Maternal antioxidant treatment protects adult offspring against memory loss and hippocampal atrophy in a rodent model of developmental hypoxia

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Abstract
Chronic fetal hypoxia is one of the most common outcomes in complicated pregnancy in humans. Despite this, its effects on the long-term health of the brain in offspring are largely unknown. Here, we investigated in rats whether hypoxic pregnancy affects brain structure and function in the adult offspring and explored underlying mechanisms with maternal antioxidant intervention. Pregnant rats were randomly chosen for normoxic or hypoxic (13% oxygen) pregnancy with or without maternal supplementation with vitamin C in their drinking water. In one cohort, the placenta and fetal tissues were collected at the end of gestation. In another, dams were allowed to deliver naturally, and offspring were reared under normoxic conditions until 4 months of age (young adult). Between 3.5 and 4 months, the behavior, cognition and brains of the adult offspring were studied. We demonstrated that prenatal hypoxia reduced neuronal number, as well as vascular and synaptic density, in the hippocampus, significantly impairing memory function in the adult offspring. These adverse effects of prenatal hypoxia were independent of the hypoxic pregnancy inducing fetal growth restriction or elevations in maternal or fetal plasma glucocorticoid levels. Maternal vitamin C supplementation during hypoxic pregnancy protected against oxidative stress in the placenta and prevented the adverse effects of prenatal hypoxia on hippocampal atrophy and memory loss in the adult offspring. Therefore, these data provide a link between prenatal hypoxia, placental oxidative stress, and offspring brain health in later life, providing insight into mechanism and identifying a therapeutic strategy.

Keywords
brain, fetal hypoxia, hippocampus, memory, programming
1 | INTRODUCTION

During the last 40 years, significant evidence derived from human epidemiological studies as well as from preclinical animal models has accumulated to show that suboptimal intrauterine conditions can increase the risk of adverse health outcomes in the offspring; a process known as developmental programming.\(^1\)\(^-\)\(^4\) Despite a wealth of knowledge on programmed effects on multiple organ systems in the adult offspring by different suboptimal pregnancy conditions,\(^1\)\(^-\)\(^4\) how gestational hypoxia affects the long-term health of the brain in offspring is less clear. This is surprising given that chronic fetal hypoxia is one of the most common consequences of complicated pregnancy in humans, resulting from conditions such as placental insufficiency, preeclampsia, maternal respiratory disease, maternal obesity, and pregnancy at high altitude.\(^5\)

Studies in human populations at high altitude\(^6\)\(^-\)\(^8\) and in animal models of hypoxic pregnancy have reported fetal and postnatal growth stunting,\(^2\)\(^-\)\(^6\)\(^,\)\(^1\)\(^-\)\(^1\)\(^1\) and alterations in neurologic development in the offspring.\(^1\)\(^-\)\(^6\)\(^,\)\(^1\)\(^-\)\(^1\)\(^6\) However, it has not been possible to isolate the mechanisms driving these effects, preventing insight into plausible intervention. This is because studies at high altitude cannot differentiate between the effects of pre- and post-natal hypoxia on brain health outcomes in the adult offspring, as exposure to hypobaric hypoxia is constant before and after birth. Further, most high-altitude populations are highly impoverished with a high prevalence of maternal malnutrition.\(^1\)\(^7\)\(^,\)\(^1\)\(^8\) Similarly, exposure of pregnant rats or mice to hypoxia can lead to a significant fall in maternal food intake.\(^1\)\(^9\)\(^-\)\(^2\)\(^1\)\(^)\(^ Therefore, the extent to which the adverse effects on the brain of the offspring of highland pregnancy in humans or of hypoxic pregnancy in rodents is due to a reduction in fetal nutrition or fetal oxygenation, with or without fetal growth restriction, is difficult to disentangle.

We have established a rodent model of early-onset hypoxic pregnancy in rats that does not affect maternal food intake during pregnancy or lead to fetal growth restriction.\(^2\)\(^2\)\(^,\)\(^2\)\(^3\) Therefore, this model allows identification of effects of prenatal hypoxia on programming of neurodevelopment that are independent of fetal growth restriction and alterations in maternal food intake. Gestational hypoxia promotes placental oxidative and mitochondrial stress,\(^2\)\(^2\)\(^,\)\(^2\)\(^3\) and it is known that oxidative stress is associated with adverse brain health outcomes.\(^2\)\(^4\)\(^-\)\(^2\)\(^6\) Therefore, placental oxidative stress could provide a mechanism linking prenatal hypoxia with offspring neurodevelopmental disorders. As hypoxia activates the hypothalamic-pituitary-adrenal axis in the fetus\(^2\)\(^7\) and, in turn, fetal exposure to excess glucocorticoids during pregnancy can also program adverse neurodevelopmental consequences in the adult offspring,\(^2\)\(^8\)\(^-\)\(^3\)\(^0\) we investigated whether alterations in maternal or fetal glucocorticoid levels could provide a second underlying mechanism. Therefore, in this study, we tested the hypothesis that hypoxic pregnancy programs neurodevelopmental deficits in the adult offspring by increasing placental oxidative stress and exposure to excess glucocorticoids. The hypothesis was tested by adopting an interventional study design, combining behavioral experiments in vivo with endocrinology, molecular biology, stereology, and histology using our established rat model of pre-natal hypoxia. We investigated the effects of prenatal hypoxia with and without maternal treatment with the antioxidant vitamin C on placental indices of oxidative stress, on maternal and fetal hematocrit and stress hormones, on fetal and adult brain oxidative stress, and on the structure and function of the brain in adult offspring.

2 | MATERIALS AND METHODS

2.1 | Animals and experimental design

The study used an established rat model of early-onset hypoxic pregnancy.\(^2\)\(^2\)\(^,\)\(^2\)\(^3\) Wistar rats (Charles River Limited, UK) were housed in individually ventilated cages (60% humidity, 21°C, 12:12 hour light-dark cycle) with free access to food (maintenance diet, Charles River, UK) and water. After 10 days of acclimatization, virgin female Wistar rats (10–12 weeks of age) were paired with male Wistar rats (minimum 12 weeks of age). The presence of a copulatory plug was considered day 0 of pregnancy, after which the dam was housed individually. Maternal weight, food, and water consumption were monitored throughout gestation.

On day six of pregnancy (term is 21 days), rats were randomly divided into four groups (n = 13-15 dams/group): normoxic (N) or hypoxic (H) pregnancy, with or without maternal vitamin C treatment (HC and NC). Pregnant rats assigned to hypoxia were placed inside a transparent hypoxic chamber that could house nine rat cages in a tranquil environment, as previously described in detail.\(^2\)\(^2\)\(^-\)\(^2\)\(^3\)\(^,\)\(^3\)\(^1\)\(^,\)\(^3\)\(^2\) The chamber was maintained at an inspired fraction of oxygen of 13%. This level of hypoxia simulates ~ 3600 m above sea level, the altitude at which pregnancy complications have been demonstrated to be significantly increased in human populations.\(^6\)\(^-\)\(^8\)\(^,\)\(^3\)\(^3\)\(^-\)\(^3\)\(^5\) In experiments in sheep, in which the fetus can be catheterized long term, we have also shown that this level of reduced maternal oxygenation during pregnancy yields fetal arterial PO\(^2\) values between 12 and 14 mmHg.\(^7\)\(^,\)\(^3\)\(^6\)\(^,\)\(^3\)\(^7\) This is the PO\(^2\) measured by cordocentesis in human fetuses in pregnancy affected by chronic fetal hypoxia.\(^3\)\(^8\) Uterine wall invasion and the establishment of the placenta commences after day 9.25 of gestation in rat pregnancy,\(^3\)\(^9\) meaning placentalion in this model also takes place under hypoxic conditions. Therefore, in the present study, this level of maternal hypoxia is relevant to human sea level pregnancy complicated by chronic hypoxia in early or late gestation as well as human pregnancy at high altitude. The third and fourth groups of dams underwent pregnancy under normoxic or
hypoxic conditions, respectively, but received vitamin C (0.5 mg.100 mL−1) in the drinking water, which was prepared fresh every day. The dose of vitamin C used was derived from previous studies in our laboratory, which reported elevations in maternal circulating ascorbate concentrations of human clinical relevance,40 and those that confer successful antioxidant protection in the offspring.32,41

On day 20 of gestation, a subset of dams (n = 7-9/group) from each group was randomly chosen and anesthetized with isoflurane via a cone and then maintained with ketamine (40 mg/kg, Fort Dodge Animal Health, U.K) and xylazine (5 mg/kg, Millpledge Veterinary, U.K) injected intraperitoneally. The pregnant uterus was exposed through a midline section, and the anesthetized pups were killed via cervical transection. In all pups, the ano-genital distance was measured with digital callipers for determination of sex. Maternal blood was taken by cardiac puncture into a 1 ml syringe. Fetal blood was taken via capillarity into hematocrit tubes. From 1 male fetus per litter, body weight was recorded, and the placenta and brain were collected. The placenta was immersion fixed in 4% paraformaldehyde and the brain snap frozen in liquid nitrogen and maintained at −80°C. For the remaining dams, treatment (hypoxia and/or vitamin C) was ceased at day 20 of gestation, and they were allowed to deliver naturally. Within 3-5 hours after birth, pups were sexed, and litters reduced to 8 pups to standardize nutrition and maternal care. Pups remained with their mothers until weaning (postnatal day 21). At three and a half months of age, behavioral testing commenced in n = 10-12 adult male offspring per group. At four months of age, when behavioral testing was complete, adult male offspring were deeply anesthetized (0.2 mL total volume, i.p., 100 mg/mL ketamine, and 20mg/mL xylazine), then perfused intracardially with NaCl followed by 4% paraformaldehyde (PFA) under physiological pressure.42,43 Brains were collected, weighed, and stored overnight at 4°C in 4% PFA for subsequent stereological analysis. In a separate subset of adult male offspring (n = 6/group), the brain was isolated and snap frozen in liquid nitrogen and maintained at −80°C for subsequent analysis.

2.2 Placental studies

The fixed placentae were embedded in paraffin wax, then completely sectioned at 7 μm perpendicular to the chorionic plate (Leica RM 2235 microtome, Leica Microsystems, Germany).

2.2.1 Immunohistochemistry

To assess placental indices of oxidative and nitrosative stress, immunohistochemistry was performed on sections near the placental midline. Sections were dewaxed and washed in tap water for 10 minutes, before incubating with 3% H₂O₂ in distilled water. Antigen retrieval was performed by incubating sections in Tris-EDTA (pH9.0, Sigma Aldrich, UK), after which the sections were washed for 15 minutes in phosphate-buffered saline with 0.1% Tween 20 and 0.1% Triton X (PBS- TT, all Sigma-Alrdich, UK). Nonspecific binding was blocked with 5% BSA in PBS for one hour. Sections were then incubated with primary antibody (nitrotyrosine, NT, 1:500, Merck, UK, 06-284; heat shock protein 70, Hsp70, 1:500, Enzo Life Sciences, UK, ADI-SPA-812; Hsp90, 1:300, Enzo Life Sciences, UK, ADI-SPA-830) in 5% BSA in PBS-TT (Sigma Aldrich, U.K.) overnight. The next day, sections were washed in PBS, incubated with secondary antibody (1:200 in 5% BSA in PBS, Vector Laboratories, USA), and then washed in PBS. Sections were incubated in AB in PBS (Vector Laboratories, U.K.) and then washed in PBS. Staining was visualized with DAB (Sigma Aldrich, UK). Tissue was then dehydrated through increasing concentrations of alcohol before being cover slipped with DPX (Sigma Aldrich, UK).

2.2.2 Optical density quantification

Using a calibrated optical density step tablet (ImageJ, National Institutes of Health), the optical density (OD) of NT, Hsp70, and Hsp90 staining was measured in the labyrinth transport zone of the placenta, as this is where oxygen and nutrients are exchanged between maternal and fetal circulations. For each antibody, 10 regions within the labyrinth zone were randomly selected for quantification.

2.3 Maternal and fetal hematocrit and endocrinology

Maternal and fetal blood samples collected at the end of pregnancy were used to calculate maternal and fetal hematocrit using a Hawksley centrifuge.32 Remaining blood was dispensed into K+-EDTA-treated tubes and centrifuged immediately at 4000 r.p.m. for 4 minutes at 4°C. Plasma samples were stored at −80°C until analyses. The concentrations of corticosterone in plasma were quantified using a commercially available ELISA kit, according to the manufacturer’s instructions (IBL International, Hamburg, Germany). The inter-assay and intra-assay coefficients of variation for corticosterone were 6.1.1% and 5.2%, respectively.

2.4 Adult offspring behavioral analyses

2.4.1 Morris water maze

Spatial learning and reference memory were evaluated in offspring using the water maze.44 The maze, surrounded
by visual cues, consisted of a white circular tank filled with water (1 m diameter, 1 m depth, 24-25°C), which was made opaque with nontoxic white paint. An escape platform was submerged 2 cm below the surface. Rats were given four trials per day over four consecutive days. The platform remained in the same quadrant (target quadrant) during the entire experiment. Rats that failed to find the platform were given a score of 60 seconds, then physically placed on the platform for 20 seconds. Intertrial intervals ranged from 15 seconds to 10 minutes. Escape latency, path length, swimming speed, and thigmotaxis (an animal’s propensity to swim along the edge of the water maze) were quantified using video tracking software (HVS Image 2020, Hampton, UK). Twenty-four hours after the last training day, animals were subjected to a probe trial, which assessed spatial reference memory, in which rats were allowed to swim without the platform for 60 seconds. Swimming distance, path length, and the percentage of time spent in each area were recorded (HVS Image 2020, Hampton, UK).

2.4.2 | Open field

The open field task determines novel environment exploration and general locomotor activity, and provides an initial screen for anxiety-related behavior. Rats were placed in a circular arena (1 m diameter) for 5 minutes. The arena was virtually divided into the periphery, middle, and center. Path length, speed, and the percentage of time spent in each area were recorded (HVS Image 2020, Hampton, UK).

2.5 | Adult offspring brain histological and stereological analyses

2.5.1 | Volumetric analysis

The cerebrum (n = 5/group) was exhaustively sectioned at 50µm using a vibratome (Leica RM 2235, Germany). Sections were then stored in cyroprotectant at −20°C. To assess the volume of the cerebrum and its compartments, systematic random sampling was used to select 10 equally spaced sections per animal. Selected sections were stained with 1% Cresyl Violet. A point grid was superimposed on the Cresyl Violet-stained sections and viewed using a x1.25 objective (Olympus BX-50 microscope and CAST). Points falling on each compartment were counted and the Cavalieri principle was applied to determine estimated volumes:

\[ V_{\text{obj}} = t \times \sum a = t \times a(p) \times \sum P \]

where \( V_{\text{obj}} \) represents the estimated volume of the brain region, \( t \) is the total length of the brain (\( t = \text{no. of sections} \times \text{section thickness} \)), \( a(p) \) is the area associated with each point, and \( \sum P \) is the sum of points for that region.

2.5.2 | Immunohistochemistry and lectin histochemistry

Sections were washed for 30 minutes in PBS (Sigma-Aldrich, UK), incubated with 0.3% H2O2 in methanol for 20 minutes, and then washed in PBS for 15 minutes. Nonspecific binding was blocked with 4% BSA in PBS for 30 minutes. Sections were then incubated with primary antibody (myelin basic protein, MBP; 1:400, three sections per animal, MAB386; neuronal nuclei, NeuN, 1:400, four-five sections per animal containing the entire hippocampus, MAB377; presynaptic marker synaptophysin 1:1000, two sections per animal, MAB5258-I; all from Millipore, UK) in 2% BSA in PBS containing 0.3% Triton (Sigma Aldrich, UK) overnight. The following day, sections were washed for 15 minutes in PBS, incubated for one hour with secondary antibody (1:400 in 2% BSA in PBS, Vector Laboratories, USA) then washed for 15 minutes in PBS. Sections were incubated for one hour in AB in PBS (Vector Laboratories, UK), then washed in PBS for 15 minutes. Staining was visualized with metal DAB in peroxide buffer (Thermo Scientific, UK) for two minutes. Tissue was then mounted with 0.5% gelatine in PBS on slides. The same protocol was used for lectin histochemistry (two sections per animal), with the exception that sections were incubated in biotinylated Lycopersicin esculentum (tomato) lectin overnight (1:200, Vector Laboratories, UK, B-1175-1), and the secondary antibody omitted.

2.5.3 | Neuronal number and soma volume quantification

Each area of the hippocampus (CA1, CA2/3 and dentate gyrus) has its own cellular structure and distinctive function. Therefore, neuronal number within each of these areas was estimated in all NeuN-positive-stained sections containing the hippocampus using the fractionator method and an Olympus BX-50 microscope and CAST. Step motors on the microscope were used to randomly sample a known fraction of the tissue. An unbiased counting frame was superimposed on the tissue image and nuclei within the counting frame, or those touching the permitted lines of the counting frame, were counted. Total neuronal number was determined in each hippocampal brain region using the formula:

\[ \text{est} N = \sum n \times f_1 \times f_2 \]

where \( \text{est} N \) is the estimated total number of nuclei, \( \Sigma n \) is the sum of nuclei counted, \( f_1 \) is the reciprocal of the sampling
fraction, and \( f_2 \) is the areal sampling fraction. The soma volume of neurons was calculated using the vertical dissector tool in the CAST program\(^{42} \) and the following formula:

\[
V_n = \frac{4\pi}{3} \times I_3n
\]

where \( I_n \) is the distance from the midpoint of the neuron to the cell membrane.

### 2.5.4 Percent of blood vessels and synaptophysin-positive puncta quantification

The percent of lectin-positive blood vessels and synaptophysin-positive puncta were measured within the CA1 (stratum lacunosum moleculare [SL-M] and radiatum [SR]), CA2/3 (stratum lucidum [SL], SL-M and SR), and the dentate gyrus (DG) (molecular layer [ML]) of the hippocampus. A point grid was superimposed on two equally spaced sections containing the dorsal hippocampus and viewed using a \( \times60 \) objective (Olympus BX-50 microscope and CAST). Points falling on lectin-positive blood vessels and synaptophysin-positive puncta versus the parenchyma (10-20 fields of view across both sections and hemispheres) were counted, to calculate the percent of positive staining.\(^{42,43} \)

### 2.5.5 Optical density of myelin-basic protein and extent of myelination

Due to importance of myelin in the process of memory consolidation,\(^{50,51} \) the OD of MBP-stained fibers was measured in the corpus callosum and the cortex using a calibrated optical density step tablet (ImageJ, National Institutes of Health). On the basis of anatomical landmarks, equivalent sections from all rats were chosen.\(^{42,43} \) The area of MBP-positive fibers in the cortex, relative to cortical size, was calculated using the same software.\(^{42,43} \) Eight fields of view within the corpus callosum and the cortex were examined in three sections per animal.

### 2.6 Cerebral oxidative stress

Indices of oxidative stress in fetal brains were determined in frozen tissue via ELISA (\( n = 7-9 \)/group). For consistency, the same method was used to determine indices of oxidative stress in brains from adult offspring (\( n = 6/ \) group). Therefore, lipid peroxidation was analyzed in frozen fetal and adult brain tissue using an OxiSelect 4-hydroxynoneal (4-HNE) Adduct ELISA kit (Cambridge Biosciences, UK). Protein oxidation was assayed using a Nitrotyrosine (3-NT) ELISA kit (MitoSciences, UK). Both ELISAs were performed according to the manufacturer’s instructions.

### 2.7 Statistical analysis

To control for sex and within-litter variation, no more than 1 male offspring per litter and associated placenta and brain tissue were randomly chosen per outcome variable for the fetal studies. Similarly, 1-2 male offspring per litter were randomly chosen per outcome variable for the studies at adulthood. Female offspring were not studied, and they were donated to other projects. It is well accepted that the estrous cycle affects memory and alters anxiety-related behavior.\(^{52-55} \) Therefore, this confounder was excluded using this study design. Experimental groups were assigned using a random choice generator. All quantitative analyses were performed with the observer (E.J.C.) blind to the treatment groups. All data sets were first assessed for normality of distribution using the Shapiro-Wilk test. Potential outliers were determined using the Grubb’s test. There were no outliers removed from the maternal, fetal, or adult offspring data sets. Data are presented as mean ± SD. Differences between treatment groups were compared statistically using a two-way ANOVA (factors: environment, treatment), or a repeated measures two-way ANOVA (within subject factor: day of training; between subject factors: environment, treatment), followed by the Tukey’s post-hoc test where appropriate. For all comparisons, statistical significance was accepted when \( P < .05 \) (IBM SPSS Statistics 23).

### 3 RESULTS

#### 3.1 Effects of gestational hypoxia and maternal antioxidant treatment on pregnancy characteristics and biometry

Values for maternal food intake, maternal body weight, gestational length, litter size, and offspring sex ratio were not different between groups (Table 1, \( P > .05 \)). There was a significant main effect of environment on maternal (\( F(1, 18) = 81.121, P < .001 \)) and fetal (\( F(1, 27) = 230.187, P < .001 \)) hematocrit values, and placental weight (\( F(1, 27) = 74.305, P < .001 \)). Relative to normoxic pregnancies, hypoxic pregnancies with or without vitamin C treatment had similarly increased hematocrit and placental values (Table 1). There were no significant environment \( \times \) treatment interactions (all \( P > .05 \)). In the fetuses, body weight, absolute, and relative brain weight values were not different between groups (Table 1, \( P > .05 \)). In adult offspring (4 months of age), values for body weight, absolute, or relative brain weight were also not different between groups (Table 1,
TABLE 1 Maternal and offspring data

|                | N       | H      | HC     | NC     | Statistical Significance |
|----------------|---------|--------|--------|--------|--------------------------|
| Mother Food intake (g/day) | 24.7 ± 2.4 (n = 6) | 25.4 ± 4.9 (n = 6) | 26.7 ± 2.8 (n = 6) | 26.0 ± 3.0 (n = 5) | P<sub>ENV</sub> = 0.387 P<sub>TRT</sub> = 0.135 P<sub>INT</sub> = 0.971 |
| Body weight (g) | 307.0 ± 42.2 (n = 6) | 321.4 ± 47.8 (n = 6) | 319.0 ± 44.2 (n = 6) | 319.0 ± 44.1 (n = 5) | P<sub>ENV</sub> = 0.526 P<sub>TRT</sub> = 0.663 P<sub>INT</sub> = 0.513 |
| Gestation length (days) | 22.0 ± 0.7 (n = 6) | 22.2 ± 0.8 (n = 6) | 22.4 ± 0.5 (n = 6) | 21.8 ± 0.4 (n = 5) | P<sub>ENV</sub> = 0.185 P<sub>TRT</sub> = 0.953 P<sub>INT</sub> = 0.446 |
| Litter size | 13.0 ± 2.9 (n = 6) | 14.3 ± 2.8 (n = 6) | 12.4 ± 2.7 (n = 6) | 11.8 ± 2.0 (n = 5) | P<sub>ENV</sub> = 0.416 P<sub>TRT</sub> = 0.195 P<sub>INT</sub> = 0.756 |
| Offspring sex ratio (% males) | 53.3 ± 3.7 (n = 6) | 48.9 ± 13.2 (n = 6) | 44.1 ± 11.2 (n = 6) | 42.5 ± 12.2 (n = 5) | P<sub>ENV</sub> = 0.769 P<sub>TRT</sub> = 0.119 P<sub>INT</sub> = 0.537 |
| Hematocrit (%) | 30.9 ± 1.1 (n = 6) | 36.8 ± 2.2 (n = 6)* | 36.1 ± 1.0 (n = 6) | 30.5 ± 1.6 (n = 5) | P<sub>ENV</sub>: <0.001 P<sub>TRT</sub> = 0.386 P<sub>INT</sub> = 0.731 |
| Plasma Corticosterone (ng/mL) | 499.9 ± 193.8 (n = 6) | 483.8 ± 219.7 (n = 6) | 389.4 ± 57.5 (n = 6) | 467.9 ± 166.5 (n = 5) | P<sub>ENV</sub> = 0.517 P<sub>TRT</sub> = 0.388 P<sub>INT</sub> = 0.668 |
| Fetal Offspring Body weight (g) | 3.60 ± 0.16 (n = 9) | 3.61 ± 0.25 (n = 7) | 3.63 ± 0.28 (n = 7) | 3.61 ± 0.23 (n = 8) | P<sub>ENV</sub> = 0.836 P<sub>TRT</sub> = 0.865 P<sub>INT</sub> = 0.915 |
| Placental weight (g) | 0.603 ± 0.007 (n = 9) | 0.676 ± 0.034 (n = 7)* | 0.670 ± 0.036 (n = 7)† | 0.586 ± 0.016 (n = 8) | P<sub>ENV</sub>: <0.001 P<sub>TRT</sub> = 0.223 P<sub>INT</sub> = 0.561 |
| Brain weight (g) | 0.171 ± 0.005 (n = 9) | 0.171 ± 0.009 (n = 7) | 0.171 ± 0.006 (n = 7) | 0.170 ± 0.006 (n = 8) | P<sub>ENV</sub> = 0.839 P<sub>TRT</sub> = 0.860 P<sub>INT</sub> = 0.860 |
| Brain/Body weight (%) | 4.8 ± 0.3 (n = 9) | 4.8 ± 0.4 (n = 7) | 4.7 ± 0.4 (n = 7) | 4.7 ± 0.3 (n = 8) | P<sub>ENV</sub> = 0.997 P<sub>TRT</sub> = 0.880 P<sub>INT</sub> = 0.972 |
| Hematocrit (%) | 34.4 ± 0.9 (n = 9) | 41.4 ± 1.7 (n = 7)* | 42.1 ± 1.5 (n = 7)† | 33.8 ± 1.5 (n = 8) | P<sub>ENV</sub>: <0.001 P<sub>TRT</sub> = 0.996 P<sub>INT</sub> = 0.180 |
| Plasma Corticosterone (ng/mL) | 269.7 ± 144.7 (n = 7) | 246.1 ± 120.6 (n = 7) | 254.5 ± 115.7 (n = 6) | 302.4 ± 156.9 (n = 6) | P<sub>ENV</sub> = 0.509 P<sub>TRT</sub> = 0.703 P<sub>INT</sub> = 0.821 |
| Adult Offspring Body weight (g) | 550.8 ± 32.7 (n = 12) | 553.6 ± 68.8 (n = 10) | 550.5 ± 44.9 (n = 12) | 531.1 ± 56.3 (n = 10) | P<sub>ENV</sub> = 0.479 P<sub>TRT</sub> = 0.468 P<sub>INT</sub> = 0.596 |
| Brain weight (g) | 1.95 ± 0.15 (n = 12) | 1.87 ± 0.08 (n = 10) | 1.92 ± 0.10 (n = 10) | 1.94 ± 0.08 (n = 10) | P<sub>ENV</sub> = 0.159 P<sub>TRT</sub> = 0.490 P<sub>INT</sub> = 0.374 |
| Brain/Body weight (%) | 0.354 ± 0.029 (n = 12) | 0.342 ± 0.038 (n = 10) | 0.351 ± 0.032 (n = 10) | 0.369 ± 0.038 (n = 10) | P<sub>ENV</sub> = 0.161 P<sub>TRT</sub> = 0.262 P<sub>INT</sub> = 0.803 |

Note: Values are mean ± SD in dams, male fetuses or male adult offspring from normoxic (N), hypoxic (H), hypoxic + vitamin C (HC), and normoxic + vitamin C (NC) pregnancies. The average daily maternal food intake and body weight was calculated between days 6 to 20 of gestation when hypoxia was induced. The maternal data were generated from 5–6 dams/group. The adult male offspring were generated from these dams, with 1–2 offspring per litter studied (n = 10–12/group). The fetal data were generated from a separate group of dams (n = 7–9/group) in which 1 male offspring per litter and associated placenta and fetal brain tissue were collected. Maternal data were not collected from all dams. *vs. N, †vs. NC, both P < .05, two-way ANOVA, and post-hoc Tukey test. Statistical significance following a two-way ANOVA examining the effects of environment (P<sub>ENV</sub>), treatment (P<sub>TRT</sub>), and their interaction (P<sub>INT</sub>) are presented.

P > .05. Maternal and fetal corticosterone concentrations at the end of gestation were not different between groups (Table 1, P > .05).

3.2 | Effects of gestational hypoxia and maternal antioxidant treatment on indices of oxidative stress in the placenta and the brain of the offspring

The intensity of 3-NT, Hsp70, and Hsp90 immunostaining in the labyrinth zone of the placenta at day 20 of gestation was determined as an indicator of placental nitrosative and oxidative stress. There was a significant interaction between environment and treatment for 3-NT (F(1,27) = 9.586, P = .005), Hsp70 (F(1,27) = 4.519, P = .043), and Hsp90 (F(1,11) = 7.550, P = .025, Figure 1A-C). Higher staining intensity was observed for all markers of nitrosative and oxidative stress in the placentae from hypoxic pregnancy relative to normoxic pregnancy (3-NT: P = .008; Hsp70: P = .002; Hsp90: P = .002). Maternal vitamin C treatment in hypoxic pregnancies prevented the upregulation of placental 3-NT (P = .019), Hsp70 (P = .012), and Hsp90 (P = .016). No significant changes in staining intensity were observed...
in the placentas from normoxic pregnancies treated with vitamin C. In marked contrast, levels of 4-HNE and 3-NT in fetal and adult brain tissue, assessed using ELISA, were all below the limit of detection suggesting that if present, levels of oxidative damage were very low in the brain. As a positive control, fetal liver tissue was also processed for indices of oxidative stress. 3-NT could be detected and measured in fetal liver, and values were similar for normoxic and hypoxic pregnancy whether treated or untreated with vitamin C (N: 17.4 ± 14.6 nmol/mg protein; H: 15.2 ± 9.8 nmol/mg protein; HC: 21.0 ± 4.4 nmol/mg protein; NC: 19.1 ± 8.9 nmol/mg protein, \( P > .05 \)).

3.3 Effects of gestational hypoxia and maternal antioxidant treatment on behavioral analyses in the adult offspring

3.3.1 Morris Water Maze

All offspring learned the position of the submerged platform, evidenced by the progressive decrease in escape latency \( (F(2.562, 102.488) = 50.478, P < .001) \) and path length \( (F(3, 120) = 49.921, P < .001) \) during the four-day training period (Figure 2A,B). Swimming speed \( (F(3, 120) = 11.183, P < .001) \) and percent time in thigmotaxis also decreased during the training period \( (F(3, 120) = 87.431, P < .001, \) Figure 2C,D). No significant main effect of environment was observed in relation to latency, path length, swimming speed, or percent time in thigmotaxis (all \( P > .05 \)). However, there was a significant main effect of treatment on path length \( (F(1, 40) = 4.292, P = .045) \), and percent time in thigmotaxis \( (F(1, 40) = 12.153, P = .001) \), which were reduced in offspring whose mothers were treated with vitamin C compared to those who were treated with water (both \( P < .05 \)). There were no significant environment \( \times \) treatment interactions (all \( P > .05 \)).

To assess spatial memory, a probe test was performed following the four-day training period. There was a significant effect of environment on the percentage of time searching in the target quadrant that had previously contained the submerged platform \( (F(1, 40) = 6.781, P = .013, \) Figure 3A), and in the opposite quadrant \( (F(1, 40) = 4.582, P = .038, \) Figure 3B). Taken together, relative to normoxic offspring, offspring of hypoxic pregnancy spent less time searching in the target quadrant \( (P = .011) \) and more time in the opposite quadrant \( (P = .019) \). Maternal treatment with vitamin C in hypoxic pregnancy prevented this effect \( (P > .05, \) Figure 3A,B). There was neither significant effect of environment or treatment on path length \( (P > .05, \) Figure 3C) and swimming speed \( (P > .05, \) Figure 3D), nor any significant environment \( \times \) treatment interactions (all \( P > .05 \)). Maternal treatment with vitamin C in normoxic pregnancy did not alter spatial memory performance during the probe test (all \( P > .05 \)).

3.3.2 Open field

There was no significant difference between offspring of normoxic or hypoxic pregnancy in percent time spent in the
FIGURE 2  Morris water maze training. Training: (A) latency, (B), path length, (C) swimming speed, and (D) percent time in thigmotaxis during the four-day training period. Values are mean ± SD in normoxic (N, white bars), hypoxic (H, black bars), hypoxic + vitamin C (HC, red bars), and normoxic + vitamin C (NC, blue bars) offspring (n = 10-12/group). Statistical significant examining the effects of environment (PENV), treatment (PTRT), and their interaction (PINT) are indicated below each figure, two-way ANOVA repeated measures ANOVA and post-hoc Tukey test. The data for these outcomes were generated from 1 to 2 male offspring per litter.
CAMM et al.

periphery (N: 80.5 ± 10.1%, H: 81.8 ± 5.1%) or center (N: 3.2 ± 3.2%, H: 2.7 ± 0.9%) of the open field arena, or in path distance (N: 34.5 ± 7.1 m, H: 36.3 ± 5.2 m), or speed (N: 0.116 ± 0.025 m/s, H: 0.121 ± 0.017 m/s, all P > .05).

Similarly, maternal vitamin C treatment in normoxic or hypoxic pregnancy had no effect on percent time spent in the periphery (HC: 76.8 ± 6.6%, NC: 76.2 ± 11.3%), center (HC: 4.1 ± 2.69%, NC: 4.9 ± 3.3%), path distance (HC: 32.2 ± 5.1 m, NC: 34.9 ± 2.9 m), or speed (HC: 0.106 ± 0.018 m/s, NC: 0.115 ± 0.011 m/s, all P > .05).

3.4 | Effects of gestational hypoxia and maternal antioxidant treatment on histology and stereological analyses of the brain in the adult offspring

3.4.1 | Gross morphology and regional brain volumes

At four months of age, examination of Cresyl Violet-stained sections did not reveal any gross alterations in cerebral cytoarchitecture or absolute (data not shown) or relative (Table 2, all P > .05) regional volumes between the treatment groups.

3.5 | Hippocampal morphology

3.5.1 | CA1

Within the CA1 region of the hippocampus, there was a significant interaction between environment and treatment on neuronal number (F(1,16) = 5.722, P = .029, Figure 4A) and the percent of the parenchyma occupied by lectin-positive blood vessels (F(1,16) = 4.899, P = .042, Figure 5A). Further, there was a significant effect of environment on the percent of synaptophysin-positive puncta (F(1,16) = 7.287, P = .016, Figure 6A). Relative to normoxic pregnancy, offspring of hypoxic pregnancy had less pyramidal neurons (P = .007, Figure 4A) and reduced percentage of lectin-positive blood vessels (P = .002, Figure 5A) and synaptophysin-positive puncta (P = .004, Figure 6A). The soma volume of the pyramidal neurons in the CA1 was unaltered (N: 1562 ± 66 µm³; H: 1495 ± 120 µm³, P > .05). Maternal vitamin C treatment in hypoxic pregnancy protected against alterations in hippocampal morphology. Relative to hypoxic pregnancy, neuronal number (P = .004, Figure 4A) and the percent of lectin-positive blood vessels (P < .001, Figure 5A) were significantly increased in hypoxic offspring whose mothers received vitamin C. Soma volumes were not altered (1509 ± 41 µm³,
TABLE 2  Offspring cerebral stereological data

| Region                           | N          | H          | HC         | NC         | P_{ENV} | P_{TRT} | P_{INT} |
|----------------------------------|------------|------------|------------|------------|---------|---------|---------|
| Cortex (% total)                 | 47.4 ± 1.7 | 46.5 ± 2.1 | 46.8 ± 2.3 | 47.2 ± 1.1 | 0.498   | 0.947   | 0.776   |
| Hippocampus (% total)            | 7.7 ± 0.6  | 8.5 ± 0.8  | 7.8 ± 0.8  | 8.5 ± 0.8  | 0.884   | 0.820   | 0.05    |
| White matter (% total)           | 1.0 ± 0.5  | 1.1 ± 0.3  | 1.2 ± 0.4  | 1.1 ± 0.2  | 0.586   | 0.740   | 0.778   |
| Corpus callosum (% total)        | 6.7 ± 0.2  | 6.4 ± 0.9  | 7.3 ± 1.4  | 6.6 ± 0.5  | 0.688   | 0.326   | 0.248   |
| Deep grey matter (% total)       | 37.2 ± 2.4 | 37.6 ± 2.3 | 36.9 ± 2.9 | 36.7 ± 1.2 | 0.784   | 0.572   | 0.973   |

Note: Values are mean ± SD in male adult offspring (n = 5/group) from normoxic (N), hypoxic (H), hypoxic + vitamin C (HC), and normoxic + vitamin C (NC) pregnancies. Relative brain volumes were estimated using Cresyl Violet-stained coronal sections of the cerebrum and the Cavalieri principle. Statistical significance following a two-way ANOVA examining the effects of environment (P_{ENV}), treatment (P_{TRT}), and their interaction (P_{INT}) are presented. The data for these outcomes were generated from 1 to 2 male offspring per litter.

$P > .05$). Vitamin C treatment in normoxic pregnancy increased the percentage of lectin-positive blood vessels relative to untreated normoxic pregnancy ($P = .004$, Figure 5A). The number of neurons, percentage of synaptophysin-positive puncta (Figure 6A), and soma volumes (1555 ± 67 $\mu$m$^3$) were unchanged (all $P > .05$).

### 3.5.2 | CA2/3

While there was no effect of environment or treatment on neuronal number in the CA2/3 region of the hippocampus (both $P > .05$), there was a significant main effect of environment on the percent of lectin-positive blood vessels ($F(1,16) = 5.365, P = 0.034$, Figure 5B) and synaptophysin-positive puncta ($F(1,16) = 5.711, P = 0.030$, Figure 6B). In addition, there was a significant main effect of treatment on the percent of lectin-positive blood vessels ($F(1,16) = 48.375, P < .001$, Figure 5B). Relative to normoxic pregnancy, offspring of hypoxic pregnancy had a significant reduction in the percentage of lectin-positive blood vessels ($P = 0.013$, Figure 5B) and synaptophysin-positive puncta ($P = 0.008$, Figure 6B). Soma volumes were unaltered (N: 2696 ± 52 $\mu$m$^3$, H: 2536 ± 142 $\mu$m$^3$; $P > .05$). Maternal vitamin C treatment in hypoxic pregnancy significantly increased the volume fraction of lectin-positive blood vessels ($P < .001$, Figure 5B) and synaptophysin-positive puncta ($P = 0.034$, Figure 6B), relative to hypoxic pregnancy. Soma volumes were unchanged (2895 ± 185 $\mu$m$^3$, $P > .05$). Vitamin C treatment in normoxic pregnancy increased the percentage of lectin-positive blood vessels relative to untreated normoxic pregnancy ($P = .002$, Figure 5B), with no changes in the percentage of synaptophysin-positive puncta (Figure 6B) or soma volumes (2403 ± 157 $\mu$m$^3$; all $P > .05$). No environment x treatment interactions were significant within the CA2/3 (all $P > .05$).

3.5.3 | Dentate gyrus (DG)

Within the dentate gyrus, there was a significant interaction between environment and treatment ($F(1,15) = 5.676, P = .031$, Figure 4C), and a main effect of treatment ($F(1,15) = 7.903, P = .013$, Figure 4C), on neuronal number. In addition, there was a significant interaction ($F(1,16) = 4.719, P = .045$, Figure 5C) and main effects of environment ($F(1,16) = 10.038, P = .006$, Figure 5C) and treatment ($F(1,16) = 30.854, P < .001$) on percentage of lectin-positive blood vessels (Figure 5C), and a significant main effect of environment on the percentage of synaptophysin-positive puncta ($F(1,16) = 9.976, P = .006$, Figure 6C). Relative to normoxic pregnancy, offspring of hypoxic pregnancy had a reduction in the number of granule cells ($P = 0.010$, Figure 4C) as well as the percentage of lectin-positive blood vessels ($P = 0.022$, Figure 5C) and synaptophysin-positive puncta ($P = 0.006$, Figure 6C). Granule cell soma volumes were unaltered (N: 733 ± 29 $\mu$m$^3$, H: 722 ± 45 $\mu$m$^3$, $P > .05$). Relative to hypoxic pregnancy, maternal vitamin C treatment in hypoxic pregnancy significantly increased granule cell number ($P = 0.022$, Figure 4C) and the volume fraction of lectin-positive blood vessels ($P < .001$, Figure 5C). The volume fraction of synaptophysin-positive puncta (Figure 6C) and soma volumes were unchanged (875 ± 99 $\mu$m$^3$, both $P > .05$). Vitamin C treatment in normoxic pregnancy increased the percentage of lectin-positive blood vessels when compared to untreated normoxic pregnancy ($P = 0.030$, Figure 5C). Granule cell number, soma volume (805 ± 37$\mu$m$^3$), and the percentage of synaptophysin-positive puncta (Figure 6C) were unaltered (all $P > .05$). There were no other significant environment x treatment interactions.

3.5.4 | Myelination

There was a significant effect of environment ($F(1,16) = 5.927, P = .027$) and treatment ($F(1,16) = 5.562, P = .031$) on the
OD of MBP-positive immunostaining in the cortex. There was no significant interaction between environment and treatment ($P > .05$). Relative to normoxic offspring, no changes were observed in hypoxic offspring in the OD of MBP (cortex- N: 0.38 ± 0.05 AU, H: 0.42 ± 0.06 AU; corpus callosum- N: 0.49 ± 0.07 AU, H: 0.50 ± 0.07 AU), or the extent of myelination in the cortex (N: 74.4 ± 1.8%, H: 74.4 ± 2.8%, all $P > .05$).
However, relative to hypoxic pregnancy, maternal vitamin C treatment in hypoxic pregnancy significantly increased the OD of MBP in the cortex (0.50 ± 0.08 AU, \( P = .046 \)). The OD of MBP in the corpus callosum (0.57 ± 0.14 AU) and the extent of myelination in the cortex (73.4 ± 7.1%) were unaltered (all \( P > .05 \)). Vitamin C treatment in normoxic pregnancies did not alter the OD of MBP (corpus callosum: 0.49 ± 0.06 AU; cortex: 0.42 ± 0.04 AU), nor the extent of myelination (76.7 ± 2.0% AU, all \( P > .05 \)).

**DISCUSSION**

The data show that prenatal hypoxia reduced neuronal number, vascularity, and synaptic density in the hippocampus and impaired memory function in the adult male offspring. These adverse effects of hypoxic pregnancy were independent of reductions in maternal food intake, fetal growth restriction, or elevations in maternal and fetal plasma corticosterone concentrations. Levels of oxidative stress in the placenta, but not in the fetal or adult offspring brain, were increased in hypoxic pregnancy. Maternal supplementation with vitamin C during hypoxic pregnancy protected against oxidative stress in the placenta and prevented the adverse effects of prenatal hypoxia on hippocampal atrophy and memory loss in the adult offspring (Figure 7). Therefore, the data partially support the hypothesis tested showing that hypoxic pregnancy programs neurodevelopmental deficits in the adult offspring by increasing placental oxidative stress but not via enhanced fetal exposure to excess glucocorticoids. Therefore, maternal treatment with antioxidants in pregnancy complicated by fetal hypoxia may confer neurodevelopmental protection in the offspring.

Evidence derived from human clinical studies as well as from preclinical animal models supports the concept that exposure to maternal stress during pregnancy or fetal exposure to excess glucocorticoids during gestation can program neurodevelopmental problems in the progeny.\(^{26-30}\) Indeed, the hippocampus is very sensitive to the regulatory effects of glucocorticoids.\(^{36,57}\) For this reason, we measured plasma concentrations of corticosterone in the present study. However, the endocrine data show that this model of early-onset hypoxic pregnancy was not associated with elevations in glucocorticoids either in the maternal or fetal circulation. This outcome is in keeping with studies of ovine pregnancy.
at high altitude,\textsuperscript{58} and with studies of ovine pregnancy exposed to isobaric chronic hypoxia at sea level.\textsuperscript{36,59} It has been proposed that hypoxic pregnancy downregulates adrenocortical sensitivity in fetal life, as an adaptive response to protect against preterm birth induced by chronic hypoxic stress.\textsuperscript{58} Therefore, the data in the present study highlight that neurodevelopmental deficits in the adult offspring programmed by early-onset hypoxic pregnancy are not secondary to fetal glucocorticoid overexposure during gestation.

Many studies in humans and experimental animal models have reported previously that hypoxic pregnancy can slow fetal growth and lead to a reduction in birth.\textsuperscript{7,8,10,11} In rodent models, significant fetal growth restriction is triggered particularly when hypoxic pregnancy occurs in the last third of gestation.\textsuperscript{2,9} However, other studies in rat pregnancy have also reported that early-onset hypoxia in the first third of pregnancy, as the model used in this study, minimizes any reduction in fetal growth because of placental adaptive responses that protect against the adverse effects of hypoxia on fetal growth, such as an increase in the fetal capillary surface area.\textsuperscript{23} Therefore, the data in the present study highlight that neurodevelopmental deficits in the adult offspring programmed by early-onset hypoxic pregnancy are also independent of fetal growth restriction.

Impairments in learning ability,\textsuperscript{60} memory consolidation,\textsuperscript{61-63} and problem-solving behavior\textsuperscript{64,65} have been reported previously in juvenile and adult rodent offspring of hypoxic pregnancy. Conversely, fewer studies have determined the long-term impact of developmental hypoxia on cerebral structure in adult offspring. Golan et al. reported a reduction in neuronal cell density and size in the primary motor cortex and cerebellum in adult mice following acute hypoxia in late gestation.\textsuperscript{66} Chronic prenatal hypoxia from mid-gestation also resulted in a reduction in the density of neural cell adhesion molecule (NCAM) polysialylated neurons in the hippocampus of adult rats, which the authors attributed to an impairment of synaptic plasticity.\textsuperscript{61} Data in the present study show that in addition to an impairment of spatial reference memory, there was a significant reduction in neuronal number, vascularity, and synaptic density in different regions of the hippocampus in offspring of hypoxic pregnancy. Significant differences exist between each region of the hippocampus regarding function, cell morphology, ability for neurogenesis, and vulnerability to various insults.\textsuperscript{49} Because on these fundamental differences, each hippocampal region was analyzed separately in the present study. To give further insight into potential mechanisms underlying the effects of prenatal hypoxia on memory function in the adult offspring in the present study, additional investigations were performed focused on synaptophysin. Synaptophysin is a specific presynaptic marker commonly used as an index of synaptic density and neuronal transmission.\textsuperscript{67,68} Importantly, loss of hippocampal synaptophysin correlates strongly with cognitive decline in Alzheimer’s disease\textsuperscript{69} and impaired learning and memory in rats.\textsuperscript{70} Reductions in synaptophysin-immunoreactivity have been reported in the hippocampus of

**FIGURE 7** Summary Illustration. Prenatal hypoxia reduced neuronal number, vascularity, and synaptic density in the hippocampus and impaired memory function in the adult offspring. These adverse effects of hypoxic pregnancy were independent of reductions in maternal food intake, fetal growth restriction or elevations in maternal and fetal plasma corticosterone concentrations. Levels of oxidative stress in the placenta, but not in the fetal or adult offspring brain, were increased in hypoxic pregnancy. Maternal supplementation with vitamin C during hypoxic pregnancy protected against oxidative stress in the placenta and prevented the adverse effects of prenatal hypoxia on hippocampal atrophy and memory loss in the adult offspring.
fetal sheep following betamethasone exposure,⁷¹ and in rats following prenatal stress.⁷⁰ We show that the percentage of synaptophysin-positive staining was also reduced within the hippocampus in adult offspring of hypoxic pregnancy. Taken together, the reduction in neuronal number, impaired vascularity, and reduced synaptic density in the hippocampus in adult offspring of hypoxic pregnancy is contributing mechanisms underlying their measured impairment in memory function.

The current study also demonstrated that maternal treatment with vitamin C in hypoxic pregnancy protected against adverse changes in hippocampal structure and memory loss in the adult offspring, underscoring the conceptual advance of the data reported in the present study. The mechanism underlying the protective effect of maternal vitamin C treatment in hypoxic pregnancy on the cerebral structure and function of the offspring appears to be at the level of the placenta. It is established that chronic hypoxia during pregnancy leads to a fall in maternal and fetal arterial PO₂, triggering the activation of the hypoxia inducible factor (HIF).⁷³ In turn, HIF regulates the expression of hundreds of target genes including erythropoietin or EPO, which enhances red blood cell production and an indication of this effect is determined by an increase in hematocrit.⁷³ We show that exposure of pregnant rats to 13% O₂ for most of gestation leads to an increase in maternal and fetal hematocrit levels, indicative of HIF activation in both the mother and fetus of treated and untreated hypoxic pregnancy. Another line of evidence supports that increased oxidative stress will limit NO bioavailability, promoting an increased redox vascular tone that can impair blood flow in most circulations, particularly in those which are highly sensitive to NO, such as the placental vascular bed.⁴⁰,⁷⁴,⁷⁵ Therefore, in this model of hypoxic pregnancy, the measured levels of increased oxidative stress in the placenta will compound the effects of hypoxia on the offspring, further limiting fetal oxygen delivery. Additional data in the present study show that this reduction in fetal oxygenation does not trigger oxidative stress in the fetal brain, but it is sufficient to impact brain development, promoting neurodevelopmental deficits in the adult offspring. Further, we show that maternal treatment with vitamin C during hypoxic pregnancy restores oxidative stress in the placenta to normal levels. Combined, therefore, data in the present study support that maternal treatment with the antioxidant vitamin C ameliorates the fall in fetal oxygen delivery by virtue of protecting placental perfusion rather than by improving fetal PO₂. The maintained fall in fetal PO₂ in hypoxic pregnancy with vitamin C treatment will therefore still stimulate an increase in fetal hematocrit, as measured. However, the reduced placental oxidative stress will confer protection on fetal brain development, preventing the programming of the neurodevelopmental deficits, such as memory loss, in the adult offspring (Figure 7). Protective effects on the placenta during hypoxic pregnancy of maternal treatment with antioxidants, such as the mitochondria-targeted antioxidant MitoQ, have been previously described by independent laboratories.⁷⁶,⁷⁷ Data in these reports, and others which have utilized the same rodent model described in the current study, support that maternal treatment with antioxidants may be beneficial in complicated pregnancy via mechanisms protecting against placental stress and enhancing placental perfusion.²²,²³,⁷⁶,⁷⁷ Data in the present study also show that maternal treatment with vitamin C in normoxic pregnancy relative to untreated normoxic pregnancy also increased blood vessel density in all hippocampal areas studied. This suggests that maternal treatment with vitamin C may increase placental perfusion above basal levels even in control pregnancy and that this effect may favor hippocampal development in the progeny. Accordingly, previous data confirm that maternal treatment with vitamin C even in healthy pregnancy does indeed increase umbilical blood flow above basal levels.⁴⁰

The dose of vitamin C used in the current study was derived from our previous work, demonstrating elevations in circulating ascorbate concentrations in ovine pregnancy within the range required for it to act as an antioxidant in vivo.⁴²,⁴¹,⁷⁴ We are aware that the dose of vitamin C given to the rodent dams (ca. 500 mg/day/kg) far exceeds that given to pregnant women, for instance, in clinical trials to ameliorate preeclampsia (ca. 14 mg/day/kg).⁷⁸–⁸⁴ In addition, high doses of vitamin C can promote kidney stones.⁸⁵ Therefore, the present study provides proof of principle that maternal antioxidant therapy can protect the offspring brain from the adverse effects of prenatal hypoxia. However, future studies should focus on alternative antioxidant therapy of improved human translational potential, such as with melatonin, allopurinol, or the mitochondria-targeted antioxidant MitoQ.¹⁰,²⁵,²⁶,⁴⁰,⁷⁶,⁸⁶–⁸⁹ The present study focused on male offspring from hypoxic pregnancy to control for sex differences. Female offspring were not investigated, which is a limitation. There is growing evidence for the importance of addressing sex differences in the developmental programming of adult disease. Therefore, to further increase the human translational impact of the findings, future studies should incorporate sex-specific effects of hypoxic pregnancy, with or without antioxidant treatment, on cerebral structure and function in female as well as male offspring.

In conclusion, our study establishes a link between the direct effects of developmental hypoxia, independent of fetal growth restriction and fetal exposure to excess glucocorticoids, with placental oxidative stress and programming of hippocampal atrophy and memory loss in the adult offspring. Further, maternal antioxidant treatment in hypoxic pregnancy restored placental oxidative stress to normal levels and prevented the programmed adverse effects on cerebral structure and function in the adult offspring, implicating placental oxidative stress as a mediating factor. Therefore, the data link developmental conditions complicated by oxidative stress
with later brain health in the offspring, both offering insight into underlying molecular mechanisms and plausible intervention strategies. These findings have important clinical implications in relation to pregnancies complicated by reduced oxygen delivery, such as during placental insufficiency, pre-eclampsia, or pregnancy at high altitude.

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CONFLICT OF INTEREST
The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS
E.J. Camm, D. A. Giussani and S.E. Ozanne designed the research. E.J. Camm, C. Cross, A.D. Kane, J.L. Tarry-Adkins, S.E. Ozanne and D.A. Giussani performed the research and analysed the data. E.J. Camm and D.A. Giussani drafted the paper. All authors edited and approved the paper. D.A. Giussani and S.E. Ozanne obtained funding.

ETHICS
The study was approved by the Cambridge University Animal Welfare and Ethical Review Board. All procedures were carried out under the UK Animals (Scientific Procedures) 1986 Act.

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