OBSTETRICS

Neurocognitive sequelae of antenatal corticosteroids in a late preterm rabbit model

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BACKGROUND: Late preterm birth is associated with short-term respiratory and adaptive problems. Although antenatal corticosteroids seem to reduce the respiratory burden, this may come at the cost of adverse neuropsychological outcomes later in life. This impact has not been investigated.

OBJECTIVE: Herein, we investigate what the short- and long-term neurodevelopmental effects of a single course of betamethasone in simulated late preterm birth.

STUDY DESIGN: Time-mated pregnant does received 0.1 mg/kg betamethasone (n=8) or 1 mL saline intramuscular (n=6) at the post-conceptional ages of 28 and 29 days. The antenatal corticosteroid dose and scheme were based on previous studies and were comparable with routine clinical use. Cesarean delivery was done on postconceptional age 30 days (term=31 days), and new-born rabbits were foster-cared for 28 days and were thereafter cared for in group housing. Neonatal lung function testing and short-term neurobehavioral testing was done. Open field, spontaneous alternation, and novel object recognition tests were subsequently performed at 4 and 8 weeks of age. On postnatal day 1 and at 8 weeks, a subgroup was euthanized and transcardially perfuse fixated. Ex vivo high-resolution Magnetic Resonance Imaging was used to calculate the Diffusion Tensor Imaging-derived fractional anisotropy and mean diffusivity. Fixated brains underwent processing and were serial sectioned, and a set of 3 coronal sections underwent anti-NeuN, Ki67, and terminal deoxynucleotidyl transferase dUTP nick end labeling staining.

RESULTS: Antenatal corticosteroid exposure was associated with improved neonatal lung function, yet resulted in a long-term growth deficit that coincided with a persistent neurobehavioral deficit. We demonstrated lower neonatal motor scores; a persistent anxious behavior in the open field test with more displacements, running, and self-grooming episodes; persistent lower alternation scores in the T-Maze test; and lower discriminatory indexes in the novel object recognition.

On neuropathological assessment, antenatal corticosteroid exposure was observed to result in a persistent lower neuron density and fewer Ki67+ cells, particularly in the hippocampus and the corpus callosum. This coincided with lower diffusion tensor imaging-derived fractional anisotropy scores in the same key regions.

CONCLUSION: Clinical equivalent antenatal corticosteroid exposure in this late preterm rabbit model resulted in improved neonatal lung function. However, it compromised neonatal and long-term neurocognition.

Key words: antenatal corticosteroid, betamethasone, late preterm, neurobehavior, neurocognition

Introduction

Preterm birth (PTB), that is, birth before 37 weeks' gestational age, is the most significant clinical problem in contemporary obstetrics. PTBs total 5% to 18% of worldwide deliveries, with 70% of those occurring late, that is, between 34 and 37 weeks. Up to a third of late PTB infants experience short-term respiratory morbidity.[1] There is currently little doubt that the maternal administration of a single course of antenatal corticosteroids (ACS), for example, 2 doses of 12 mg intramuscular (IM) betamethasone 24 h apart substantially reduces the risks of neonatal death and respiratory distress syndrome (RDS) in children born after early PTB, that is, before 34 weeks.[2] However, there are some concerns about the potential effects of ACS on other fetal organ systems, in particular, the central nervous system.[3,4] This concern may be even more relevant to late PTB, wherein the absolute proportion of RDS is low, and most of the respiratory problems are temporary.[1] Still ACS is often used in the setting of late PTB but with mixed results, as there is only 1 study to date demonstrating a clear reduction in RDS.[5] The other studies either showed minimal benefit[6,7] or no benefit at all.[8,9] Given that no study has evaluated the neurodevelopmental effect of ACS and the lesser obvious respiratory benefit of ACS, routine administration of ACS in the setting of late PTB may be questioned.

It is reasonable to reflect on animal studies, as the available clinical data cannot easily define the precise effect of ACS on future neurodevelopment. In a systematic review on the long-term neurodevelopmental effects of ACS therapy in preclinical models,[10] deleterious long-term neurocognitive effects were consistently present, but most studies used repetitive and/or a high total dose of mostly dexamethasone. This is unfortunate, as this does not cover the clinical scenario of ACS administration in the context of late PTB. Another challenge is the fact that no specific animal model can replicate the complex physiology of human gravidity and fetal development, as variations in maternal physiology, fetal maturation, and parturition all play a role in determining the validity of the animal model.[11] However, the rabbit is best suited to bridge the gap

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AJOG at a Glance

Why was this study conducted?

Late preterm birth is associated with short-term respiratory and adaptive problems.
Antenatal corticosteroids (ACS) seem to reduce the respiratory burden, but they also affect other fetal organs, in particular, the central nervous system.
No study has evaluated the neurodevelopmental effects of antenatal ACS in the setting of late preterm birth.

Key findings

In the late preterm rabbit model, ACS exposure improved neonatal lung function.
It also resulted in a long-term growth deficit that coincided with a neurobehavioral deficit.
Specific neuropathological changes were seen in the hippocampus and the corpus callosum, which play a vital role in short-term and spatial memory.

What does this add to what is known?

This is the first study to evaluate the short- and long-term effects of late preterm ACS exposure.

between small and large animals, being widely available, having low housing needs, a short reproductive cycle, and a timed gestation with large litters and a placental development close to that in humans.[12] In translational neuroscience, rabbits have the advantage of having a more complex brain structure with a greater white matter proportion than rodents, and the timing of perinatal brain white matter maturation is comparable with that of humans. Hence, the rabbit appears to be an ideal model to examine prenatal insults in a controlled manner.[13,14] We earlier characterized the early neuropathological consequences of both early and late preterm birth in the rabbit model.[15,16]

Herein, we investigate the question of what the short- and long-term neurodevelopmental effects of a single course of betamethasone in the context of simulated late PTB are.

Materials and Methods

Animal delivery and care

Animals were treated according to the current guidelines[17]. The experiments were approved by the Ethics Committee for Animal Experimentation of the Faculty of Medicine (P112/2017). Timemated pregnant rabbit does (*Oryctolagus cuniculus*; hybrid of New Zealand White/Black and Flemish Giant rabbit) were housed in separate cages at 21°C, 42% humidity, 12 h day-night cycle, and free access to water and food. The does received either 0.1 mg/kg betamethasone (Celestone Chronodose, Schering-Plough Labo N.V., Belgium) or 1 mL saline IM at 24 h and 48 h before delivery. Celestone Chronodose is a 2-component preparation of betamethasone acetate and phosphate, and the dose was diluted into a total volume of 1 mL. The dosage and scheme of ACS administration were based on previous rabbit studies and were comparable to use clinically.[18,19] On postconceptional age (PCA) 30 days, a cesarean rather than vaginal delivery was done (term=31 days). Neurobehavioral assessments were done on postnatal (PN) day 1 (preterm corrected age) and at 4 and 8 weeks. Euthanasia and tissue collection for ex vivo Magnetic Resonance Imaging (MRI) and histologic evaluation were done at PN + 1 day or PN + 63 day, equivalent to neonatal and prepubertal assessments in humans. A subgroup of animals underwent neonatal lung function testing (Supplemental Material 1-Lung function testing). The experimental setup is depicted in Figure 1.

Before delivery, the does were weighed and premedicated with IM ketamine (15 mg/kg, Nimatek; Eurovet Animal Health BV, Bladel, The Netherlands) and medetomidine (25 mg/kg, Domintor, Orion Pharma, Aartselaar, Belgium). They were then placed in the supine position and were administered local anesthesia (2% lidocaine hydrochloride, Xylocaine, AstraZeneca, Brussel, Belgium). The does were euthanized after delivery (1 mL intravenous T61; Intervet International BV, Boxmeer, The Netherlands).

New-born kittens were dried, stimulated, weighed, and placed in an incubator (TLC-50 Advance, Brinsea Products, Weston Super Mare, United Kingdom) at 32°C with 60% humidity, as described.[15] Afterward, they were subcutaneously microchipped (Compact Max Datamars, AgnTho's AB, Lidingö, Sweden) and randomized (http://www.randomizer.org) to either a short-term (PCA 32 days) or long-term evaluation (28 and 56 days). The kittens were mixed and were randomly allocated to be raised by cross fostering in groups of 8−10 by a doe that did not undergo any kind of manipulation and spontaneously delivered the day before. The foster mother was housed in a double cage with a nesting box (54×31×27 cm). After 4 weeks, when they were fully weaned, the rabbits were randomly allocated to a group housing of 4−6 in a double cage with free access to water and food.

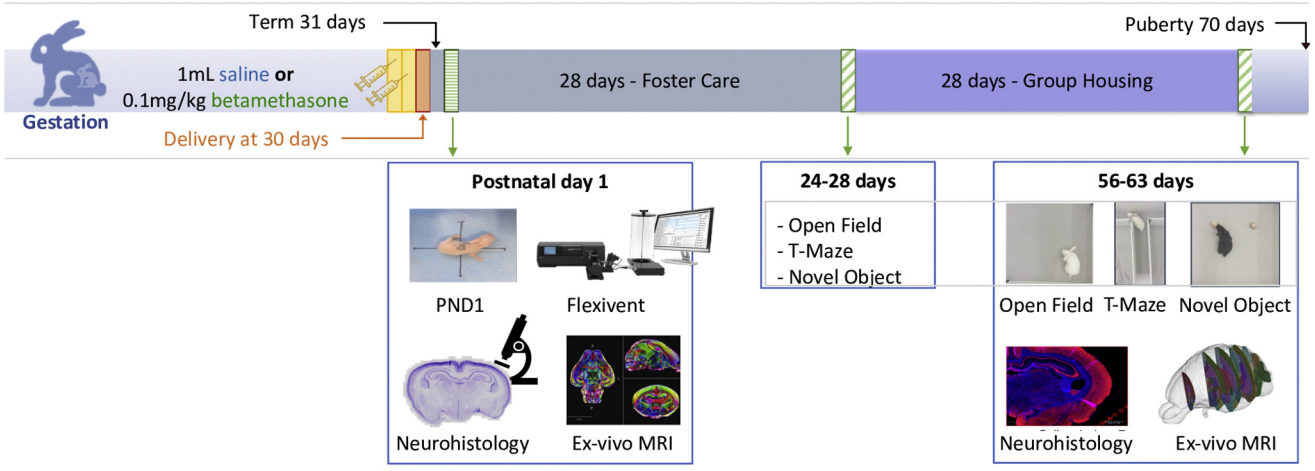
Neurobehavioral evaluations were carried out in the mornings and in a designated space with auditory and olfactory contamination kept to a minimum. The health of experimental animals was assessed beforehand. The testing was video-recorded, and all videos were anonymized and scored by an observer who was blinded to the group allocation.

The details of neurobehavioral evaluations are provided in the supplemental material. (Supplemental Material 1—Neurobehavioral testing).

Euthanasia and tissue collection

After neurobehavioral testing, the rabbits were anesthetized with IM ketamine (35 mg/kg, Nimatek; Eurovet Animal Health BV, Bladel, The Netherlands) and xylazin (6 mg/kg, XYL-M; VMD, Arendonk, Belgium) and were transcardially

FIGURE 1
Study setup



Time-mated pregnant does received 0.1 mg/kg IM betamethasone or saline 1 mL IM at postconceptional age 28 and 29 days. Does were electively delivered at a postconceptional age 30 days. Assessments were done on corrected postnatal day 1, at 4 weeks and 8 weeks of age.

IM, intramuscular.

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perfused with 0.9% saline and heparin (100 u/mL), followed by 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer (pH, 7.4). After extraction, the brain was further immerse fixated for 48 h, whereupon the whole brain volumes were determined using the fluid displacement method.[20]

Histopathology

Paraffin-embedded brains were microtomed at 4 μm. A set of 5 serial coronal sections at 100 μm intervals was taken starting at (1) medial septal nucleus and (2) hippocampal formation. The primary antibodies used included mouse monoclonal anti-NeuN (MAB377, Millipore, Billerica, MA), anti-NG2 chondroitin sulfate proteoglycan (MAB5384, Millipore), and mouse monoclonal anti-human Ki67 (M724001-2, Agilent, Diegem, Belgium). The secondary antibody was Alexa Fluor 488 goat antimouse conjugate (Invitrogen, Sigma-Aldrich). The degenerating nuclei were visualized by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method for fluorescent in situ end labeling of double-stranded DNA fragmentation (Apoptag S7110; Millipore). The sections were counterstained with Hoechst 33342 (Sigma-Aldrich).

Imaging acquisition and quantification

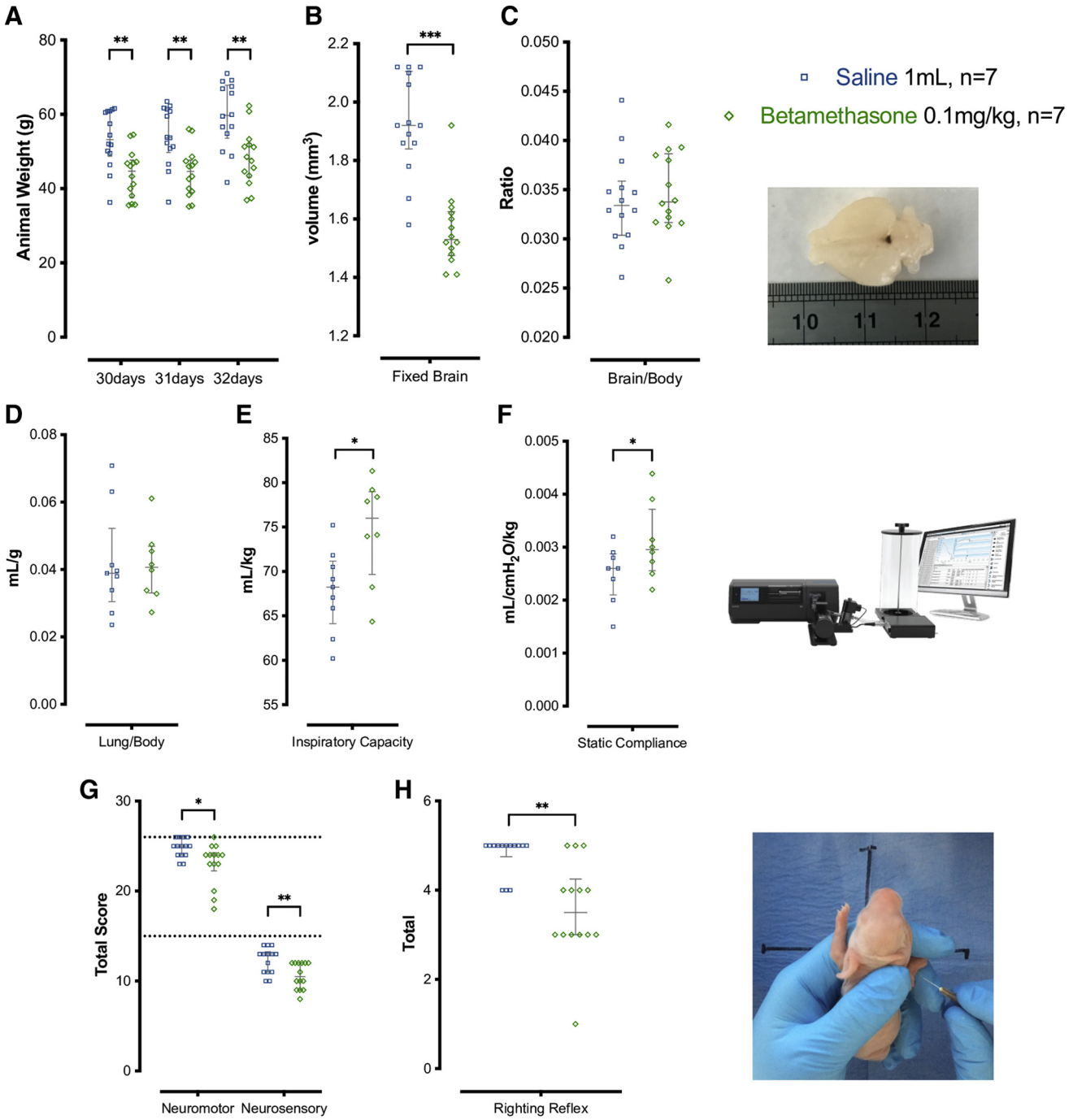
The histologic slides were digitized using the Zeiss AxioScan Z1 (AxioScan Slide Scanner, Carl Zeiss MicroImaging GmbH, Munich, Germany) using a 20× Plan Apochromat objective coupled to a 3-Chip charged-coupled device Camera (Hamamatsu Photonics, Hamamatsu, Japan). The quantification profiles of the digitized whole slide images were done using the QuPath open-source software (https://qupath.github.io)[21] under either bright-field fast cell counting or fluorescent positive cell detection settings. To differentiate between the cellular types in each stain, the detection classifier function[22] was used.

Quantification of the immunohistochemistry positive cells with the NeuN, TUNEL, and Ki67 stains was done with the positive cell detection function. Herein, the whole region of interest was annotated and quantified, and positive cell counts were expressed as a percentage of positive cells per total cells detected.

Magnetic resonance imaging

Ex vivo MRI was performed on perfused fixed brains using the active staining technique as described.[23] Briefly, a Bruker Biospec 9.4 Tesla small animal MRI scanner (Bruker Biospin, Ettlingen, Germany; horizontal bore, 20 cm) equipped with actively shielded gradients (600 mT/m) was used. Data were acquired using a 72 mm internal diameter quadrature volume coil for transmission decoupled with a rat brain quadrature shaped surface coil for signal reception (volume resonator, Rapid Biomedical, Rimpar, Germany). Data were acquired with a high-resolution 3D Flash sequence (TE/TR 5.5/50 ms; flip angle 70; slice thickness 0.35 mm with no interslice gap, data matrix 392×392×392; isotropic resolution 89 μm; 4 averages, acquisition time 1 h 11 mins) and a spin echo-echo planar imaging sequence (8 segments; TE/TR: 25/150 ms; slice thickness 0.4 mm with no interslice gap, data matrix 192×160×160; isotropic spatial resolution of 208 μm; 64-directions and 3 b-values per direction of 800, 1000, 1500 s/mm^2, acquisition time 10 hours 26 minutes). Subsequent T1 volumetric data and diffusion tensor imaging-derived fractional anisotropy (FA) and mean diffusivity (MD) were calculated from automated template propagation.

FIGURE 2
Neonatal assessments on corrected postnatal day 1



New-born rabbit body weight (**A**), brain volumes (**B**), brain(g)/body ratio(g) (**C**). Neonatal lung-to-body ratios (**D**), inspiratory capacity per body weight (**E**) and static compliance per body weight (**F**). Neonatal neurobehavioral assessment with total motor score (**G**) and sensory score (**H**). Saline n=14, Betamethasone n=14. Data displayed as median and IQR with significance compared with the saline group indicated as *single asterisk*.05≥*P*>.01; *double asterisks*.01≥*P*>.001; *triple asterisks P*<.001.

IQR, interquartile range.

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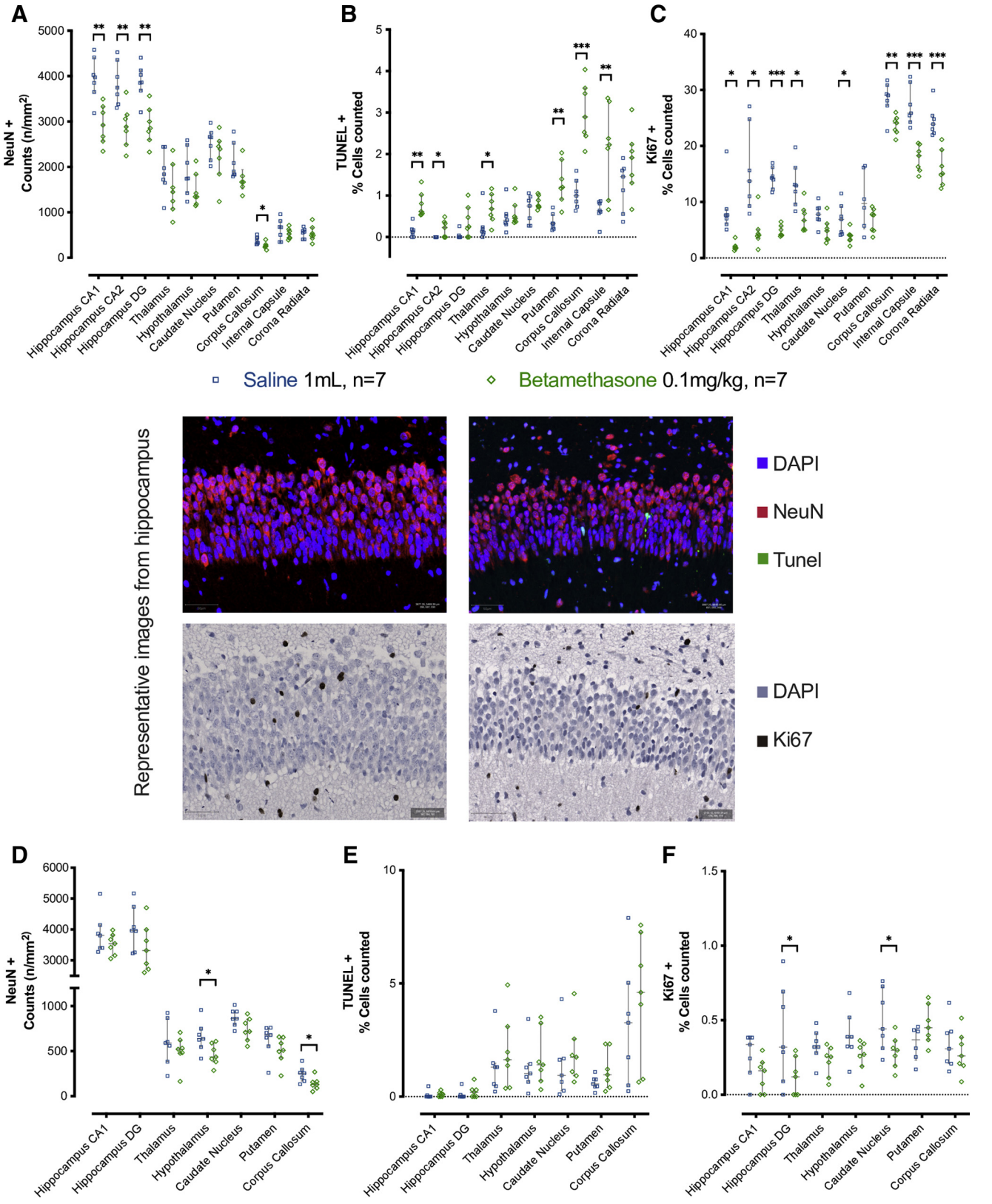
FIGURE 3
Neuropathological outcomes



Statistics

As no previous data existed, a power calculation was done on the basis of pilot neurobehavioral data. A power analysis using G*Power (Faul & Erdfelder, 1992) indicated that 5−8 rabbits per group will be required if an anticipated 5% or 10% effect size is expected when α and power $(1-\beta)$ was set to 0.05 and 0.80, respectively, using a 2-tailed t test. The data were analyzed using Prism for Windows version 8.0 (GraphPad, San Diego, CA). The normality of distribution was assessed by the D'Agostino-Pearson omnibus test. The data were presented as mean with standard deviations or median and interquartile ranges. A Grubbs' test with α 0.05 was used to identify outliers. Comparison was done by an unpaired student's t test or the Mann Whitney test. The group means were compared by 1-way analysis of variance or the Kruskal Wallis test. A P value $<.05$ was considered significant. Adjustment for multiple testing was applied with 1% Benjamini-Hochberg false discovery rate for >5 comparisons. The survival curves are presented as Kaplan-Meier graphs with Mantel-Cox test groups comparisons.

Results

There were no differences in the perinatal maternal and litter characteristics between the betamethasone and the saline groups (Supplemental Table 1). In the betamethasone group, there was a higher proportion of stillbirths per litter (5.8±7.8% vs 32.0±26.2%; P=.008).

Antenatal corticosteroid exposure is associated with augmented lung functionality

Betamethasone-exposed neonatal rabbits had increased inspiratory capacity (67.7 ± 6.5 mL/kg vs 73.8±5.7 mL/kg; P=.033) and static compliance ($2.5\pm1.0\times10^{-3}$ mL/cmH_2O/kg vs $3.1\pm1.0\times10^{-3}$ mL/cmH_2O/kg; P=.038) (Figure 2). Yet, there was no clear difference in the lung-to-body weight ratio ($3.4\pm0.8\times10^{-2}$ vs $3.8\pm1.2\times10^{-2}$; P=.414) (Supplemental Table 2).

Antenatal corticosteroid exposure is associated with significant neonatal morbidity

Betamethasone exposure led to lower placental (5.9±0.8 g vs 5.0±0.8 g; P=.010) and birth weights (53.3±7.8 g vs 43.4±6.5 g; $P\leq.001$). Yet, the brain-to-body ratio ($3.3\pm0.5\times10^{-2}$ vs $3.4\pm0.4\times10^{-2}$; P=.538) remained similar (Figure 2).

Furthermore, betamethasone exposure had a clear neurobehavioral impact with a lower motoric score (24.7±1.1 vs 23.1±2.3; P=.021) because of an impaired gait and locomotion ability and a lower sensory score (12.1±1.5 vs 10.7±1.5; P=.017) (Supplemental Table 3).

The neurobehavioral impact of antenatal corticosteroid exposure coincides with region-specific histopathological and magnetic resonance imaging alterations

In the betamethasone neonatal rabbits, there were lower neuron densities, especially in the hippocampus (3954±449 vs 2932±402; P=.001) and the corpus callosum (383±84 vs 265±70; P=.014). The lower neuron densities were accompanied by more apoptosis demonstrated by TUNEL percentages and less proliferation demonstrated by Ki67 percentages (Supplemental Table 4). Moreover, these region-specific differences were observed in MRI-derived diffusion tensor imaging (DTI) data, with lower FA and higher MD, especially in the frontal cortex, hippocampus, and corpus callosum. Figures 3 and 4 (Supplemental Table 5).

Antenatal corticosteroid exposure leads to a longstanding weight deficit and neurobehavioral impact

Betamethasone-exposed rabbits had a mortality risk up to the first week of life, and though their body weight deficits waned over time, it remained significant up to 7 weeks of life (Figure 5). At the time of their final assessments by 8 weeks of life, no significant body (1960 ±235 vs 1821±265; P=.149) or brain weight (9.5±0.8 g vs 9.2±1.4 g; P=.617) differences were seen (Supplemental Table 6). Betamethasone-exposed rabbits exhibited different patterns of behavior on neurobehavioral assessments at both 4 and 8 weeks of life. In the open field behavior test, they had more overall and corner displacements, which coincided with more running episodes, more self-grooming, and more biting events. These behaviors were seen at 4 weeks of age and persisted up to 8 weeks (Supplemental Table 7). Furthermore, on specific short-term and spatial memory testing, the betamethasone-exposed rabbits had lower spontaneous alterations scores in the T-Maze Test (TMT) and lower discriminate index in the Novel Object Recognition Test (NORT). These differences were once again seen at both 4 and 8 weeks of life, though to a lesser degree (Figure 5) (Supplemental Table 7).

The long-term neurobehavioral deficit coincides with specific neuropathological alterations

At 8 weeks of life, the betamethasone-exposed rabbits had a persistently lower expression of Ki67+ cells, specifically in the caudate nucleus (0.5±0.2 vs 0.3±0.1; P=.039) (Figure 3). Lower NeuN+ cell counts were present in the

Outcomes per region of interest with representative images of the hippocampus. Neuron density (**A**), TUNEL positive percentage (**B**) and Ki67 positive percentage (**C**) on corrected day 1 and neuron density (**D**), TUNEL positive percentage (**E**) and Ki67 positive percentage (**F**) on day 63. Saline n=7, Betamethasone n=7. Data displayed as median and IQR with significance compared with the saline group indicated as *single asterisk*.05≥P>.01; *double asterisks*.01≥P>.001; *triple asterisks* P<.001.

IQR, interquartile range; *TUNEL*, terminal deoxynucleotidyl transferase dUTP nick end labeling

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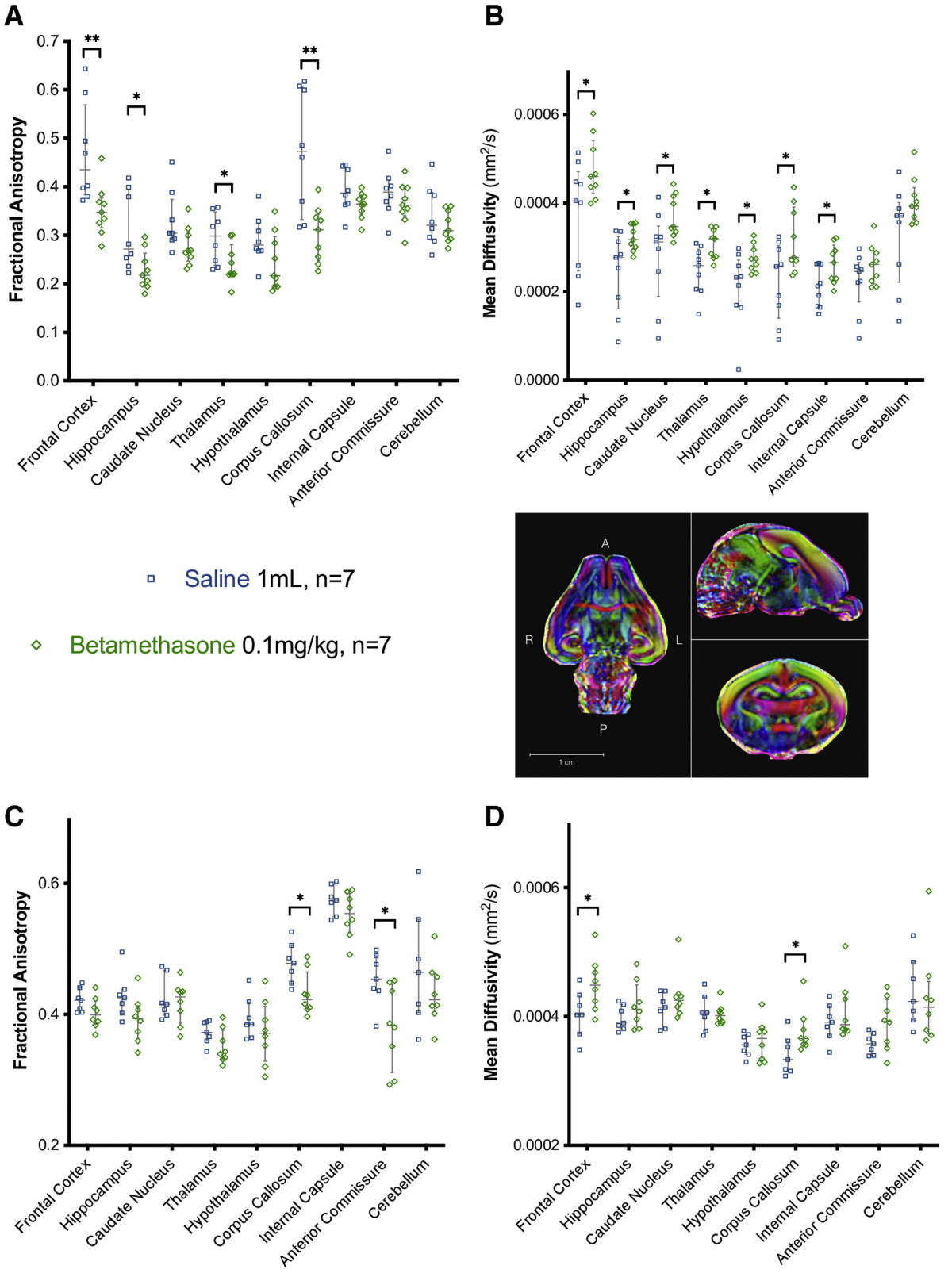
FIGURE 4
Ex-vivo MRI-derived DTI outcomes



hypothalamus (659±168 vs 462±116; *P*=.027), caudate nucleus (875±98 vs 740±128; *P*=.049) and corpus callosum (245± 85 vs 140±68; *P*=.028). Nevertheless, there were no specific differences seen in the expression of TUNEL+ cells (Supplemental Table 8).

MRI-derived DTI data showed lower FA estimates in the corpus callosum (0.478±0.031 vs 0.431±0.033; *P*=.015) and the anterior commissure (0.453±0.040 vs 0.380±0.064; *P*=.019) of the betamethasone-exposed rabbits, whereas the MD was higher in the frontal cortex (4.0±1.2 vs 4.5±0.7; *P*=.042) and corpus callosum (3.4±0.9 vs 3.8±0.7; *P*=.042) (Figure 4) (Supplemental Table 9).

Comment

Principal findings

In the late preterm rabbit model, antenatal betamethasone exposure improved neonatal lung function. However, it also compromised neurocognition, both in the neonatal and in the long-term period. We demonstrated a substantial neuropathological deficit that coincided with altered MRI-derived DTI deviations in key regions such as the hippocampus and the corpus callosum.

Results in context of what is known

Although many previous studies investigated the effect of maternal ACS administration in rabbits, none of them seem fitted to answer the question whether ACS in the setting of late PTB influences future neurodevelopment. Most studies investigated the effects of ACS given before 27 days PCA,[19,24–28] and they used *repetitive*[25] or *higher* doses[19,29], whereas others reported only on *short-term* neuropathological outcomes.[18] From a neonatal pulmonary function viewpoint, the optimal dosage of betamethasone is 0.1 mg/kg.[26] We went on to use this dosage and confirmed the previously reported beneficial pulmonary effects, but our aim was to review the short- and long-term secondary effects on the brain. Herein, ACS exposure led not only to anxiety-like behavior but also impaired spatial memory. These findings have also been observed in rodents even after a single course of ACS.[30,31] Furthermore, a dose-dependent effect of ACS was clearly associated with a long-term abnormal memory and socialization behaviors in rats.[32] Therefore, it seems that the developing brain, with the meso-cortico-limbic system as a focus point, is particularly sensitive to exogenous glucocorticoids.[33] The hippocampus plays a central role in this system and facilitates a myriad of functions within the brain. These include cognition, behavior, memory, coordination of the autonomic activity, and regulation of a number of endocrine systems.[34] For a better comprehension of the impact of ACS in the rabbit model, a multiregional neuropathological analysis was done to better understand the underlying neuronal milieu.

We noted an overwhelmingly persistent effect on cellular proliferation in the brain by the expression of Ki67+ cells. This finding is in line with previous reports in rodents, wherein ACS exposure resulted in the long-term suppression of proliferation.[35,36] Even a clinically relevant dose of ACS in rats significantly reduced neuronal proliferation, especially in the hippocampus.[37] In the current study, concurring lower neuron densities were seen in the neonatal period. This finding can be explained by the reprogramming of the protective negative feedback loop of the hypothalamic–pituitary–adrenal axis axis by ACS, leading to lower neuron expression in the hypothalamus, hippocampus, and frontal cortex.[38] In retrospect, it was foreseeable that most of the alterations in the current study were found in these specific regions. Lastly, our results are also in keeping with studies in primates, where ACS has been shown to induce hippocampal neural degeneration, which is still evident at 9 months of age.[39]

This is the first study to utilize MRI-derived DTI data to substantiate the neuropathological readouts. Previous studies only employed MRI-derived volumetric data, and in both studies, higher concentrations of ACS were used.[39,40] Through the application of MD and FA, we could demonstrate the persistent impact of ACS in the corticothalamic tracts as observed in sheep.[41]

Research implications

This work motivates future research to identify fetuses with the highest risk of respiratory problems so that we can try to limit ACS exposure to them. This is potentially feasible, as cutting-edge image analysis technology has been shown to effectively predict the degree of fetal lung maturity with accuracy.[42] If we could apply this assessment to those at risk of late PTB, we could help identify a subgroup in which the risk of respiratory morbidity is so high that the potential side effects of ACS may be acceptable.

Strengths and limitations

The main strengths of this study lie within the study setup, that is, using a relevant model and critically appraising the outcomes with a multimodal assessment. All the assessments were blinded and evaluated by a team experienced with the methods used.[15,16,43] Furthermore, the construct validity seems plausible, as the apparent outcomes are in keeping with findings in other models studying the effects of maternal ACS exposure.

Data per region of interest with DTI FA (**A**) and MD (**B**) on corrected day 1 and FA (**C**) and MD (**D**) on day 63. One representative example for acquisition quality (center). Saline n=9, betamethasone n=9. Data displayed as median and IQR with significance compared with the saline group indicated as *single asterisk*.05≥*P*>.01; *double asterisks*.01≥*P*>.001; *triple asterisks P*<.001.

DTI, diffusion tensor imaging; *FA*, fractional anisotropy; *IQR*, interquartile range; *MD*, mean diffusivity; *MRI*, magnetic resonance imaging.

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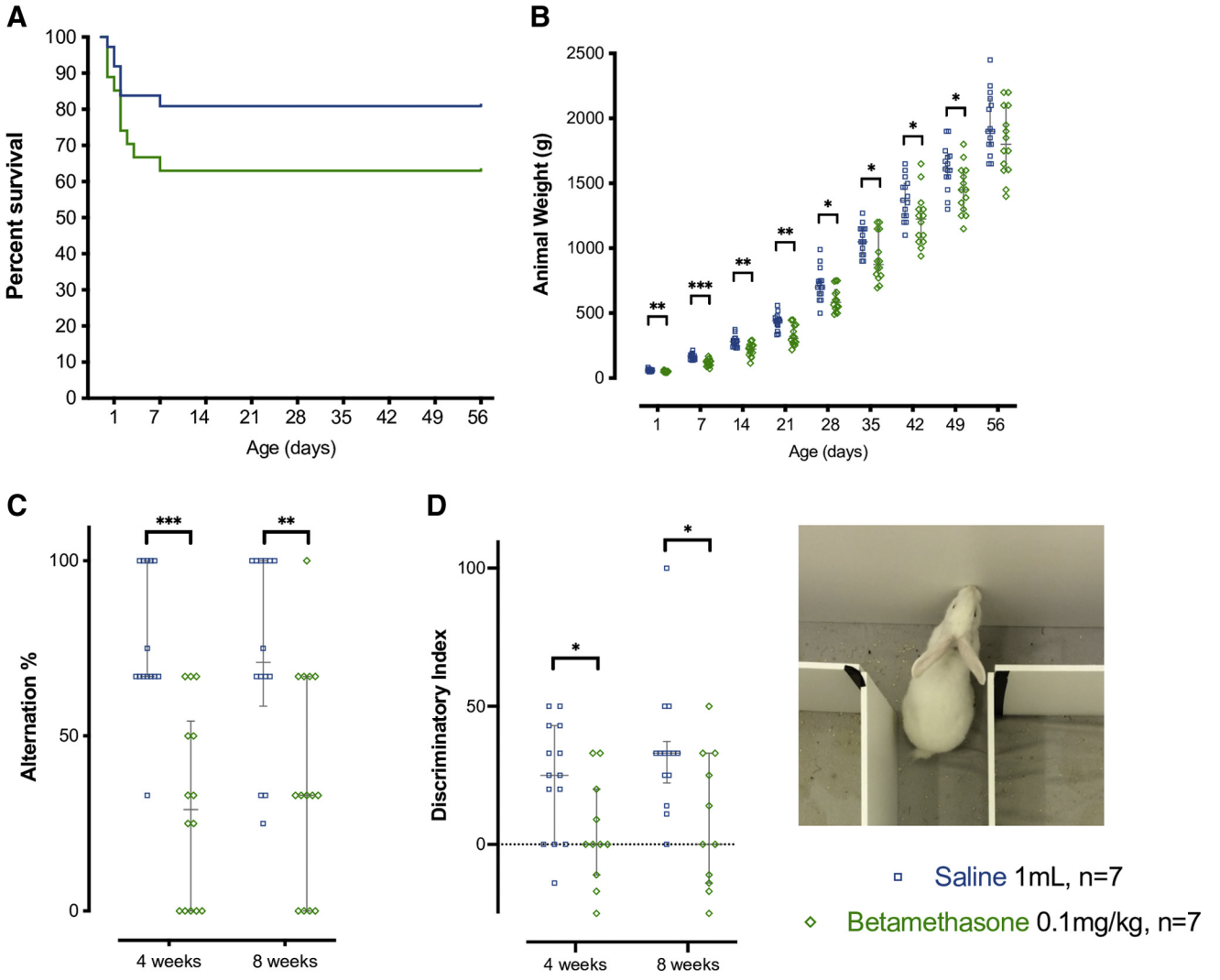
FIGURE 5
Neurobehavioral assessment at 4 and 8 weeks of life



The general health outcomes are depicted in the survival curve (**A**) and body weight (**B**) of rabbits (Saline n=14, Betamethasone n=14) The neurobehavioral outcomes are represented in the spontaneous alternation percentage during the T-Maze test (**C**) and discriminatory index in the novel object recognition test (**D**) (saline n=11, betamethasone n=11). Data displayed as median and IQR with significance compared with the saline group indicated as *single asterisk*.05≥*P*>.01; *double asterisks*.01≥*P*>.001; *triple asterisks P*<.001.

IQR, interquartile range.

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Lastly, through the utilization of cross fostering, we aimed to control the maternal and litter-associated biases. We do acknowledge several limitations in our study. First, no animal model can perfectly mimic the clinical condition in humans, and in the rabbit model, a shorter dose interval closer to the delivery could have been more physiological for this animal. However, we wanted to conform to previous studies. In addition, the mortality observed herein is clinically not present. That is typical for this animal model, however, direct causation has not yet been established.[19,25] Furthermore, the phenotypic variation in the long-term outcomes can be considered as an integral weakness. However, this variation also occurs in humans, and it should be considered when designing studies on antenatal interventions, as it could impact the variation of the severity, recovery, repair, and treatment response. Lastly, we did not investigate the potentially underlying mechanistic footprint, as this was not our aim.

We have demonstrated that antenatal betamethasone exposure leads to abnormal neonatal neurocognition, which persists in the longer term. This was paralleled by structural brain changes. We think that this justifies a

scrutinous review of the outcomes of children who were born late preterm and were exposed to ACS. ■

Acknowledgments

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Supplemental Material 1: Methods

Lung function testing

A subgroup of neonatal rabbits underwent lung function testing 2 h after birth as described before.[1] The FlexiVent 5.2 (SCIREQ, Montreal, Canada) ventilator was used to perform pulmonary function tests on deeply anesthetized pups. The trachea was surgically exposed, and an 18G metal cannula was inserted in the trachea and connected to the ventilator (120 breaths/min ventilation; tidal volume 8 mL/kg). Three pressure volume perturbations were performed to measure the inspiratory capacity and the static compliance. The mean value was used in the analysis and was corrected for body weight. Thereafter, the animals were euthanized and the lungs were harvested and pressure fixed on 25 cmH_2O of paraformaldehyde for 24 h. The volume of the fixed lungs was measured by water immersion (Scherle's principle).

Neurobehavioral testing

Neurobehavioral evaluations were carried out in the mornings and were done in a designated space with auditory and olfactory contamination kept to a minimum. The health of experimental animals was assessed before neurobehavioral testing by checking for their health and weight. The testing was video-recorded, and all videos were anonymized and scored by an observer who was blinded to the group allocation.

Postnatal day 1 evaluation

In the short-term group, the postnatal day 1 (PND1) evaluation was performed on the corrected postnatal day 1, that is, postconceptional age 32 days in a designated 30×30 cm area. Before handling, the pups were left undisturbed for a minimum of 60 secs in the assessment area, and thereafter, the experiment was videotaped. We first assessed motor function (tone, motor activity, and locomotion and gait), and afterward, sensation, (touching the whiskers with cotton swab and a mild pin prick to evaluate pain sensation on the hind limbs) including cranial nerves (olfaction, sucking and swallowing and head turn to feeding and righting reflex), as previously described.[2] The sum of these scores represented the total neuromotor (maximum score=26) and neurosensory evolution (maximum score=15).

In the long-term group, the open field, T-maze, and NORTs were performed at the postnatal age of 24 to 28 days and again at 56 to 63 days in a designated room that was insulated from sound and was temperature-controlled at 21°C with full overhead illumination at 300 lux. The area was cleared of any urine and feces after each test. The rabbits were conditioned daily for the testing conditions, and on the day before the testing, they were habituated in the test room in the open field for 30 minutes without any objects or interference. The sequence of assessments was habituation on day 1, open field on day 2, T-maze on days 3 and 4, and NORT on day 5.

Open field test

The Open Field Test (OFT) served as an evaluation of emotional reactivity and anxiety and exploratory- and non-exploratory locomotion. The OFT was designed and used as previously described[3] and was performed on a designated 80×80 cm surface with 80 cm-high polyvinyl chloride walls. It was divided into 9 numbered squares. Each rabbit was individually placed in a starting box (20×20 cm at 4 weeks; 25×25 cm at 8 weeks), which was removed after 60 seconds. The animal was then left free to explore the open field over a period of 5 minutes. Afterward, it was returned to its cage. The following aspects of behavior were scored:

1. Latency time of leaving the starting point (seconds)
2. Total and central displacements, number of squares crossed (n)
3. Running episodes (n)
4. Escape attempts (n)
5. Exploration events, moving with forelegs or standing while sniffing and looking around inside the same square (n)
6. Hops, the number of times the rabbit completely displaced its body by a hop (n)
7. Standing still, with fore and hind legs not stretched (n)
8. Resting, totally inactive with the body touching the floor and fore and/or hind legs stretched out (n)
9. Rearing, number of times the rabbit upheaves on its hind legs (n)
10. Self-grooming events (n)
11. Digging events (n)
12. Biting, biting any elements of the pen (n)
13. Defecation and urination (n)

Novel object recognition test

The NORT served as an evaluation of the rabbit's response to novelty and object-directed exploration to indirectly assess recognition and declarative memory. The NORT was performed in the OFT pen and was adapted from the original description[4] only in terms of the stimulus used. Instead of using a visual stimulus, an odor-based stimulus was used, that is, by placing pieces of apple or orange inside perforated plastic containers, as olfactory sensitivity is highly developed in rabbits.[5] This is in agreement with the notion that the type of stimulus presented must be one in which the sensory perception of the species chosen is adequate.

Each session was composed of 3 phases. During the "sample phase," the rabbit was allowed to explore the pen for 5 minutes, with 2 containers that had the same odor-based stimulus (apple). Afterward, in the "retention phase," the rabbit was returned to the transport box for a 30-minute interval. Finally, during the "testing phase," 2 different odor-based stimuli (apple and orange) were presented to the animal for 5 minutes more. At the end of the test, the rabbit was returned to its cage.

For both the phases, exploration of the object was considered when the rabbit showed sniffing, touching, and having moving vibrissae while directing the nose toward the object <1 cm.

Cumulative events exploring each object in the 2 sessions was recorded. Finally, the discrimination index (DI)[6], which represents the ability to

discriminate the novel from the familiar object, will be calculated as follows:

$$DI = \frac{(Novel\ Object\ Exploration\ Time - Familiar\ Object\ Exploration\ Time)}{(Novel\ Object\ Exploration\ Time + Familiar\ Object\ Exploration\ Time)}$$

T-Maze test

The TMT was used to assess the rabbits' spatial learning and short-term memory and free-choice exploration. The TMT was performed in accordance with the published methods for rodents[7] and was modified for rabbits.[8] Each rabbit was tested once daily and for 2 consecutive days.

The maze was composed of a common corridor and 2 goal arms (80×15 cm at 4 weeks and 80×20 cm at 8 weeks). The 2 goal arms were not separated, so the animal could freely choose either of them, and at the end of each arm, a black triangle or square was placed as a visual cue. Each testing session was composed of a sample run and a testing run with a 15-min interval in between.

The rabbit was placed in the starting box for 30 secs before it was allowed to choose a goal arm. Once the animal had entered the common corridor, the starting box was closed. We considered that it had made a choice when all 4 paws were placed in the arm. At this point, the chosen arm was closed to prevent access to the opposite one, and the rabbit was allowed to explore it for 30 seconds. If the animal failed in the sample run when it did not choose any arm within 3 minutes from opening the starting box, a second sample run was allowed after 15 minutes. The testing run occurred with the same conditions as described for the sample run, but no second testing run was allowed in the case of failure. For this test, we evaluated the time spent in the starting area, the total spontaneous alternation (expressed as %), and the failure rate in the 2 testing sessions.

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Supplemental Material 2: Results

SUPPLEMENTAL TABLE 1
Maternal observations at delivery and litter outcomes

Observations and outcomes	Preterm saline	Preterm betamethasone	*P* value
Maternal weight (kg)	4.9±0.7	4.7±0.6	.466
Heart rate (bpm)	133±19	154±24	.062
Saturation (%)	94±2.2	94±1.4	.618
Temperature (°C)	38.2±0.3	38.4±0.2	.113
Litter size (n)	10±2.1	9±2.8	.855
Stillbirth rate (%)	5.8±7.8	32.0±26	.008[a]

Saline n=8, betamethasone n=12. Data are presented as mean and standard deviation with significance compared with the term birth group.

bpm, beats per minute.

[a] $01 \geq P > .001$.

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SUPPLEMENTAL TABLE 2
Early neonatal biometric and pulmonary outcomes

Outcomes	Preterm saline	Preterm betamethasone	*P* value
Biometrics	n=14	n=14	
Birthweight (g)	53.3±7.8	43.4±6.5	<.001[a]
Placental weight (g)	5.9±0.8	5.0±0.8	.010[b]
Brain volume (mL)	1.9±0.2	1.6±0.1	<.001[a]
Brain weight (g)	1.9±0.2	1.6±0.1	<.001[a]
Brain/body ratio	0.033±0.005	0.034±0.004	.538
Pulmonary evaluation	n=8	n=8	
Lung volume (mL)	1.6±0.4	1.3±0.3	.180
Lung (mL)/body(g)	0.034±0.008	0.038±0.012	.414
Inspiratory capacity (mL/kg)	67.7±6.5	73.8±5.7	.033[b]
Static Compliance (mL/cmH_2O/kg)	0.0025±0.001	0.0031±0.001	.038[b]

Data are presented as mean and standard deviation with significance compared with the term birth group.

[a] $P < .001$; [b] $05 \geq P > .01$.

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SUPPLEMENTAL TABLE 3
Early neonatal neurobehavioral outcomes

Outcomes	Preterm saline	Preterm betamethasone	*P* value
Neuromotor score	n=14	n=14	
Posture	3.0±0	2.9±0.4	.153
Gait	3.4±0.5	2.7±0.8	.010[a]
Locomotion	2.5±0.5	2.1±0.6	.058
Motor activity fore limb	3.0±0	3.0±0	
Motor activity hind limb	3.0±0	3.0±0	
Motor activity head	2.8±0.4	2.5±0.7	.140
Activity duration	3.0±0	3.0±0	
Limb tone	4.0±0	3.9±0.3	.325
Total motor score	24.7±1.1	23.1±2.3	.021[a]
Neurosensory score	n=14	n=14	
Sensation touch	2.2±0.8	1.5±0.9	.031[a]
Suck swallow	2.7±0.5	2.5±0.5	.145
Head turning	3.0±0	2.9±0.3	.326
Odor aversion	2.1±0.4	2.0±0.7	.492
Pain sensation	2.1±0.9	1.8±0.8	.386
Total sensory score	12.1±1.5	10.7±1.5	.017[a]
Righting reflex	4.8±0.4	3.5±1.1	<.001[b]

Data are presented as mean and standard deviation with significance compared with the term birth group.

[a] $05 \geq P > .01$; [b] $P < .001$.

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SUPPLEMENTAL TABLE 4

Early neonatal neuropathological outcomes

Outcomes	Preterm saline	Preterm betamethasone	*P* value
NeuN+counts	n=7	n=7	
Hippocampus CA1	3954±449	2932±402	.001[a]
Hippocampus CA2	3842±458	2984±385	.002[a]
Hippocampus DG	3879±367	2912±393	<.001[b]
Thalamus	1880±553	150± 541	.195
Hypothalamus	1903±530	1487±204	.107
Caudate nucleus	2523±288	2223±665	.239
Putamen	2107±353	1737±339	.096
Corpus callosum	383±84	265±70	.014[c]
Internal capsule	615±231	530±117	.405
Corona radiata	547±105	537±171	.895
TUNEL+percentage	n=7	n=7	
Hippocampus CA1	0.14±0.15	0.7± 0.30	<.001[b]
Hippocampus CA2	0	0.20±0.20	.039
Hippocampus DG	0.04±0.10	0.38±0.38	.057
Thalamus	0.26±0.36	0.69±0.35	.040[c]
Hypothalamus	0.48±0.33	0.57±0.11	.611
Caudate nucleus	0.67±0.33	0.83±0.16	.270
Putamen	0.36±0.13	1.31±0.38	.001[a]
Corpus callosum	1.04±0.34	3.00±0.72	<.001[b]
Internal capsule	0.64±0.26	2.13±1.05	.008[a]
Corona radiata	1.24±0.58	1.82±0.76	.135
Ki67+Percentage	n=7	n=7	
Hippocampus CA1	8.75±4.68	2.06±0.76	.008[c]
Hippocampus CA2	15.64±7.53	4.78±2.94	.007[a]
Hippocampus DG	14.29±1.90	4.77±1.01	<.001[b]
Thalamus	13.10±3.88	7.14±2.44	.006[a]
Hypothalamus	7.81±1.92	4.93±2.14	.021[c]
Caudate nucleus	6.88±2.80	3.84±1.19	.029[c]
Putamen	10.26±5.27	6.77±2.14	.171
Corpus callosum	28.41 ± 2.96	23.71±1.65	.005[a]
Internal capsule	27.15±3.49	17.81±2.33	<.001[b]
Corona radiata	24.47±2.71	16.05±3.19	<.001[b]

Data are presented as mean and standard deviation with significance compared with the term birth group.

CA, cornu ammonis; *DG*, dentate gyrus, *TUNEL*, terminal deoxynucleotidyl transferase dUTP nick end labeling.

[a] $01 \geq P > .001$; [b] $P < .001$; [c] $05 \geq P > .01$.

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SUPPLEMENTAL TABLE 5

Early neonatal magnetic resonance imaging diffusion tensor imaging results

Outcomes	Preterm saline	Preterm betamethasone	*P* value
Fractional anisotropy	n=9	n=9	
Frontal cortex	0.469±0.102	0.352±0.052	.015[a]
Hippocampus	0.307±0.079	0.228±0.040	.028[a]
Caudate nucleus	0.329±0.061	0.277±0.039	.066
Thalamus	0.294±0.053	0.241±0.040	.037[a]
Hypothalamus	0.291±0.049	0.244±0.059	.095
Corpus callosum	0.472±0.127	0.302±0.056	.006[b]
Internal capsule	0.393±0.046	0.363±0.027	.128
Anterior commissure	0.388±0.049	0.365±0.043	.335
Cerebellum	0.338±0.063	0.319±0.033	.451
Mean diffusivity ($mm^2/sec \times 10^{-4}$)	n=9	n=9	
Frontal cortex	3.741±1.223	4.771±0.701	.047[a]
Hippocampus	2.455±0.898	3.215±0.291	.037[a]
Caudate nucleus	2.794±0.105	3.715±0.486	.036[a]
Thalamus	2.474±0.534	3.081±0.337	.012[a]
Hypothalamus	2.085±0.828	2.753±0.306	.046[a]
Corpus callosum	2.237±0.861	3.156±0.733	.027[a]
Internal capsule	2.104±0.453	2.627±0.441	.024[a]
Anterior commissure	2.218±0.664	2.604±0.459	.173
Cerebellum	3.277±0.112	4.054±0.517	.084[a]

Data are presented as mean and standard deviation with significance compared with the term birth group.

[a] $05 \geq P > .01$; [b] $01 \geq P > .001$.

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SUPPLEMENTAL TABLE 6
Long-term biometric outcomes

Outcomes	Preterm saline	Preterm betamethasone	*P* value
Biometrics	n=14	n=14	
Body weight day 1 (g)	60±9	50±6	.003[a]
Body weight day 7 (g)	165±22	120±27	<.001[b]
Body weight day 14 (g)	281±42	217±51	.001[a]
Body weight day 21 (g)	435±61	325±75	<.001[b]
Body weight day 28 (g)	720±123	603±93	.008[a]
Body weight day 35 (g)	1068±112	923±181	.018[c]
Body weight day 42 (g)	1377±161	1220±203	.029[c]
Body weight day 49 (g)	1619±170	1452±185	.018[c]
Body weight day 56 (g)	1960±235	1821±265	.149
Brain volume (mL)	9.1±0.7	8.8±1.3	.622
Brain weight (g)	9.5±0.8	9.2±1.4	.617
Brain/body ratio	0.0047±0.0003	0.0050±0.0003	.057

Data are presented as mean and standard deviation with significance compared with the term birth group.

[a] $01 \geq P > .001$; [b] $P < .001$; [c] $05 \geq P > .01$.

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SUPPLEMENTAL TABLE 7
Long-term neurobehavioral outcomes

Outcomes		Preterm saline	Preterm betamethasone	*P* value
Open field behavior		n=14	n=14	
Latency time (s)	4 wk	3 (1–7)	4 (2–20)	.247
	8 wk	1 (1–2)	2 (1–10)	.311
Total displacements (n)	4 wk	29 (27–41)	49 (30–54)	.039[a]
	8 wk	26 (24–38)	40 (29–51)	.049[a]
Central displacements (n)	4 wk	7 (6–8)	5 (4–9)	.329
	8 wk	7 (5–8)	3 (2–5)	<.001[b]
Corner displacements (n)	4 wk	9 (7–11)	17 (12–20)	.004[c]
	8 wk	9 (6–12)	18 (15–23)	<.001[b]
Exploration (n)	4 wk	8 (6–9)	6 (6–7)	.011[a]
	8 wk	7 (6–8)	5 (4–7)	.011[a]
Standing still (n)	4 wk	3 (2–4)	7 (5–8)	.025[a]
	8 wk	2 (2–4)	3 (2–7)	.127
Running (n)	4 wk	0	2 (1–2)	.018[a]
	8 wk	1 (0–4)	1 (0–1)	.014[a]
Resting (n)	4 wk	0	0	
	8 wk	0	0	

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SUPPLEMENTAL TABLE 7
Long-term neurobehavioral outcomes *(continued)*

Outcomes		Preterm saline	Preterm betamethasone	*P* value
Rearing (n)	4 wk	5 (1–7)	5 (1–11)	.370
	8 wk	7 (4–9)	6 (5–12)	.537
Self-grooming (n)	4 wk	1 (0–2)	3 (1–5)	.019[a]
	8 wk	1 (0–1)	2 (1–4)	.010[a]
Digging (n)	4 wk	0	0	
	8 wk	0	1 (0–2)	.051
Biting (n)	4 wk	0	1 (0–1)	.031[a]
	8 wk	0	2 (1–2)	.006[c]
Urinating (n)	4 wk	0	0	
	8 wk	0	0	
Defecating (n)	4 wk	0	0	
	8 wk	0	1 (1–3)	.021[a]
T-Maze		n=14	n=14	
Total runs (n)	4 wk	3	3	
	8 wk	3	3	
Failed runs (n)	4 wk	0 (0–1)	1 (0–1)	.027[a]
	8 wk	0 (0–1)	1 (0–1)	.563
Alternation %	4 wk	76 (67–100)	29 (0–55)	<.001[b]
	8 wk	73 (58–100)	38 (0–67)	.003[c]
Novel object recognition		n=14	n=11	
Sample phase interactions (n)	4 wk	7 (5–7)	9 (4–13)	.138
	8 wk	8 (5–11)	11 (9–13)	.001[a]
Testing phase interactions (n)	4 wk	7 (4–8)	8 (4–11)	.488
	8 wk	7 (4–9)	8 (6–9)	.273
Novel object interactions (n)	4 wk	3 (3–5)	3 (2–5)	.541
	8 wk	4 (3–6)	4 (3–5)	.717
Discriminatory index	4 wk	25 (0–43)	0 (−11 to 20)	.020[a]
	8 wk	33 (22–37)	0 (−14 to 33)	.014[a]

Data are presented as mean and standard deviation with significance compared with the term birth group.

[a] $05 \geq P > .01$; [b] $P < .001$; [c] $01 \geq P > .001$.

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SUPPLEMENTAL TABLE 8
Long-term neuropathological outcomes

Outcomes	Preterm saline	Preterm betamethasone	*P* value
NeuN+counts	n=7	n=7	
Hippocampus CA1	3864±647	3542±336	.247
Hippocampus DG	4054±711	3410±758	.126
Thalamus	593±247	501±169	.432
Hypothalamus	659±168	462±116	.027[a]
Caudate nucleus	875±98	740±128	.049[a]
Putamen	623±174	508±150	.211
Corpus callosum	245±85	140±68	.028[a]
TUNEL+percentage	n=7	n=7	
Hippocampus CA1	0.08±0.17	0.10±0.11	.075
Hippocampus DG	0.09±0.21	0.22±0.28	.334
Thalamus	1.30±1.17	1.97±1.61	.399
Hypothalamus	1.26±1.05	1.70±1.23	.490
Caudate nucleus	1.37±1.43	1.91±1.33	.478
Putamen	0.61±0.30	1.17±0.85	.140
Corpus callosum	3.19±2.70	4.39±2.82	.432
Ki67+percentage	n=7	n=7	
Hippocampus CA1	0.27±0.15	0.10±0.11	.080
Hippocampus DG	0.41±0.33	0.12±0.13	.059
Thalamus	0.33±0.11	0.22±0.10	.076
Hypothalamus	0.40±0.17	0.26±0.11	.090
Caudate nucleus	0.50±0.21	0.29±0.11	.039[a]
Putamen	0.34±0.12	0.47±0.13	.090
Corpus callosum	0.34±0.16	0.28±0.14	.535

CA, cornu ammonis; *DG*, dentate gyrus.

Data are presented as mean and standard deviation with significance compared with the term birth group.

[a] $05 \geq P > .01$.

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SUPPLEMENTAL TABLE 9

Long-term magnetic resonance imaging outcomes

Outcomes	Preterm saline	Preterm betamethasone	*P* value
Fractional anisotropy	n=9	n=9	
Frontal cortex	0.422±0.018	0.402±0.023	.090
Hippocampus	0.428±0.034	0.392±0.035	.070
Caudate nucleus	0.425±0.033	0.418±0.032	.691
Thalamus	0.373±0.018	0.351±0.026	.077
Hypothalamus	0.395±0.032	0.373±0.048	.315
Corpus callosum	0.478±0.031	0.432±0.033	.015[a]
Internal capsule	0.574±0.022	0.552±0.034	.152
Anterior commissure	0.454±0.040	0.381±0.064	.019[a]
Cerebellum	0.470±0.088	0.433±0.048	.338
Mean diffusivity ($mm^2/sec \times 10^{-4}$)	n=9	n=9	
Frontal cortex	4.047±1.223	4.501±0.701	.042[a]
Hippocampus	3.975±0.898	4.175±0.291	.201
Caudate nucleus	4.119±0.105	4.318±0.486	.248
Thalamus	4.055±0.534	4.036±0.337	.875
Hypothalamus	3.558±0.828	3.625±0.306	.623
Corpus callosum	3.401±0.861	3.778±0.733	.042[a]
Internal capsule	3.909±0.453	4.096±0.441	.366
Anterior commissure	3.575±0.664	3.887±0.459	.075
Cerebellum	4.387±0.112	4.284±0.517	.764

Data are presented as mean and standard deviation with significance compared with the term birth group.

[a] $05 \geq P > .01$.

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