2008), indicating an increase in pregnancies at risk for altered intrauterine environments. Although we administered only a moderate CR during pregnancy, we still saw a positive effect on hippocampal *Igf2* expression in a sex specific manner. Should our future research confirm that natural prenatally programmed changes in hippocampal *Igf2* levels are beneficial to the mother and her progeny, the present data would significantly contribute to our understanding of how early epigenetic changes effect adult cognition and behavior.

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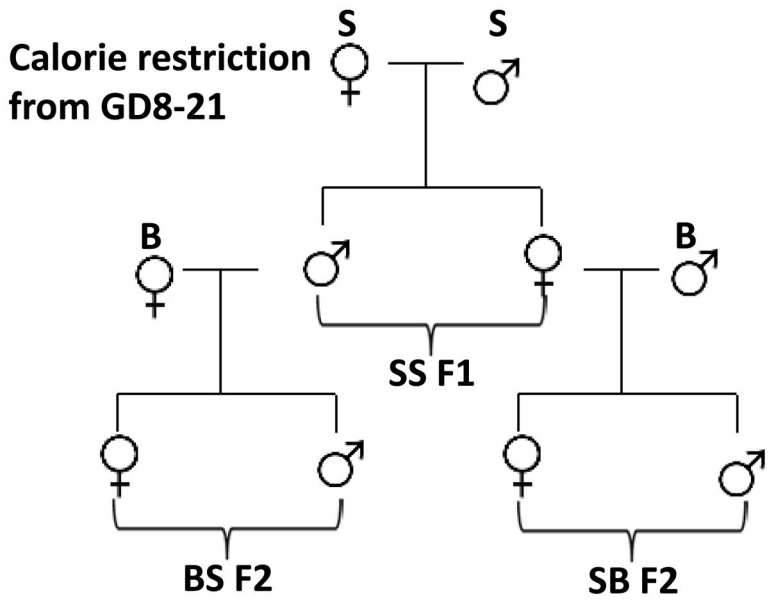

Figure 1. Schematic experimental design

Sprague Dawley (S) females were mated with S males. From gestational day 8 through 20 these dams were exposed to one of 2 prenatal treatments (Control or Calorie Restricted). The resulting SS F1 males and females were mated with naive female and male Brown Norway (B) rats to generate the BS F2 and SB F2 progeny, respectively.

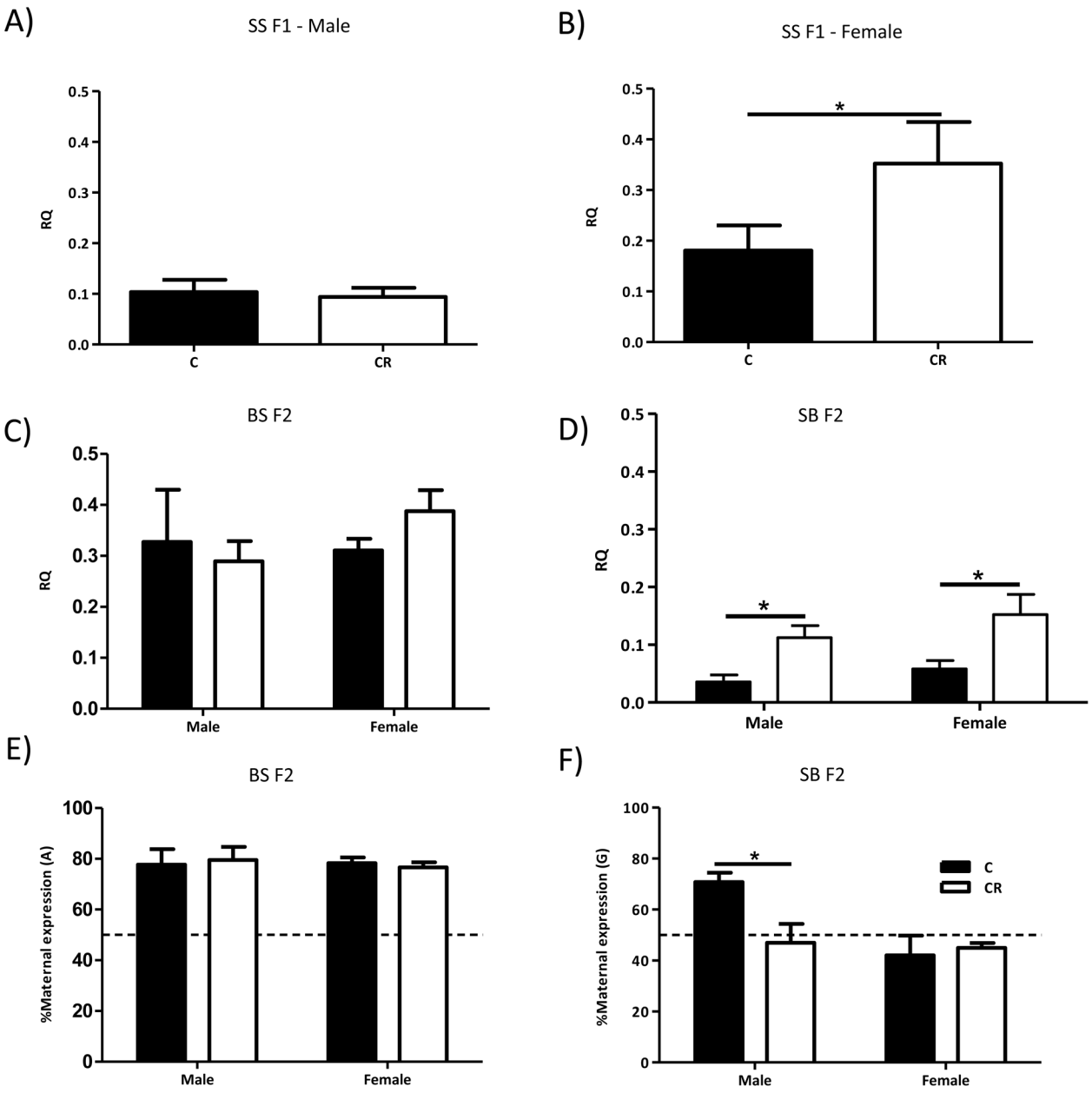

Figure 2. Calorie restriction (CR) during pregnancy alters total and allele-specific expression of hippocampal *Igf2* only through the female line

A) CR has no effect on *Igf2* transcript levels in adult SS F1 male offspring, while B) SS F1 female offspring of CR dams have a significant increase in hippocampal *Igf2* transcript levels. C) Grandmaternal CR has no effect on hippocampal *Igf2* expression in adult BS F2 male and female progeny, and D) Grandmaternal CR results in increased hippocampal *Igf2* in SB F2 male and female offspring. E) In BS F2 offspring, hippocampal *Igf2* is preferentially expressed from the maternal allele with no changes by grandmaternal CR, but F) Grandmaternal CR affects allele-specific expression only in the male SB F2 animals.

Data are represented as mean +/− SEM. qPCR experiments: N=9–12/group; pyrosequencing N=3–4/group.