Brain Pathology

RESEARCH ARTICLE

Antenatal low-intensity pulsed ultrasound reduces neurobehavioral deficits and brain injury following dexamethasone-induced intrauterine growth restriction

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Abstract

Intrauterine growth restriction (IUGR) is a leading cause of perinatal mortality and morbidity, and IUGR survivors are at increased risk of neurodevelopmental deficits. No effective interventions are currently available to improve the structure and function of the IUGR brain before birth. This study investigated the protective effects of low-intensity pulsed ultrasound (LIPUS) on postnatal neurodevelopmental outcomes and brain injury using a rat model of IUGR induced by maternal exposure to dexamethasone (DEX). Pregnant rats were treated with DEX (200 µg/kg, s.c.) and LIPUS daily from gestational day (GD) 14 to 19. Behavioral assessments were performed on the IUGR offspring to examine neurological function. Neuropathology, levels of neurotrophic factors, and CaMKII-Akt-related molecules were assessed in the IUGR brain, and expression of glucose and amino acid transporters and neurotrophic factors were examined in the placenta. Maternal LIPUS treatment increased fetal weight, fetal liver weight, and placental weight following IUGR. LIPUS treatment also increased neuronal number and myelin protein expression in the IUGR brain, and attenuated neurodevelopmental deficits at postnatal day (PND) 18. However, the number of oligodendrocytes or microglia was not affected. These changes were associated with the upregulation of brain-derived neurotrophic factor (BDNF) and placental growth factor (PlGF) protein expression, and

enhancement of neuronal CaMKII and Akt activation in the IUGR brain at PND 1. Additionally, LIPUS treatment promoted glucose transporter (GLUT) 1 production and BDNF expression in the placenta, but had no effects on GLUT3 or amino acid transporter expression. Our findings suggest that antenatal LIPUS treatment may reduce IUGR-induced brain injury via enhancing cerebral BDNF/CaMKII/Akt signaling. These data provide new evidence that LIPUS stimulation could be considered for antenatal neuroprotective therapy in IUGR.

KEYWORDS
brain injury, CaMKII, intrauterine growth restriction, neurotrophic factor, ultrasound

1 | INTRODUCTION

Intrauterine growth restriction (IUGR) affects approximately 10% of pregnancies worldwide and is a leading cause of perinatal mortality and morbidity (1, 2). IUGR survivors are at increased risk of neurodevelopmental deficits, with lifelong neurological deficits ranging from behavioral and motor disabilities to cerebral palsy (3). Underlying these functional impairments, IUGR also compromises brain development and is commonly associated with reduced myelination and decreased total brain cell number (4, 5). However, to date, no effective methods are available to reverse the pathological intrauterine condition or to accelerate the growth of fetuses in pregnancies complicated by IUGR. Thus, intervention applied before birth to improve placental function and protect the fetal brain is important to reduce subsequent neurodevelopmental impairment in IUGR infants.

The leading causes of IUGR are maternal malnutrition and chronic maternal diseases, followed by placental insufficiency due to vascular or circulatory placental damage (6, 7). Hypoxia, infections during pregnancy, fetal malformations, smoking, pollution, and chronic alcoholism are also causative factors, though less common (6, 7). The appropriate growth of the fetal brain is highly dependent on the availability of growth factors. BDNF, a member of the neurotrophin family, is widely expressed in the developing fetal brain and plays a vital role in neuronal survival, neuronal differentiation, and synaptic plasticity (8). BDNF is downregulated in cord blood from IUGR neonates clinically (9) and lower levels of BDNF receptors have been demonstrated in cortical neurons of IUGR rats, leading to reduced cell viability and synaptic function (10). PlGF and vascular endothelial growth factor (VEGF), which are both present during early neuronal development, have also been found to exhibit protective effects against various types of neuronal damage (11–13) and are involved in neuronal plasticity and neuronal repair by augmenting neurogenesis and angiogenesis (14, 15). Glial cell line-derived neurotrophic factor (GDNF) is another important neurotrophic factor that is present in high concentrations during brain development and promotes neuronal survival in neonatal models (16, 17). This evidence suggests that interventions that increase cerebral growth factors have a therapeutic role in IUGR-induced brain damage.

LIPUS is a form of ultrasound with a non-thermal mechanism and it can produce a pulse wave that is delivered at a much lower intensity (less than 3 W/cm^2) than that of traditional ultrasound energy (18). LIPUS is known to accelerate bone healing and soft tissue regeneration following injury (19, 20). In the brain, LIPUS can increase the electrical activity of neurons in the cortex without causing any damage to the brain tissue (21). Our previous studies have demonstrated that LIPUS exerts neuroprotective effects in animal models of traumatic brain injury (22), cerebral ischemia (23), and vascular dementia (24). The mechanisms by which LIPUS protects the brain from damage are multi-modal and involve the enhancement of neurotrophic factor release and anti-apoptosis with the upregulation of apoptosis-related signaling (22, 23, 25, 26). Furthermore, in healthy human subjects, LIPUS causes a significant increase in soft tissue microcirculation and oxygen saturation of the hindfoot (27). More recently, a multicenter, randomized, double-blind, sham-treated, controlled clinical study showed that LIPUS can effectively treat patients with mild to moderate erectile dysfunction without significant adverse effects, likely through the mechanical force of LIPUS and restoration of the pathological changes in the corpus cavernosum (28). Together, these therapeutic effects of LIPUS hold promise for improving placental function and reducing IUGR-related brain injury. Therefore, the objective of this study was to test the effects of LIPUS on fetal growth and childhood neurodevelopmental outcomes using a rat model of IUGR induced by maternal exposure to DEX.

2 | MATERIALS AND METHODS

2.1 | Animals

All animals were maintained according to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the experimental procedures were approved by the Institutional Animal Care and Use Committee at Cheng Hsin General Hospital (Animal permit number CHIACUC-107-13). Time-pregnant Wistar rats were purchased from BioLASCO (Taipei, Taiwan) and were allowed free access to water and maintained on a 12-h/12-h light-dark cycle at a controlled temperature (22–25°C) and humidity (40%–60%).

2.2 | Intrauterine growth restriction model

A DEX-induced IUGR model was used as previously described (29). Briefly, after anesthetization (1.5 L/min oxygen in 4% isoflurane), pregnant rats were treated with DEX (Sigma Aldrich, St. Louis, MO, USA; 200 μg/kg in 0.9% saline) or an equal volume of saline (control) by subcutaneous injection from GD 14 to 19. Pregnant rats were then placed in a heated cage to maintain body temperature during recovery from anesthesia.

2.3 | Pulsed ultrasound apparatus and setting

LIPUS exposure was generated by a 1.0-MHz, single element plane transducer (ME7410: 9.3 cm^2 effective radiating area (ERA); Mettler Electronics, Anaheim, CA) with 5-ms burst lengths at a 50% duty cycle and a repetition frequency of 100 Hz. The LIPUS was transmitted from the plane transducer to the abdominal wall of the pregnant rats. The rat's abdominal region was shaved and ultrasound gel was used to maximize the transmission of ultrasound between the transducer and the skin surface. Under anesthetization with 1.5 L/min oxygen in 4% isoflurane, pregnant rats were exposed to a total of 15 min LIPUS in three intervals of 5 min duration each with 5 min of non-exposure between sonications. LIPUS was applied at a spatial average intensity (SAI) over the plane transducer head was 500 mW/cm^2. The control group and the DEX group had the same anesthesia protocol as the DEX + LIPUS group.

2.4 | Experimental design

Pregnant rats were randomly assigned into the following three groups: 1) DEX group, rats receiving daily subcutaneous injections of DEX from GD 14 to GD 19; 2) DEX + LIPUS group, pregnant rats receiving daily DEX followed by LIPUS treatment from GD 14 to GD 19; and 3) control group, rats treated with daily subcutaneous injection of 0.9% saline (1 ml/kg) from GD 14 to GD 19. A total of 21 pregnant rats (n = 7/group) were used for this experiment. All outcome measurements (Figure 1A) and analyses were performed in a blinded manner.

To investigate the effects of DEX with and without LIPUS on the pregnant rats and growth performance of their pups, maternal weight was recorded every day from GD 13 to GD 20. On GD 20, placentas and offspring rats were harvested via cesarean section. After obtaining the placental weight, fetal weight, and fetal liver weight, the placentas were snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde for further assessment. Animals that were allocated for post-natal evaluation were born through vaginal delivery. To assess the effects of DEX with and without LIPUS on the neurodevelopment of newborn rats, behavioral assessments were performed on a PND 18. In addition, brain tissues were obtained from PND 1 or PND 18 rats, snap frozen in liquid nitrogen or fixed in 4% paraformaldehyde for further studies on the changes in factors associated with neurodevelopment. Assessments included western blot analysis and (IF) labeling. A total of 29 PND 1 rats and 21 PND 18 rats (n = 7–10 /group) were used for this experiment.

2.5 | Behavioral testing

A variety of behavioral tests were conducted to evaluate the motor function of PND 18 offspring rats as previously described.(31) The definitions of the tests and associated scoring systems are briefly stated as follows.

2.5.1 | Ambulation

Walking abilities (no movement = 0; crawling with asymmetric limb movement = 1; slow crawling but symmetric limb movement = 2; and fast crawling/walking = 3).

2.5.2 | Surface righting

The release time for a pup to return from a back position to a prone position.

2.5.3 | Negative geotaxis

The time for a pup to turn its face up on a downward slope.

2.5.4 | Hind limb suspension

The pup was placed in a cage with its hind legs gently suspended from the edge of the cage to assess its hind
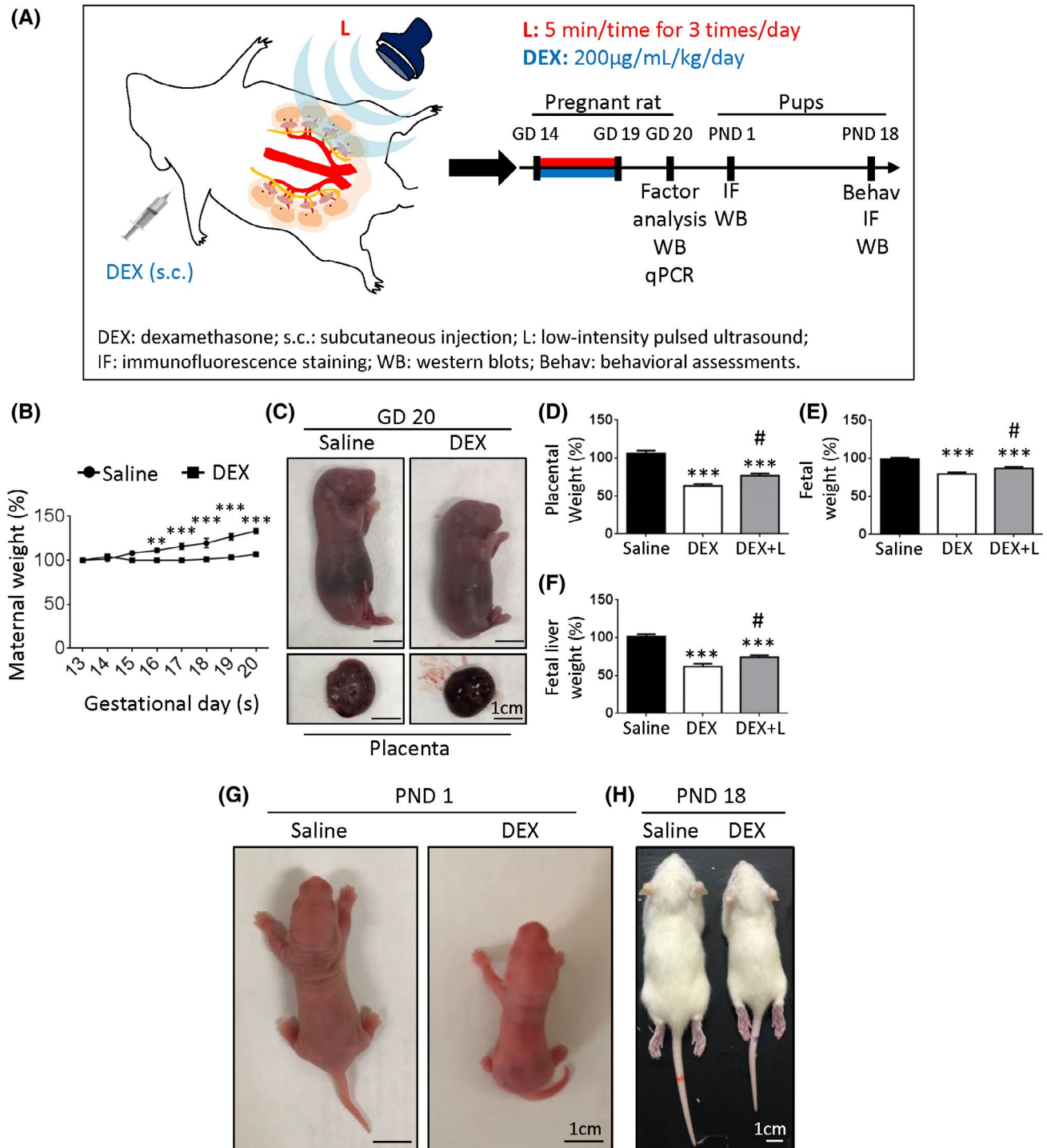
(A)

L: 5 min/time for 3 times/day
DEX: 200μg/mL/kg/day

DEX: dexamethasone; s.c.: subcutaneous injection; L: low-intensity pulsed ultrasound; IF: immunofluorescence staining; WB: western blots; Behav: behavioral assessments.

(B) **(C)** **(D)** **(E)** **(F)** **(G)** **(H)**

FIGURE 1 **Maternal LIPUS treatment increased maternal weight gain, placenta weight, fetal body weight, and liver weight**. (**A**) The experimental design scheme is used to study the effect of LIPUS treatment in rat IUGR model. (**B**) Body weight changes in pregnant rats from GD 13 to 20. (**C**) Representative pictures of fetuses and placentas on GD 20. (**D**) Placenta weight, (**E**) fetal weight, and (**F**) fetal liver weight measured at GD 20. Representative pictures of rat pups at (**G**) PND 1 and (**H**) PND 18. Values are presented as mean ±standard error of mean; **$p < 0.01$ and ***$p < 0.001$ versus saline group; #$p < 0.05$ versus DEX group (n = 7/group; one-way analysis of variance). Behav, behavioral assessments; DEX, dexamethasone; GD, gestational day; IF, immunofluorescence staining; IUGR, intrauterine growth restriction L, low-intensity pulsed ultrasound; PND, postnatal day; s.c., subcutaneous injection; WB, western blots

limb strength (normal separation of the hind limbs with a raised tail = 4; weakness with closer hind limbs = 3; weakness with hind limbs frequently touching each other = 2; hind limbs almost always clenched with the tail raised = 1; and hind limbs always clenched with the lowered tail or unable to hold onto the edge of the cage = 0).

2.5.5 | Cliff aversion

With its forepaws and snout positioned over the edge initially, the time for a pup to turn away from the edge.

2.6 | Tissue processing and histology

After terminal anesthesia, offspring rats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed in 4% paraformaldehyde overnight, cryoprotected with 30% sucrose, and then coronally sectioned (10 μm) from the level of the olfactory bulbs to the visual cortex.

2.7 | Immunofluorescence staining

IF staining was performed as previously described (22). To determine different cell types or structural protein expression, the sections were incubated with anti-myelin basic protein (MBP) antibody (1:200; ab40390, Abcam, Cambridge, UK), anti-Olig2 antibody (1:200; NBP1-28667, Novus, Centennial, CO, USA), anti-neuronal nuclei antigen (NeuN) antibody (1:1000; MAB377, Millipore, Billerica, MA, USA), or anti-Iba1 antibody (1:1000; No. 019-19741, Wako, Richmond, VA, USA) overnight after 1 h of normal goat serum blocking. To assess the neuronal expression of pAkt or pCaMKII, sections were incubated overnight at 4°C with anti-pAkt Ser473 antibody (1:200; #9271, Cell Signaling, Danvers, MA, USA) or anti-pCaMKII (1:200; #12716, Cell Signaling), together with anti-NeuN antibody (1:1000, Millipore). The sections were then washed, followed by incubation with Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (1:500; Molecular Probes, Eugene, OR, USA) for 2 h. All sections were observed and photographed under a fluorescence microscope (Olympus BX-51, Tokyo, Japan).

2.8 | Quantification of IF staining

NeuN, Olig2, Iba1, and MBP staining was quantified on three consecutive sections from the cortex, striatum, or corpus callosum at a level of 0.62 mm from the bregma as previously described (25). NeuN-, Olig2-, and Iba1-positive cells were counted at a magnification of 200× in three randomly selected, non-overlapping fields with areas of $920 \times 860 \ \mu m^2$ in the cortex and striatum. For MBP staining, optical densit0000000D) was measured from digitized sections at a magnification of 200×. Regions of interest encompassing the corpus callosum were manually outlined and OD values were calculated. To minimize variability due to tissue processing, average values of OD were calculated in each field of view. Quantification of MBP staining was expressed as (OD of corpus callosum /mean OD of per field of view) × 100%. NeuN-, Olig2-, and Iba1-positive cells were expressed as cells/field. Analysis was performed by two experimenters who were blinded to all animal groups. Inter-rater reliability was within 10%.

2.9 | Protein extraction and western blots

Western blot analyses were performed as follows. Protein samples obtained from brain or placenta tissue homogenates were separated on 8%–12% sodium dodecyl sulfate-polyacrylamide gels, transferred to Immobilon-P membranes (Millipore), and probed overnight at 4°C with primary antibodies including anti-BDNF (1:1000; GTX132621), anti-GDNF (1:1000; GTX17447), and anti-VEGF (1:1000; GTX102643) from GeneTex (Irvine, CA, USA); anti-pCaMKII (1:1000; #12716), anti-total CaMKII (1:1000; #11945), anti-pAkt S473 (1:1000; #9271), anti-pAktT308 (1:1000; #4056), anti-total Akt (1:1000; #9272), anti-pERK (1:1000; #9101), and anti-total ERK (1:1000; #9102) from Cell Signaling; anti-PlGF (1:1000; ab196666), anti-glucose transporter (GLUT) 3 (ab41525), anti-GLUT1 (ab115730), anti-SLC38A1 (ab134268), anti-SNAT2 (ab90677), and anti-SLC38A4 (ab58785) from Abcam; and anti-β-actin (1:10000; A5441, Sigma-Aldrich). Afterward, the membranes were washed and incubated with anti-rabbit or anti-mouse horseradish peroxidase-linked secondary antibodies (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 1 h. The relative intensity of protein signals was normalized to the corresponding β-actin intensity and was quantified by densitometric analysis with the use of ImageJ 1.50i software (National Institutes of Health, Bethesda, MD, USA).

2.10 | Real-time quantitative reverse transcriptase-polymerase chain reaction

After extraction using the RNeasy Mini Kits (QIAGEN, Valencia, CA, USA), RNA samples from the placenta were subjected to reverse transcription with SuperScript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed with an ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA, USA). Thermal cycling was initiated with a 2 min incubation at 50°C, followed by a 10 min denaturation step at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min. The primers and probes for GLUT1 (Rn01417099), GLUT3 (Rn00567331), and β-actin (Rn00667869) were obtained from Applied Biosystems. Relative quantities of GLUT1, GLUT3, and β-actin mRNA were calculated using the previously described comparative threshold cycle method (32).

2.11 | Statistical analysis

Data are presented as the mean and standard error of the mean (mean ± SEM). One-way or two-way analysis of variance (ANOVA) followed by post hoc Bonferroni evaluation was used for multiple groups to determine significant differences. A Student's t test was used to test the differences between the two groups. Statistical significance was set at $p < 0.05$.

3 | RESULTS

3.1 | Maternal LIPUS treatment increased maternal weight gain, placenta weight, fetal body weight, and liver weight

DEX administration from GD 14 to GD 19 led to an arrest in maternal body weight gain immediately after the beginning of injection. The body weight of DEX-treated pregnant rats was significantly lower than that of the saline-treated control rats from GD 16 to GD 20 (all $p < 0.01$, Figure 1B). When rats were sacrificed on GD 20, the number and weights of fetuses and placentas were recorded. There was an overall reduction in fetal (Figure 1C) and placental sizes (Figure 1C) accompanied by a 40.6% decrease in placental weight ($p < 0.001$; Figure 1D) and a 20.2% decrease in fetal weight ($p < 0.001$; Figure 1E) following DEX administration. Liver weight, an indicator of fetal nutrition, was also reduced following DEX exposure (60.9% of the control level, $p < 0.001$; Figure 1F). The body size of pups born to DEX-treated mothers was also smaller than that of the control group on PND 1 and PND 18 (Figure 1G,H). These data indicate that subcutaneously injected DEX during the last third of the gestation period led to the induction of IUGR. We then used the DEX-induced IUGR model to explore the protective effect of LIPUS on maternal placenta function and fetal growth. The results showed that maternal LIPUS treatment (DEX + L) significantly increased placental weight by 21.3% ($p = 0.025$; Figure 1D) and fetal weight by 9.7% ($p = 0.029$; Figure 1E) compared with those of rats treated with DEX alone. Likewise, fetal liver weight also increased by 20.2% following LIPUS treatment ($p = 0.048$; Figure 1F). These findings suggest that maternal LIPUS stimulation improves placenta function and fetal growth in the DEX-induced IUGR model.

3.2 | Maternal LIPUS treatment ameliorated motor disabilities in IUGR offspring

To investigate the protective efficacy of maternal LIPUS treatment in IUGR offspring, we first assessed body weight changes. At PND 18, the body weight of pups born to mothers treated with DEX alone was significantly reduced compared with those of the control group (27.15 ± 0.36 g vs. 37.33 ± 0.32 g, 27.3% reduction, $p < 0.001$; Figure 2A). However, treatment with LIPUS (DEX + L) improved IUGR-induced body weight changes (DEX vs. DEX + L, 30.65 ± 0.96 g vs. 27.15 ± 0.46 g, $p = 0.003$; Figure 2A). As IUGR is associated with a high risk for neurological deficits, we further explored the effect of maternal LIPUS treatment on the motor function of IUGR offspring using five different behavioral tests. The results showed that at PND 18, most pups born to mothers treated with DEX alone had a "crawling" gait with symmetric limb movement, while their control counterparts had transitioned to walking (average ambulation score: 2.14 ± 0.14 vs. 3.00 ± 0.00, $p < 0.001$; Figure 2B). However, significant improvement in ambulation was observed in the DEX + L group offspring compared with pups born to mothers treated with DEX alone (2.71 ± 0.18 vs. 2.14 ± 0.14, $p = 0.003$; Figure 2B). The latencies of surface righting, an indicator of trunk control, and negative geotaxis, an indicator of balance and coordination, in the DEX-treated offspring were also longer than that in the control group (surface righting latency: 1.68 ± 0.12 s vs. 1.23 ± 0.01 s, $p < 0.001$; Figure 2C; negative geotaxis latency: 3.98 ± 0.26 s vs. 3.03 ± 0.10 s, $p = 0.0021$; Figure 2D). Both surface righting and negative geotaxis walk latencies were shorter in the DEX + L offspring compared with pups born to mothers treated with DEX alone (surface righting latency: 1.21 ± 0.02 s vs. 1.68 ± 0.12 s, $p < 0.001$; Figure 2C; negative geotaxis latency: 3.09 ± 0.08 s vs. 3.98 ± 0.26 s, $p = 0.002$; Figure 2D). Hindlimb strength was assessed using the hindlimb suspension test. DEX-treated offspring showed hindlimb weakness, as demonstrated by a decrease in hanging score (hindlimb hanging score: DEX vs. control, 2.43 ± 0.20 vs. 3.86 ± 0.14, $p < 0.001$, Figure 2E). Likewise, maternal DEX + L treatment increased hindlimb strength of the IUGR offspring compared to that of pups born to mothers treated with DEX alone (3.43 ± 0.20 vs. 2.43 ± 0.20, $p = 0.004$; Figure 2E). The cliff aversion test measures labyrinthine function, motor strength, and coordination. The DEX-treated offspring exhibited longer mean latency compared with the control group (2.66 ± 0.12 s vs. 1.85 ± 0.06 s, $p < 0.001$, Figure 2F) and maternal LIPUS treatment improved the performance (DEX + L vs. DEX, 2.11 ± 0.09 vs. 2.66 ± 0.16, $p = 0.002$, Figure 2F).

3.3 | Maternal LIPUS treatment reduced neuronal loss and demyelination in IUGR offspring

To determine whether the above differences in behavioral function were owing to different levels of brain tissue damage, histological outcomes were evaluated at PND 18. We examined the expression of healthy mature neurons labeled with NeuN, a neuronal nuclei marker, in the different groups. Compared with the control group, the number of NeuN-positive cells in the cortex of rats treated with DEX alone was significantly reduced (229.65 ± 6.21 vs.
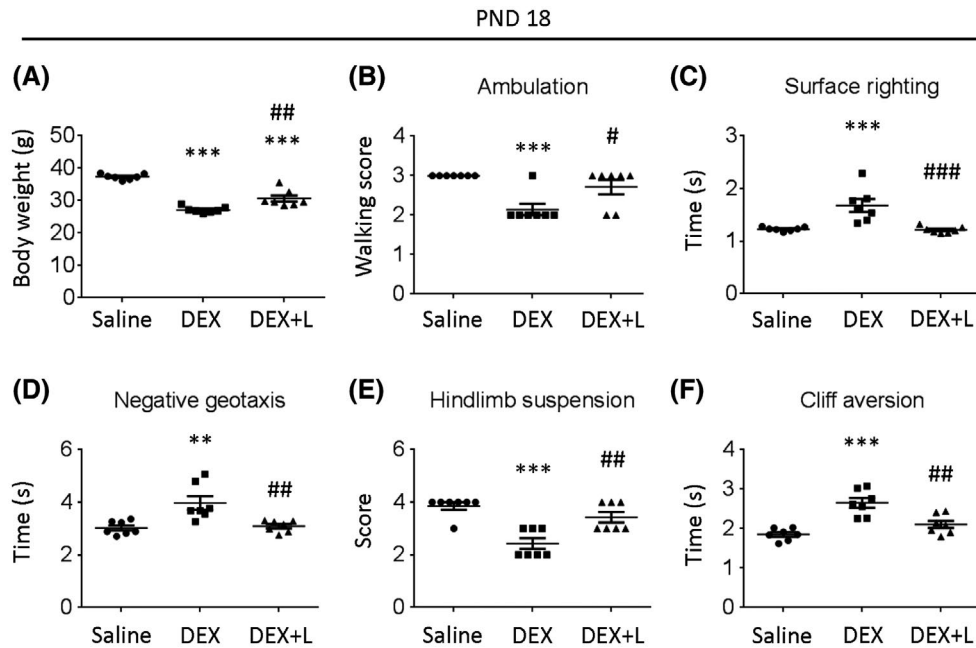
PND 18

FIGURE 2 Maternal LIPUS treatment ameliorated motor disabilities in IUGR offspring. (A) Body weight and assessments of (B) ambulation, (C) surface righting, (D) negative geotaxis, (E) hindlimb suspension, and (F) cliff aversion at PND 18 in IUGR offspring. Values are presented as mean ±standard error of mean; **$p < 0.01$ and ***$p < 0.001$ versus saline group; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ versus DEX group (n = 7/group; one-way analysis of variance). DEX, dexamethasone; IUGR, intrauterine growth restriction; L, low-intensity pulsed ultrasound; PND, postnatal day

296.75 ± 17.07, respectively, cells/field, $p = 0.002$, Figure 3A) at PND 18. However, maternal LIPUS treatment significantly increased the number of NeuN-positive cells (DEX + L vs. DEX, 274.81 ± 2.84 vs. 229.65 ± 6.21 cells/field, $p = 0.044$, Figure 3A). Nevertheless, no statistical differences were observed in the number of NeuN-positive cells among the three groups in the striatum (all $p > 0.05$, Figure 3A). Olig2 and Iba1 expressions were used to identify oligodendrocytes and microglia, respectively. There was no difference in the number of Olig2-positive cells or Iba1-positive cells in both the cortex (all $p > 0.05$) and striatum (all $p > 0.05$) among the three groups (Figure 3B,C). MBP staining was employed to assess white matter myelin sheath density. While myelin density in the brains of pups born to mothers treated with DEX alone was significantly reduced by 17.5% compared with that of the control in the corpus callosum at PND 18 ($p = 0.004$, Figure 3D), but maternal LIPUS treatment significantly increased myelin density by 26.7% compared to that of DEX treated pups ($p = 0.018$, Figure 3D).

3.4 | Maternal LIPUS treatment increased neurotrophic factor expression in IUGR brains

The above data indicate that the improvement of motor function in IUGR offspring following maternal LIPUS treatment was associated with a reduction in the neuronal loss and preservation of myelin. We then explored the possible mechanisms of LIPUS-mediated protection in IUGR brains. Neurotrophic factors have been shown

to promote CNS myelination and neuronal survival (33, 34). We have previously shown that LIPUS can enhance protein levels of neurotrophic factors in cultured astrocytes and rat brains (22, 35). To determine whether LIPUS triggers neurotrophic factor production in IUGR brains, the protein levels of BDNF, PlGF, VEGF, and GDNF were measured in the brain (Figure 4A–D). The protein levels of BDNF and PlGF were significantly decreased in the brains of pups born to mothers treated with DEX alone at PND 1 compared with the control group, but LIPUS treatment caused a significant increase in the protein levels of BDNF (59.9%, $p = 0.01$; Figure 4A) and PlGF (35.8%, $p = 0.023$; Figure 4B) compared with those of the DEX alone group. Similarly, maternal DEX exposure decreased the GDNF level in the pup brain compared with the controls, and LIPUS treatment increased the level compared with that of DEX treated pups although these trends did not reach statistical significance (all $p > 0.05$; Figure 4D). However, there was no difference in VEGF levels among the control, DEX alone, and DEX + L-treated groups (all $p > 0.05$; Figure 4C).

3.5 | Maternal LIPUS treatment enhanced the activation of CaMKII and downstream Akt signaling, but did not affect ERK signaling in IUGR brains

Since LIPUS increased the protein levels of BDNF, we next assessed whether LIPUS enhanced the phosphorylation of CaMKII, which is a downstream target of
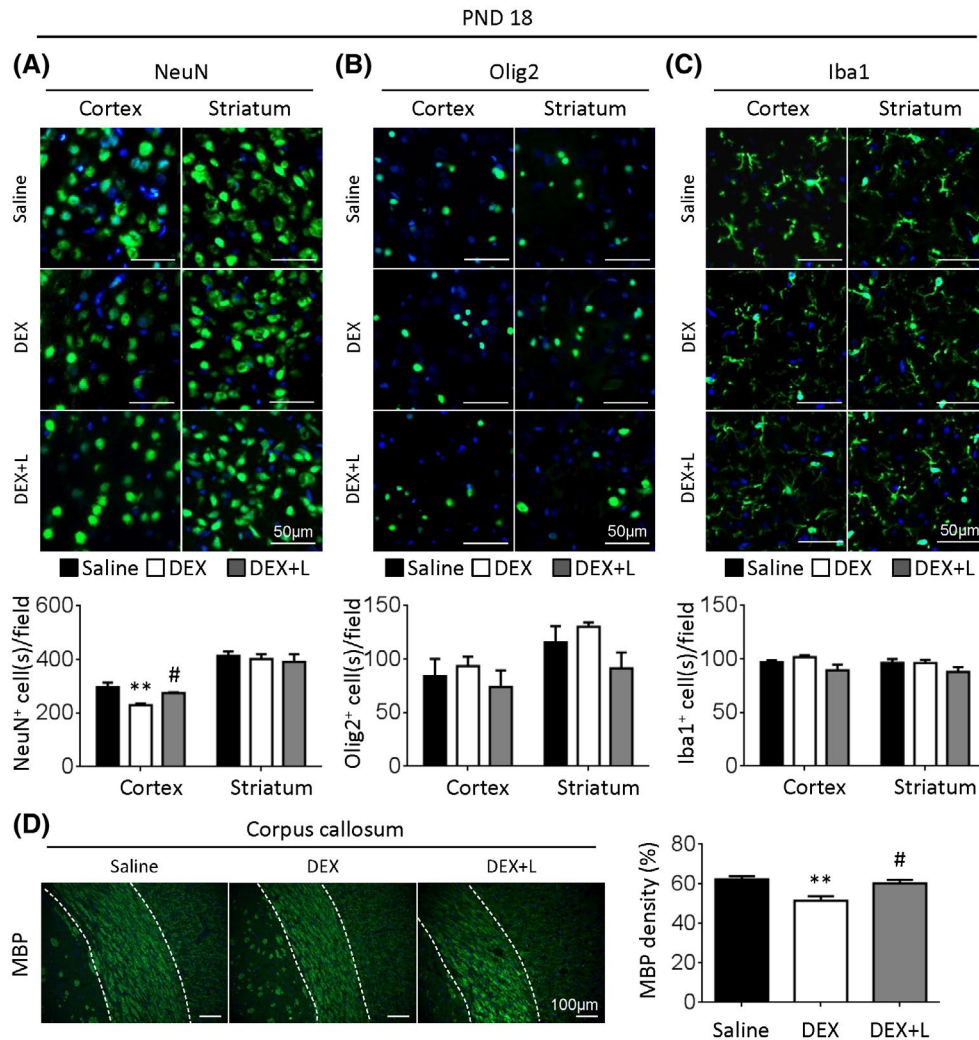
FIGURE 3 **Maternal LIPUS treatment reduced neuronal loss and demyelination in IUGR offspring**. Representative immunostaining images and quantitative data of (**A**) neuron number of neuronal nuclei (NeuN$^+$), (**B**) oligodendrocyte number (Olig2$^+$), (**C**) microglia number (Iba1$^+$), and (**D**) myelin density (MBP intensity) at PND 18 after IUGR. The number of NeuN-, Olig2-, and Iba1-positive cells is expressed as the mean number per field of view (0.8 mm^2). Scale bar = 50 μm. Quantification of MBP staining is expressed as (OD of corpus callosum / mean OD of per field of view) × 100%. Scale bar = 100 μm. Values are mean ± standard error of mean; **$p < 0.01$ versus saline group; #$p < 0.05$ versus DEX group (n = 5–6/group; one-way ANOVA). DEX, dexamethasone; IUGR, intrauterine growth restriction; L, low-intensity pulsed ultrasound; MBP, myelin basic protein; PND, postnatal day

BDNF involved in neuronal survival and CNS myelination.(36, 37) Maternal exposure to DEX induced a significant decrease in CaMKII phosphorylation in pup brains at PND 1 compared with the control group ($p = 0.002$; Figure 5A). LIPUS treatment significantly increased CaMKII phosphorylation to 183.7% of the DEX alone-level ($p < 0.001$; Figure 5A). We also examined the phosphorylation of Akt and ERK, both of which can be activated by CaMKII (38, 39) and are important mediators of neuronal survival and CNS myelination. (40, 41) The level of Akt S473 phosphorylation in pup brains was significantly reduced in the DEX alone group compared to the control group ($p < 0.001$, Figure 5B), and maternal LIPUS treatment (DEX + L) upregulated Akt S473 phosphorylation at PND 1 (48.5% of the DEX alone-level, $p = 0.034$; Figure 5B). However, there were

no differences in phosphorylated levels of Akt T308 and ERK among the three groups (all $p > 0.05$; Figure 5B,C). We used double IF to further analyze whether LIPUS affected phospho-CaMKII and phospho-Akt S473 expression in neurons. Immunoreactivity of phospho-CaMKII and phospho-Akt S473 decreased in the neurons of DEX-treated offspring compared to those of the control group at PND 1, whereas maternal LIPUS treatment increased neuronal expression of phospho-CaMKII and phospho-Akt S473 compared to the DEX-treated group (Figure 5D,E). The percentage of phospho-Akt S473 positive neurons also increased following LIPUS treatment (Figure 5E). These data suggest that the activation of neuronal CaMKII and Akt S473 in IUGR brains contributes to the protective effects of maternal LIPUS treatment.
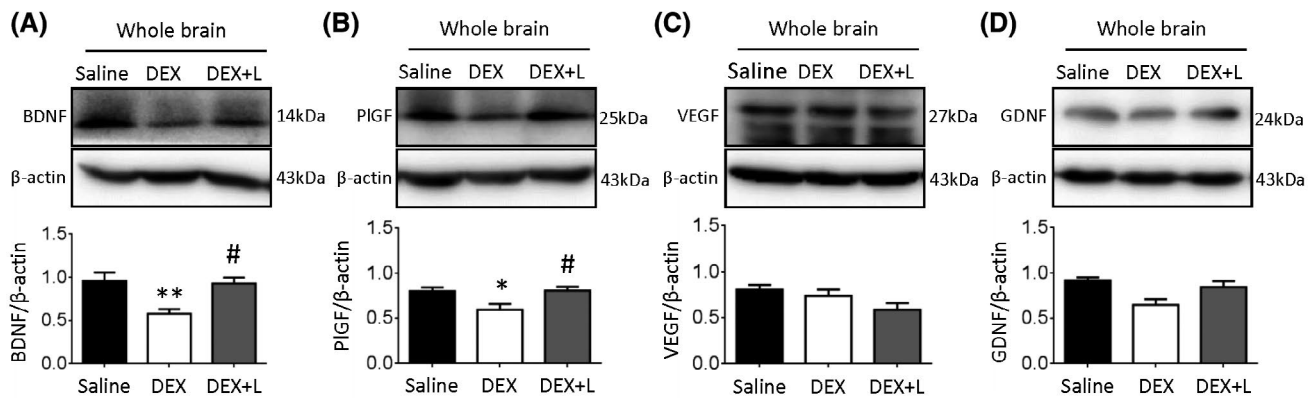
PND 1

FIGURE 4 **Maternal LIPUS treatment increased neurotrophic factor expression in IUGR brains**. Representative immunoblots and quantitative data of (A) BDNF, (B) PlGF, (C) VEGF, and (D) GDNF at PND 1 after IUGR. Values are presented as mean ±standard error of mean; *$p < 0.05$ and **$p < 0.01$ versus saline group; #$p < 0.05$ versus DEX group (n = 9–10/group; one-way analysis of variance). BDNF, brain-derived neurotrophic factor; DEX, dexamethasone; GDNF, glial cell line-derived neurotrophic factor; IUGR, intrauterine growth restriction; L, low-intensity pulsed ultrasound; PlGF, placental growth factor; PND, postnatal day; VEGF, vascular endothelial growth factor

3.6 | Maternal LIPUS treatment increased expression of GLUT1 and neurotrophic factors in the placenta

Fetal growth is dependent on nutrient availability, which is linked to the capacity of the placenta to transport these nutrients. Changes in the expression and activity of nutrient transporters in the placenta have been implicated in the pathophysiology of IUGR.(42) We investigated whether LIPUS treatment affected the expression of glucose and amino acid transporters. As glucose is the primary nutrient required for the growth of the fetus and placenta, we assessed the expression of GLUT1 and GLUT3, which are the primary glucose transporters in both human and rodent placentas. Compared with the placental homogenates from the control group, significantly lower levels of GLUT1 protein were noted in the placental homogenates from rats treated with DEX alone ($p = 0.007$; Figure 6A). Nevertheless, rats simultaneously treated with DEX and LIPUS had an increased GLUT1 level in the placenta compared to those of rats treated with DEX alone ($p = 0.047$; Figure 6A). The GLUT3 protein level in the placenta demonstrated a decreasing trend in rats treated with DEX alone compared with the control group and an increasing trend following LIPUS treatment, although the difference was not statistically significant (all $p > 0.05$; Figure 6B). In contrast, no change in response to DEX or LIPUS treatment was noted in the protein levels of the three Na^+-dependent amino acid transporters SNAT1, SNAT2, and SNAT4 (all $p > 0.05$; Figure 6C–E). We further examined the effect of LIPUS on *GLUT1* and *GLUT3* gene translation. In the placental homogenates from rats treated with DEX alone, there was a significant decrease in *GLUT1* and *GLUT3* mRNA

levels compared with those of the placental homogenates from the control group (Figure 6F,G). The decrease in *GLUT1* mRNA level significantly increased following LIPUS treatment, and *GLUT1* mRNA levels in the placental homogenates from DEX + L-treated rats was 228.8% of the level found in rats treated with DEX alone ($p = 0.046$; Figure 6F). Similarly, LIPUS treatment increased *GLUT3* mRNA levels compared with those of rats treated with DEX alone; however, this trend did not reach statistical significance ($p > 0.05$; Figure 6G).

GLUT1 immunohistochemistry indicated that in the placenta of the control group, immunoreactivity of GLUT1 was noted in the labyrinth, and to a lesser extent, in the junctional zone (also known as the trophospongium) of the placenta (Figure 6H,K). The labyrinth contains fetal and maternal blood channels. Within these channels, maternal and fetal blood circulate independently, while at the same time physiological exchange occurs between the two. The junctional zone includes maternal blood channels through which maternal blood flows into and out of the labyrinth. Compared with the placentas of the control group, GLUT1 immunostaining was less intense in the labyrinth and junctional zone of the placentas than those of rats treated with DEX alone (Figure 6H–M). In contrast, immunostaining of GLUT1 was stronger in the placentas from DEX + L-treated rats than in the placentas from rats treated with DEX alone.

We also examined the placental expression of neurotrophic factors, which are involved in the development of the placenta-fetal unit. In the placental homogenates from rats treated with DEX alone, there was a significant decrease in BDNF protein and an increase in VEGF expression compared with the placental homogenates from the control group (Figure 6N,O). However, LIPUS caused a significant
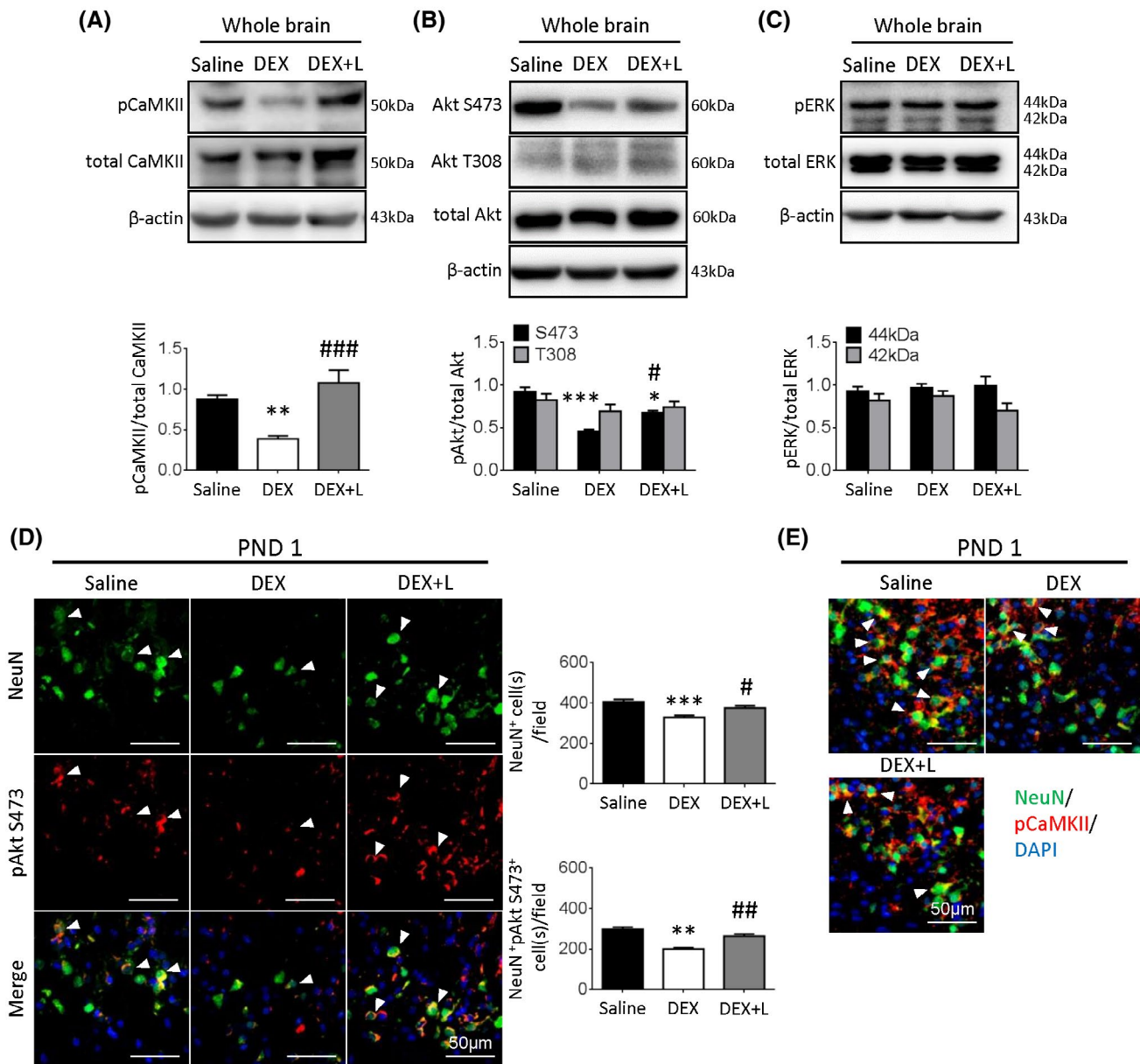
PND 1

FIGURE 5 **Maternal LIPUS treatment enhanced the activation of CaMKII and downstream Akt signaling in IUGR brains**. Representative immunoblots and quantitative data of (**A**) CaMKII, (**B**) Akt, and (**C**) ERK phosphorylation and total protein expression at PND 1 after IUGR. Representative images of double immunostaining and quantitative data of (**D**) neuron number (NeuN[+]) and pAkt S473-positive neurons, and (**E**) pCaMKII-positive neurons at PND 1 after IUGR. Sections were stained with DAPI (blue) to visualize the nuclei. Scale bar = 50 μm. Values are mean ±standard error of mean; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus saline group; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ versus DEX group (n = 7–8/group; one-way ANOVA). DAPI, 4'-6-Diamidino-2-phenylindole; DEX, dexamethasone; IUGR, intrauterine growth restriction; L, low-intensity pulsed ultrasound; PND, postnatal day

increase in the protein levels of BDNF (201.4% of the DEX level, $p = 0.005$; Figure 6N) and a decrease in VEGF (64.7% of the DEX level, $p = 0.037$; Figure 6O) compared with the DEX alone group. Nevertheless, there was no difference in PlGF or GDNF level among the control, DEX alone, and DEX + L-treated groups (all $p > 0.05$; Figure 6P,Q).

4 | DISCUSSION

This is the first study to provide evidence that maternal LIPUS administration promotes fetal growth in the DEX-induced IUGR model. Motor disabilities, neuronal damage, and demyelination in IUGR offspring were also reduced following maternal LIPUS stimulation.
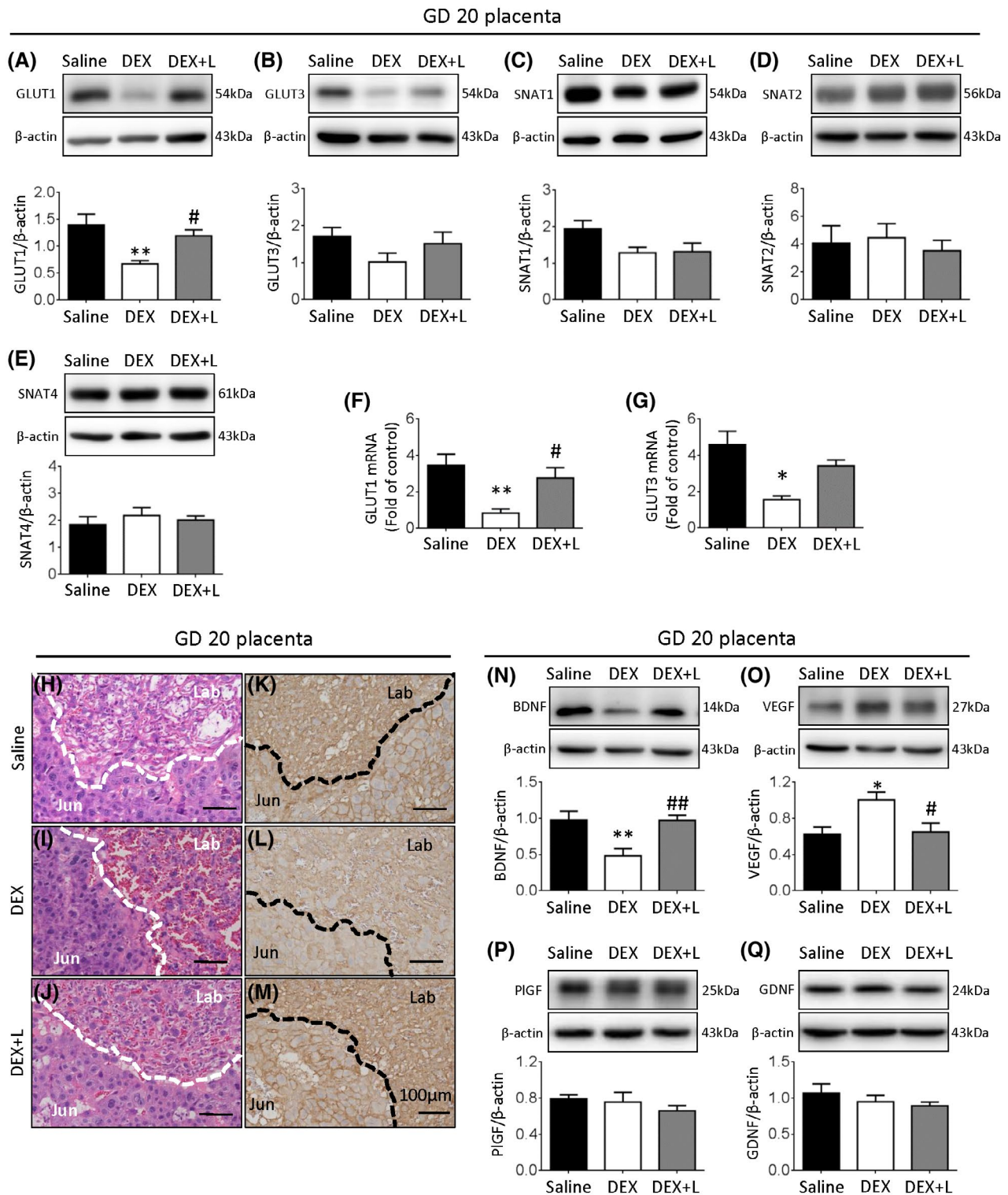
FIGURE 6 **Maternal LIPUS treatment increased expression of GLUT1 and neurotrophic factors in IUGR placentas**. Representative immunoblots and quantitative data of (**A**) GLUT1, (**B**) GLUT3, (**C**) SNAT1, (**D**) SNAT2, and (**E**) SNAT4 protein expressions, and (**F**) GLUT1 and (**G**) GLUT3 mRNA expressions in the placentas at GD 20 after IUGR. Representative hematoxylin & eosin staining (**H-J**) and immunoreactivity of GLUT1 (**K-M**) in the labyrinth and junctional zone of rat placentas. Lab, labyrinth; Jun, junctional zone Scale bar = 100 μm. Representative immunoblots and quantitative data of (**N**) BDNF, (**O**) VEGF, (**P**) PlGF, and (**Q**) GDNF in the placentas at GD 20 after IUGR. Values are presented as mean ±standard error of mean; *$p < 0.05$, **$p < 0.01$ versus saline group; #$p < 0.05$ and ##$p < 0.01$ versus DEX group (n = 6–8/group; one-way ANOVA). BDNF, brain-derived neurotrophic factor; DEX, dexamethasone; GD, gestational day; GDNF, glial cell line-derived neurotrophic factor; GLUT, glucose transporter; IUGR, intrauterine growth restriction; L, low-intensity pulsed ultrasound; PlGF, placental growth factor; PND, postnatal day; VEGF, vascular endothelial growth factor

Mechanistically, LIPUS increased BDNF and PlGF expression and enhanced the activation of CaMKII and downstream Akt signaling in IUGR brains. Additionally, LIPUS increased GLUT1 and BDNF expression in the placenta. Our results suggest that maternal LIPUS treatment is a potential therapy for IUGR. The promotion of neurological recovery is of great clinical relevance, since to date, there are no therapies that can cure the neurological deficits that arise from IUGR (43).

Our results showed that fetal growth was restricted and placental weight was reduced after maternal DEX exposure. Motor activities of offspring were also decreased following antenatal DEX administration, associated with a reduction in neuronal number and myelin density, and maternal DEX exposure also decreased expression of GLUT1 and BDNF in the placenta. Based on these findings, we speculate that maternal DEX exposure may impair placental function, subsequently leading to fetal growth restriction and brain damage. Our results confirmed previous reports that maternal DEX treatment from the mid-to-late gestation period in rats caused fetal growth retardation and delayed neurodevelopment (29, 44). Different animal models have been created and widely used to investigate the pathophysiology of IUGR and the mechanisms by which IUGR predisposes fetuses to later progress of metabolic or neurodevelopmental disorders (45).

Although several animal models have been developed to study the mechanisms of IUGR, the majority of them manifest most but not all the symptoms seen in humans (45). In the current study, pregnant rats exposed to DEX at mid-to-late gestation were used as the model of IUGR. One major reason for using rats is that they have larger uteri and litters than mice. Consequently, the device used for LIPUS treatment can be applied to the gravid uterus to reduce its effects on other intra-abdominal organs. Furthermore, there are additional reasons for using DEX administration instead of other interventions to induce IUGR. First, the administration of DEX is simple, and the model is less traumatic and complicated than surgical interventions such as uterine artery ligation or occlusion and hypoxic insult (45). Second, it has been demonstrated that placentas from DEX-treated animals have disturbances of angiogenesis and are smaller than those that are untreated (46). These changes are similar to those seen in the human placenta from women with pregnancies complicated by IUGR. Third, various animals with maternal exposure to DEX have been widely used to study the link between low birth weight and later development of cardiovascular or metabolic diseases and neurodevelopmental deficits (47–50). One limitation of this study is that we only investigated the effect of LIPUS on DEX-induced IUGR in rats. Compared with other rat IUGR models created by maternal or fetal interventions such as uterine artery ligation or occlusion, L-NAME administration, hypoxic treatment, and nutrient or protein restriction, antenatal exposure to DEX is exceptional in humans and the extent of growth restriction is less severe (51). Moreover, it has been reported that the DEX-induced IUGR model is less sensitive than food restriction in rat studies aimed at investigating the link between low birth weight, rapid postnatal catch-up growth, and later development of glucose intolerance and other metabolic diseases (48). Therefore, further studies using other animal models with naturally occurring IUGR or IUGR created by maternal or fetal intervention, or genetic manipulation, are needed to confirm the beneficial effects of LIPUS.

Neurological impairments such as motor disability are common complications of IUGR (52). These motor disabilities are caused due to IUGR associated with reduced myelination and decreased neuronal numbers in the brain (52). We found that antenatal LIPUS treatment attenuated DEX-induced motor deficits as evidenced by improvement in ambulation, balance, and coordination, better trunk control, and increased hindlimb strength in offspring at PND 18. This protective effect was accompanied by an increase in the number of NeuN-positive cells and myelin density, indicating a reduction in neuronal loss and white matter disruption. We have previously reported that LIPUS treatment decreases neuronal apoptosis and reduces the upregulation of apoptosis-related signaling molecules in animal models of traumatic brain injury (22, 25) and cerebral ischemia (23). As the IUGR infant brain displayed decreased neuronal numbers that were associated with increased neuronal degeneration and apoptotic cell death experimentally, the preservation of neuronal number with LIPUS treatment in the current work is possible due to a reduction in neuronal degeneration. In addition, white matter disruption is reported to be a major neuropathological feature of the IUGR brain (52). We showed that LIPUS treatment attenuated DEX-induced demyelination, which is in agreement with our previous work showing that LIPUS reduced myelin disruption in animal models of vascular dementia (24) and AlCl3-induced cerebral damage (53). However, neither DEX exposure nor LIPUS treatment affected the number of oligodendrocytes. It is possible that LIPUS improves myelin maturation or restores the quantity of myelin without affecting the total number of oligodendrocytes, or it might be related to time points or intensity of LIPUS treatment. The mechanisms involved in LIPUS-induced myelin protection warrant further investigation.

BDNF is a known major neurotrophic protective factor, involved in the regulation of several critical neuronal functions and neuronal survival in patients with nervous system diseases such as traumatic brain injury, Parkinson's disease, and Alzheimer's disease (54, 55). The role of BDNF in IUGR has also been documented in a previous clinical study showing that BDNF is downregulated in cord blood from IUGR neonates (9). In the current study, we showed that maternal exposure to DEX

induced a decrease in cerebral BDNF level in offspring and that maternal LIPUS treatment promoted increase cerebral BDNF protein levels in offspring exposed to DEX. This observation expanded our previous finding that LIPUS upregulates cerebral BDNF protein expression in animals subjected to traumatic brain injury (22), cerebral ischemia (23) or AlCl3-induced cerebral damage (24). Along with increased cerebral BDNF levels, we showed that antenatal LIPUS treatment enhanced CaMKII phosphorylation and increased the number of phospho-CaMKII-positive neurons in the brain of IUGR offspring at PND 1. CaMKII, a downstream target of BDNF (36), has been reported to be abundantly expressed in neurons (56) and promote neuronal survival by activating the downstream pathway of PI3 K/Akt (57). Accumulating evidence has demonstrated that the PI3 K/Akt pathway is a major survival pathway in various neurological disorders (22, 58). Akt inhibits apoptosis by phosphorylating and inactivating pro-apoptotic factors to maintain mitochondrial integrity, which prevents the inhibition of anti-apoptotic proteins (51). We observed that antenatal LIPUS treatment increased cerebral Akt Ser473 phosphorylation and Ser473 phospho-Akt-positive neuron number in the IUGR brain, which was accompanied by increased neuronal number and improved neurological function. These findings suggest that antenatal LIPUS treatment may reduce IUGR-induced brain injury by enhancing cerebral BDNF/CaMKII/Akt signaling. As the systemic use of exogenous BDNF is impeded by its short serum half-life and poor blood-brain barrier (59), our results provide a novel strategy for enhancing endogenous BDNF in the brain via a non-invasive approach.

We also observed that LIPUS enhanced the protein level of PlGF in IUGR brains in addition to increased BDNF protein expression. Upregulation of *PlGF* mRNA and protein has been detected in the ischemic brain (60), suggesting an important role for PlGF in pathological conditions in the brain. Both in vitro and in vivo studies have demonstrated neuroprotective properties of PlGF under ischemic conditions (11, 61). As a homolog of VEGF, PlGF has also been shown to play a role in vessel stabilization and angiogenesis under pathological conditions (30). It is possible that PIGF-mediated neurovascular protection contributed to the improvement of neurobehavioral outcomes following LIPUS stimulation. This point remains to be examined in future studies.

Most causes of IUGR involve the placenta (2). There is evidence that the dysregulation of placental BDNF and VEGF plays a role in the pathophysiology of IUGR (62, 63). Similar to findings of a previous report using maternal undernutrition as a model of IUGR in rats (63). we found that DEX treatment induced a significant reduction in the placental levels of BDNF. A glucocorticoid receptor-binding site has been identified in the promoter region of exon IV of the *BDNF* gene (64). It is likely that DEX downregulates *BDNF* expression through direct binding to its regulatory sequences (64). However, DEX treatment upregulated placental levels of VEGF. Our finding is consistent with an observation of placental samples from a large series of patients, which revealed that women with pregnancies complicated by IUGR had significantly higher expression of VEGF in the placenta than women with normal pregnancies (62). Placentas from IUGR pregnancies also had higher levels of basic fibroblast growth factor and endothelial nitric oxide synthase, suggesting that hypoxia is a potential stimulus for this pathology (65). One of the most striking findings of our study is that we found that LIPUS treatment restored, to some extent, the changes in placental levels of BDNF and VEGF induced by DEX administration. However, further studies are needed to explore the mechanisms underlying the effects of LIPUS effects on these molecules.

Changes in the expression and activity of nutrient transporters in the placenta have been implicated in the pathophysiology of pregnancies complicated by IUGR (42). GLUT1 is the main glucose transporter in both human and rodent placentas. In this study, pregnant rats simultaneously treated with DEX and LIPUS had significantly higher fetal liver weight, birth weight, and placental weight than rats treated with DEX alone; this was accompanied by the upregulation of GLUT1 mRNA and protein expression. Our previous study demonstrated that LIPUS stimulation enhances GLUT1 protein expression in brain astrocyte cells. Upregulation of GLUT1 by LIPUS likely leads to an increased transfer of glucose to the fetal circulation, which subsequently stimulates the production of fetal growth factors responsible for the intensification of fetal growth.

The therapeutic effects of LIPUS have been demonstrated in several organ systems in both animal and human studies. LIPUS is used clinically to enhance bone healing for patients who have a fracture or osteotomy (19, 20). Experimental studies have also demonstrated that LIPUS exerts neuroprotective effects in the central nervous system (22–24) and promotes peripheral nerve regeneration (66). Additional clinical benefits of LIPUS in the management of women with pregnancies complicated by IUGR include non-invasiveness and ease of application. Nevertheless, several limitations of LIPUS treatment in IUGR pregnancies merit attention. First, although we and others did not find any detrimental effects of LIPUS on the growth of fetuses in rats or on the targeted tissues in patients undergoing LIPUS treatment, its bioenergic effects on the developing fetus and placenta in humans remain to be clarified. This is particularly important when developing the therapeutic program for LIPUS treatment in women with pregnancies complicated by IUGR. Second, LIPUS is very likely not able to exert its effect on the placenta in women in which the placenta is located at the posterior uterine wall or in women with placentation underneath uterine fibroids, as the
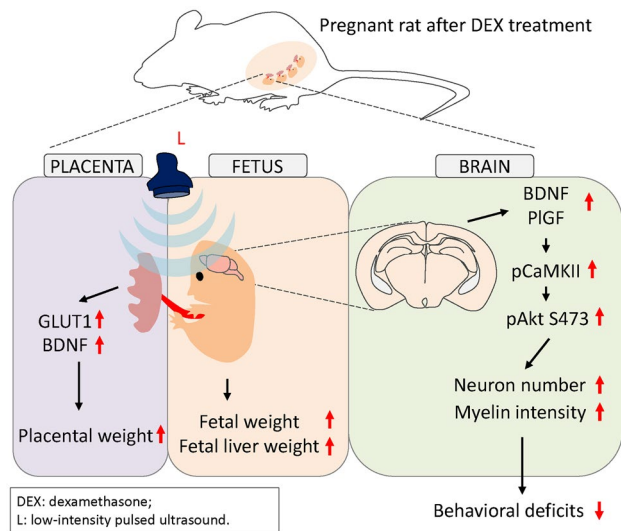
FIGURE 7 The schematic diagram indicates the potential mechanisms of LIPUS treatment-induced neuroprotection in IUGR offspring brain. Our results demonstrate that antenatal LIPUS treatment promotes neuronal survival and myelination, and reduces neurodevelopmental deficits in the postnatal IUGR rats. These effects are related to increasing BDNF and PlGF expression, and subsequently enhancing neuronal CaMKII-Akt signaling activation in the newborn IUGR brain. Meanwhile, LIPUS treatment promotes GLUT1 production and BDNF expression in the placenta. Antenatal LIPUS treatment also increases fetal weight, fetal liver weight, and placental weight after IUGR. Our findings suggest that the enhancement of cerebral BDNF-CaMKII-Akt signaling, and placental GLUT1 and BDNF expression by LIPUS could be a potential strategy for the management of IUGR. BDNF, brain-derived neurotrophic factor; GLUT, glucose transporter; IUGR, intrauterine growth restriction; L, low-intensity pulsed ultrasound; PlGF, placental growth factor.

fetus or uterine fibroids obstruct the transmission of ultrasound. Similar difficulties may be encountered in fetuses whose heads are positioned away from the maternal abdominal surface when targeting the fetal brain to improve its development.

5 | CONCLUSIONS

In conclusion, as summarized in Figure 7, we demonstrated that LIPUS treatment significantly reduced neurological deficits and brain damage in an animal model of IUGR. These beneficial effects of LIPUS might be due to the enhancement of BDNF/CaMKII/Akt signaling pathways in the offspring brain and an increase in BDNF and GLUT1 expression in the placenta. Our findings suggest that LIPUS stimulation could be a novel therapeutic technique for the treatment of IUGR-related brain injury.

CONFLICTS OF INTEREST
The authors have no competing financial interests to disclose.

AUTHOR CONTRIBUTIONS
THH, CHW, FYY, and SFC participated in the design and coordination of the study, performed experiments, analyzed data, and contributed to the writing of the manuscript. THH, YCL, and CCC participated in the design and coordination of the study as well as helped to draft the manuscript. YCL, CCC, and HC performed experiments and analyzed data. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available within the article and from the corresponding author upon reasonable request.

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