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Intrauterine growth restriction promotes hypothalamic circadian dysregulation in adult mouse offspring

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Original Article

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Abstract

Adverse prenatal conditions can induce intrauterine growth restriction (IUGR) and increase the risk of adulthood metabolic disease. Mechanisms underlying developmentally programmed metabolic disease remain unclear but may involve disrupted postnatal circadian rhythms and kisspeptin signalling. We investigated the impact of maternal hypoxia-induced IUGR on hypothalamic and hepatic expression of clock genes (*Bmal1*, *Per2* and *Reverbα*), metabolic genes (*Pparα*, *Pparγ* and *Pgc1α*) and kisspeptin genes (*Kiss1* and *Kiss1r*) in adult offspring. Pregnant BALB/c mice were housed in hypoxic conditions (10.5% oxygen) from gestational day 11 to 17.5 and then returned to normoxic conditions until term (gestational day ~ 21). Control animals were housed in normoxic conditions throughout pregnancy. Offspring were weighed at birth. At 8 weeks of age, body, liver and brain tissues were collected and weighed. Relative clock gene, metabolic gene and kisspeptin signalling gene expression were measured using qPCR. The IUGR offspring were lighter at birth and remained lighter at 8 weeks but with higher brain relative to body weight. The IUGR offspring had decreased hypothalamic *Bmal1* and *Reverbα* expression, but unchanged hepatic clock gene expression and no change in hypothalamic or hepatic *Per2* expression, compared with Control offspring. This tissue-specific change in clock gene expression suggests circadian dysregulation. There were no IUGR-related changes to metabolic gene expression in the hypothalamus or liver, but IUGR offspring had increased hypothalamic *Kiss1r* expression. These results demonstrate IUGR offspring from hypoxia pregnancies show central circadian misalignment and potentially disrupted hypothalamic *Kiss1*/*Kiss1r* signalling, which may contribute to developmentally programmed metabolic disease.

Introduction

Human and animal studies have demonstrated that individuals who experienced intrauterine growth restriction (IUGR) and low birth weight have an increased risk of metabolic disease in adulthood.[1,2] The underlying mechanisms for the association between IUGR and metabolic disease remain poorly understood, but one potential mechanism could involve prenatal insults affecting the development of organs important for circadian rhythms and metabolism.

Circadian rhythms are natural cycles that align behaviour and physiology over the 24-h light–dark cycle.[3] These rhythms are regulated by the central 'master clock' located in the suprachiasmatic nucleus of the hypothalamus, which coordinates peripheral clocks in almost all organs and tissues.[4] Circadian rhythms modulate metabolic activity and play important roles in the functioning of metabolic organs including the liver.[5]

The structure and function of organs associated with circadian rhythms and metabolism, such as hypothalamus and liver, are determined during development *in utero*.[6,7] In mice, hypothalamic neurogenesis occurs between gestational day (GD) 10 and 16,[8] and hepatoblast differentiation occurs from GD 13.5 to 18.5.[9] Adverse prenatal conditions can therefore affect hypothalamic and hepatic development, and subsequently circadian rhythms in adulthood.[10] Such circadian disruptions are implicated in the pathophysiology of a variety of diseases including metabolic disorders such as obesity and type II diabetes.[11,12] In particular, misalignment between the endogenous circadian clocks across multiple organs is associated with impaired glucose tolerance and decreased insulin sensitivity,[13,14] similar to the symptoms of developmentally programmed metabolic disease in IUGR offspring.[15,16]

Genes involved in circadian rhythms, known as clock genes, are rhythmically expressed across the 24 h day as part of a transcription-translation feedback loop (TTFL) in virtually every nucleated cell of the body.[17] The positive arm of the circadian TTFL consists of brain and muscle Arnt-like 1 (*Bmal1*) and circadian locomotor output cycles kaput (*Clock*[17]). The BMAL1 and CLOCK proteins heterodimerise and induce transcription of period circadian regulators

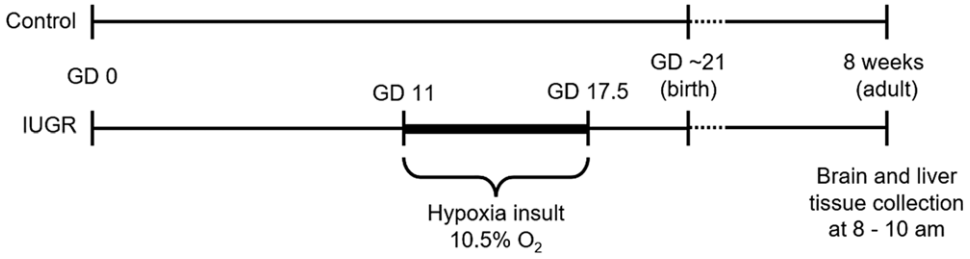

Figure 1. *Experimental timeline.* Pregnant BALB/c mice in the Control group were maintained in normoxic conditions (21% O_2) throughout gestation. Mice in the IUGR group were exposed to hypoxic conditions (10.5% O_2) from GD 11 to GD 17.5. Offspring were raised until euthanised at 8 weeks of age for brain and liver tissue collection. GD, gestational day; IUGR, intrauterine growth restriction; O_2, oxygen.

(*Per1-3*) and cryptochrome circadian regulators (*Cry1-2*[17]). Nuclear receptor subfamily 1 group D member 1 (*Reverbα*) and retinoic acid receptor-related orphan receptor alpha (*Rora*) repress and stimulate *Bmal1* transcription respectively, thereby regulating the TTFL.[17] Additionally, the peroxisome proliferator-activated receptors (PPARs) and their heterodimeric partner Pparγ coactivator 1 α (Pgc1α) are key to the bidirectional relationship between circadian rhythms and energy metabolism, and display diurnal rhythmic expression.[18,19] Various adverse conditions *in utero* are associated with increased *Pparα*, *Pparγ* and *Pgc1α* expression that could contribute to metabolic disease.[20,21]

Another potential player in the association between adverse prenatal conditions, circadian disruption and metabolic disease is the kisspeptin (*Kiss1*) signalling pathway in the hypothalamus, and in other metabolic organs including the liver and pancreas.[22] Kisspeptin neurons in the hypothalamus signal via the kisspeptin receptor (*Kiss1r)* to play key roles in reproduction, energy metabolism and circadian rhythms of behaviour.[22–25] Adverse prenatal conditions, such as maternal under- or over-nutrition, alter *Kiss1* and *Kiss1r* expression in the hypothalamus and liver, and drive sex-specific changes in glucose and insulin levels.[26,27]

The aim of this project was therefore to characterise the impact of mid-gestation maternal hypoxia-induced IUGR on hypothalamic and hepatic clock gene, metabolic gene and *Kiss1*/*Kiss1r* gene expression in both male and female adult mice.

Materials and methods

Maternal hypoxia-induced IUGR mouse model and tissue collection

This project was approved by the Telethon Kids Institute (Project Number #264) and was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition).

Pregnant female BALB/c mice were obtained from Animal Resources Centre (Murdoch, WA, Australia) at GD 7 and randomly allocated into Control or IUGR groups. All pregnant mice were housed at 23 ± 1 °C with a 15:9 h light:dark cycle and access to an allergen-free diet (Specialty Feeds, Glen Forrest, WA, Australia) and water *ad libitum*. Dams in the Control group were housed in normoxic conditions (21% oxygen) throughout gestation. As shown in Fig. 1, dams in the IUGR group were housed in a hypoxic chamber (10.5% oxygen maintained by continuous infusion of excess nitrogen gas) from GD 11 to 17.5 and then returned to normoxic conditions (21% oxygen) until birth at GD ~ 21.[28,29] Offspring from dams exposed to hypoxia are referred to as IUGR offspring and offspring from dams exposed to normoxic conditions are referred to as Control offspring.

Offspring were weighed at birth. After weaning, offspring were separated by sex to different cages, with each cage housing between 1 and 4. At 8 weeks of age, between 8 - 10 am (2 - 4 h after lights on), one male and one female offspring from each litter (Control male, $n = 11$; Control female, $n = 12$; IUGR male, $n = 12$; IUGR female, $n = 11$) were euthanised in randomised order with ketamine (240 mg/kg) and xylazine (12 mg/kg) by intraperitoneal injection.[30] Body weight, abdominal circumference, crown-rump length, liver and brain weight were recorded at post-mortem. Brain tissues were flash frozen with isopentane, liver tissues were snap frozen with liquid nitrogen, and all tissues were stored at −80°C.

RNA extraction

Whole brain samples were dissected to isolate the hypothalamus which was homogenised.[25] Liver sections (approximately 50 mg) were isolated and homogenised. Total RNA for both tissues was extracted using QIAzol Lysis Reagent (Qiagen Pty Ltd., Melbourne, VIC, Australia) according to manufacturer's instructions and then resuspended in an appropriate quantity of nuclease-free water (approximately 30 µL) and stored at −80°C.[25] The RNA samples were assessed for yield and quality using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Reverse transcription

The RNA was DNAse treated (Promega, Madison, WI, USA) to remove any genomic DNA contamination. Hypothalamic RNA (1 µg) was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Liver RNA (5 µg) was reverse transcribed to cDNA using Moloney Murine Leukemia Virus reverse transcriptase (Promega), purified using the QIAquick Column PCR Purification Kit (Qiagen) and eluted into nuclease-free water.[25]

Real-time polymerase chain reaction (PCR)

Analyses of relative mRNA expression levels for *Bmal1*, *Per2, Reverbα, Pparα*, *Pparγ, Pgc1α, Kiss1* and *Kiss1r* were performed through standard quantitative polymerase chain reaction (PCR) on the Rotorgene 6000 (Qiagen) using QuantiNova SYBR Green PCR Master Mix (Qiagen). Primer sequences are listed in Table 1 with *Pparα* and *Pparγ* designed using Primer-BLAST (National Centre for Biotechnology Information, Bethesda, MD, USA). The PCR cycling conditions were as follows: an initial denaturation step of 95 °C for 2 min, followed by at least 40 cycles of denaturation at 95 °C for 5 s and annealing of primers for 15 s . Melt curve analysis and DNA sequencing were performed to confirm amplification specificity.

Relative mRNA expression levels of clock (*Bmal1, Per2, Reverbα*), metabolic (*Pparα, Pparγ, Pgc1α*) and kisspeptin (*Kiss1* and *Kiss1r*) were measured in both the liver and hypothalamus samples. Expression of the target genes in each sample was normalised using geometric means calculated from the expression

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Table 1. Forward and reverse primer sequences for real-time PCR

Gene	Forward primer sequence (5'–3')	Reverse primer sequence (5'–3')	Product length (base pairs)
Bmal1[31]	CGTGCTAAGGATGGCTGTTC	CTTCCCTCGGTCACATCCTA	166
Per2[31]	AACAAATCCACCGGCTACTG	CTCCGGTGAGACTCCTCTTG	145
Reverbα[31]	CGGGGCTCACTCGTCTCCCT	GCTCGGGGAGGAGCCACTAGA	185
Pparα	GCATTTGGGCGTATCTCACC	CTTGACCAGCCACAAACGTC	119
Pparγ	AAGAAGCGGTGAACCACTGA	GGAATGCGAGTGGTCTTCCA	154
Pgc1α[32]	TCTGGAACTGCAGGCCTAACTC	GCAAGAGGGCTTCAGCTTTG	96
Kiss1[33]	CTCTGTGTCGCCACCTATGG	AGGCTTGCTCTCTGCATACC	126
Kiss1r[33]	CTGTCAGCCTCAGCATCTGG	AGCAGCGGCAGCAGATATAG	172
Hprt[34]	TGAAGTACTCATTATAGTCAAGGGCA	CTGGTGAAAAGGACCTCTCG	109
Ppia[33]	AGCATACAGGTCCTGGCATC	TTCACCTTCCCAAAGACCAC	127
Sdha[33]	TGGGGAGTGCCGTGGTGTCA	CTGTGCCGTCCCCTGTGCTG	149
Tbp[33]	GGGAGAATCATGGACCAGAA	CCGTAAGGCATCATTGGACT	113

Bmal1, brain and muscle Arnt-like 1; F, forward; Hprt, hypoxanthine-guanine phosphoribosyltransferase; Kiss1, kisspeptin; Kiss1r, kisspeptin receptor; PCR, polymerase chain reaction; Per2, period circadian regulator 2; Pgc1α, peroxisome proliferator-activated receptor γ coactivation 1 α; Ppar, peroxisome proliferator-activated receptor; Ppia, peptidylprolyl isomerase A; Rev-erbα, nuclear receptor subfamily 1 group D member 1; R, reverse; Sdha, succinate dehydrogenase complex subunit A; Tbp, TATA box binding protein.

Table 2. Body characteristics at 8 weeks of age

	Control		IUGR	
	Male ($n = 11$)	Female ($n = 12$)	Male ($n = 12$)	Female ($n = 11$)
Body weight (g)	23.00 ± 2.40	20.15 ± 1.95#	21.15 ± 2.05*	18.60 ± 0.97 #*
Crown-rump length (cm)	10.13 ± 0.15	9.88 ± 0.10#	9.65 ± 0.14*	9.28 ± 0.13#*
Abdominal circumference (cm)	7.50 ± 0.18	7.23 ± 0.07	7.50 ± 0.15	7.31 ± 0.07

Body weight data are median ± interquartile range, crown-rump length and abdominal circumference data are mean ± SEM.
*Denotes significantly different compared with Control ($p < 0.05$, two-way ANOVA or Kruskal–Wallis one-way ANOVA for body weight).
#Denotes significantly different compared with males ($p < 0.05$, two-way ANOVA or Kruskal–Wallis one-way ANOVA for body weight). IUGR, intrauterine growth restriction.

of reference genes: hypoxanthine phosphoribosyltransferase (*Hprt*), peptidylprolyl isomerase A (*Ppia*), succinate dehydrogenase complex subunit A (*Sdha*) and TATA box binding protein (*Tbp*) in the liver; and *Hprt, Ppia* and *Tbp* in the hypothalamus.[35]

Statistical analysis

Data were transformed where necessary to ensure that the assumptions of normality and homoscedasticity of variances for the parametric tests were satisfied. Birth weight was compared using a *t*-test to determine the effect of treatment (Control vs IUGR). Body characteristics and gene expression in the hypothalamus and liver were compared using two-way ANOVAs to determine the effect of treatment (Control vs IUGR) and sex (male vs female) on mRNA expression of *Bmal1*, *Per2*, *Reverbα*, *Pparα*, *Pparγ*, *Pgc1α*, *Kiss1* and *Kiss1r*. Data which do not fit a normal distribution (body weight at 8 weeks, hepatic *Bmal1* expression and hypothalamic *Per2* expression) were compared using two Kruskal–Wallis one-way ANOVAs. All normally distributed data are presented as mean ± SEM, data which do not fit a normal distribution are displayed as median ± interquartile range, with *p*-values < 0.05 considered statistically significant for all analyses. All statistical analysis was performed in SigmaPlot (Version 14.5, Grafiti LLC, Palo Alto, CA, USA). Graphs were generated using GraphPad PRISM (Version 10.3, GraphPad Software, Boston, MA, USA).

Results

Growth outcomes

Following mid-gestation hypoxia, the IUGR offspring had lower birth weight compared with Control offspring (Control ($n = 57$), 1.45 ± 0.02 g; IUGR ($n = 49$), 1.37 ± 0.03 g, $p = 0.047$). There was no significant difference in litter size between IUGR and Control offspring (Control ($n = 16$), 3.56 ± 0.38 offspring per litter; IUGR ($n = 15$), 3.27 ± 0.25 offspring per litter; $p = 0.522$).

At 8 weeks of age, IUGR offspring remained smaller than Control offspring, with lower body weight ($p < 0.001$, Table 2) and shorter crown-rump length ($p < 0.001$, Table 2), but similar abdominal circumference between groups ($p = 0.722$, Table 2). At 8 weeks of age, female offspring had lower body weight ($p < 0.001$, Table 2) and shorter crown-rump length ($p = 0.023$, Table 2) than male offspring, but abdominal circumference was comparable between sexes ($p = 0.098$, Table 2). There was no interaction between treatment and sex in crown-rump length ($p = 0.662$, Table 2) or abdominal circumference ($p = 0.757$, Table 2).

At 8 weeks of age, absolute brain weight was similar between IUGR and Control offspring ($p = 0.141$, Table 3), but IUGR offspring had greater brain weight relative to body weight than Control offspring ($p = 0.015$, Table 3). There was no effect of sex on absolute brain weight ($p = 0.810$, Table 3), but female offspring had greater brain weight relative to body weight than male offspring ($p < 0.001$, Table 3). At 8 weeks of age, IUGR offspring had lower absolute liver weight than Controls ($p = 0.016$, Table 3), but no difference in liver weight relative to body weight between the groups ($p = 0.874$; Table 3). Female offspring had lower absolute liver weight than male offspring ($p < 0.001$, Table 3) and also lower

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Table 3. Brain and liver weights at 8 weeks of age

	Control		IUGR	
	Male ($n = 11$)	Female ($n = 12$)	Male ($n = 12$)	Female ($n = 11$)
Brain weight (g)	0.39 ± 0.01	0.38 ± 0.01 ($n = 10$)	0.37 ± 0.01	0.38 ± 0.00
Brain:body weight (%)	1.69 ± 0.05	1.86 ± 0.04# ($n = 10$)	1.75 ± 0.05*	2.03 ± 0.04*#
Liver weight (g)	1.32 ± 0.05	1.00 ± 0.03#	1.21 ± 0.03*	0.92 ± 0.04*#
Liver:body weight (%)	5.68 ± 0.10	5.00 ± 0.12#	5.79 ± 0.33	4.99 ± 0.17#

Data are mean ± SEM.
*Denotes significantly different compared with Control ($p < 0.05$, two-way ANOVA).
#Denotes significantly different compared with males ($p < 0.05$, two-way ANOVA). IUGR, intrauterine growth restriction.

liver weight relative to body weight than male offspring ($p < 0.001$, Table 3). There was no interaction between treatment and sex in absolute brain weight ($p = 0.115$, Table 3), brain relative to body weight ($p = 0.203$, Table 3), absolute liver weight ($p = 0.695$, Table 3) or liver relative to body weight ($p = 0.785$, Table 3).

Clock gene expression

Expression of the core clock gene *Bmal1* was lower in in the hypothalamus of IUGR offspring compared with Control ($p < 0.001$, Fig. 2A), but there was no difference in hepatic *Bmal1* expression between the groups ($p = 0.668$, Fig. 2B). There was no sex effect on *Bmal1* expression in either the hypothalamus ($p = 0.317$, Fig. 2A) or liver ($p = 0.057$, Fig. 2B). There was no interaction of sex and treatment on *Bmal1* expression in the hypothalamus ($p = 0.176$, Fig. 2A).

Hypothalamic *Per2* expression was comparable between treatment groups ($p = 0.565$, Fig. 2C) and sexes ($p = 0.991$, Fig. 2C). In the liver, there was no difference in *Per2* expression between IUGR and Control offspring ($p = 0.214$, Fig. 2D), but female offspring had higher hepatic *Per2* expression than male offspring ($p = 0.005$, Fig. 2D). There was no interaction between sex and treatment on *Per2* expression in the liver ($p = 0.605$, Fig. 2D).

Hypothalamic *Reverbα* expression was lower in IUGR compared with Control offspring ($p = 0.047$, Fig. 2E), but hepatic *Reverbα* expression was similar between groups ($p = 0.532$, Fig. 2F). In both the hypothalamus and liver, *Reverbα* expression was similar between sexes (hypothalamus, $p = 0.509$, Fig. 2E; liver, $p = 0.084$, Fig. 2F). There was no interaction between sex and treatment on *Reverbα* expression in either the hypothalamus ($p = 0.606$, Fig. 2E) or liver ($p = 0.686$, Fig. 2F).

Metabolic gene expression

There were no differences in *Pparα* expression between Control and IUGR offspring in either the hypothalamus or liver (hypothalamus, $p = 0.415$, Fig. 3A; liver, $p = 0.066$, Fig. 3B). Similarly, hypothalamic and hepatic *Pparγ* expression were also comparable between IUGR and Control offspring (hypothalamus, $p = 0.081$, Fig. 3C; liver, $p = 0.623$, Fig. 3D). Further, there were no changes in hypothalamic or hepatic *Pgc1α* expression between Control and IUGR offspring (hypothalamus, $p = 0.096$, Fig. 3E; liver, $p = 0.213$, Fig. 3F).

Metabolic gene expression was also similar between sexes in the hypothalamus (*Pparα*, $p = 0.754$, Fig. 3A; *Pparγ*, $p = 0.977$, Fig. 3C; *Pgc1α*, $p = 0.855$, Fig. 3E) and in *Pparγ* expression in the liver ($p = 0.236$, Fig. 3D). However, female offspring had higher hepatic expression of *Pparα* ($p < 0.001$, Fig. 3B) and *Pgc1α* ($p = 0.010$, Fig. 3F) than male offspring, irrespective of treatment.

There was no interaction between treatment and sex in any of the metabolic genes in the hypothalamus (*Pparα*, $p = 0.727$, Fig. 3A; *Pparγ*, $p = 0.057$, Fig. 3C; *Pgc1α*, $p = 0.766$, Fig. 3E) or liver (*Pparα*, $p = 0.537$, Fig. 3B; *Pparγ*, $p = 0.736$, Fig. 3D; *Pgc1α*, $p = 0.843$, Fig. 3F).

Kisspeptin gene expression

There was no difference in hypothalamic *Kiss1* expression between IUGR and Control offspring ($p = 0.220$, Fig. 4A), but female offspring had increased hypothalamic *Kiss1* expression compared with male offspring ($p < 0.001$, Fig. 4A). There was no interaction between treatment and sex on hypothalamic *Kiss1* expression ($p = 0.145$, Fig. 4A). The IUGR offspring had decreased hypothalamic *Kiss1r* expression compared with Control offspring ($p = 0.018$, Fig. 4B), and male offspring had increased hypothalamic *Kiss1r* expression compared with female offspring ($p = 0.003$, Fig. 4B). There was no interaction between treatment and sex on hypothalamic *Kiss1r* expression ($p = 0.128$, Fig. 4B). In the liver samples, *Kiss1* and *Kiss1r* expression were too low to obtain quantifiable data.

Discussion

With the interest of understanding the mechanism underlying the strong associations found between IUGR and the development of metabolic disease in later life,[36] we proposed that mid-gestation maternal hypoxia-induced IUGR may disrupt circadian rhythms and *Kiss1*/*Kiss1r* signalling leading to developmentally programmed metabolic disease. Our main finding that hypothalamic, but not hepatic, *Bmal1* and *Reverbα* expression were decreased in IUGR offspring from hypoxic pregnancies compared with Controls suggests dysregulation between the hypothalamic and hepatic circadian clocks in IUGR offspring. Furthermore, IUGR offspring had decreased hypothalamic *Kiss1r* expression, possibly indicating disrupted *Kiss1*/*Kiss1r* signalling in the hypothalamus and therefore altered hypothalamic control over metabolism and circadian rhythms.

Intrauterine programming of adulthood outcomes occurs during key windows of developmental plasticity, and the timing and duration of a prenatal insult can alter its long-term effects.[21,37] The IUGR mouse model used in this study, mid-gestation maternal hypoxia-induced IUGR, targeted key developmental windows for the liver and hypothalamus.[8,9] Consistent with previous publications, IUGR offspring were smaller at birth and 8 weeks of age compared with Control offspring.[28–30,38,39] Of relevance to this study, we reported that the IUGR offspring had greater brain relative to body weight at 8 weeks of age, consistent with asymmetric IUGR.[40] Asymmetric IUGR refers to nonuniform growth restriction wherein brain volume is spared at the expense of the viscera, caused by preferential redirection of energy to the brain when substrate delivery to a fetus is restricted in the latter half of gestation.[41] The IUGR offspring had smaller absolute liver weights at 8 weeks of age, but there was no difference in liver relative to

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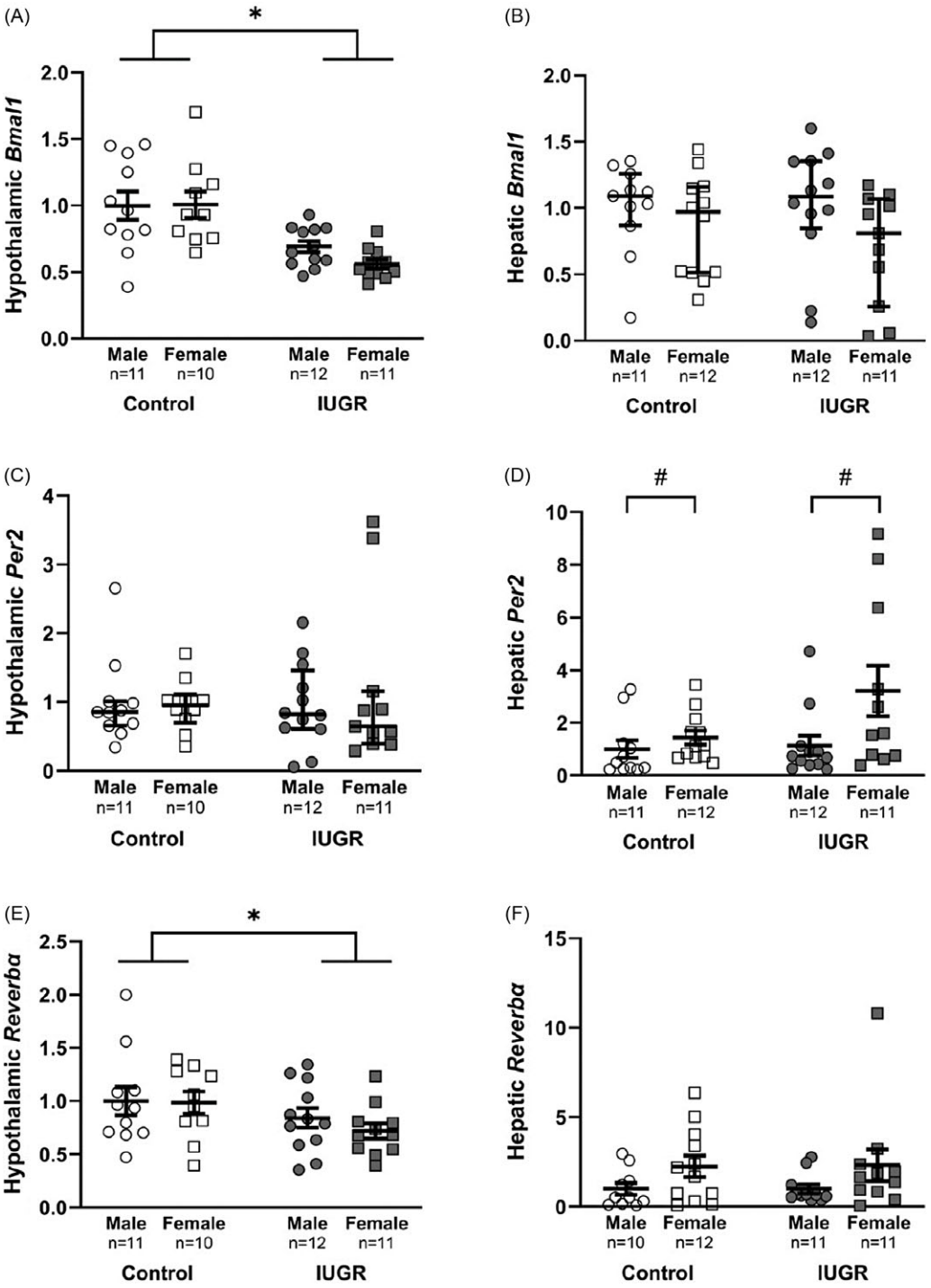

Figure 2. *Relative clock gene expression in mouse hypothalamus and liver.* Gene expression of hypothalamic *Bmal1* (*A*), hepatic *Bmal1* (*B*), hypothalamic *Per2* (*C*), hepatic *Per2* (*D*), hypothalamic *Reverbα* (*E*) and hepatic *Reverbα* (*F*). * denotes significantly different compared with Control ($p < 0.05$, two-way ANOVA or Kruskal–Wallis one-way ANOVA for hepatic *Bmal1* and hypothalamic *Per2* expression); # denotes significantly different compared with males ($p < 0.05$, two-way ANOVA or Kruskal–Wallis one-way ANOVA for hepatic *Bmal1* and hypothalamic *Per2* expression). All data are mean ± SEM except hepatic *Bmal1* (*B*) and hypothalamic *Per2* (*C*) expression are median ± interquartile range. Open circles, Control males; open squares, Control females; closed circles, IUGR males; closed squares, IUGR females. Bmal1, brain and muscle Arnt-like 1; IUGR, intrauterine growth restriction; Per2, period circadian regulator 2; Reverbα, nuclear receptor subfamily 1 group D member 1.

body weight between Control and IUGR offspring. Asymmetric IUGR is commonly associated with decreased liver weight relative to body weight[41]; however, decreased liver relative to body size in IUGR animals can resolve by early adulthood, potentially due to catch-up growth.[42,43] Therefore, any decreased liver weight in the present study was likely resolved by the 8-week time point of tissue collection. Similar to previous findings that we reported in respiratory studies,[28–30,38,39] we observed sex-dependent effects whereby somatic and liver growth outcomes were reduced in female offspring compared with male offspring. In sum, growth parameters in IUGR compared with Control offspring were consistent with asymmetric restricted growth *in utero*, suggesting that hypothalamic and hepatic development *in utero* were likely affected by maternal hypoxia.

Exposure to adverse prenatal conditions, including hypoxic conditions, can disrupt postnatal circadian rhythms.[11,44–46] In the present study, hypothalamic *Bmal1* and *Reverbα* expression were lower in IUGR offspring compared with Control offspring,

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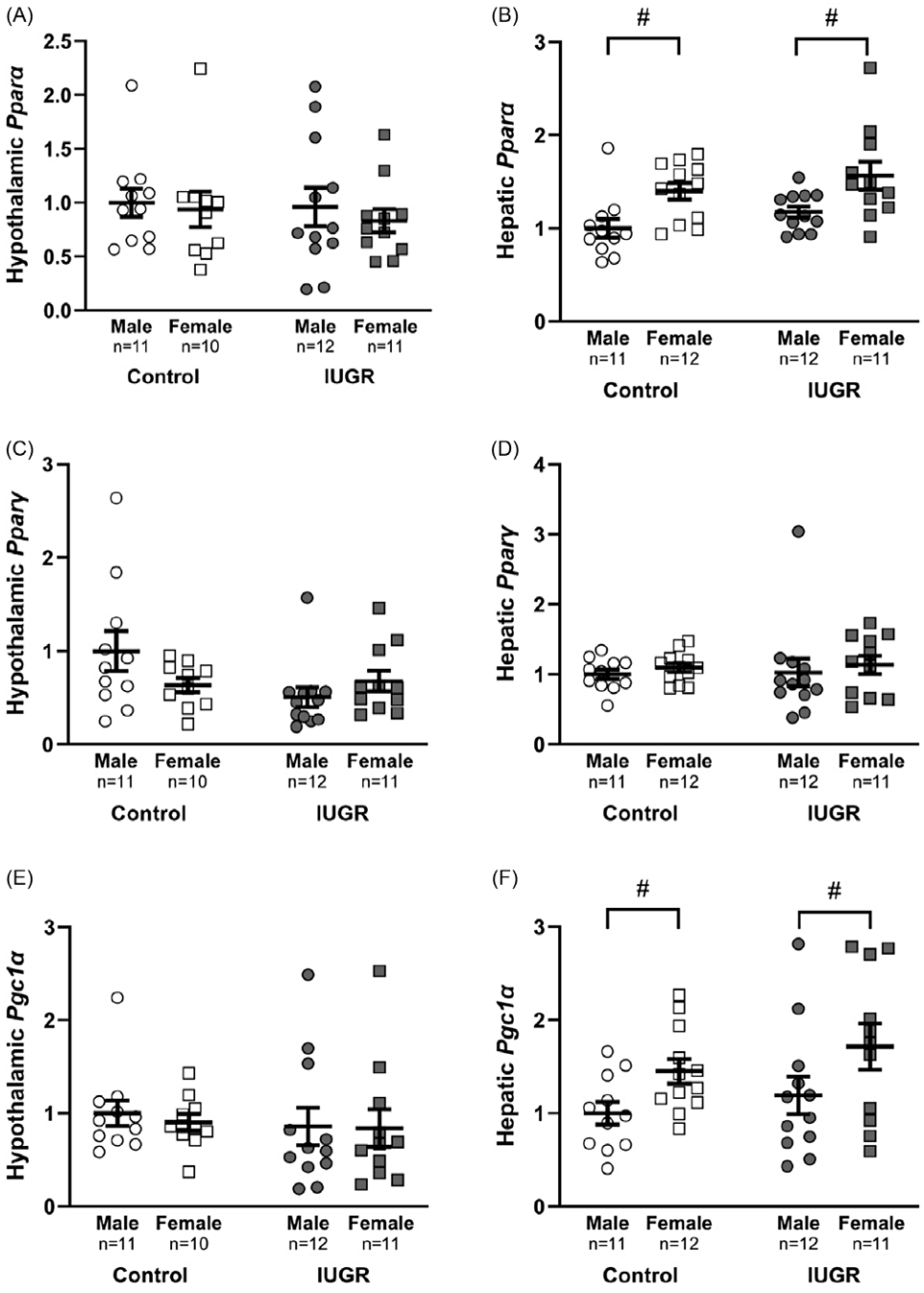

Figure 3. Relative metabolic gene expression in the hypothalamus and liver. Gene expression of hypothalamic *Pparα* (*A*), hepatic *Pparα* (*B*), hypothalamic *Pparγ* (*C*), hepatic *Pparγ* (*D*), hypothalamic *Pgc1α* (*E*) and hepatic *Pgc1α* (*F*). # denotes significantly different compared with males ($p < 0.05$, two-way ANOVA). Data are mean ± SEM. Open circles, Control males; open squares, Control females; closed circles, IUGR males; closed squares, IUGR females. IUGR, intrauterine growth restriction; Pgc1α, peroxisome proliferator-activated receptor γ coactivation 1α; Ppar, peroxisome proliferator-activated receptor.

although hypothalamic *Per2* expression was unaffected by prenatal hypoxia at this time of collection (i.e., around 2 - 4 h after lights on). Altered expression of only two of the three clock genes was unexpected as the components of the core circadian TTFL interact to regulate each other's expression, and changes in *Bmal1* expression are generally reflected in both *Per2* and *Reverbα*.[46,47] It is possible that *Per2* expression is impacted at other times of day, or changes are obscured by concomitant changes in amplitude, mesor and/or acrophase of *Per2* expression. A decrease in both *Bmal1* and *Reverbα* expression was also unexpected, given that these clock genes have opposing patterns of rhythmic oscillation whereby *Bmal1* expression peaks at the end of the dark phase, while *Reverbα* expression peaks in the middle of the light phase and *Reverbα* inhibits *Bmal1* expression.[17,18] The decrease in both *Bmal1* and *Reverbα* therefore suggests a potential disarrangement of the circadian TTFL in the hypothalamus of IUGR offspring, which would likely be associated with both desynchronisation of endogenous circadian clocks and metabolic disease.[48]

Although relatively subtle, significant changes in hypothalamic clock gene rhythmicity were observed in IUGR offspring at 8 weeks of age. The possibility remains that these programmed changes may be exacerbated as offspring age, or through metabolic challenges such as consumption of high energy diets.[46,49] More comprehensive, circadian studies are necessary to investigate whether the decreased *Bmal1* and *Reverbα* expression in IUGR offspring was due to changes in amplitude, mesor, acrophase of

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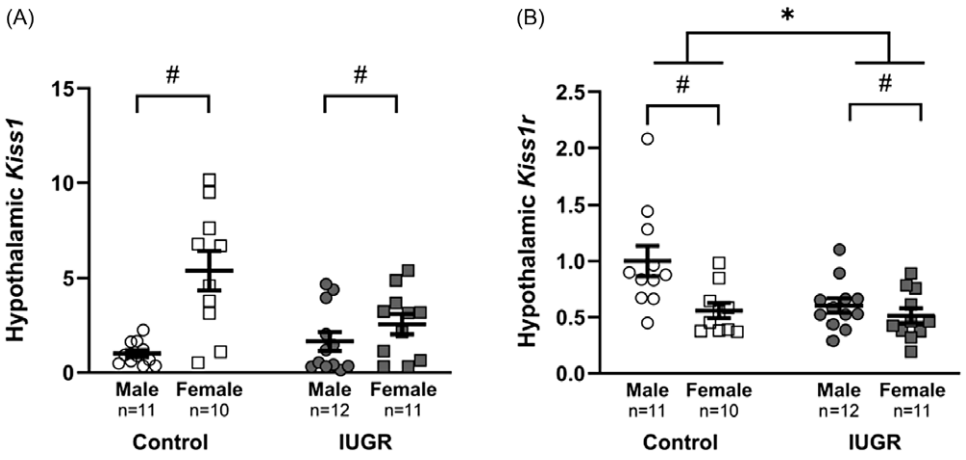

Figure 4. *Relative kisspeptin gene expression in the hypothalamus.* gene expression of hypothalamic *Kiss1* (*A*) and hypothalamic *Kiss1r* (*B*).* denotes significantly different compared with Control ($p < 0.05$, two-way ANOVA); # denotes significantly different compared with males ($p<0.05$, two-way ANOVA). Data are mean ± SEM. Open circles, Control males; open squares, Control females; closed circles, IUGR males; closed squares, IUGR females. Kiss1, kisspeptin; Kiss1r, kisspeptin receptor.

expression, or combinations thereof. Gene expression of other components of the core circadian TTFL including *Clock* and *Cry* should be investigated in future studies.

There were no differences in hepatic clock gene expression of IUGR and Control offspring at this single time of day (around 2 - 4 h after lights on), however changes in circadian networks could still be occurring at other times of day. Independent of treatment group, female offspring had higher hepatic *Per2* expression than male offspring; this sexual dimorphism was likely caused by lower amplitude of *Per2* rhythmic expression in the female compared with male liver.[50] These results suggest that the hepatic clock was not affected by mid-gestation maternal hypoxia-induced IUGR, potentially due to lower susceptibility to hypoxia as a circadian disruptor.[51] Hypoxia exerts tissue-specific effects and, consistent with the present study, causes more pronounced disturbances to rhythmic clock gene expression in the brain than the liver.[44,52] Interestingly, IUGR induced by a maternal protein-deficient diet from two weeks before mating and throughout pregnancy disrupts hepatic and cortical (*Bmal1, Clock, Per2* and *Reverbα*), but not hypothalamic, clock gene expression in 8-week-old mouse offspring, which may be due to differences in the type and timing of the prenatal insult between mouse models.[46]

Peripheral circadian clocks can function independently from the central clock, so disruptions to the central clock do not necessarily disrupt the liver.[53] Tissue-specific change in clock gene expression suggests that the hypothalamic and hepatic circadian clocks may be misaligned in young adult IUGR offspring. Mice with misalignment between their central and peripheral circadian clocks in different tissues develop metabolic symptoms including impaired glucose tolerance, hypoinsulinemia and hyperglycaemia.[53,54] Therefore interventions aimed at restoring circadian alignment may provide a potential treatment for developmentally programmed metabolic disease in adulthood.[55]

The *Pparα, Pparγ* and *Pgc1α* genes are recognised as links between the circadian system and energy metabolism and are sensitive to varied prenatal insults.[56–59] To our best knowledge, the present study is the first to investigate hypothalamic and hepatic *Pparα, Pparγ* and *Pgc1α* expression using this mouse model of mid-gestation maternal hypoxia-induced IUGR. The lack of change in metabolic gene expression in IUGR offspring may be due to the specific nature, timing or duration of prenatal insult. Moreover, like circadian dysregulation, metabolic symptoms programmed *in utero* may be exacerbated as offspring age or when offspring face metabolic challenges such as a high fat diet.[42,57,60] At 8 weeks old, the young adult mice in this study may have been too young to display changes in *Pparα, Pparγ* or *Pgc1α* expression. Rhythmic expression of Pparα, Pparγ and Pgc1α could also have obscured changes in gene expression as tissues were only collected at a single time point (8 - 10 am, i.e., early in the light phase) when hepatic *Pparα* and *Pparγ* expression is low.[18] Metabolic genes, including others such as *Rorα* and *Pparδ*, should be assessed in other metabolic tissues such as the pancreas or skeletal muscle to understand how these tissues may be differentially affected by adverse prenatal conditions and interact to contribute to metabolic disease.

The higher hepatic *Pparα* and *Pgc1α* expression in female offspring was likely caused by sexual dimorphism in overall rhythmic expression of these genes, that is, higher mesor in the female compared with male liver.[61] This sexual dimorphism may arise because Pparα protects against oestrogen-mediated hepatotoxicity,[62] and Pgc1α is required for oestrogen-dependent reactive oxygen species detoxification in the liver.[63]

Adverse prenatal conditions can program age- and sex-specific changes in postnatal *Kiss1* and *Kiss1r* expression,[27,64] for example, prenatal undernutrition has been shown to decrease hypothalamic *Kiss1* at juvenile (2-3 weeks in rats) and mature (29 weeks in rats) ages.[26,64] Though hypothalamic *Kiss1* expression was similar in IUGR and Control offspring at 8 weeks of age, there was a prenatal hypoxia-driven decrease in hypothalamic *Kiss1r* expression of IUGR compared with Control offspring. Kisspeptin signalling via Kiss1r modulates the activity of orexigenic and anorexigenic neurons in the hypothalamus, thus Kiss1r is key to regulating hunger/satiety and energy balance.[23,65] Decreased hypothalamic *Kiss1r* expression in IUGR offspring may therefore represent a deficit in central regulation of glucose homoeostasis and may contribute to developmentally programmed metabolic disease.

We also observed sexual dimorphism in hypothalamic *Kiss1* and *Kiss1r* expression. Higher hypothalamic *Kiss1* expression in female offspring may reflect the greater number of kisspeptin neurons found in the female compared with male hypothalamus.[66] The *Kiss1* neurons in the anteroventral periventricular nucleus of the hypothalamus are involved in generating the preovulatory gonadotropin-releasing hormone/luteinising hormone surge, which only occurs in females.[67,68] Lower hypothalamic *Kiss1r* expression in female compared with male offspring is consistent with previous evidence that female rats have lower hypothalamic *Kiss1r* expression than male rats at 45 days of age and evidence of a more pronounced effect of *Kiss1r* knockout on male sexual

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differentiation than female.[69,70] Moreover, a rise in Kiss1 mRNA in female offspring (and potentially kisspeptin expression) could be the stimulus for the decline in *Kiss1r* expression seen in our studies. Kisspeptin-induced desensitisation of Kiss1r signalling has been seen in rats,[71] and specifically results in a decline in Kiss1r mRNA expression in sheep.[72] Previous studies have reported that both *Kiss1* and *Kiss1r* are expressed only at low levels in liver tissue,[73,74] which may explain why we were unable to quantify hepatic *Kiss1* or *Kiss1r* expression.

In summary, our data align with our proposal that mid-gestation maternal hypoxia-induced IUGR can program disrupted circadian rhythms and *Kiss1/Kiss1r* signalling, which may subsequently result in developmentally programmed metabolic disease. The present study focused on mRNA expression as a reflection of developmentally programmed changes in gene expression following adverse prenatal conditions.[75–77] With these findings, future studies could incorporate metabolic parameters *in vivo* such as intraperitoneal glucose tolerance testing to confirm symptoms of metabolic disease, measure protein abundance in addition to mRNA data, or employ a transcriptomic approach to potentially identify a wider range of genes developmentally programmed by mid-gestation maternal hypoxia. Further research could also measure gene expression across multiple circadian time points, at an older age (e.g., 6 months old in mice) or after a metabolic challenge to investigate the onset and persistence of IUGR-related changes in clock genes and metabolism.

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition)) and has been approved by the institutional committee (Telethon Kids Institute Animal Ethics Committee (Project Number #264)).

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