Impaired miR449a-induced downregulation of *Crhr1* expression in low-birth-weight rats

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Abstract

Low birth weight (LBW) is related to increased incidence of common cardiovascular and metabolic disorders, and psychopathologies later in life. Recent studies have suggested that maternal malnutrition affects fetal hypothalamic–pituitary–adrenal (HPA) axis programming although the mechanism is unknown. We demonstrated that LBW offspring delivered from malnourished dams showed prolonged elevated plasma corticosterone concentrations when compared with those of normal-birth-weight (NBW) offspring and impaired downregulation of corticotropin-releasing factor receptor type 1 (CRF-R1, *Crhr1*) in the anterior pituitary in restraint. Restraint increased expression of miR449a, which we had previously demonstrated to be involved in *Crhr1* downregulation, in the anterior pituitary and serum exosomal miR449a contents through glucocorticoids in NBW offspring, but not in LBW offspring. Although plasma corticosterone concentrations were higher at 2000 h than at 0800 h in both LBW and NBW offspring, they were significantly higher in LBW offspring than in NBW offspring at 2000 and 0200 h. There were no significant diurnal changes in miR449a expression levels in the anterior pituitary of either NBW or LBW offspring, but the expression was significantly lower in LBW offspring than in NBW offspring at 1400, 2000, and 0200 h. The expression levels of GAS5, which inhibits glucocorticoid receptor (GR) binding to glucocorticoid-responsive element, in the anterior pituitary of LBW offspring were elevated when compared with those of NBW offspring. The downregulation of GR found in NBW offspring did not occur in restrained LBW offspring. These results indicate that impaired miR449a expression, probably induced by increased GAS5 expression, causes dysregulation of *Crhr1* expression in the anterior pituitary, resulting in prolonged HPA axis activation in restrained LBW offspring.

Key Words

- ▶ low birth weight
- ▶ psychopathology
- ▶ stress
- ▶ HPA axis
- ▶ CRF receptor
- ▶ miRNA

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Introduction

Low birth weight (LBW) is reportedly related to an increased prevalence of common cardiovascular and metabolic disorders later in life, including hypertension, coronary heart disease, type 2 diabetes, and hyperlipidemia (Levitt *et al.* 2000, Reynolds *et al.* 2001, Davies *et al.* 2004, Irving *et al.* 2004). Furthermore, accumulating

evidence indicates that LBW is associated with psychopathologies such as depression, schizophrenia, and autism (Wahlbeck *et al.* 2001, Indredavik *et al.* 2004, Raikkonen *et al.* 2008, Schendel & Bhasin 2008). These disorders are associated with abnormalities of the hypothalamic–pituitary–adrenal (HPA) axis such as disturbed circadian

rhythm, impaired cortisol suppression by dexamethasone (Holsboer-Trachsler *et al.* 1987, Hoshino *et al.* 1987, Vreeburg *et al.* 2009, Hempel *et al.* 2010), and abnormal adrenocorticotropin (ACTH) and cortisol responses to corticotropin-releasing factor (CRF) or stress (Young *et al.* 2000, Ehnvall *et al.* 2004, Corbett *et al.* 2006). Recent animal studies have indicated that maternal malnutrition-induced impairment of fetal programming of the HPA axis function is associated with adult-onset dysregulation of the axis (Chadio *et al.* 2007, Rumball *et al.* 2008, Buhl *et al.* 2010). Dysregulation of the HPA axis function is the most common and consistently reported abnormality in depression (Reus *et al.* 1985). In human studies, LBW has been shown to be related to alternations in HPA axis function in adult life, such as elevated basal morning cortisol levels, enhanced cortisol responses to ACTH₍₁₋₂₄₎ or CRF challenge test, and increased urinary cortisol metabolite excretion (Levitt *et al.* 2000, Ward *et al.* 2004, de Rooij *et al.* 2006). In addition to maternal malnutrition, prenatally stressed animals show LBW, enhanced basal levels of corticosterone and prolonged responses of the HPA axis to stress, impaired feedback regulation by glucocorticoids, and fewer glucocorticoid receptors (GRs) in the hippocampus (Clarke *et al.* 1994, Koehl *et al.* 1999, Bhatnagar *et al.* 2005, Viltart *et al.* 2006, Owen & Matthews 2007, Brunton & Russell 2010). Stress activates the synthesis and release of hypothalamic CRF, pituitary ACTH, and adrenal glucocorticoids. Corticotropes in the anterior pituitary are key elements in the regulatory mechanisms of the HPA axis: glucocorticoids down-regulate not only *Crf* (*Crh*) mRNA expression at the hypothalamus, but also CRF type 1 receptor (*Crf-r1*, *Crhr1*) and proopiomelanocortin (*Pomc*) mRNA expression and ACTH secretion in the anterior pituitary (Birnberg *et al.* 1983, Kovacs & Mezey 1987, Zhou *et al.* 1996, Ochedalski *et al.* 1998). To our knowledge, no reports have addressed the pathophysiological role of the anterior pituitary in dysregulation of the negative feedback system of the HPA axis under stress in LBW offspring. Therefore, in this study, we used rats delivered from malnourished dams to explore the role of the anterior pituitary in the mechanism underlying the abnormal HPA axis response to stress, as such abnormal responses may be associated with psychopathologies such as depression, schizophrenia, and autism in humans with LBW.

In this study, LBW offspring, who had been delivered from calorie-restricted (60% of that of control) dams, showed delayed restoration of plasma ACTH and corticosterone to basal levels and impaired *Crhr1* downregulation in the anterior pituitary during restraint. We then examined

the expression of miR449a, which we have previously demonstrated to be involved in *Crhr1* downregulation (Nemoto *et al.* 2013), in the anterior pituitary of restrained LBW or normal-birth-weight (NBW) rats. As microRNAs (miRNAs) have been recently shown to be present in exosomes released from cells and those exosomal miRNAs are stable in the blood or extracellular fluids (Hunter *et al.* 2008, Turchinovich *et al.* 2011), we attempted to detect exosomal miR449a in blood and culture medium of rat anterior pituitary cells. Finally, we examined the expression of *Gas5* non-coding RNA (ncRNA), which inhibits GR binding to glucocorticoid-responsive elements in DNA and blocks glucocorticoid-induced changes in gene expression (Kino *et al.* 2010), in order to explore its role in the regulation of miR449a expression.

Materials and methods

Animals

Wistar rats were maintained at $23 \pm 2^\circ\text{C}$ under a 12 h light:12 h darkness cycle (lights on at 0800 h and off at 2000 h) and were allowed to access laboratory chow and distilled water *ad libitum*. All experimental procedures were approved by the laboratory animals ethics review committee of Nippon Medical School.

Twenty proestrus female rats (age, 9 weeks) were mated with normal male rats. Dams were housed individually with free access to water and were divided into two groups; food intake of food-restricted (FR) dams was restricted to 60% of the control group, while control dams freely accessed food during the entire gestational period. Both dam groups delivered at gestational day 22; average numbers of offspring from control and FR dams were 15.0 (range 12–20) and 13.8 (range 12–17), and male to female ratios were 5:6 and 6:5 respectively. Mean body weights at birth of male offspring delivered from control dams (NBW) and offspring delivered from FR dams (LBW) were 5963 ± 5 mg ($n=25$) and 4618 ± 63 mg ($n=65$) ($P<0.001$) respectively. After parturition, FR dams were allowed to access food *ad libitum*. Both offspring groups were weaned at 21 days of age and were allowed to access laboratory chow and water *ad libitum*. Only male offspring were used in experiments, as female rats showed menstrual cycle-dependent responses to the stress (Iwasaki-Sekino *et al.* 2009). Each experimental group consisted of offspring from at least three litters. Mean body weights of NBW and LBW males used in this study at day 42 were 202.3 ± 3.5 and 193.2 ± 2.9 g ($P=0.145$, un-paired *t*-test), $n=60$ respectively.

Diurnal change analysis

Six-week-old NBW and LBW ($n=24$) offspring were killed by decapitation at 0200, 0800, 1400, and 2000 h. Trunk blood and their anterior pituitaries were collected.

Restraint stress exposure

Six-week-old rats were wrapped in a flexible wire mesh (12×12 mm) and kept for 30, 60, 90, or 120 min between 0900 and 1200 h in an isolated room. Rats were killed in the adjacent room immediately after restraint and their trunk blood and anterior pituitaries were collected. Non-stressed control rats were housed in a separate room and were killed in the same way and subjected to identical processing.

Primary culture of anterior pituitary cells

Thirty normal rats (age, 6 weeks) were killed by decapitation and their anterior pituitaries were removed under sterile conditions. Anterior pituitary cells were cultured as described previously (Nemoto *et al.* 2009, 2010). Cells were allowed to attach to the plating surfaces at 37 °C in a humidified 5% CO₂–95% air incubator for 4 days. Cells were then treated with dexamethasone at concentrations of 10 and 100 ng/ml for 1 h.

Exosome purification

Serum samples or culture media (250 µl) were mixed with ExoQuick exosome precipitation solution (System Bioscience, Inc., Mountain View, CA, USA). Purified exosome samples were resuspended in ultrapure water.

RNA extraction and real-time RT-PCR analysis

Total RNA was extracted from cultured pituitary cells and rat pituitaries using RNAiso Plus (Takara, Shiga, Japan).

The absorbance of each sample at wavelengths of 260 and 280 nm was assayed, and RNA purity was estimated to be the 260 nm/280 nm ratio (the 260 nm/280 nm ratio of all samples used in this study was higher than 1.7). For miRNA expression analysis, first-strand cDNA was synthesized at 37 °C for 1 h using 1 µg of denatured total RNA and then terminated at 85 °C for 5 min using Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR kit (Clontech Laboratories, Inc.). For mRNA and *Gas5* ncRNA expression analyses, first-strand cDNA was generated using 0.5 µg of denatured total RNA; the reaction mixture was incubated at 37 °C for 15 min, 84 °C for 5 s, and 4 °C for 5 min using a PrimeScript RT reagent kit with gDNA Eraser (Takara). PCR was performed by denaturation at 94 °C for 5 s and annealing extension at 60 °C for 30 s for 40 cycles, using SYBR premix Ex Taq (Takara) and specific primer set for rat *Pomc*, *Gr*, *Crhr1*, *Gas5*, or *Gapdh* (Table 1). To normalize each sample for RNA content, GAPDH, a house-keeping gene, and U6 small nuclear RNA (Clontech Laboratories, Inc.) were used for mRNA, ncRNA, and miRNA expression analyses respectively. Diluted normal rat pituitary cDNA and the second derivative method were used as the standard and for calculating Ct values respectively (Nolan *et al.* 2006).

Western blotting of *Crhr1*

The anterior pituitary tissues were lysed with TNE buffer (10 mM Tris–HCl, pH 7.8, 1% NP-40, 150 mM NaCl, and 1 mM EDTA) containing the Complete proteinase inhibitor cocktail (Roche Diagnostics). After centrifugation to remove debris, protein concentrations in the supernatant were measured. Protein extracts (20 µg) were mixed with 3 × SDS sample buffer (200 mM Tris–HCl, pH 6.8, 30% glycerol, 6% SDS, 0.03% bromophenol blue, and 3% β-mercaptoethanol) and boiled for 5 min. They were then separated by SDS–PAGE and transferred onto

Table 1 Primer sequences of the studied genes

Genes	Primers	Length (bp)	Accession number (GenBank)
<i>Pomc</i>	CCTCCATAGACGTGTGGAGCTG AAGGGCTGTTTCATCTCCGTTG	151 (180–330)	NM_139326
<i>Gr</i> (<i>Nr3c1</i>)	CATGTTAGGTGGGCGTCAAGTG GGTCATTTGGTCATCCAGGTGTAAG	91 (1756–1846)	NM_012576
<i>Crhr1</i> (<i>Crhr1</i>)	TCGGCTTTTCATCCTACGCAAC AATTGTAGGCGGCTGTCACCA	112 (478–589)	NM_030999
<i>Gas5</i>	AACTGAAGGCATGGCAAGCTC CACTGCACTGTCCACTTGTGAGA	81 (244–324)	NR_002704
<i>Gapdh</i>	GGCACAGTCAAGGCTGAGAATG ATGGTGGTGAAGACGCCAGTA	143 (242–384)	NM_017008

PVDF membranes. The membranes were blocked with 10% nonfat dried milk and primed with an anti-*Crhr1* antibody (C-20) (1:500) (Nemoto *et al.* 2013). Membranes were washed and incubated with HRP-conjugated anti-goat IgG (Jackson Immuno Research Laboratory, West Grove, PA, USA). Immunoreactivity was detected and quantified with ChemiDoc XRS (Bio-Rad Laboratories, Inc.) using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA). After detecting *Crhr1* signals, antibodies were stripped from membranes by incubation in Restore PLUS western blot stripping buffer (Thermo Scientific), followed by re-priming with anti- β -actin MAB (GeneScript, Piscataway, NJ, USA) and HRP-conjugated anti-mouse-IgG (Jackson Immuno Research Laboratory). Signals were then detected and quantified to normalize for differences in blotting efficiency and protein loading. Expression levels were calculated as follows: (intensity of 60-kDa band + the intensity of 40-kDa band)/intensity of β -actin. Data are given as a percentage of non-stressed or control values.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Tukey's *post hoc* test or using two-way ANOVA with the Bonferroni multiple comparisons post test using the Prism 5.0 software (GraphPad

Software, Inc., La Jolla, CA, USA). All results were expressed as mean $\pm$ S.E.M. Statistical significance was set at the level of $P < 0.05$.

Results

LBW offspring showed impaired feedback regulation of HPA axis

There were no significant differences in basal ACTH or corticosterone levels between NBW and LBW offspring when the restraint stress session started at 0900 h. Restraint stress significantly increased plasma ACTH and corticosterone levels at 30 ($n=6$, $P < 0.001$ for both ACTH and corticosterone, non-stressed vs 30 min) and 60 min ($n=6$, $P < 0.001$ for both ACTH and corticosterone, non-stressed vs 60 min), and both hormones were significantly lower at 90 ($n=6$, $P < 0.001$, for both ACTH and corticosterone, 60 min vs 90 min) and 120 min ($n=6$, $P < 0.001$, for both ACTH and corticosterone, 60 min vs 90 min) in NBW (Fig. 1A and B) offspring. Restraint stress exposure also significantly increased plasma ACTH and corticosterone levels in LBW offspring ($n=6$, $P < 0.001$, for both ACTH and corticosterone, non-stressed vs 30 and 60 min), and they remained elevated at significantly higher levels than those of NBW offspring at 90 min ($n=6$, $P < 0.001$ for both ACTH and corticosterone, NBW vs LBW offspring) and 120 min ($n=6$, $P < 0.0001$ for ACTH, NBW vs LBW) (Fig. 1A and B).

Figure 1

Impaired HPA axis regulation in restrained LBW offspring. NBW and LBW offspring were restrained for 30, 60, 90, or 120 min. Their plasma ACTH (A) and corticosterone (B) concentrations, and *Pomc* (C) and *Crhr1* (D) mRNA expression in the anterior pituitary were assayed. *Crhr1* protein expression in the anterior pituitary was assayed (E for typical image, F and G for three

independent blot images for NBW and LBW offspring respectively, and H for quantification results). mRNA and protein expression levels are shown as % of non-stressed (non) NBW offspring. $n=6$. *Indicates significance vs non using Turkey's *post hoc* analysis, #indicates significance vs NBW using Turkey's *post hoc* analysis.

Restraint stress significantly increased *Pomc* mRNA expression in the anterior pituitary at 30, 60, and 90 min in NBW offspring ($n=6$, $P<0.05$, $P<0.01$, and $P<0.05$ for non-stressed vs 30, 60, and 90 min respectively) and at 60 and 90 min LBW offspring ($n=6$, $P<0.05$, and $P<0.01$ for non-stressed vs 60 and 90 min respectively), and *Pomc* mRNA expression levels were restored to basal level at 120 min in NBW offspring while those of LBW offspring remained significantly high even at 120 min ($n=6$, $P<0.01$, NBW vs LBW) (Fig. 1C). Restraint stress significantly decreased *Crhr1* mRNA expression in the anterior pituitary at 60, 90, and 120 min in NBW offspring ($n=6$, $P<0.01$, $P<0.001$, and $P<0.0001$ for non-stressed vs 60, 90, and 120 min respectively), but *Crhr1* mRNA expression at 120 min was significantly higher in LBW offspring than in NBW offspring ($n=6$, $P<0.05$, NBW vs LBW) (Fig. 1D).

We detected *Crhr1* protein expression using anti-CRF receptor antibodies as described previously (Nemoto *et al.*

2013; Fig. 1E, F, and G). Restraint stress significantly decreased *Crhr1* protein at 60, 90, and 120 min in NBW offspring ($n=6$, $P<0.001$, $P<0.0001$, and $P<0.0001$ for non-stressed vs 60, 90, and 120 min respectively), and significantly decreased *Crhr1* protein only at 120 min in LBW offspring ($n=6$, $P<0.0001$ for non-stress vs 120 min) (Fig. 1H). The *Crhr1* protein levels at 90 min were significantly higher in LBW offspring than in NBW offspring ($n=6$, $P<0.01$, NBW vs LBW) (Fig. 1H).

Restraint-induced miR449a expression is impaired in LBW rats

Restraint stress significantly increased miR449a expression in the anterior pituitary and serum exosome in NBW offspring ($n=6$, $P<0.05$ for pituitary expression and serum exosome content, non-stressed vs 90 min) but not in LBW offspring ($n=6$, $P<0.05$ for pituitary expression and serum exosome content, NBW vs LBW) (Fig. 2A and B).

Figure 2

miR449a is expressed and secreted by anterior pituitary cells and stress- and dexamethasone-induced increase in expression of miR449a is abolished in LBW offspring. NBW and LBW offspring were restrained for 30, 60, 90, or 120 min, and miR449a in the anterior pituitary (A) and serum exosomes (B) were assayed. Expression of miR449a and exosomal miR449a contents from culture media of dexamethasone-treated anterior pituitary cells (C for expression and D for exosomal contents) and expression of *Crhr1* mRNA (E)

in cells were measured. Dexamethasone (30 μ g/kg body weight) was intraperitoneally injected into naive rats ($n=8$), and miR449a expression in the anterior pituitary (F), serum exosome miR449a contents (G), and *Crhr1* mRNA expression (H) in the anterior pituitary were assayed after 120 min of injection. *Indicates significance vs control (Co) or vs non-stressed group (non) using Turkey's *post hoc* analysis, #indicates significance vs NBW using Turkey's *post hoc* analysis.

Expression of miR449a and contents of exosomal miR449a from culture media of anterior pituitary cells were increased with dexamethasone at a concentration of 100 ng ($n=6$, $P<0.01$ for expression and $P<0.05$ for exosome contents, control vs 100 ng/ml), which significantly suppressed *Crhr1* mRNA expression in the anterior pituitary cells ($n=6$, $P<0.05$, control vs 100 ng/ml) (Fig. 2C, D, and E). Administration of dexamethasone (i.p., 30 μ g/kg body weight (BW)) to naive rats significantly increased miR449a expression in the anterior pituitary and serum exosome content ($n=6$, $P<0.001$ for anterior pituitary expression and $P<0.05$ for serum exosome content, saline vs 30 μ g/kg BW), which significantly suppressed *Crhr1* mRNA expression in the anterior pituitary ($n=8$, $P<0.001$, saline vs 30 μ g/kg BW) (Fig. 2F, G, and I).

Diurnal changes of plasma corticosterone and miR449a expression in the anterior pituitary

Plasma corticosterone concentrations were highest at 2000 h and lowest at 0200 h in NBW offspring ($n=6$, $P<0.001$, 2000 h vs 0200 h), and were highest at 2000 h and lowest at 0800 h in LBW offspring ($n=6$, $P<0.001$, 2000 h vs 0800 h) (Fig. 3A). The plasma corticosterone concentrations at 2000 and 0200 h were significantly higher in LBW offspring than in NBW offspring ($n=6$, $P<0.05$ at 2000 h and $P<0.001$ at 0200 h, NBW vs LBW) (Fig. 3A). Although there were no significant changes in the anterior pituitary miR449a expression levels at 0800, 1400, 2000, and 0200 h in either NBW or LBW offspring, the expression levels at 2000 and 0200 h were significantly lower in LBW offspring than in NBW offspring ($n=6$, $P<0.001$ at 2000 h and $P<0.05$ at 0200 h, NBW vs LBW) (Fig. 3B).

GAS5 expression in anterior pituitary is elevated in LBW offspring

Restraint stress did not induce any significant changes in GAS5 expression in the anterior pituitary in either NBW or LBW offspring; however, GAS5 expression levels in the anterior pituitary were significantly higher in LBW offspring than in NBW offspring before and during restraint ($n=6$, $P<0.001$ for non-stressed and $P<0.05$ at 30, 60, 90, and 120 min, NBW vs LBW) (Fig. 4A).

Restraint stress significantly increased anterior pituitary *Gr* mRNA expression at 60 min in NBW offspring ($n=6$, $P<0.05$, non-stress vs 60 min), and at 60, 90, and 120 min in LBW offspring ($n=6$, $P<0.05$ at 60, 90, and

Figure 3

Diurnal change in corticosterone and miR449a expression in the anterior pituitary. NBW and LBW offspring were killed at 0800, 1400, 2000, and 0200 h. Plasma corticosterone (A) and miR449a expression in the anterior pituitary (B) were assayed. $n=6$. *Indicates significance vs 0800 h using Turkey's post hoc analysis, #indicates significance vs NBW using Turkey's post hoc analysis.

120 min, non-stress vs restraint). Although the restraint-induced increase in *Gr* mRNA expression was restored to basal levels at 90 and 120 min in NBW offspring, it remained at significantly high levels at 90 and 120 min in LBW offspring ($n=6$, $P<0.05$ at 90 min and $P<0.01$ at 120 min, NBW vs LBW) (Fig. 4B).

Discussion

This study demonstrated impaired restoration of ACTH and corticosterone secretion and *Pomc* and *Gr* mRNA expression during restraint in the anterior pituitary of LBW offspring in accordance with previous reports (Buhl et al. 2007, 2010). Although several research groups have attempted to elucidate the mechanism underlying the dysfunction of the HPA axis in LBW offspring by analyzing GRs in the hypothalamus, hippocampus, and amygdala (Lesage et al. 2002, Sebaai et al. 2002, 2004), the details of this mechanism remain unclear. In this study, we focused on the anterior pituitary, where CRF acts to stimulate POMC expression and ACTH secretion through *Crhr1* and glucocorticoids act to modulate ACTH secretion through GRs; our results indicated that mRNA and protein levels of *Crhr1* in the anterior pituitary were significantly

Figure 4

GAS5 expression and GR expression in the anterior pituitary under restraint. NBW and LBW offspring were restrained for 30, 60, 90, or 120 min, and their GAS5 (A) and GR mRNA (B) expressions in the anterior pituitary of LBW and NBW offspring were assayed. $n=6$. #Indicates significance vs NBW using Turkey's *post hoc* analysis, * indicates significance vs non-stressed group (non) using Turkey's *post hoc* analysis.

reduced by restraint in both NBW and LBW offspring, but *Crhr1* mRNA levels at 120 min and *Crhr1* protein levels at 90 min were significantly higher in restrained LBW offspring than in restrained NBW offspring. These results therefore indicate that impaired downregulation of *Crhr1* is involved in the prolonged *Pomc* mRNA expression and ACTH secretion in the anterior pituitary of LBW offspring.

The expression of miR449a in the anterior pituitary was increased by restraint in NBW offspring but not in LBW offspring. We have previously demonstrated that overexpression of miR449a suppressed mRNA and protein expression of *Crhr1*, and knockdown of *miR449a* blocked dexamethasone-induced suppression of *Crhr1* expression (Nemoto *et al.* 2013), indicating that miR449a suppresses *Crhr1* expression. Results of a previous *in vitro* study had also indicated that the expression of miR449a was positively regulated by glucocorticoids, but not by CRF. ADX completely blocked restraint-induced increases in miR449a expression. Furthermore, we had previously demonstrated that expression levels of *Crhr1* mRNA and protein were significantly higher at several points during restraint stress exposure in ADX rats than in sham rats, although the expression of *Crhr1* mRNA and protein were

significantly reduced during immobilization (Nemoto *et al.* 2013). In this study, we demonstrated that both miR449a expression in the anterior pituitary and plasma corticosterone levels were highest at 2000 h and lowest at 0200 h in NBW offspring. Taken together, our findings indicate that corticosterone-induced miR449a expression in the anterior pituitary was defected and that inhibition of miR449a consequently impairs the downregulation of *Crhr1* expression during restraint in LBW offspring.

We then examined pituitary GAS5 expression because GAS5 inhibits GR binding to glucocorticoid-responsive elements in DNA and blocks glucocorticoid-induced changes in gene expression (Kino *et al.* 2010). We actually found that the expression of GAS5 was significantly higher in LBW offspring than in NBW offspring in this study. Moreover, we found that both miR449a expression in the anterior pituitary and plasma corticosterone levels have diurnal rhythm in NBW offspring, while rhythm of plasma corticosterone levels were impaired in LBW offspring. Therefore, it seems likely that overexpression of GAS5 blocks corticosterone action, thereby causing suppression of miR449a expression in LBW offspring despite prolonged elevation of *Gr* mRNA expression in the anterior pituitary. The elevated expression of GAS5 may be an obstacle to GR autoregulation in the anterior pituitary of LBW offspring during restraint. Although GAS5 expression is reportedly increased in the hippocampus by psychogenic stress in mice (Meier *et al.* 2010), there were no changes in GAS5 expression in the pituitary during immobilization in either NBW or LBW offspring in this study. Moreover, we were unable to detect any changes in GAS5 expression in dexamethasone-treated anterior pituitary cells *in vitro* (data not shown), thus suggesting that GAS5 expression in the anterior pituitary is regulated in a glucocorticoid-independent manner. However, glucocorticoids may be involved in the regulation of GAS5 expression in the fetal pituitary as hypercortisolemia is present in FR dams, and hypercortisolemia appears to modulate the programming of the HPA axis in fetuses (Catalani *et al.* 2002).

In this study, we detected miR449a expression in both the anterior pituitary and exosomes in the culture medium of anterior pituitary cells and serum. Serum exosomal miR449a increased in parallel with miR449a mRNA expression in the anterior pituitary of restrained rats. As miR449a is expressed in the brain and various peripheral tissues (Linsen *et al.* 2010), the origin of serum exosomal miR449a is unclear. However, the results of this study indicate that the anterior pituitary is one of the original sites for miR449a-containing exosomes in peripheral circulation, as exosomal miR449a in the

culture medium of anterior pituitary cells was increased by dexamethasone. As exosomes carry proteins, RNAs, and miRNAs, mediate cell-to-cell signaling, and are released into extracellular fluids in a paracrine manner (Sahoo *et al.* 2011), miR449a-containing exosomes may have a paracrine role in the anterior pituitary. Furthermore, because restraint significantly increased serum exosomal miR449a in NBW offspring but not in LBW offspring and the sequences of miR449a and its binding region of CRFR1 3'UTR have 100% homologies between human and rats, quantification of serum exosomal miR449a would be a useful tool for evaluation of dysregulation of the HPA axis in stress in humans.

This study demonstrated that impairment of miR449a expression, which is probably caused by enhanced expression of GASS, induces prolonged elevation of plasma corticosterone levels via delayed lowering of *Crhr1* expression in the anterior pituitary of LBW offspring delivered from malnourished dams.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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