

## BASIC SCIENCE ARTICLE



# Downregulation of insulin receptor isoform A in the forebrain of fetal growth-restricted rats

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**BACKGROUND:** Children with asymmetrical fetal growth restriction (FGR), whose head size is relatively preserved, often have a poor neurodevelopmental prognosis. Insulin receptor isoform A (IR-A) is predominantly expressed in neurons and is important in neurodevelopment. This study investigated changes in brain IR-A expression in neonatal FGR model rats.

**METHODS:** FGR model rats were generated by maternal caloric restriction (CR). Glucose uptake and the expression of glucose transporter (GLUT) genes in the brain and liver, and of the IR-A gene in the brain were compared between CR and control group neonates. Gene expression in the brain was examined by RNA sequencing. Brain IR-A localization was analyzed using immunohistochemistry.

**RESULTS:** The brain-to-liver ratios for organ weight and glucose uptake were significantly higher in CR rats. GLUT gene expression was maintained in the CR brain. Whole brain IR-A expression was reduced in CR rats. Furthermore, IR-A expression was decreased in the forebrain of CR rats, but not changed in the hindbrain.

**CONCLUSION:** The regional differences in IR-A in the FGR rat brain indicate that an endocrinological mechanism regulates brain IR-A to maintain brain function under nutrient deficiency. However, a decrease in IR-A in the fetal period may cause postnatal brain impairment.

*Pediatric Research*; <https://doi.org/10.1038/s41390-025-04372-8>

### IMPACT:

- Insulin receptor isoform A (IR-A) expression is reduced in the neonatal brain of asymmetrical fetal growth restriction (FGR) model rats generated by maternal caloric restriction.
- IR-A expression is decreased in the forebrain, which is important for cognitive brain functions, whereas IR-A expression is maintained in the hindbrain, which is important for basic vital activities.
- The expression of genes related to forebrain development is significantly decreased, while the expression of genes related to hindbrain development is increased in neonatal FGR model rats. These results can explain why FGR infants have a poor neurodevelopmental prognosis despite their brain size being relatively protected.

## INTRODUCTION

Fetal growth restriction (FGR) is a condition in which the growth of the fetus *in utero* is impaired and the size of the fetus is smaller than expected for the corresponding gestational age.<sup>1</sup> FGR is one of the leading causes of perinatal mortality and morbidities,<sup>2</sup> as well as the cause of numerous long-term problems. One cause of FGR is the inability of the fetus to receive adequate amounts of nutrition *in utero*. Such fetuses are known to have an asymmetrical body proportion in which the brain size is relatively preserved compared with the trunk; this is known as “brain-sparing”.<sup>3</sup> One mechanism that might underlie this phenomenon is the redistribution of blood flow, i.e., blood flow is preferentially distributed to the brain compared with other organs.<sup>4,5</sup> In addition, Simmons et al. reported that glucose transport to the brain is maintained but not to other organs.<sup>6,7</sup> However, the detailed mechanism of “brain-sparing” is not known.

An important challenge in infants with FGR is that, even though brain size is relatively preserved, they still have a poor neurodevelopmental prognosis. A number of systematic reviews report that FGR children are at risk of poor neurodevelopmental outcomes.<sup>8,9</sup> These children also tend to suffer from learning and attention disabilities, even at school age.<sup>10,11</sup> Various animal models have demonstrated that FGR alters brain structure and functions. FGR disturbs the normal development of gray and white matter and the connectivity of developing neurons.<sup>1</sup> However, the reason for the poor neurodevelopmental prognosis, despite the relatively preserved brain size, is not known.

Insulin signaling is a key factor in the regulation of fetal growth, and insulin receptors (IRs) have specific functions in the fetal period. IRs mainly consist of two structurally distinct isoforms, IR-A and IR-B. These are both encoded by the Insulin receptor (*INSR*) gene and differ by the skipping or inclusion of exon 11. IR-B has an

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Received: 16 January 2025 Revised: 5 August 2025 Accepted: 12 August 2025

Published online: 26 September 2025

additional 12 amino acids encoded by exon 11 in the C-terminal helix of the alpha subunit, while IR-A lacks this segment.<sup>12</sup> IR-B is abundant in numerous differentiated cells and insulin-targeted tissues, such as liver and adipose tissue. IR-B enhances glucose regulation of insulin (a metabolic effect). In contrast, IR-A stimulates cell proliferation by activating ERK1/2 and AKT signaling (a mitogenic effect) rather than regulating glucose levels.<sup>12,13</sup> IR-A is predominantly expressed during fetal development and embryogenesis and regulates tissue development in response to growth factors.<sup>14</sup> Furthermore, IR-A is predominantly expressed in neurons, while IR-B is rarely expressed in the brain.<sup>15</sup> IR-A is profoundly involved in neuron development and function,<sup>16</sup> neuronal survival and neuroprotective processes,<sup>17,18</sup> cognitive function via synaptic plasticity,<sup>16</sup> and synapse formation.<sup>15,19</sup>

The purpose of this study was to examine the hypothesis that brain IR-A expression is reduced in FGR fetuses with relatively preserved brain size. This may explain why neurodevelopmental prognosis is poor in asymmetrical FGR infants.

## METHODS

### Caloric-restriction model rat

Pregnant female Wistar rats, between 10 and 12 weeks of age were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were housed under a 14:10-h light-dark cycle with free access to laboratory chow and water. Experimental procedures were conducted in accordance with the standards of humane animal care and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. Pregnant rats were divided into two groups at embryonic day (E) 14.5. One group was fed *ad libitum* with regular chow, which was ground into a fine powder (Control). The other group was restricted to 50% of the calories consumed by the control dams between E14.5 and E21.5 (caloric restriction: CR). At E21.5, dams were anesthetized with isoflurane and pups were born by Cesarean section and immediately weighed. Newborn pups to be dissected for analyses were selected according to the following criteria. Based on the data for neonates ( $n = 140$ ) born from normally fed dams, a birth weight of 3.99 g was the  $-2.0$  standard deviation level. We therefore defined pups with a birth weight  $<3.99$  g from CR dams as eligible for inclusion in the CR group, and pups with a birth weight of 4.26–4.81 g from control dams as the control group (the birth weight range among normal pups at the  $\pm 1.0$  standard deviation level). Whole brain samples were weighed. Whole livers were also sampled and weighed to calculate the brain-to-liver weight ratio, which is a major indicator of the brain-sparing effect.<sup>20</sup>

### Glucose uptake assay

The glucose uptake capacity of neonatal organs was measured by 2-deoxyglucose (2-DG) uptake assays, using a modified method described by Kaur et al.<sup>21</sup> After fasting for 4 hours, pregnant female rats at E21.5 were treated intravenously with 0.1 g/kg of 2-DG and euthanized 60 min later. Individual neonate rats ( $n = 9$  in each group) were collected and 10 mg brain and liver samples were dissected. 2-DG uptake was measured by quantifying 2-DG6P accumulation using a commercial kit (Cosmo Bio, Tokyo, Japan) following the manufacturer's instructions. To calculate the glucose uptake rate of a whole organ, not per unit sample weight, we applied the following: an organ's glucose uptake =  $2\text{DG6P concentration } (\mu\text{M}) \times [\text{weight of whole organ (mg)} / \text{sectioned sample weight (mg)}]$ .

### Gene expression analysis

Organ samples were corrected from 6 rats per group, which consisted of 2 pups per litter meeting our definition of FGR and control selected from 3 individual litters. These samples were immediately frozen in liquid nitrogen and then kept at  $-80^\circ\text{C}$  before homogenization. RNA was extracted using a TRIzol method (Invitrogen, Carlsbad, CA). cDNA was synthesized from isolated total RNA samples using a PrimeScript<sup>TM</sup> RT reagent kit (Takara Bio, Tokyo, Japan), following the manufacturer's protocols. Quantitative measurement of transcripts was performed by RT-PCR on the StepOnePlus system (Applied Biosystems, Foster City, CA) using  $10 \mu\text{mol/L}$  primer pairs and SYBR<sup>TM</sup> Green PCR Master Mix (Applied Biosystems). The primers used are listed in Supplement Table. RNA levels were normalized to those of the

Hypoxanthine phosphoribosyl transferase 1 (*Hprt1*) gene. *Ir-A* (210 bp) and *Ir-B* (246 bp) fragments were identified by being resolved on 2% agarose gels. For region-specific comparisons of brain IR isoform expression, we separated brain samples into two parts coronally, at the level of the lambda. We designated the rostral side as the forebrain, including the cerebrum, and the caudal side as the hindbrain, including the pons and the medulla. Expression levels of the IR isoforms in these two parts were quantified by qPCR, as described above. Three glucose transporter genes (*Slc2a1*, *Slc2a2*, and *Slc2a3*) were measured to compare the ability to uptake glucose in each organ. *Slc2a1*, the most critical transporter during the fetal period,<sup>22</sup> was measured in brain and liver. Based on organ-specific expression,<sup>23</sup> *Slc2a3* and *Slc2a2* were measured in brain and liver, respectively.

### RNA sequencing

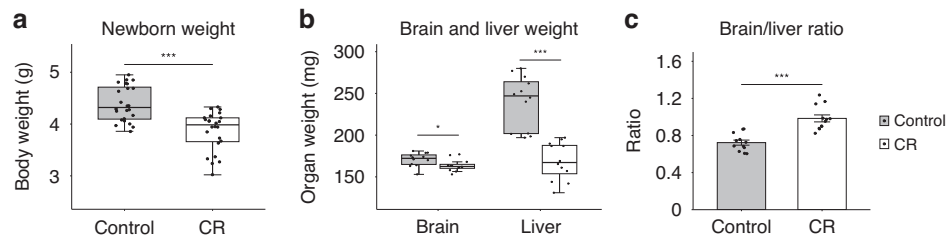
Total RNA samples from control ( $n = 3$ ) and CR ( $n = 3$ ) groups were assessed on a TapeStation system (Agilent Technologies, Santa Clara, CA) to ensure an RNA integrity number greater than 7.0. After library preparation, sequencing, mapping, and gene expression analysis were performed by DNAFORM (Yokohama, Japan). RNA-seq libraries were sequenced using paired-end reads (150 nt for read 1 and read 2) on a DNBSEQ-G400 instrument (Shenzhen, China). Obtained reads were then mapped to a reference rat genome (mRatBN7.2) using STAR (version 2.7.10a). Reads of annotated genes were counted using featureCounts (version 2.0.1). Differentially expressed genes (DEGs) with an FDR (false discovery rate,  $q$ -value)  $\leq 0.05$  were detected using the DESeq2 package (version 1.20.0). DEGs with  $\log_2[\text{Fold Change (FC)}] > 0.5$  were extracted as up-regulated genes, and those with  $\log_2(\text{FC}) > -0.5$  as down-regulated genes. Each gene set was analyzed for Gene Ontology (GO) enrichment using clusterProfiler package (version 4.10.0). Biological process subontology GO terms are listed. The data were deposited in NCBI's (National Center for Biotechnology Information's) Gene Expression Omnibus and are accessible through GEO Series accession number GSE278557.

### Immunohistochemistry

Four-micron-thick sections were prepared from paraformaldehyde (4% w/v)-fixed, paraffin embedded P0 neonatal brain tissues. We selected four regions of interest (ROI) representing cerebral cortex, hippocampus, pons, and medulla. Some of the sections were identified with Kluver-Barrera stain. Each section was obtained manually with reference to a rat brain atlas,<sup>24</sup> with six sections (6 pups) for each brain region in the CR and control groups. To assess changes in IR abundance, immunohistochemistry was performed. Paired CR and control sections generated from identical brain regions were mounted on the same slide to equalize staining conditions. After deparaffinization and rehydration, the sections were blocked with 3% bovine serum albumin and incubated overnight at  $4^\circ\text{C}$  with the primary antibodies, anti-insulin receptor beta (1:100, Cell Signaling Technology Cat# 23413, RRID:AB\_2924796), and anti-NeuN (1:500, Proteintech Cat# 66836-1-Ig, RRID:AB\_2882179). The beta subunit of the insulin receptor, to which our antibody specifically binds, is a common component between IR-A and IR-B<sup>12</sup>; therefore, this immunohistochemical staining identifies the total abundance of both IR isoforms. The sections were then incubated for 1 h at room temperature with secondary antibodies, Alexa 488 anti-rabbit (1:500, Thermo Fisher Scientific Cat# A-11034, RRID:AB\_2576217) and Alexa 594 anti-mouse (1:500, Thermo Fisher Scientific Cat# A-11032, RRID:AB\_2534091). All images were recorded using a fluorescence BZ-X microscope (Keyence, Osaka, Japan) at  $20\times$  magnification and were analyzed using ImageJ software (version. 2.14.0). To unify images between the two groups, the following representative regions were designated: the medial parietal area of the cerebrum as the cortex, the hippocampal CA1–CA3 area as the hippocampus, the area assumed to be the dorsal raphe nucleus as the pons, and the area assumed to be the solitary tract nucleus as the medulla. NeuN-stained particles were considered a marker for counting neurons. For quantitative comparison, we divided the mean IR intensity by neuron counts (IR/NeuN), so that IR abundance in each image was normalized for neuron density. IR/NeuN values were compared between the paired brain samples on the same slides and the differences were tested statistically.

### Western blotting

Brain tissue samples ( $n = 6$  in each group) were homogenized and proteins were extracted using N-PER<sup>TM</sup> reagent (Thermo Fisher Scientific) following the manufacturer's instructions. The protein concentration was determined



**Fig. 1** Changes in fetal growth in the maternal caloric-restriction (CR) model. **a** CR reduced the weight of newborn P0 pups. Two dams in each group produced 12 pups per litter, and all littermates were included in the analysis.  $n = 24$  neonates in each group. **b** CR also reduced P0 brain and liver weights significantly. Six eligible organs were obtained from each litter, and the weight data from the two litters were included.  $n = 12$  in each group. **c** CR increased the brain-to-liver ratio in individuals. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

using a Bradford protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein (20  $\mu$ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Burlington, MA). The membranes were blocked with 3% bovine serum albumin in TBS-T (0.1% Tween-20 in TBS) for 1 h at room temperature, followed by incubation for 1 h at room temperature with the following primary antibodies: anti-insulin receptor beta (1:1000, Abcam, Cambridge, UK; Cat# ab69508, RRID:AB\_1209215) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000, Fujifilm Wako Pure Chemical, Osaka, Japan; Cat# 015-25473, RRID:AB\_2665526), as a loading control. Following washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Jackson ImmunoResearch, West Grove, PA; Cat# 111-035-003, RRID: AB\_2313567) for 1 hour at room temperature and signals were visualized using enhanced chemiluminescence reagent (Bio-Rad) and imaged with a ChemiDoc imaging system (Bio-Rad). Band intensities of IR were quantified using ImageLab software (Bio-Rad). The ratio of the IR expression level in the forebrain to the hindbrain (forebrain/hindbrain ratio) was calculated using the signal values of these two brain segments obtained from the same subject rat.

### Statistical analysis

All continuous values are expressed as the mean  $\pm$  standard error. Two groups were compared by Wilcoxon's rank sum test.  $p < 0.05$  was defined as the cutoff for statistical significance. Data analysis was performed using R Project software (version 4.3.2).

## RESULTS

### Body organ weight of newborns

Three dams were prepared for each of the CR and control groups. Twelve pups were obtained from each dam, and the weight data from the pups of both dams in each group were combined for analysis. Our maternal caloric-restriction model resulted in decreased newborn weight (Fig. 1a). Compared with the control group ( $n = 24$ ), the mean weight in the CR group ( $n = 24$ ) was significantly decreased (Control  $4.3 \pm 0.28$  g vs CR  $3.8 \pm 0.30$  g,  $p < 0.001$ ). The brain and liver of newborns ( $n = 12$ ) showed significant weight reduction ( $p = 0.03$  and  $p < 0.001$ , respectively, Fig. 1b) and the brain-to-liver ratio in the CR group was significantly higher than that in the control group ( $p < 0.001$ , Fig. 1c). Fetal brain growth was relatively preserved compared with peripheral organs, indicating brain sparing in our FGR model.

### Glucose transporter gene expression and glucose uptake

The expression levels of major GLUT mRNAs in the brain and liver of newborns were compared between the CR and control groups. The level of *Slc2a1* mRNA, an essential glucose transporter in the fetal period, in the brain of CR newborns was not significantly different from that in the control group, while that in the liver of CR neonates was significantly lower than that in the control group ( $p = 0.002$ , Fig. 2a). There was no significant difference in *Slc2a3* level in the brain between the CR and control groups, while the level of *Slc2a2*

in the liver was significantly lower in the CR group than in the control group (Figs. 2b, c). Glucose uptake assays (using a 2-DG uptake protocol) showed that the brain-to-liver ratio was significantly higher in CR rats than in control rats ( $p = 0.02$ , Fig. 2d). This indicates a marked difference in glucose uptake in CR newborns between brain and liver. These results indicate that the fetal brain is prioritized over the liver in terms of glucose uptake.

### IR expression changes in neonatal brain

RT-qPCR analysis revealed that *Ir-A* was predominantly expressed in the brain (Fig. 3a), which is consistent with previous reports.<sup>15</sup> *Ir-A*, but not *Ir-B*, expression levels in the brain in the CR group were significantly lower than in the control group ( $p = 0.04$ ). DEG analysis of RNA-seq data (Fig. 3b) demonstrated a significant reduction of *Insr* ( $q = 0.016$ ) and *Ir-A* transcript counts ( $q = 0.016$ ), identical with qPCR. *Ir-B* was not detected as a differential transcript in our analysis. Moreover, a heatmap of genes known to be associated with transcription of the *Insr* gene<sup>12</sup> enabled CR and control groups to be distinguished (Fig. 3c).

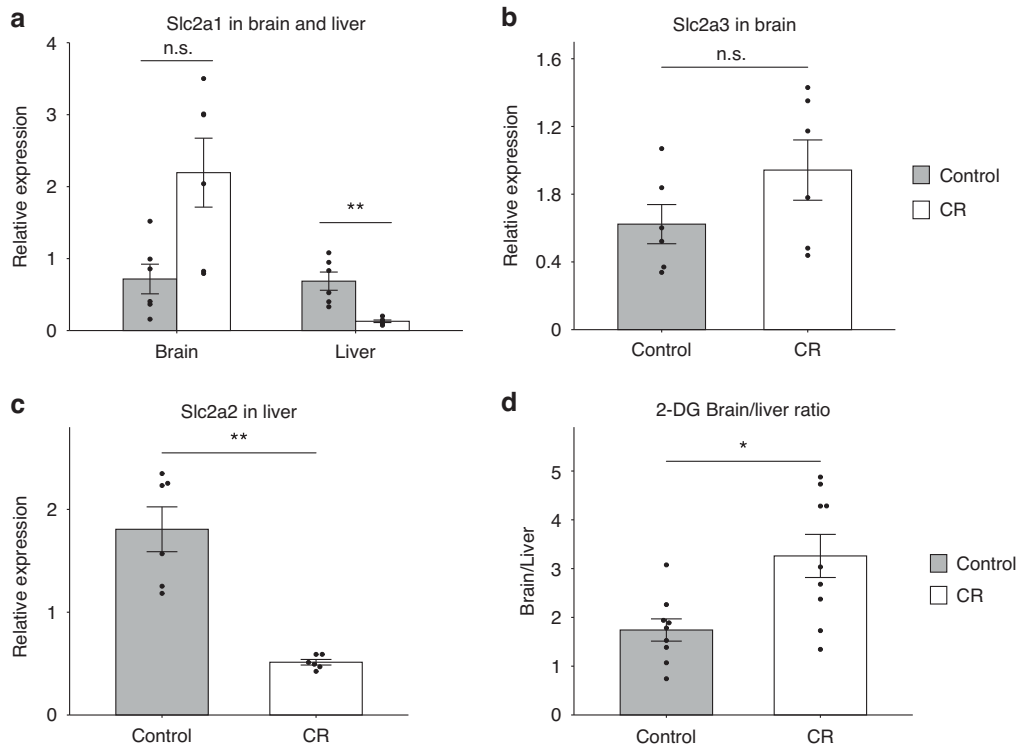
### Gene ontology

Enriched GO terms were identified for up- and down-regulated DEGs in the CR neonatal brain (Fig. 4a). For both gene groups, highly relevant biological processes mainly included those related to neurotransmitters and brain development and differentiation. Down-regulated DEGs had overlapping GO terms referring to development of the forebrain structure: telencephalon, pallium, and cerebral cortex. In contrast, hindbrain development was a significant biological process listed for up-regulated DEGs. GO terms including the word "brain" were searched for among all the statistically significant terms of the up- and down-regulated DEGs and they showed a distinctive feature (Fig. 4b). In the up-regulated genes list, three out of four terms indicated development and morphogenesis of the hindbrain. In contrast, the majority of down-regulated gene terms were related to neuron development of the forebrain.

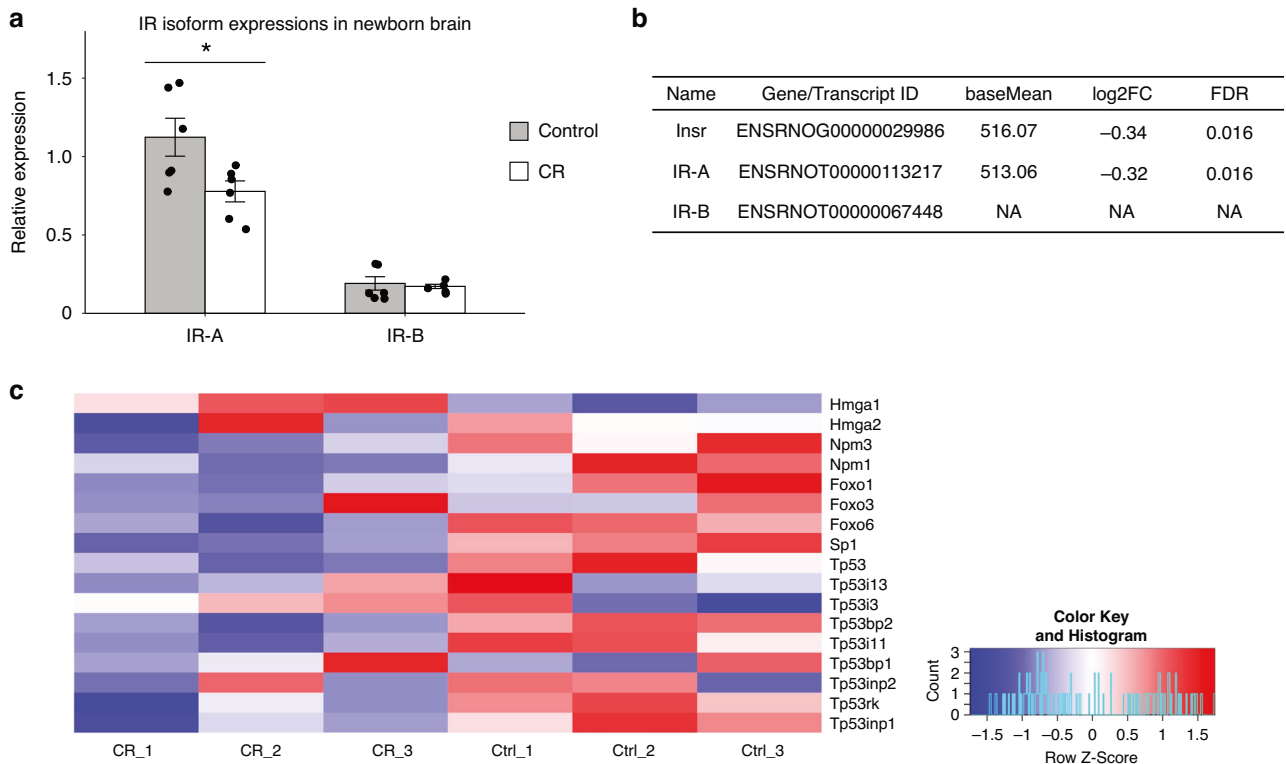
### IR expression analyses in segmented brain by RT-qPCR and western blotting

RT-qPCR analyses of the segmented brain showed that *Ir-A* expression levels in the forebrain were significantly decreased ( $p = 0.04$ ), while levels in the hindbrain were unchanged in the CR group (Fig. 5a). *Ir-B* expression levels also decreased in the hindbrain in the CR group, but the difference was smaller than for *Ir-A* expression.

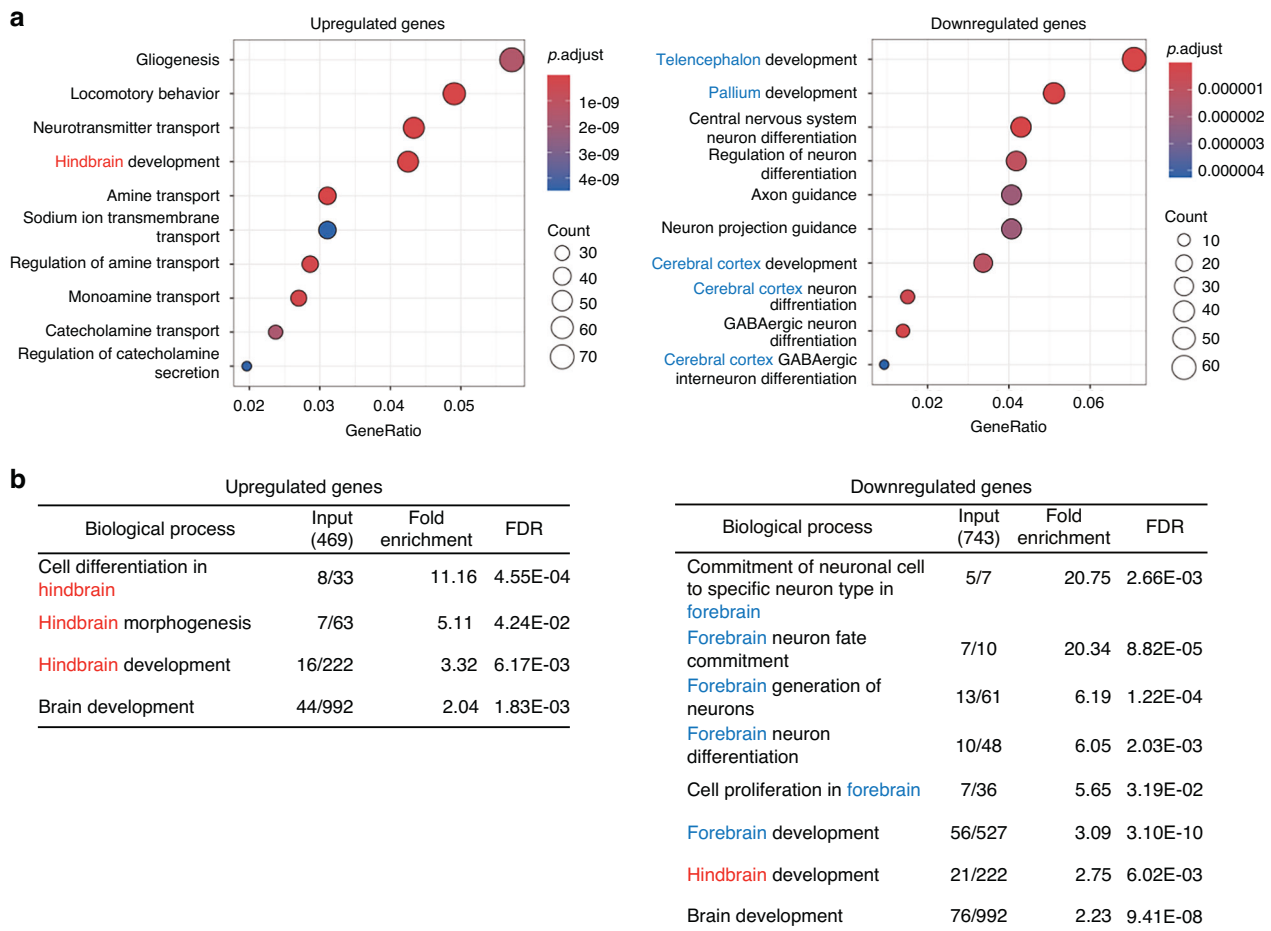
IR protein expression levels in the segmented brain, forebrain and hindbrain, were measured by western blotting. GAPDH-normalized IR values tended to be lower in the CR group, but the difference between the two groups was not significant (forebrain:  $p = 0.13$ , hindbrain:  $p = 0.13$ ) (data not shown); however, the forebrain/hindbrain ratio of IR expression was significantly decreased in the CR group ( $p = 0.04$ , Fig. 5b).



**Fig. 2** Altered glucose uptake in CR neonates. Gene expression was analyzed by qPCR and 2-deoxyglucose (2-DG) uptake assays were performed in P0 neonatal organs. **a** *Slc2a1* expression in the brain did not differ between the two groups, but was decreased in CR liver. **b** *Slc2a3* expression in the brain was not different between the two groups. **c** *Slc2a2* expression was decreased in CR liver. **d** The whole organ brain-to-liver ratio of 2-DG uptake was significantly increased in the CR group. n.s. = not significant; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Fig. 3** Changes in the levels of insulin receptor isoform mRNAs. **a** Comparison of relative gene expressions by qPCR. CR reduced the levels of *Ir-A* in newborn brain, and that of *Ir-B* did not change.  $n = 6$  in each group. **b** Differential expression analysis by RNA sequencing. *Insr* and *Ir-A* were down-regulated with false discovery rate  $< 0.05$ . NA: not applicable. **c** Heatmap of genes known to be related to the transcription of the *Insr* gene. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



**Fig. 4** Enrichment analyses by RNA-sequencing of neonatal CR brain. **a** Gene ontology analysis of the CR brain. Biological Process terms are listed. Significant terms were ranked by gene ratio and are visualized as dot plots. In these lists, the terms highlighted in red are related to the hindbrain and terms highlighted in blue are related to the forebrain. **b** Lists of all the significantly enriched terms that included the word “brain” in up- and down-regulated gene categories. False discovery rate <0.05 was defined as significant.

### Immunohistochemistry

Immunohistochemistry demonstrated differences in IR abundance among four ROIs: cerebral cortex, hippocampus, pons, and medulla (Fig. 6a-d). IR/NeuN in the cortex and hippocampus of the CR group was significantly lower than in the control group ( $p < 0.001$  and  $p = 0.002$ , respectively, Fig. 6e), but it was significantly higher in the medulla ( $p = 0.002$ , Fig. 6e). There was no significant difference between groups in IR/NeuN in the pons. There was no difference in NeuN cell counts between the two groups for any of the examined brain areas (Fig. 6f); therefore, IR/NeuN is a reliable index of the IR abundance in immunohistochemical images.

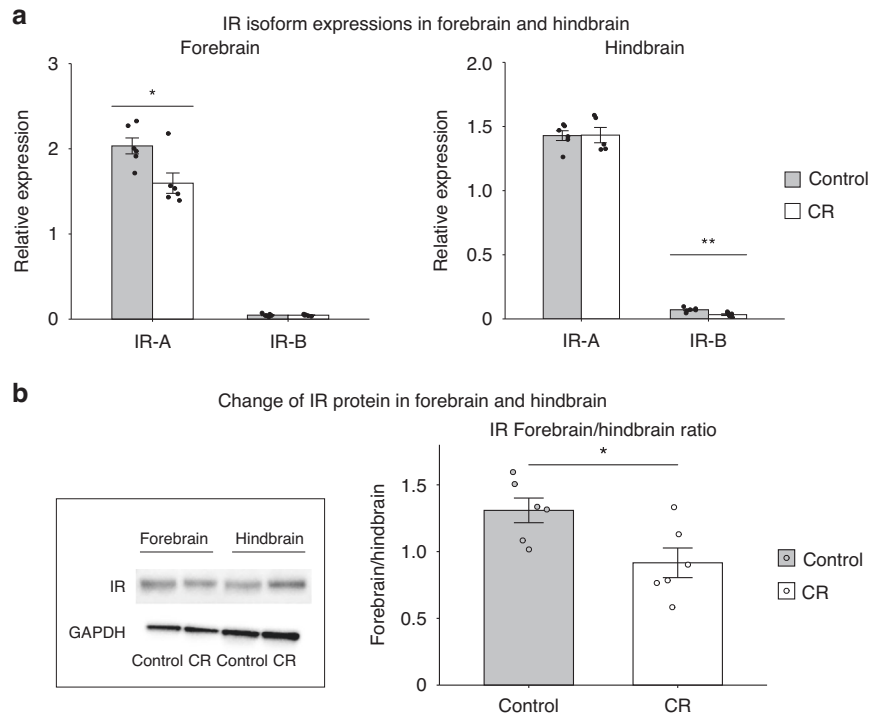
### DISCUSSION

In this study, we revealed that IR-A expression is reduced in the brain of neonatal asymmetric FGR model rats generated by maternal caloric restriction. Furthermore, we found that IR-A expression is decreased in the forebrain, which is important for cognitive brain function, whereas IR-A expression is maintained in the hindbrain, which is important for basic vital activities, such as the cardio-respiratory centers. Considering the fact that IR-A is important for neurodevelopment, our results can explain why FGR infants have a poor neurodevelopmental prognosis, despite their brain size being relatively protected.

In the fetal period, IR-A has important roles in neuronal development, especially in the central nervous system.<sup>12,14,15</sup>

Several previous studies have shown that reduced IR expression disrupts the appropriate development of neuronal tissue and is directly associated with impairment of cognitive functions.<sup>25,26</sup> Other studies using animal models that disrupt brain IR have demonstrated cognitive dysfunction and memory impairment in adulthood.<sup>27,28</sup> The impact of IR on neuronal development is thought to be through its involvement in synaptogenesis.<sup>15,16,19</sup> In response to a lack of nutrients, fetal neurons may reduce IR-A expression to enable the scarce nutrition to be used for cell survival rather than neural maturation, such as synaptogenesis. In other words, reduced IR-A expression in the FGR brain can be interpreted as an adaptive response of the fetal brain to a low-nutrition environment. However, reduced IR-A expression in the forebrain can lead to future developmental disorders. We consider that this is one of the mechanisms by which FGR infants have a poor neurodevelopmental prognosis despite their relatively preserved brain size. Although the slight reduction in brain weight in our CR model pups suggested that reduced IR-A expression might have partly suppressed neuronal proliferation, histological analysis showed no marked reduction in neuron cell counts. It is therefore plausible that postnatal brain developmental disorders are more attributable to impaired neural maturation processes, such as synaptogenesis, rather than a decrease in neuron cell counts.

In our FGR model, inhibition of brain growth was relatively mild compared with that in peripheral organs, suggesting that our model can replicate asymmetrical FGR in humans. Our analysis of



**Fig. 5 Brain-region-specific IR mRNA and protein expression levels.** **a** Relative quantification of IR isoform mRNA by qPCR. CR reduced *Ir-A* but not *Ir-B* expression in the forebrain. There was no significant difference in *Ir-A* expression in the hindbrain between the CR and control groups. *Ir-B* was decreased in the CR group.  $n = 6$  in each group. \* $p < 0.05$ ; \*\* $p < 0.01$ . **b** Quantification of IR protein levels by western blot. The forebrain/hindbrain ratio was calculated using the signal value of these two brain segments obtained from the same subject rat. The forebrain/hindbrain ratio was significantly decreased in the CR group.  $n = 6$  in each group. \* $p < 0.05$ .

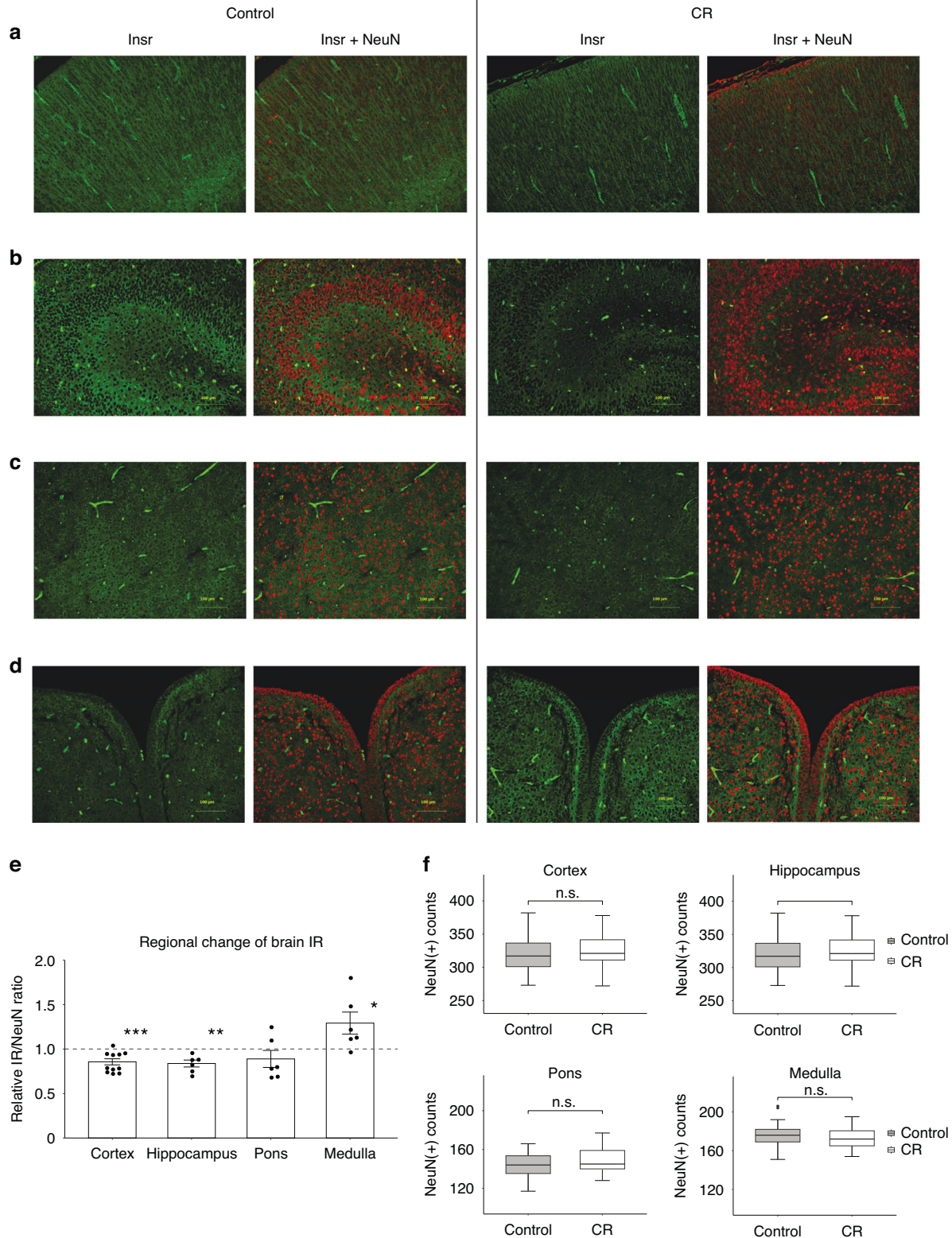
glucose transporter expression and 2-DG uptake also showed that glucose uptake into the brain was prioritized over uptake by other organs in our FGR model. These findings indicate that under low nutritional environments, the expression of glucose transporters in the fetal liver is suppressed to preferentially distribute the available glucose to the brain. IR in neonatal hepatocytes is related to glucose uptake through the regulation of GLUT 1 and 2 expression.<sup>29,30</sup> These reports suggest that modulation of IR expression affects the expression of glucose transporters and glucose utilization. Based on these findings, we considered that insulin signaling in asymmetric FGR might affect the change in GLUT expression. Such endocrinological changes may be a key to understanding the mechanism of the “brain-sparing” effect, which contributes to relatively preserved brain size and neurodevelopmental prognosis.

The analyses of RNA-seq in CR neonatal brain revealed that the expression of genes related to cell differentiation and development in the hindbrain is up-regulated, whereas the expression of genes related to cell differentiation and development in the forebrain is down-regulated. This result indicates the preferential maintenance of the hindbrain development. The hindbrain includes a number of cardio-respiratory centers and is, therefore, responsible for maintaining basic vital activities.<sup>31</sup> Our enrichment analysis indicates that fetuses in a nutrient-deficient environment try to prioritize survival by protecting the hindbrain. However, various FGR animal models have demonstrated impaired neurogenesis and synaptogenesis in the cortex and hippocampus.<sup>32–34</sup> A brain MRI study of human fetuses showed that FGR is associated with relative enlargement of the brainstem.<sup>35</sup> Our results are consistent with these previous reports, and furthermore, our RNA-seq analysis showed in more detail that fetuses try to preserve hindbrain function under nutrient-deficient conditions.

We also found regional localization of IR-A within the brain. Region-specific qPCR analysis revealed that IR-A expression was

suppressed in the forebrain but did not change in the hindbrain. Western blot analysis of the segmented brain showed that the forebrain/hindbrain ratio of IR expression was decreased in the CR group, suggesting that IR expression was more markedly reduced in the forebrain than in the hindbrain in response to CR. The results of western blotting and immunohistochemistry indicated that the regional changes in brain IR protein levels were identical to the changes in IR-A mRNA levels. Notably, the IR antibody cannot distinguish between IR-A and IR-B; however, the region-specific qPCR results and predominance of IR-A in neurons suggested that the change in IR protein within the brain reflected changes in IR-A. In terms of the neuroprotective property of IR-A, these results were consistent with the preserved hindbrain development shown by RNA-seq. This suggests an endocrinological mechanism to selectively prioritize the development of the brain areas that are most important for survival, which can be called the “endocrinological hindbrain-sparing effect”. A previous study showed that growth-restricted fetuses shift the blood perfusion of the cerebrum to deep gray matter including hindbrain.<sup>36</sup> Not only hemodynamic changes but also endocrinological mechanisms may contribute to the regulation of regional brain development under conditions of low nutrition. Since the time at which caloric restriction was started in our FGR model was nearly concordant with the developmental stage in which a rat fetus initiates brain synaptogenesis,<sup>37</sup> IR-A expression in the forebrain may be reduced to restrict synaptogenesis in exchange for maintaining development of the hindbrain. Consequently, reduction of IR-A expression in the forebrain can result in postnatal functional brain disorders.

The major strength of our study is that we showed IR-A expression to be reduced in the forebrain of the FGR model. Our study has several limitations. First, the immunohistochemistry and western blot results did not necessarily provide a complete reflection of the changes in IR isoforms, and methods for



**Fig. 6 Images and quantitative analysis of immunohistochemistry.** Representative histological images of newborn brain regions. **a** Cerebral cortex, **(b)** hippocampus, **(c)** pons and **(d)** medulla. Green staining denotes Insulin receptor (IR)-positive area. Red staining denotes neuronal nuclei (NeuN)-positive cells. Quantitative analysis of immunohistochemistry. **e** Comparison of the IR/NeuN ratio in four regions. The relative regional change in the IR/NeuN in the CR group was measured. The ratio in the counterpart control was set as 1.0. CR decreased the ratio in the cerebral cortex and hippocampus, but increased it in the medulla. There was no significant difference between groups in the IR/NeuN in the pons.  $n = 11$  in the cortex,  $n = 6$  in the hippocampus, pons and medulla. **f** Comparison of NeuN-positive cell counts per image. In all regions of interest (cortex, hippocampus, pons, and medulla), the difference between CR and control groups was not statistically significant. n.s. = not significant;  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ .

distinguishing between IR-A and IR-B at the protein level are limited. Second, this study did not prove a causal relationship between the reduction in brain IR-A expression in our FGR model rat and neurodevelopmental impairments or expression of glucose transporters. To examine causality, other experimental models are needed, such as genetically modified animals. Third, it is not known if the results of this animal model can be applied to humans, and careful interpretation is needed. There are differences in the developmental processes of the human and rat fetal brains.<sup>37</sup> The time and duration of caloric restriction in our FGR model is equivalent to the second trimester of human pregnancy.<sup>38</sup> It is unclear whether our model is also suitable as a model of human FGR because of nutritional deficiencies that occur outside of the second trimester. However, we believe that our model is important as a nutritionally restricted model of the second trimester, which is a critical time for brain development. Finally, because our analyses were based on bulk brain tissue, the mechanism by which neuronal IR expression is regionally regulated in FGR is unclear. Further investigations are warranted to elucidate cell-to-cell interactions and upstream regulatory factors for a greater understanding of brain IR-A regulation.

In conclusion, we revealed that IR-A expression is reduced in the neonatal brains of FGR model rats generated by maternal caloric restriction. We also found that there was regional localization of IR-A expression within the brain. Our results suggest an endocrinological mechanism that maintains both neuronal survival and glucose uptake in the brain through the regulation of IR-A expression, which can be called the “endocrinological hindbrain-sparing effect”. However, this adaptive process, the reduction of IR-A in the forebrain, can be associated with postnatal functional disorders of the brain, even though brain size is maintained. This can explain why FGR infants with relatively protected brain size have poor neurodevelopmental prognosis. Further research is needed to elucidate the mechanisms of IR-A reduction and to develop interventions that prevent neurodevelopmental impairment in FGR infants.

## DATA AVAILABILITY

The data sets generated during the current study are available from the corresponding author on reasonable request.

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## ACKNOWLEDGEMENTS

The authors thank the Division of Histological Study, Center for Anatomical Studies, Graduate School of Medicine, Kyoto University, for preparing histological samples for microscopic examination. We also thank Jeremy Allen, PhD, from Edanz (<https://jp.edanz.com/ac>) for editing a draft of this manuscript.

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Conceptualization: Y.T., S.T. and M.K. Methodology: Y.T., S.T., Y.Y. and K.M. Investigation: Y.T., Y.Y., and K.M. Funding acquisition: R.A. and S.T. Supervision: S.T., J.T. and M.K. Writing original draft: Y.T. Review and editing: S.T., J.T. and M.K. All authors have read and agreed to the published version of the manuscript.

## FUNDING

This work was supported by JSPS KAKENHI, Grant Numbers JP22K17798, JP24K15678, and JP24K20703, the Mother and Child Health Foundation, Grant Number R05-K1-7, and Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics, Grant Number 35-Waka1.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41390-025-04372-8>.

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