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Antenatal steroids elicited neurodegenerative-associated transcriptional changes in the hippocampus of preterm fetal sheep independent of lung maturation

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Abstract

Background Antenatal steroid therapy for fetal lung maturation is routinely administered to women at risk of preterm delivery. There is strong evidence to demonstrate benefit from antenatal steroids in terms of survival and respiratory disease, notably in infants delivered at or below 32 weeks' gestation. However, dosing remains unoptimized and lung benefits are highly variable. Current treatment regimens generate high-concentration, pulsatile fetal steroid exposures now associated with increased risk of childhood neurodevelopmental diseases. We hypothesized that damage-associated changes in the fetal hippocampal transcriptome would be independent of preterm lung function.

Methods Date-mated ewes carrying a single fetus at 122 ± 2dGA (term=150dGA) were randomized into 4 groups: (i) Saline Control Group, 4×2ml maternal saline intramuscular(IM) injections at 12hr intervals ($n=11$); or (ii) Dex High Group, 2×12mg maternal IM dexamethasone phosphate injections at 12hr intervals followed by 2×2ml IM saline injections at 12hr intervals ($n=12$; representing a clinical regimen used in Singapore); or (iii) Dex Low Group, 4×1.5mg maternal IM dexamethasone phosphate injections 12hr intervals ($n=12$); or (iv) Beta-Acetate Group, 1×0.125mg/kg maternal IM betamethasone acetate injection followed by 3×2ml IM sterile normal saline injections 12hr intervals ($n=8$). Lambs were surgically delivered 48hr after first maternal injection at 122–125dGA, ventilated for 30min to establish lung function, and euthanised for necropsy and tissue collection.

Results Preterm lambs from the Dex Low and Beta-Acetate Groups had statistically and biologically significant lung function improvements (measured by gas exchange, lung compliance). Compared to the Saline Control Group, hippocampal transcriptomic data identified 879 differentially significant expressed genes (at least 1.5-fold change and FDR < 5%) in the steroid-treated groups. Pulsatile dexamethasone-only exposed groups (Dex High and Dex Low) had three common positively enriched differentially expressed pathways related in part to neurodegeneration ("Prion Disease", "Alzheimer's Disease", "Arachidonic Acid metabolism"). Adverse changes were independent of respiratory function during ventilation.

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Conclusions Our data suggests that exposure to antenatal steroid therapy is an independent cause of damage-associated transcriptomic changes in the brain of preterm, fetal sheep. These data highlight an urgent need for careful reconsideration and balancing of how antenatal steroids are used, both for patient selection and dosing regimens.

Keywords Preterm, Steroids, Sheep, Fetus, Brain injury, Hippocampus, Lung maturation

Background

Preterm birth (delivery before 37 weeks gestation) occurs in ~ 11% of all pregnancies worldwide and is the leading cause of mortality and morbidity for children under 5 years old [1]. When accurately targeted to women at risk of preterm delivery, antenatal corticosteroid therapy (ACS) has been credited with reductions in the risk of perinatal death and respiratory disease [2–4]. The largest benefits are seen in preterm infants born at or below 32 weeks' gestation. Thereafter, treatment effect magnitude tapers with proximity to term, reflected by large increases in the number of patients needed to be treated to prevent a single case of respiratory disease. However, despite over 50 years of clinical use, ACS therapy is still not optimized, and there is no international consensus regarding the ideal drug formulation, dosing regimen, or route of administration.

Three corticosteroids (betamethasone phosphate, dexamethasone phosphate, and betamethasone acetate) comprise most ACS dosing regimens worldwide [5–8]; these steroids readily cross the placenta and exert extensive genomic and non-genomic effects on the developing fetus [9]. The two most widely studied ACS formulations, dexamethasone phosphate and betamethasone phosphate (used alone or in combination with betamethasone acetate), have significantly different pharmacokinetic profiles [10]. Unfortunately, the risk-benefits for the developing fetus remain poorly understood which is critical as antenatal steroid use has expanded over the past 20 years to include many patients at considerably lower risks than for which the therapy was originally designed [11]. Furthermore, despite receiving ACS therapy, many babies do not demonstrate any respiratory benefit from treatment (non-response) [12, 13]. Increased use persists, despite evidence from population-level cohort studies concluding an association between exposure to ACS and an elevated risk of childhood neurological deficits/behavioral disorders, serious childhood infection [14, 15], and impaired academic performance [16]. Moreover, additional reports have described ACS exposure being associated with an increased risk of neonatal hypoglycaemia [17], HPA axis dysregulation [18–22], reduced birth weight, and head circumference [23, 24]. Each of which is independently linked to diminished health outcomes.

The impact of ACS on the fetal brain is a topic of particular importance. Due to the high density of glucocorticoid receptor expression, the hippocampus could be highly susceptible to off-target, detrimental ACS effects [25–27]. These concerns are supported by evidence from clinical studies in preterm neonates showing that a standard full-dose ACS exposure was associated with reduced hippocampal neuron density [28] and bilateral cortex thinning (maximally in areas of the brain related to emotional regulation) in pre-adolescents [29]. Animal studies have also shown that ACS exposure increased neuronal degeneration [25], reduced brain weight [30], delayed central nervous system myelination [31], and suppressed normal neurodevelopmental pathway differentiation within the hippocampus [32, 33].

Based on the concept that current dosing regimens are probably excessively high to achieve the expected lung maturation and could unnecessarily increase the risk of harm, several recent and ongoing efforts to optimize ACS dosing have been proposed [34–36]. However, no study has attempted to evaluate the effects of a high dose regimen in current clinical use (2 maternal intramuscular injections of 12 mg dexamethasone phosphate spaced by 12 h, used clinically in Singapore) vs. lower dose pulsatile regimens of ACS on both fetal lung maturation and brain development.

Study aim

We hypothesized that injury-associated changes in the fetal hippocampal transcriptome would be independent of preterm lung function subsequent to antenatal steroid exposure. We test this hypothesis using the sheep model of pregnancy.

Methods

Animal model and antenatal corticosteroid treatment

Merino ewes with singleton fetuses received an intramuscular injection (IM) of 150 mg medroxyprogesterone acetate (Depo-Provera, Pfizer, New York, NY) on 117 ± 2 days gestational age (dGA) to decrease the risk of steroid-induced premature labor. Baseline maternal blood samples were collected for analysis at 0800, 24–48 h prior to allocation to treatment group. Animals were then randomly allocated to one of four groups (Fig. 1), with first treatment given at 122 ± 2 dGA (term = 150 dGA). Lung

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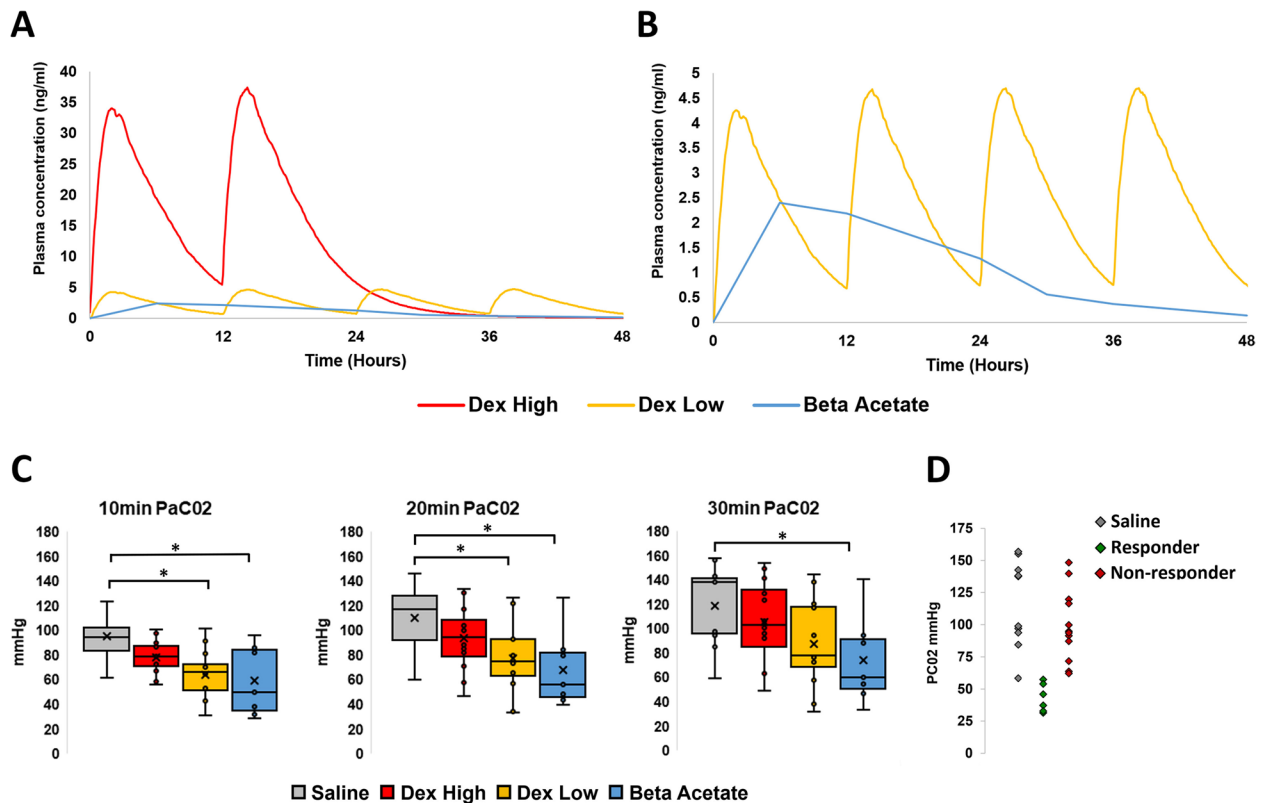
Fig. 1 Experimental design. Diagram of experimental design and intervention groups

maturation in the preterm fetal sheep at 124 days' gestation approximates that of the human at 30–32 weeks' gestation. First injections occurred at 08:00 to control for circadian effects: (i) Saline Control Group, 4×2 ml maternal saline intramuscular (IM) injections at 12-h intervals ($n=11$); or (ii) Dex High Group, 2×12 mg maternal IM dexamethasone phosphate (DBL Dexamethasone sodium phosphate 4 mg/ml, Hospira NZ, New Zealand) injections at 12-h intervals followed by 2×2 ml IM saline injections at 12-h intervals ($n=12$; this protocol simulates the standard ACS regimen used clinically in Singapore); or (iii) Dex Low Group, 4×1.5 mg maternal IM dexamethasone phosphate injections 12-h intervals ($n=12$; this protocol represents a 75% reduction on the Singapore clinical regimen); or (iv) Beta-Acetate Group, 1×0.125 mg/kg maternal IM betamethasone acetate injection followed by 3×2 ml IM sterile normal saline injections at 12-h intervals ($n=8$; this protocol represents a positive control group previously shown to elicit robust preterm lung maturation in the preterm sheep equivalent to standard courses of dexamethasone phosphate or betamethasone phosphate and acetate used worldwide).

Serial maternal plasma samples were collected at 6, 12, 18, 24, and 36 h after first maternal injection. All preterm lambs included in this study for ventilation analysis

(122–125 days gestation) were surgically delivered 48 h after first maternal injection at 122–125 dGA.

One ewe from the Saline Control group was removed from the analysis as it was found not to be pregnant at pre-delivery ultrasound. One lamb from the Beta-Acetate Group, which was incorrectly intubated and not ventilated for 15 min, was removed from the ventilation analysis.

Pharmacokinetics

Liquid chromatography-mass spectrometry (LC–MS) was performed on all maternal and fetal plasma samples to assess dexamethasone and betamethasone-acetate concentrations respectively as previously reported [37]. Betamethasone Acetate (Beta-Acetate Group) pharmacokinetic parameters were estimated by non-compartmental analysis using Phoenix WinNonlin software (version 8.1, Certara, Inc.). Dexamethasone (Dex Low and Dex High Groups) simulated pharmacokinetic parameters were generated by Dr Mark Milad based on existing ovine dexamethasone pharmacokinetic data [8].

Delivery and ventilatory assessment

Maternal plasma samples for analysis were taken immediately prior to delivery, i.e., 48 h after first maternal

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injection. Fetal plasma samples for analysis were obtained during operative delivery and immediately prior to ventilation commencing. Prior to delivery, ewes received an intravenous bolus of midazolam (0.5 mg/kg) and ketamine (10 mg/kg) for the deep induction of anaesthesia. The fetal head was delivered through abdominal and uterine incisions, and a 4.5-mm endotracheal tube was secured by tracheostomy. The fetus was then delivered, and the ewe euthanized under anaesthesia with pentobarbital. Lambs were weighed, dried, and placed in a radiant warmer (Cozy Cot, Fisher & Paykel Healthcare, New Zealand) bed under a plastic insulating wrap (Neowrap, Fisher & Paykel, NZ). Mechanical ventilation (Fabian HFO, Accutronic Medical Systems AG, Switzerland) was immediately commenced with the following settings: peak inspiratory pressure (PIP) of 35 cmH_2O, positive end expiratory pressure (PEEP) of 5 cmH_2O, respiratory rate (RR) of 50 breaths per minute, inspiratory time (iT) of 0.5 s, using 100% heated and humidified oxygen. We used 100% oxygen which allows comparison of oxygenation through the partial arterial pressure of oxygen among the groups.

Investigators operating the ventilators were blinded to the animal treatment groups. An umbilical artery was catheterized for blood sampling and administration of supplemental anaesthesia with ketamine (5 mg/kg) as necessary. Tidal volume (V_T) was continuously measured, and the PIP was adjusted to keep the V_T between 7.0 and 8.0 ml/kg, but with PIP limited to 35 cmH_2O. At 10, 20, and 30 min of ventilation we measured temperature, blood pressure, ventilator data (PIP, V_T, and compliance), and performed blood gas measurements; pH, PCO_2 (mmHg), PO_2 (mmHg), O_2 saturation (SO_2, %), and total hemoglobin (Hb, g/dL) levels (Siemens RapidPoint 500, Munich, Germany). Dynamic compliance (Cdyn, ml/cmH_2O/kg) was recorded as measured by the ventilator. The ventilation efficiency index (VEI) was calculated using the formula VEI = 3800 / (RR × (PIP − PEEP) × PCO_2 (mm Hg)) [38].

Definition of antenatal corticosteroid response
Steroid-treated lambs were defined as having responded to ACS treatment if the $PaCO_2$ level after 30 min of ventilation was two standard deviations below the mean of the Saline group. The mean and standard deviation of the $PaCO_2$ after 30 min of ventilation in the Saline Control group was 118.3 and 31.3 mmHg thus giving rise to 57.0 mmHg (= 118.3 − 2 × 31.3) as cutoff.

Lung functional assessments
After 30 min of ventilation, lambs were euthanized with pentobarbital, disconnected from the ventilator, and the endotracheal tube was clamped for 2 min to achieve atelectasis by oxygen absorption. The lambs were weighed, and the chest was opened for visual evaluation of gross lung injury–pulmonary hemorrhage, pulmonary interstitial emphysema, gas pockets within the lung, or subpleural dissection. These were all performed by a single investigator. A deflation pressure–volume curve was measured after air inflation of the lungs to a pressure of 40 cmH_2O. Volume at a pressure of 40 cmH_2O was calculated using fetal weight after ventilation (kg) as V_{40} (ml/kg).

Hippocampal and lung mRNA extraction and RNA bulk sequencing
Twenty-four animals (Six animals from each group -Saline Control, Dex High, Dex Low, Beta-Acetate) were selected for RNA bulk sequencing based on the highest maternal steroid plasma concentration prior to delivery. The same animals were used for both lung and hippocampal RNA extraction and analysis thereby allowing for matched analysis with ventilation outcomes and ACS responsiveness.

The ACS Responder Group for RNA bulk sequencing (total six animals) consisted of three animals from the Dex Low and three animals from Beta-Acetate Groups; ACS non-responder subgroup (total twelve animals) consisted of six animals from the Dex High Group, three animals from the Dex Low Group, and three animals from Beta-Acetate Group. RNA was extracted from snap-frozen hippocampal (entire hippocampus) and lung tissue (inferior aspect of right lower lobe) using RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The concentrations of extracted RNA and RNA Integrity Number (RIN) were determined using Agilent Technologies RNA Nano Chip as per manufacturer's instructions. All RNA extracts were diluted in nuclease-free water to yield a final RNA concentration of 30 ng/µl. Hippocampal and lung tissue bulk RNA sequencing was performed by Novogene Singapore.

Bulk RNA-sequencing data processing and quality control
One hundred fifty-base pair paired-end sequenced reads were processed using the nf-core/rnaseq v3.10.1 pipeline [39] with nextflow v22.10.4 [40]. Briefly, raw reads were trimmed using Trim Galore! v0.6.7 [41] to remove low-quality bases and adapters. Trimmed reads were aligned to the *Ovis aries* reference genome, ARS-UI_Ramb_v3.0 with STAR v2.6.1d aligner [42]. Finally, Salmon v1.9.0 [43] was utilized for assigning reads. Principal component analysis was conducted for quality control assessment and exploratory analysis.

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Differential gene expression and gene set enrichment analysis

All downstream analyses were performed using R statistical software version 4.2.1 [44]. Differential gene expression analysis was performed using the DESeq2 package v1.36.0 [45]. Genes with at least 1.5-fold change in expression and FDR < 0.05 were considered significant. The results were visualized using the Enhanced Volcano v1.14.0 [46] and Complex Heatmap v2.12.1 [47]. Gene set enrichment analysis (GSEA) was run on all genes ranked by their signed log *p*-value using cluster Profiler v4.9.0 [48] and KEGG database [49].

Hematological and biochemical data acquisition

Plasma isolated from maternal and fetal blood samples was used for hematological analyses, including white blood cell counts (WBC,/µl), differential leukocyte counts (%), neutrophil count (%), biochemical parameters; gamma-glutamyl transferase (GGT, U/l), alanine aminotransferase (ALT, U/l), aspartate aminotransferase (AST, U/l), albumin (g/l), cortisol (nmol/L), adrenocorticotropic hormone (ACTH, pg/ml). Those analyses were performed by an independent clinical pathology laboratory (Vetpath, Perth, Australia). Cortisol values under limit of detection (< 5.52 nmol/l) were allocated a value of 5.52 nmol/l for statistical analyses. ACTH values under limit of detection (< 5.0 pg/ml) were allocated a value of 5 pg/ml for statistical analyses.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp, Armonk, NY). Chi-square was used to test the differences of nominal values between groups. All numerical data were tested for normality with Shapiro–Wilk tests. In the comparison of the four intervention groups (Dex High, Dex Low, Beta-Acetate, Saline Control Groups) between-group differences in parametric data were tested for significance with one-way ANOVA, while Kruskal Wallis tests were used for non-parametric data. Multiple post hoc comparisons were performed with Tukey's and Dunnett *t* (2-sided) tests. *p* values < 0.05 being significant. Repeated ANOVA test and pairwise comparisons were used to analyze maternal Saline Control Group baseline cortisol and ACTH compared to serial time points.

Results

Reduced dose antenatal steroid regimens accelerate fetal lung maturation

There were no significant differences in delivery data among treatment groups (Additional File 1 and Additional Table 1).
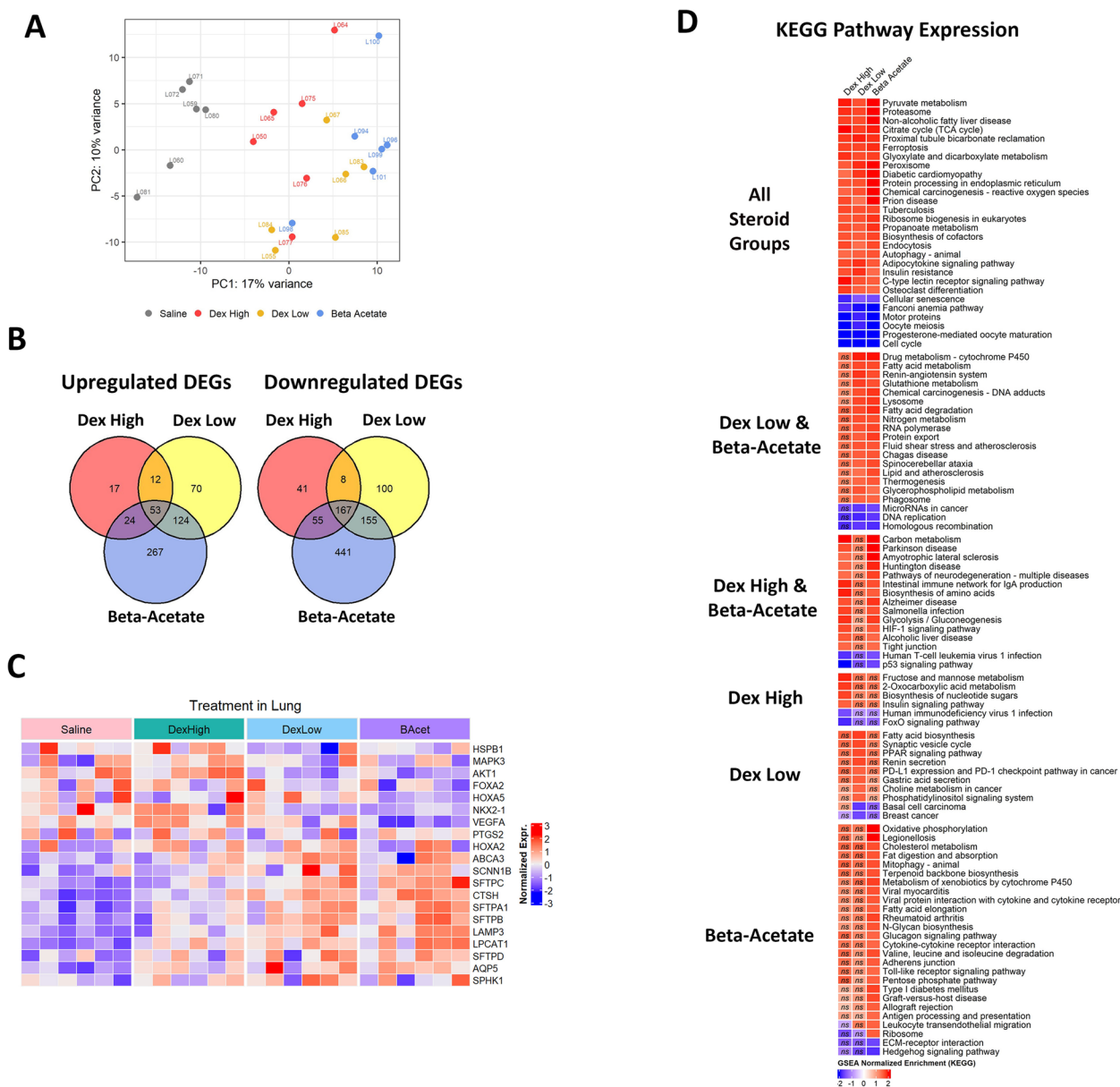
Simulated neonatal steroid plasma concentrations for each steroid-treated group highlight the pulsatile nature of the dexamethasone regimens compared to a constant exposure Beta-Acetate group (Fig. 2A, B with pharmacokinetic profile illustrated in Additional Table 2) [8]. Maternal and cord (delivery) steroid plasma measurements and pharmacokinetic parameters are described in Additional File 1.

Arterial umbilical cord gas $PaCO_2$ values serve as a robust and well-established measure of acute preterm functional lung maturation [50–53]. The Beta-Acetate Group animals had significantly improved cord $PaCO_2$ values compared to Saline Control Group animals at 10, 20, and 30 min of ventilation ($p < 0.05$) (Fig. 2C (Additional File 1)). The Dex Low Group animals had significantly improved cord $PaCO_2$ values compared to Saline Control Group animals at 10 and 20 min ($p < 0.05$) of ventilation, but not significant at 30 min ($p = 0.094$/NS) (Fig. 2C). There were no statistically significant differences in cord $PaCO_2$ between Dex High Group animals and Saline Control Group at any ventilation timepoint. Additional lung function data are shown in Additional Figs. 1A-C,2. Our data show, for the first time, that the high-dose, pulsatile, and rapidly cleared dexamethasone ACS regimen (Dex High Group) currently used clinically in Singapore did not convey statistically significant improvements in preterm ovine neonatal lung function. The greatest improvements in preterm neonatal lung function were actually associated with constant, low-concentration and extended-duration fetal steroid exposures represented by the Positive Control (Beta-Acetate) Group.

The effects of different antenatal steroid regimens on fetal lung transcriptome related to lung maturation

Principal component analysis (PCA) plots of the fetal lung transcriptome profile showed that ACS-treated animals were distinct from Saline Control animals (Fig. 3A). Differential analysis identified 1533 significant genes (with at least 1.5-fold change and FDR < 5%) in the steroid-treated groups (Fig. 3B; Supplementary GSEA Table 1). Of these significant differentially expressed genes (DEGs), 567 (37%) were upregulated while 967 (63%) were downregulated (Fig. 3B). There was a marked difference between upregulated and downregulated genes. Upregulated genes common to all steroid-treated groups related to inflammation and immune regulation (*IL1B, MARCO, C7, CXCL16, FGL1, TNFRSF13B*). In comparison, downregulated genes common to all steroid-treated groups were related to cellular division/ cell cycle (*PIMREG, PRR11, FOXM1, PRC1, KIF18B, ASPM, MKI67, CDCA3, CDCA8, CDC20*) (Fig. 4B).

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Fig. 2 Simulated fetal plasma ACS concentrations and arterial PCO_2 at 10, 20, and 30 min of ventilation. **A** Simulated fetal plasma steroid concentrations comparing Dex High, Dex Low, and Beta-Acetate dosing regimens based on maternal plasma concentration and known pharmacokinetics of dexamethasone and betamethasone (Additional Table 2) dosing regimens [8]. **B** Simulated fetal plasma steroid concentrations comparing Dex Low and Beta-Acetate dosing regimens based on maternal plasma concentration and known pharmacokinetics of dexamethasone and betamethasone dosing regimens (Additional Table 2) [8]. **C** Umbilical $PaCO_2$ at 10, 20, and 30 min of ventilation (Saline control, Dex High, Dex Low, Beta-Acetate groups). **D** ACS Responder, ACS Non-responder and Saline Control group $PaCO_2$ at 30 min of ventilation. The Asterix indicates a significant difference among the groups. Error bars represent ± 1 standard deviation

Animals in the Positive Control Beta-Acetate Group (demonstrating the highest degree of lung maturation) had the greatest number of group-specific significant DEGs (708 genes), compared to Dex Low (170 genes) and Dex High (58 genes) (Fig. 3B). Dex Low and Beta-Acetate Group animals, which had the longest predicted duration of fetal ACS-exposure and functional lung maturation,

had upregulation of established markers of lung maturation including surfactant production and transport (*SFT-PAI, SFTPB, SFTPC, CTSH, LPACT1, LPCAT3, SCNN1A* [54]) genes related to immune response/inflammation (*LAMP3, C5*), and increased vasculature (*HIF3A, EPAS1*) (Figs. 3C and 4B). Dex Low and Beta-Acetate Group animals also had common downregulation of genes
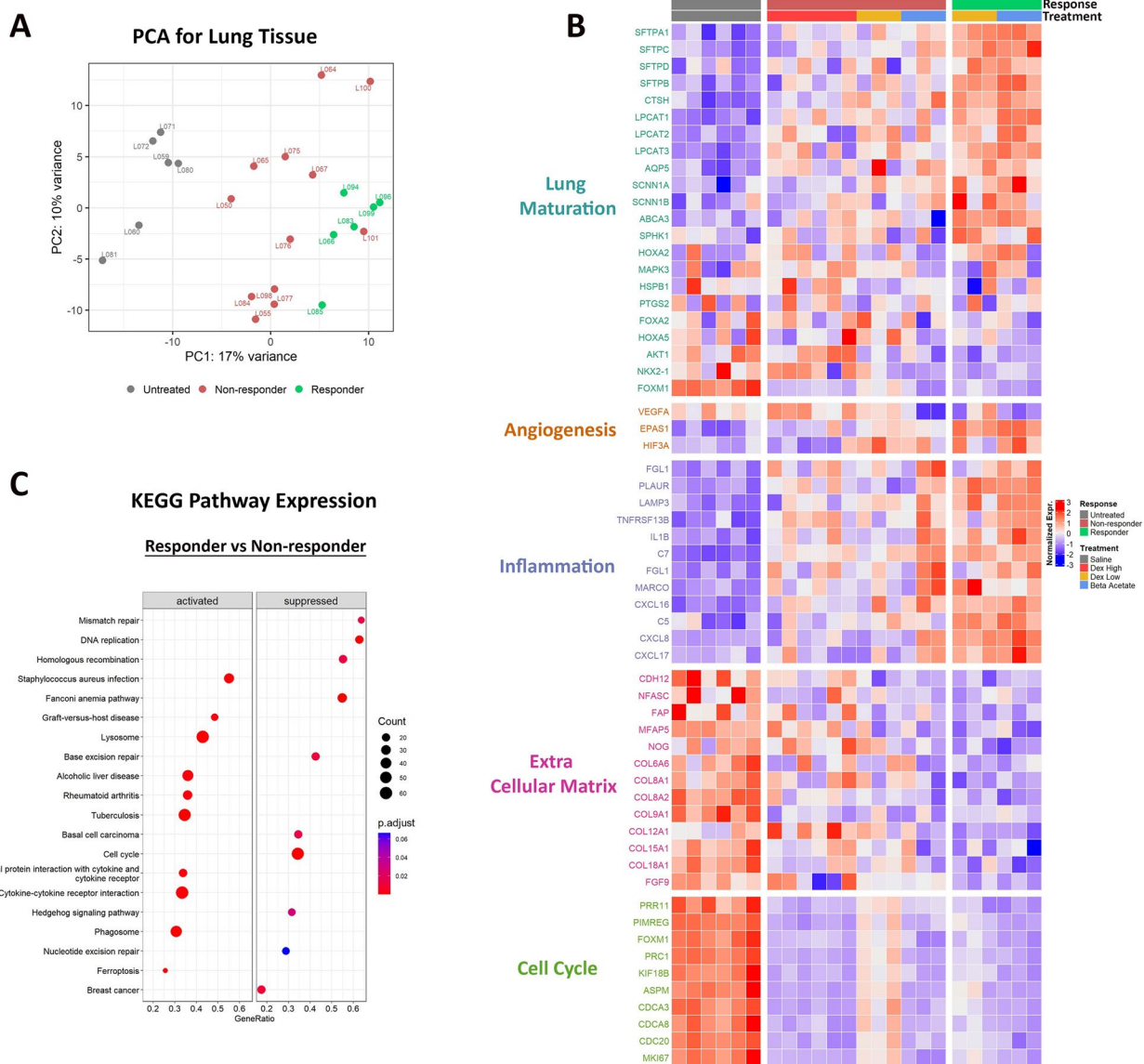
Fig. 3 Fetal lung RNA bulk sequencing for treatment groups. **A** Principal component analysis (PCA) plot of differential gene expression for intervention groups Dex High / Dex Low / Beta-Acetate/ Saline Control Groups. **B** Venn diagram demonstrating differential up and down gene regulation between Dex High/ Dex Low/ Beta Acetate group vs Saline Control group. **C** Heat map displaying differential gene expression for twenty different genes related to fetal lung maturation [54] for each intervention group (Saline Control, Dex High, Dex Low, Beta-Acetate). **D** Heatmap demonstrating the significant positively and negatively enriched KEGG Pathways (DEPs) (Fold change > 1.5, False Discovery Rate FDR < 5%) between steroid treated groups (Dex High, Dex Low, Beta-Acetate) compared to Saline Control Group

related to extracellular matrix and cell adhesion (*MFAP5, CDH12, NFASC, FAP*) (Fig. 4B). Consistent with functional studies, these markers of lung maturation were not significantly differentially expressed in Dex High Group animals (Figs. 3C and 4B).

To understand the biological themes associated with the DEGs, we conducted gene set enrichment analysis (GSEA) [48] with KEGG pathways [49] on all genes ranked by the signed negative log *p*-value. Steroid-treated groups were

compared to Saline Control Group (Fig. 3D, Supplementary GSEA Table 2). Overall, 93 (82.8%) KEGG pathways were positively enriched while 16 (17.2%) KEGG pathways were negatively enriched. We identified 22 positively enriched KEGG pathways common to all steroid-treated groups that are related to cellular metabolism, inflammation, and human disease (Fig. 3D). Six negatively enriched pathways were common to all steroid-treated groups related to cellular homeostasis and reproduction (Fig. 3D).

Table 1 Summary of the clinically relevant functional lung maturation status, differential gene and KEGG pathway expression for each treatment group compared to Saline Control group

Treatment group	Functional lung maturation	Lung tissue Genes related to lung maturation	Hippocampal tissue Genes related to neurodegeneration	Hippocampal tissue KEGG pathways related to neurodegenerative disorders
Dex High	NOT SIGNIFICANTLY IMPROVED	NOT SIGNIFICANTLY EXPRESSED	SIGNIFCANTLY EXPRESSED	- Prion disease - Alzheimer's disease - AA metabolism - Spinocerebellar ataxia
Dex Low	VARIABLE IMPROVEMENT	SIGNIFICANTLY EXPRESSED	SIGNIFICANTLY EXPRESSED	- Prion disease - Alzheimer's disease - AA metabolism - Parkinson disease - Huntington disease - Pathways of neurodegeneration – multiple diseases - Amyotrophic lateral sclerosis
Beta-Acetate	SIGNIFCANTLY IMPROVED	SIGNIFICANTLY EXPRESSED	SIGNIFICANTLY EXPRESSED	Nil

Understanding the transcriptomic fetal lung maturation response to ACS exposures

ACS treated animals were defined and grouped as ACS Responder if the $PaCO_2$ after 30 min of ventilation was more extreme than two standard deviations below the mean $PaCO_2$ value for the Saline Control Group [12]. ACS response rates observed between steroid-treated groups were Dex High Group 8.33% (1/12), Dex Low Group 25% (3/12), and Beta-Acetate Group 42.86% (3/7) (Fig. 2D). When compared to Saline Control Group animals, the ACS response rate was only significantly higher in the Beta-Acetate Group animals ($p = 0.048$).

PCA plots revealed distinct clusters of Saline Control, ACS Responder, and ACS Non-Responder Groups (Fig. 4A). Comparative analysis comparing DEGs of ACS Responders to Non-Responders showed 101 significant DEGs (Supplementary GSEA Table 3). Responder Group animals had significant upregulation of genes known to be associated with fetal lung maturation and surfactant production (*SFTPA1*, *SFTPC*, and *SFTPB*), inflammation and immune response (*CXCL8*, *CXCL17*, *C5*, *PLAUR*), and downregulation of genes related to extracellular matrix remodelling (*COL12A1*, *NOG*) (Fig. 4B). Analysis of differential KEGG pathway expression between ACS Responders and Non-Responders are presented in Fig. 4C (Supplementary GSEA Table 4). Responder Group animals had positive enrichment of 50 KEGG pathways (related to inflammation, lipid, and sugar metabolism) and negative enrichment of nine KEGG pathways (related to cell and DNA replication).

Antenatal steroid exposure causes neurodegenerative-associated changes to the fetal hippocampal transcriptome

PCA plots for all treatment groups as well as ACS Responder and Non-Responder Groups are shown in Fig. 5A. When compared to Saline Control Group, differential analysis identified 879 significant DEGs (at least 1.5-fold change and FDR < 5%) in the steroid-treated groups (Fig. 5B, Supplementary GSEA Table 5). Common to all steroid-treated groups were 41 upregulated DEGs and 26 downregulated DEGs (Fig. 5B, Additional Fig. 3). The Positive Control Beta-Acetate Group animals had the greatest number of group specific, significant DEGs (371 genes) compared to Dex Low (169 genes) and Dex High (67 genes) (Fig. 5B).

Analyses were conducted for genes related to neurodevelopment and neurodegeneration. Upregulated genes common to all steroid-treated groups included *NMB*, *CCN3*, *APOD*, and *NPPC*, while downregulated genes common to all steroid groups included *ISLR2* and *NTNG2* (Fig. 5C,D). Dex High and Positive Control Group animals had common upregulation of *AZIN2* and *C4A*. Dex Low and Positive Control Group animals had common upregulation of *DRD5* and downregulation of *SVB2*. Finally, Positive Control Group animals had downregulation of *GLDN*, which was not seen in the other steroid-treated groups.

Steroid-treated groups were compared to Saline Control Group animals in terms of differential KEGG pathway expression. Overall, the majority (75.8%) of KEGG
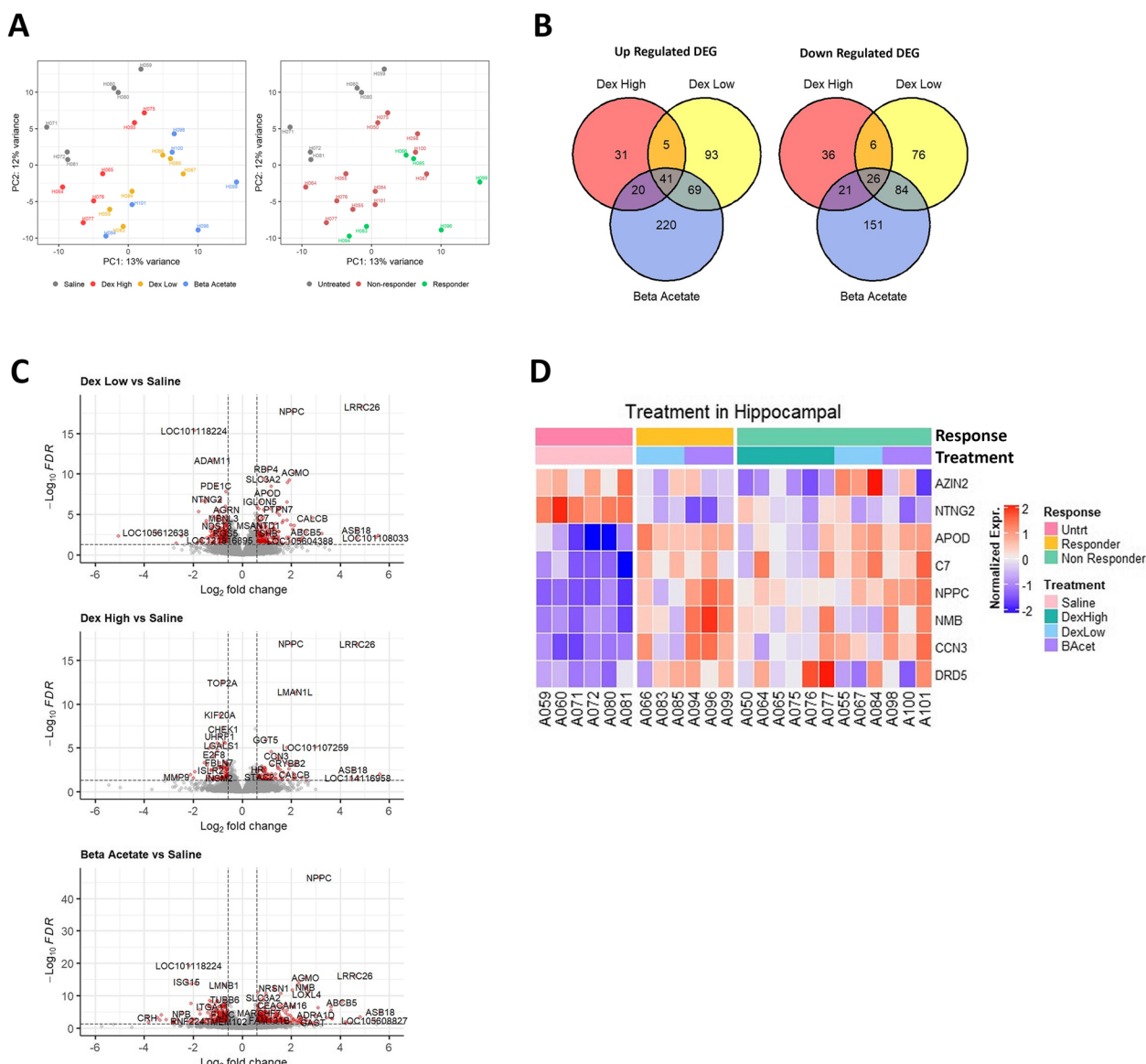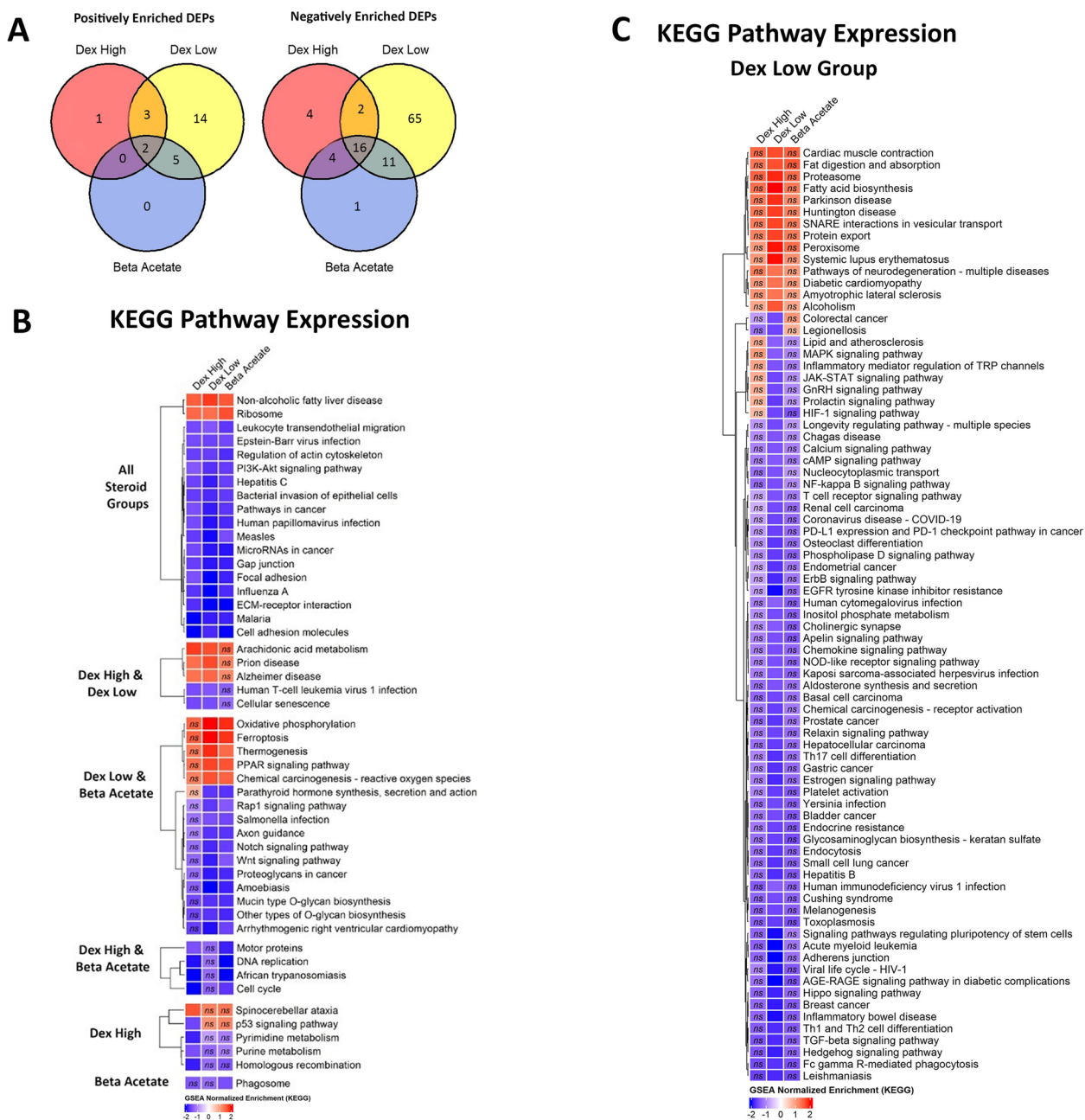
Fig. 4 Fetal lung RNA bulk sequencing for ACS responder vs non-responder groups. **A** Principal component analysis (PCA) plot of differential gene expression for ACS Responder, ACS Non-Responder and Saline Control groups. **B** Heat map displaying differential gene expression for genes related to fetal lung maturation, angiogenesis, inflammation, extra-cellular matrix, and cell cycle for ACS Responder vs ACS Non-Responder groups and by specific treatment group (Dex High, Dex Low, Beta-Acetate) compared to Saline Control. **C** Wrap plot demonstrating the significant positively and negatively enriched KEGG Pathways (DEPs) (Fold change > 1.5, False Discovery Rate FDR < 5%) for ACS Responder vs ACS Non-responder

pathways in the steroid-treated groups were negatively enriched (Fig. 6A, Additional File 1 and Supplementary GSEA Table 6). We identified two positively enriched ("Non-Alcoholic Fatty Liver Disease" & "Ribosome") and 16 negatively enriched DEPs (pathways related to cellular homeostasis & infection) common to all steroid-exposed groups (Fig. 6B).

Pulsatile dexamethasone-only exposed groups (Dex High and Dex Low) had three common positively

enriched DEPs related to neurodegeneration ("Prion Disease", "Alzheimer's Disease", "Arachidonic Acid metabolism") and two common negatively enriched DEPs ("Human T-cell leukemia virus 1 infection", "Cellular Senescence") (Fig. 6B). Furthermore, in the pulsatile, high amplitude Dex High Group, we identified unique positive enrichment of the KEGG pathway "Spinocerebellar Ataxia" (Fig. 6B) and group specific positive enrichment in the low amplitude, pulsatile Dex Low Group of
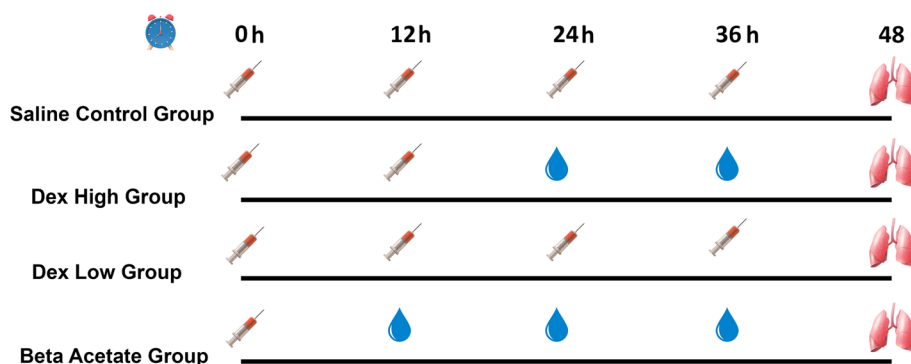
Fig. 5 Hippocampal bulk RNA sequencing data for differentially expressed genes. **A** Principal component analysis (PCA) plot of differential gene expression for intervention groups Dex High / Dex Low / Beta Acetate/ Saline Control groups and ACS Responder / ACS Non-Responder subgroups. **B** Venn diagram demonstrating differential up and down gene regulation (DEG) between Dex High/ Dex Low/ Beta Acetate group vs Saline Control group. **C** Volcano plots of differential gene expression (DEG) for each treatment group Dex High, Dex Low, and Beta Acetate compared to Saline Control group. **D** Heat map displaying significant differential gene expression of eight different genes related to neurodegenerative/ neuropsychiatric disorders for ACS Responder vs ACS Non-Responder and by specific treatment group (Dex High, Dex Low, Beta-Acetate) compared to Saline Control

"Parkinson Disease," "Huntington disease," "Pathways of neurodegeneration – multiple diseases," and "Amyotrophic lateral sclerosis" (Fig. 6C).

Comparisons between Dex Low and Positive Control (Beta-Acetate) Group animals had five common

positively enriched DEPs ("Oxidative Phosphorylation" and Chemical carcinogenesis – ROS") and 11 common negatively enriched DEPs (including "Wnt Signaling") (Fig. 6B). Dex High and Beta-Acetate Group comparison had no common positively enriched and only four

Fig. 6 Hippocampal bulk RNA sequencing data for differentially expressed KEGG pathways. **A** Venn diagram demonstrating differential up and down KEGG pathway expression (DEP) between Dex High / Dex Low / Beta Acetate groups vs Saline Control group. **B** Heat map demonstrating the significantly positively enriched and significantly negatively enriched differentially expressed KEGG Pathways (DEP) (Fold change > 1.5, False Discovery Rate FDR < 5%) in common between all steroid-treated groups (Dex High/ Dex Low/ Beta Acetate), Dex High group specific and Beta Acetate group specific, compared to Saline Control. **C** Heat map demonstrating the Dex Low group specific 14 significantly positively enriched and 65 significantly negatively enriched differentially expressed KEGG Pathways (DEP) (Fold change > 1.5, False Discovery Rate FDR < 5%) compared to Saline Control group

common negatively enriched DEPs ("Motor proteins," "DNA replication," "African Trypanosomiasis," "cell cycle") (Fig. 6B). The lack of commonality between these groups may reflect the significant differences in pharmacokinetic profiles between these groups (high amplitude pulsatile vs. low amplitude constant exposure) respectively (Fig. 2A). Surprisingly, the constant, low amplitude exposure Beta-Acetate Group had only one additional unique KEGG pathway ("Phagosome"), despite having the greatest number of significant DEGs (Fig. 6B).

Neurodegenerative changes in the fetal hippocampal transcriptome relate to steroid exposure and not ventilation outcome

Analysis of ACS Responder compared to ACS Non-Responder Groups for significant DEGs surprisingly revealed limited differential expression, none of which related specifically to neurodegeneration (one uncharacterized upregulated gene and 37 downregulated genes) (Supplementary GSEA Table 7). By comparison, both steroid-exposed groups (Responder and Non-Responder Groups) when compared to saline control respectively, demonstrated the same pattern of upregulation in the genes related to neurogenerative and neuropsychiatric disorders being *NMB, CCN3, APOD, NPPC, AZIN2, DRD5* and downregulation of *NTNG2* (Fig. 5D, Supplementary GSEA Table 8). Notably, these genes were not significantly differentially expressed when ACS Responders were compared to Non-Responder Groups.

Finally, KEGG pathway analysis of ACS Responders *vs.* Non-Responder subgroups did not demonstrate positive or negative enrichment for any pathways related to neurodegenerative disorders (Supplementary GSEA

Table 9 and Supplementary GSEA Table 10). These findings therefore suggest that the observed adverse effects of ACS exposure on neurodevelopment are related to ACS drug exposure and not impacted by lung maturation status and subsequent ventilation outcomes.

Cardiac, endocrine, immunological, and hepatic effects

Data describing the effects of different steroid dosing regimens on key homeostatic processes are shown in Additional Figs. 4–6. No outcomes likely to impact the lung maturation or transcriptomic data presented herein were identified.

Discussion
Principal findings
The principal findings of this study are that:

(i) An ACS dosing regimen used clinically in Singapore, conveying a high amplitude, pulsatile, and rapidly cleared dexamethasone ACS regimen (represented by the Dex High Group) did not demonstrate statistically significant improvements in fetal lung maturation. Furthermore, consistent with earlier reports [50], a single dose of 0.125 mg/kg Beta-Acetate induced robust fetal lung maturation based on significantly improved $PaCO_2$ (Fig. 2C), dynamic lung compliance, VEI, and lung tidal volumes (Additional Fig. 1). The comparably poor maturation effect observed in the high-dose dexamethasone group is consistent with our in silico modelling and in vivo measurements of maternofetal dexamethasone exposures following two 12 mg maternal intramuscular injections given at a 12-h interval [8, 11]. In this regimen, dexametha-

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sone is rapidly cleared from the fetal compartment, with fetal plasma concentrations likely at sub-therapeutic levels within 24 h of treatment commencing. These data are consistent with our previous findings, which showed that robust preterm lung maturation in the sheep requires an uninterrupted exposure of 1–2 ng/ml plasma betamethasone for at least ~ 30 h when delivering animals at a 48 h treatment to delivery interval [53, 55, 56]. The most robust maturation was seen in animals that had this level of steroid exposure at the time of delivery and ventilation [56]. These data further support optimizing of ACS therapy by utilizing a reduced dose, extended-duration regimens;

(ii) Significant patterns of gene expression corresponding with fetal lung maturation were only observed in animals from the reduced dose, and extended duration ACS regimens (Dex Low and Beta-Acetate Groups). Examples of this pattern of gene expression include the upregulation of genes related to surfactant production/function (*SFTPA1, SFTPB, SFTPC, CTSH, LPCAT1, LPCAT3, SCNN1A*) [57], and increased vasculature (*HIF3A, EPAS1*) as well as downregulation of genes related to extracellular remodelling (*MFAP5, CDH12, NFASC, FAP*). This pattern of transcriptome changes relating to lung maturation was not observed in the Dex High group, which was also demonstrated at a physiological level, with poor ventilation outcomes and therefore poor functional lung maturation. These data are especially relevant since the Dex High regimen is recommended for clinical use in patients at risk of preterm birth in many countries.

(iii) ACS-exposed animals had significantly dysregulated hippocampal RNA transcriptomes compared to Saline Control Group animals, suggesting the potential for altered neurodevelopment irrespective of the dose or pharmacokinetic profile of the ACS regimen employed. Despite being exposed to hypercapnia, hypoxia, and more pronounced acidosis, animals in the Saline Control Group from a neurodevelopmental injury perspective, demonstrated a far more benign transcriptome, compared to both ACS-responding and non-responding animals; and

(iv) The most concerning findings were seen in the animals exposed to both high and low-dose, pulsatile dexamethasone regimens (Dex High and Dex Low Groups). They had the greatest degree of significant enrichment of KEGG pathways associated with neurodegenerative disorders. For example, both pulsatile dexamethasone exposed groups (Dex High and Dex Low) exhibited positive enrichment of pathways related specifically to neurodegenerative disorders, including "Prion Disease," "Alzheimer's Disease," and "Arachidonic Acid (AA) Metabolism" (Fig. 6B/C). By comparison, none of these pathways were activated in the constant exposure, low-dose Beta-Acetate Group (Table 1).

Clinical implications

ACS therapy was introduced into clinical practice progressively over the past 50 years although there was initially much resistance to it. In fact, there was so much concern about failure to use it, that the NICHD held a consensus conference to generate publications to warn obstetricians that failure to use steroids for premature labor would be very problematic medicolegally [58, 59]. Recently there has been clinical concern about the overuse of such treatments and deleterious consequences of such. In current practice, ACS is still very commonly administered to women at risk of preterm delivery. Although some 11% of all babies are born preterm, many more pregnant women are judged to be at risk of preterm delivery and are given ACS therapy. As such, a very large (perhaps as much as 20%) of mothers and fetuses are now exposed to high-dose ACS.

Despite the very common usage, optimization of how and when to use ACS has never been achieved, and the effects of drug pharmacokinetics on the brain, lungs, and other vital fetal systems, remains poorly understood.

Understanding the molecular mechanisms and transcriptomic changes behind the fetal lung maturation responses to ACS, as well as the risk of injury to the developing fetal brain, are critical to deciding the proper usage of such powerful pharmaceuticals [12].

We now have molecular tools capable of answering many questions that were not possible when ACS was introduced. In this study, we have highlighted the critical role of the surfactant genes *SFTPA1, SFTPB,* and *SFTPC* in the fetal lung maturation response by demonstrating significant upregulation of these genes in the ACS Responder group compared to the non-responder group and Saline control. The late stages of fetal lung development require mesenchymal thinning to promote gas exchange [60]. Correspondingly, we observed significant downregulation in genes related to extracellular matrix (ECM) remodelling and collagen synthesis in the fetal lungs of the Responder group compared to the non-responder group and Saline control which fits with this established process of mesenchymal thinning. Schmidt et al., utilizing a non-human primate model of lung maturation, also observed downregulation in genes related to ECM remodelling and mesenchymal growth factors

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[61]. These findings help inform on further targeted approaches to inducing preterm fetal lung maturation to improve the response rates to treatment.

Despite concerning associations observed in large population cohort studies, indicating an elevated risk of neurobehavioral/learning disorders in children exposed to ACS in utero, the neurodevelopmental effects of various ACS exposures on the fetal brain are still not well understood [14, 15]. In this study we have demonstrated that all steroid-treated groups had common upregulation of genes related to dysregulation of neurodevelopment including Neuromedin B (*NMB*), Cellular Communication Network Factor 3 (*CCN3*), Apolipoprotein D (*APOD*), Natriuretic Peptide C (*NPPC*). *NMB* is a neuropeptide that has been associated with the acute stress response and anxiety in animals [62], while animal models have demonstrated that elevated *CCN3* impaired hippocampal differentiation and normal hippocampal neurogenesis [63]. Similarly, elevated levels of *APOD* have been found in the CSF and hippocampus of patients with Alzheimer's disease [64], while elevated *NPPC* has been shown to negatively affect hippocampal neuroplasticity and memory in rat models [65].

All steroid-treated groups also demonstrated downregulation of the genes Immunoglobulin superfamily containing leucine rich repeat 2 (*ISLR2)* and Netrin G2 *(NTNG2). ILSR2* is a key factor for axon guidance and brain development [66, 67]. Deficiency of *ILSR2* is associated with the development of hydrocephalus in mice and clinical studies [66, 67]. Reduced mRNA levels of *NTNG2* have also been demonstrated in the brains of patients with bipolar disorder and schizophrenia, thereby implicating NTNG2 in the pathophysiology of these diseases [68].

Further, groupwise comparisons of Dex High and Beta-Acetate Group animals revealed common significant upregulation of the genes Antizyme Inhibitor 2-like *(AZIN2)* and Complement Component 4A *(C4A). C4A* is elevated in the CSF and brain tissue of patients with schizophrenia and is associated with increased synaptic pruning [69, 70], while *AZIN2* has been found in high levels in the brains of Alzheimer's patients [71]. Dex Low and Beta-Acetate group animals both had significant upregulation of Dopamine Receptor D5 (*DRD5*), which has also been described in association with Attention-Deficit/Hyperactivity Disorder (ADHD) [72] and common downregulation of Synaptic Vesicle Glycoprotein 2B (*SVB2*) which is known to be downregulated in the brains of patients with temporal lobe epilepsy (TLE) [73]. In particular, *SVB2* has been shown to be downregulated in the brains of patients with pharmaco-resistant TLE which paralleled increased synaptic loss [74]. Finally, although overall of less concern from a developmental perspective,

animals in the Beta Acetate group had unique downregulation of Gliomedin (*GLDN*) which is an important factor in the development of nodes of Ranvier in the peripheral nervous system [75].

Taken together, these data reveal the impact of ACS treatment on the hippocampal transcriptome, with a material number of differentially regulated genes that are associated with neurodegenerative disorders increasingly identified in large, population-level cohort studies [14–16]. Further research is needed to establish a causal relationship of these findings with the epidemiological studies that have shown the development of learning difficulties and neurobehavioral disorders in individuals exposed to ACS [14, 15]. Data from our study and others certainly suggest potential connections which warrant further exploration in long-term clinical trial follow up studies.

Research implications

Understanding the molecular pharmaco-molecular relationships between ACS exposure and brain injury is important to the optimisation of ACS intervention. In addition to differential gene expression in the fetal hippocampus, all steroid exposed groups in the present study had downregulation of the KEGG pathways "Gap junction," "Focal adhesion," and "ECM -receptor activation" which are known to be involved in normal neuron migration, gene expression, cell proliferation, differentiation, and survival [76–80]. Further analysis of these negatively enriched pathways revealed common downstream signaling processes including PI3K-AKT, Ras-MAPK-ERK, JAK-STAT, and NFκB signaling. PI3K-AKT signaling is known to be integral for normal brain development, sympathetic neuron growth, and survival [81] with clinical studies demonstrating that *AKT* gene deletion syndromes exhibit microcephaly and agenesis of the corpus callosum phenotypes [82]. Furthermore, Ras-MAPK-ERK signaling is an established master regulator of neurodevelopment, important for normal neural tissue proliferation and differentiation. Specifically, ERK signaling is an integral component of learning, memory, and behavior [83], with animal studies demonstrating that inhibition and downregulation of ERK signaling can result in repressed long-term potentiation, memory consolidation, depression-like behavior, and impaired fear memory consolidation [83, 84]. Additionally, JAK/STAT signaling is important in CNS synaptic plasticity, memory, and learning [85], with downregulation in the JAK/STAT pathways (in particular JAK2/STAT3) being implicated in age-related memory decline and Alzheimer's disease [86]. Finally, NFκB signaling is also important to normal neurodevelopment, with deficits in neural plasticity and memory formation a consequence of negatively

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enriched NFκB functioning [87]. These relationships need further clarification.

In summary, our data demonstrate that all ACS exposed groups, including those with substantial dose reductions, had significant downregulation within the preterm hippocampus of crucial signaling pathways (PI3K-AKT, Ras-MAPK-ERK, JAK-STAT, and NFκB) for normal neurogenesis and development. These findings may also help explain a causal link between ACS exposure and associated increased risks of learning deficits, mental, and behavioral disorders seen in population cohort studies of ACS exposed children [14–16].

Limitations of this study

One key limitation for clinical application at this point is our inability to differentiate between potential differences in effect between betamethasone and dexamethasone. Due to agent availability and the clinical regimen of interest, we assessed rapid-release dexamethasone phosphate (pulsatile exposures at high and low doses) against betamethasone acetate which has achieved a slow-release, constant exposure profile. Although both drugs act via the glucocorticoid receptor, and the projected C_{max} for the Beta Acetate and Dex Low Groups were approximately equal, the differences in transcriptional effects seen in this study may stem from differences in the activation profile of each drug. This is not a well-studied area of glucocorticoid pharmacogenomics, and aside from differences in receptor affinity, betamethasone and dexamethasone are viewed as being interchangeable from the perspective of signalling action. Whether or not dexamethasone and betamethasone elicit markedly different transcriptional responses at identical drug exposures is an intriguing question. However, as dexamethasone is the front-line ACS agent for much of the world, our data showing that dexamethasone exposure was associated with an increased risk of adverse transcriptional changes in the fetal brain, may have serious implications for how this drug is used in obstetric practice, as well as the supply of the less readily available betamethasone-based preparations. It is also important to note that our studies relate to the acute transcriptomic effects of ACS on the brain and lung. Although we have identified injury-associated changes in the hippocampus independent of respiratory function, it is not clear if these alterations are maintained into childhood or contribute to a neurological phenotype consistent with that reported in the epidemiological and trials literature. It is also important to note that all animals were delivered at a standardized gestational age, following a fixed exposure interval. As lung and brain responses to glucocorticoid exposure are dynamic processes, it is important to note that the different findings may be observed following delivery at different gestations or treatment to delivery intervals. We also acknowledge that we did not do further analysis on fetal cardiac function beyond heart rate and blood pressure (Additional File 1, Additional Figure 4); therefore, we cannot exclude that undetected adverse cardiovascular instability due to ANS exposure may have impacted other organ systems as well as respiratory and neurological development.

Conclusions

In summary, our findings show that, in the preterm fetal sheep, a therapeutically beneficial (for lung maturation) dose of glucocorticoids delivered via the maternal compartment cannot be accomplished without materially disrupting key homeostatic systems in both mother and fetus.

In this study, we show for the first time that a high-dose regimen currently in clinical use in Singapore, fails to achieve its primary goal of improving functional maturation of the preterm fetal sheep lung, but causes marked, neurodegenerative/psychiatric disease-associated transcriptional changes in the preterm hippocampus, and severely disrupts the materno-fetal HPA axis. We additionally show, also for the first time, that adverse changes in the hippocampal transcriptome were not affected by significant dose reduction in the dexamethasone ACS groups, and that these changes occurred independent of functional lung maturation status. These indicate that administering ACS at a dose necessary for functional maturation of the preterm lung may not be achievable without an elevated risk of adverse impacts on the developing brain.

Additionally, although transcriptional changes seen in the hippocampal tissue of Beta-Acetate Group animals were less extreme (to pulsatile ACS-exposures), they still represented material changes consistent with increased risk of harm. Together with additional findings of poor lung maturation in animals exposed to very brief, high amplitude, pulsatile dexamethasone treatments, these data strongly underscore the importance of judicious patient selection, appropriately treatment targeting, and the need to develop constant, extended-duration glucocorticoid regimens to maximize benefit and minimize risk of harm deriving from ACS therapy.

Both the magnitude (dose) of materno-fetal antenatal steroid exposure, and the pharmacokinetic profile of that exposure (i.e., constant vs. pulsatile) contribute quantitatively and qualitatively to transcriptomic perturbation in the preterm fetal hippocampus. Since ACS dosing worldwide is based on pulsatile dosing regimens and high-dose dexamethasone is widely utilized as the antenatal steroid of choice, our data have significant implications for on-going efforts to optimize the safety

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and efficacy of this important obstetric intervention. Just as the dosing of oxytocin for labor induction and augmentation underwent a complete rethink a few decades ago with dramatic reduction in dosage, we these data strongly suggest a need to conceptualize a similar process for antenatal steroids.

Abbreviations

ACS	Antenatal corticosteroids
DEG	Differentially expressed genes
DEP	Differentially expressed pathways
Dex P	Dexamethasone phosphate
dGA	Days gestations age
FDR	False Discovery Rate
GSEA	Gene set enrichment analysis
HPA	Hypothalamic pituitary adrenal
IM	Intramuscular
LC-MS	Liquid chromatography-mass spectrometry
PEEP	Positive end expiratory pressure
PIP	Peak inspiratory pressure
PTB	Preterm birth
RR	Respiratory rate
VEI	Ventilation Efficiency Index
V_T	Tidal volume

Supplementary Information

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Additional File 1. (Additional Tables 1-2, Additional Figures 1-6): Maternal and neonatal plasma steroid concentrations, Neonatal delivery data, Neonatal respiratory physiological data at 10, 20 and 30 minutes of ventilation,Neonatal ventilation data lung maturation analysis, Fetal Hippocampal transcriptome changes after antenatal steroid exposure, Maternal and fetal plasma endocrine assessments, Maternal and fetal immune modulation, Fetal Liver Function. Additional Table 1: Summary of delivery data. Additional Table 2: Pharmacokinetic data for Betamethasone Acetate following single 0.125mg/ kg dose. Additional Figure 1: Preterm neonatal respiratory physiological data at 10, 20 and 30 minutes of ventilation. Additional Figure 2: Preterm neonatal ventilation data lung maturation analysis. Additional Figure 3: Hippocampal bulk RNA sequencing data for differentially expressed genes common to all steroid treated groups. Additional Figure 4: Preterm neonatal cardiovascular data at 10, 20, 30 minutes of ventilation. Additional Figure 5: Maternal and fetal plasma endocrine data. Additional Figure 6: Plasma Immune and liver function data.

Authors' contributions

Study design and conceptualisation: SC, MK Animal studies SC, HU, EF, YT, TT, YK, HI, YS, ZA, BA, MS, MK Laboratory studies SC, MK, QW, XL, Data analysis SC, MK, AR, GO, AHJ Manuscript preparation SC Manuscript review SC, SI, MK, ME, AR, CM, MC, AHJ Manuscript approval SC, MK, HU, EF, YT, TT, CM, BA, ZA, GO, AR, QW, XL, YK, YS, HI, MS, MC, SI, AHJ, ME. All authors read and approved the final manuscript.

Availability of data and materials

The raw data and processed data for RNA-sequencing experiments discussed in this publication have been deposited in the NCBI's Gene Expression Omnibus [88] and are publicly available as of the date of publication through the GEO Series accession number GSE248786 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE248786). All supplementary GSEA tables, physiological and haematological data presented in figures and text within this manuscript is available for review in the Supplementary GSEA Tables and Supporting Data Values Files at https://doi.org/https://doi.org/10.6084/m9.figshare.25532740.v1.

Declarations

Ethics approval and consent to participate
Our studies were approved by The University of Western Australia's Animal Ethics Committee (2021/ET000974) and adhered to the NIH Guide for the Care and Use of Laboratory Animals.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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References
1. Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, et al. Born too soon: the global epidemiology of 15 million preterm births. Reprod Health. 2013;10 Supp 1(Suppl 1):S2.
2. McGoldrick E, Stewart F, Parker R, Dalziel SR. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database Syst Rev. 2020;12:CD004454.
3. Roberts D, Dalziel S. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database Syst Rev. 2007;4:1–144.
4. Roberts D, Brown J, Medley N, Dalziel SR. Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. Cochrane Database Syst Rev. 2017;3:1–191.
5. Norman J, Shennan A, Jacobsson B, Stock SJ, Birth FWGfP. FIGO good practice recommendations on the use of prenatal corticosteroids to improve outcomes and minimize harm in babies born preterm. Int J Gynaecol Obstet. 2021;155(1):26–30.
6. Practice CoO. Committee Opinion No. 713: antenatal corticosteroid therapy for fetal maturation. Obstet Gynecol. 2017;130(2):e102-e9.
7. Panel ACCPG. Antenatal corticosteroids given to women prior to birth to improve fetal, infant, child and adult health: Clinical Practice Guidelines. 2015.
8. Gosavi A, Amin Z, Carter SWD, Choolani MA, Fee EL, Milad MA, et al. Antenatal corticosteroids in Singapore: A clinical and scientific assessment. Singapore Med J. 2023(0):0
9. Kemp MW, Newnham JP, Challis J, Jobe AH, Stock S. The clinical use of corticosteroids in pregnancy. Hum Reprod Update. 2016;22(2):240–59.

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10. Jobe AH, Milad MA, Peppard T, Jusko WJ. Pharmacokinetics and pharmacodynamics of intramuscular and oral betamethasone and dexamethasone in reproductive age women in India. Clin Transl Sci. 2020;13(2):391–9.

11. Jobe AH, Kemp M, Schmidt A, Takahashi T, Newnham J, Milad M. Antenatal corticosteroids: a reappraisal of the drug formulation and dose. Pediatr Res. 2021;89(2):318–25.

12. Takahashi T, Jobe AH, Fee EL, Newnham JP, Schmidt AF, Usuda H, et al. The Complex Challenge of Antenatal Steroid Therapy Non-Responsiveness. Am J Obstetr Gynecol. 2022;227(5):96–704.

13. Rodriguez A, Wang Y, Ali Khan A, Cartwright R, Gissler M, Järvelin M-R. Antenatal corticosteroid therapy (ACT) and size at birth: A population-based analysis using the Finnish Medical Birth Register. PLoS Med. 2019;16(2): e1002746.

14. Räikkönen K, Gissler M, Kajantie E. Associations between maternal antenatal corticosteroid treatment and mental and behavioral disorders in children. JAMA. 2020;323(19):1924–33.

15. Lin Y-H, Lin C-H, Lin M-C, Hsu Y-C, Hsu C-T. Antenatal Corticosteroid Exposure Is Associated with Childhood Mental Disorders in Late Preterm and Term Infants. J Pediatr. 2022;253:245–51.

16. Stutchfield PR, Whitaker R, Gliddon AE, Hobson L, Kotecha S, Doull IJ. Behavioural, educational and respiratory outcomes of antenatal betamethasone for term caesarean section (ASTECS trial). Arch Dis Child Fetal Neonatal Ed. 2013;98(3):F195–200.

17. Gyamfi-Bannerman C, Thom EA. Antenatal Betamethasone for Women at Risk for Late Preterm Delivery. N Engl J Med. 2016;375(5):486–7.

18. Niwa F, Kawai M, Kanazawa H, Iwanaga K, Matsukura T, Shibata M, et al. Limited response to CRH stimulation tests at 2 weeks of age in preterm infants born at less than 30 weeks of gestational age. Clin Endocrinol. 2013;78(5):724–9.

19. Waffarn F, Davis EP. Effects of antenatal corticosteroids on the hypothalamic-pituitary-adrenocortical axis of the fetus and newborn: experimental findings and clinical considerations. Am J Obstet Gynecol. 2012;207(6):446–54.

20. Schäffer L, Luzi F, Burkhardt T, Rauh M, Beinder E. Antenatal betamethasone administration alters stress physiology in healthy neonates. Obstet Gynecol. 2009;113(5):1082–8.

21. Tegethoff M, Pryce C, Meinlschmidt G. Effects of intrauterine exposure to synthetic glucocorticoids on fetal, newborn, and infant hypothalamic-pituitary-adrenal axis function in humans: a systematic review. Endocr Rev. 2009;30(7):753–89.

22. Weiss SJ, Keeton V, Richoux S, Cooper B, Niemann S. Exposure to antenatal corticosteroids and infant cortisol regulation. Psychoneuroendocrinology. 2023;147: 105960.

23. Davis E, Waffarn F, Uy C, Hobel C, Glynn L, Sandman C. Effect of prenatal glucocorticoid treatment on size at birth among infants born at term gestation. J Perinatol. 2009;29(11):731–7.

24. Asztalos E, Willan A, Murphy K, Matthews S, Ohlsson A, Saigal S, et al. Association between gestational age at birth, antenatal corticosteroids, and outcomes at 5 years: multiple courses of antenatal corticosteroids for preterm birth study at 5 years of age (MACS-5). BMC Pregnancy Childbirth. 2014;14(1):272.

25. Uno R, Lohmiller L, Thieme C, Kemnitz JW, Engle MJ, Roecker EB, et al. Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques I Hippocampus. Dev Brain Res. 1990;53(2):157–67.

26. Velísek L. Prenatal corticosteroid impact on hippocampus: implications for postnatal outcomes. Epilepsy Behav. 2005;7(1):57–67.

27. Whitelaw A, Thoresen M. Antenatal steroids and the developing brain. Arch Dis Child Fetal Neonatal Ed. 2000;83(2):F154–7.

28. Tijsseling D, Wijnberger LDE, Derks JB, van Velthoven CTJ, de Vries WB, van Bel F, et al. Effects of antenatal glucocorticoid therapy on hippocampal histology of preterm infants. PLoS ONE. 2012;7(3):e33369.

29. Davis EP, Sandman CA, Buss C, Wing DA, Head K. Fetal glucocorticoid exposure is associated with preadolescent brain development. Biol Psychiat. 2013;74(9):647–55.

30. Huang W, Beazley L, Quinlivan J, Evans S, Newnham J, Dunlop S. Effect of corticosteroids on brain growth in fetal sheep. Obstet Gynecol. 1999;94(2):213–8.

31. Dunlop SA, Archer MA, Quinlivan JA, Beazley LD, Newnham JP. Repeated prenatal corticosteroids delay myelination in the ovine central nervous system. J Matern Fetal Med. 1997;6(6):309–13.

32. Schmidt AF, Kannan PS, Bridges JP, Filuta A, Lipps D, Kemp M, et al. Dosing and formulation of antenatal corticosteroids for fetal lung maturation and gene expression in rhesus macaques. Sci Rep. 2019;9(1):1–10.

33. Schmidt AF, Schnell DJ, Eaton KP, Chetal K, Kannan PS, Miller LA, et al. Fetal maturation revealed by amniotic fluid cell-free transcriptome in rhesus macaques. JCI Insight. 2022;7(18):1–19.

34. Usuda H, Fee EL, Carter S, Furfaro L, Takahashi T, Takahashi Y, et al. Low-dose antenatal betamethasone treatment achieves preterm lung maturation equivalent to that of the World Health Organization dexamethasone regimen but with reduced endocrine disruption in a sheep model of pregnancy. Am J Obstetr Gynecol. 2022;227(6):903–e1.

35. Organization WH. ACTION III: A multi-country, multi-centre, three-arm, parallel group, double-blind, placebo-controlled, randomized trial of two doses of antenatal corticosteroids for women with a high probability of birth in the late preterm period in hospitals in low-resource countries to improve newborn outcomes: WHO; 2021 [Available from: https://cdn. who.int/media/docs/default-source/mca-documents/nbh/action-iii---a-multi-country-multi-centre-three-arm-parallel-group-double-blind-place bo-controlled-randomized-trial-of-two-doses-of-antenatal-corticoste riods.pdf?sfvrsn=c255b253_1&download=true.

36. Takahashi T, Fee EL, Takahashi Y, Saito M, Yaegashi N, Usuda H, et al. Betamethasone phosphate reduces the efficacy of antenatal steroid therapy and is associated with lower birthweights when administered to pregnant sheep in combination with betamethasone acetate. Am J Obstetr Gynecol. 2022;226(4):5641.e1-e14.

37. Kemp MW, Saito M, Usuda H, Molloy TJ, Miura Y, Sato S, et al. Maternofetal pharmacokinetics and fetal lung responses in chronically catheterized sheep receiving constant, low-dose infusions of betamethasone phosphate. Am J Obstetr Gynecol. 2016;215(6):775 (e1- e12).

38. Pillow JJ, Musk GC, McLean CM, Polglase GR, Dalton RGB, Jobe AH, et al. Variable ventilation improves ventilation and lung compliance in preterm lambs. Intensive Care Med. 2011;37(8):1352–9.

39. Ewels PA, Peltzer A, Fillinger S, Patel H, Alneberg J, Wilm A, et al. The nf-core framework for community-curated bioinformatics pipelines. Nat Biotechnol. 2020;38(3):276–8.

40. Di Tommaso P, Chatzou M, Floden EW, Barja PP, Palumbo E, Notredame C. Nextflow enables reproducible computational workflows. Nat Biotechnol. 2017;35(4):316–9.

41. Krueger F. Trim Galore!: A wrapper around Cutadapt and FastQC to consistently apply adapter and quality trimming to FastQ files, with extra functionality for RRBS data. Babraham Institute. 2015.

42. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29(1):15–21.

43. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 2017;14(4):417–9.

44. R Core Team R. R: A language and environment for statistical computing. 2013.

45. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):1–21.

46. Blighe K, Rana S, Lewis M. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. R package version. 2019;1:10–18129.

47. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 2016;32(18):2847–9.

48. Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. Innovation. 2021;2(3):100141.

49. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 2000;28(1):27–30.

50. Schmidt AF, Kemp MW, Rittenschober-Böhm J, Kannan PS, Usuda H, Saito M, et al. Low-dose betamethasone-acetate for fetal lung maturation in preterm sheep. Am J Obstetr Gynecol. 2018;218(1):132 (e1-e9).

51. Usuda H, Fee EL, Carter S, Furfaro L, Takahashi T, Takahashi Y, et al. Low-dose antenatal betamethasone treatment achieves preterm lung maturation equivalent to that of the World Health Organization dexamethasone regimen but with reduced endocrine disruption in a sheep model of pregnancy. Am J Obstetr Gynecol. 2022;227(6):903 (e1-e16).

52. Takahashi T, Saito M, Schmidt AF, Usuda H, Takahashi Y, Watanabe S, et al. Variability in the efficacy of a standardized antenatal steroid treatment was independent of maternal or fetal plasma drug levels: evidence from

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a sheep model of pregnancy. Am J Obstetr Gynecol. 2020;223(6):921 (e1-e10).

53. Takahashi T, Takahashi Y, Fee EL, Saito M, Yaegashi N, Usuda H, et al. Continuous but not pulsed low-dose fetal betamethasone exposures extend the durability of antenatal steroid therapy. Am J Physiol Lung Cell Mole Physiol. 2022;322(6):L784–93.

54. Kamath-Rayne BD, Du Y, Hughes M, Wagner EA, Muglia LJ, DeFranco EA, et al. Systems biology evaluation of cell-free amniotic fluid transcriptome of term and preterm infants to detect fetal maturity. BMC Med Genomics. 2015;8:67.

55. Fee EL, Takahashi T, Takahashi Y, Carter S, Furfaro L, Clarke MW, et al. One percent of the clinical dose used for antenatal steroid therapy is sufficient to induce lung maturation when administered directly to the preterm ovine fetus. Am J Physiol Lung Cell Mole Physiol. 2022;322(6):L853–65.

56. Fee EL, Takahashi T, Takahashi Y, Carter SW, Clarke MW, Milad MA, et al. Respiratory benefit in preterm lambs is progressively lost when the concentration of fetal plasma betamethasone is titrated below two nanograms per milliliter. Am J Physiol Lung Cell Mole Physiol. 2023;325(5):L628–37.

57. Polin RA, Carlo WA, Fetus Co, Newborn, Papile L-A, Polin RA, et al. Surfactant replacement therapy for preterm and term neonates with respiratory distress. Pediatrics. 2014;133(1):156–63.

58. Gilstrap LC, Christensen R, Clewell WH, D'Alton ME, Davidson EC, Escobedo MB, et al. Effect of corticosteroids for fetal maturation on perinatal outcomes: NIH consensus development panel on the effect of corticosteroids for fetal maturation on perinatal outcomes. JAMA. 1995;273(5):413–8.

59. Evans MI, Britt DW. Resistance to change. Reprod Sci. 2023;30(3):835–53.

60. Willet KE, McMenamin P, Pinkerton KE, Ikegami M, Jobe AH, Gurrin L, et al. Lung morphometry and collagen and elastin content: changes during normal development and after prenatal hormone exposure in sheep. Pediatr Res. 1999;45(5):615–25.

61. Schmidt AF, Kannan PS, Bridges J, Presicce P, Jackson CM, Miller LA, et al. Prenatal inflammation enhances antenatal corticosteroid–induced fetal lung maturation. JCI insight. 2020;5(24):e139452.

62. Merali Z, Kent P, Anisman H. Role of bombesin-related peptides in the mediation or integration of the stress response. Cell Mole Life Sci. 2002;59:272–87.

63. Luan Y, Zhang H, Ma K, Liu Y, Lu H, Chen X, et al. CCN3/NOV Regulates Proliferation and Neuronal Differentiation in Mouse Hippocampal Neural Stem Cells via the Activation of the Notch/PTEN/AKT Pathway. Int J Mol Sci. 2023;24(12):10324.

64. Terrisse L, Poirier J, Bertrand P, Merched A, Visvikis S, Siest G, et al. Increased levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients. J Neurochem. 1998;71(4):1643–50.

65. Decker JM, Wójtowicz AM, Bartsch JC, Liotta A, Braunewell KH, Heinemann U, et al. C-type natriuretic peptide modulates bidirectional plasticity in hippocampal area CA1 in vitro. Neuroscience. 2010;169(1):8–22.

66. Abudureyimu S, Asai N, Enomoto A, Weng L, Kobayashi H, Wang X, et al. Essential Role of Linx/Islr2 in the Development of the Forebrain Anterior Commissure. Sci Rep. 2018;8(1):7292.

67. Alazami AM, Maddirevula S, Seidahmed MZ, Albhlal LA, Alkuraya FS. A novel ISLR2-linked autosomal recessive syndrome of congenital hydrocephalus, arthrogryposis and abdominal distension. Hum Genet. 2019;138(1):105–7.

68. Eastwood SL, Harrison PJ. Decreased mRNA Expression of Netrin-G1 and Netrin-G2 in the Temporal Lobe in Schizophrenia and Bipolar Disorder. Neuropsychopharmacology. 2008;33(4):933–45.

69. Gracias J, Orhan F, Hörbeck E, Holmén-Larsson J, Khanlarkani N, Malwade S, et al. Cerebrospinal fluid concentration of complement component 4A is increased in first episode schizophrenia. Nat Commun. 2022;13(1):6427.

70. Yilmaz M, Yalcin E, Presumey J, Aw E, Ma M, Whelan CW, et al. Overexpression of schizophrenia susceptibility factor human complement C4A promotes excessive synaptic loss and behavioral changes in mice. Nat Neurosci. 2021;24(2):214–24.

71. Mäkitie LT, Kanerva K, Polvikoski T, Paetau A, Andersson LC. Brain neurons express ornithine decarboxylase-activating antizyme inhibitor 2 with accumulation in Alzheimer's disease. Brain Pathol. 2010;20(3):571–80.

72. Li D, Sham PC, Owen MJ, He L. Meta-analysis shows significant association between dopamine system genes and attention deficit hyperactivity disorder (ADHD). Hum Mol Genet. 2006;15(14):2276–84.

73. Van Vliet EA, Aronica E, Redeker S, Boer K, Gorter JA. Decreased expression of synaptic vesicle protein 2A, the binding site for levetiracetam, during epileptogenesis and chronic epilepsy. Epilepsia. 2009;50(3):422–33.

74. Crèvecœur J, Kaminski RM, Rogister B, Foerch P, Vandenplas C, Neveux M, et al. Expression pattern of synaptic vesicle protein 2 (SV2) isoforms in patients with temporal lobe epilepsy and hippocampal sclerosis. Neuropathol Appl Neurobiol. 2014;40(2):191–204.

75. Eshed Y, Feinberg K, Poliak S, Sabanay H, Sarig-Nadir O, Spiegel I, et al. Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier. Neuron. 2005;47(2):215–29.

76. Berman A, Kozlova N, Morozevich G. Integrins: structure and signaling. Biochem Mosc. 2003;68:1284–99.

77. van der Flier A, Sonnenberg A. Function and interactions of integrins. Cell Tissue Res. 2001;305(3):285–98.

78. Milner R, Campbell IL. The integrin family of cell adhesion molecules has multiple functions within the CNS. J Neurosci Res. 2002;69(3):286–91.

79. Benson DL, Schnapp LM, Shapiro L, Huntley GW. Making memories stick: cell-adhesion molecules in synaptic plasticity. Trends Cell Biol. 2000;10(11):473–82.

80. Roerig B, Feller MB. Neurotransmitters and gap junctions in developing neural circuits. Brain Res Rev. 2000;32(1):86–114.

81. Virdee K, Xue L, Hemmings BA, Goemans C, Heumann R, Tolkovsky AM. Nerve growth factor-induced PKB/Akt activity is sustained by phosphoinositide 3-kinase dependent and independent signals in sympathetic neurons. Brain Res. 1999;837(1–2):127–42.

82. Wang L, Zhou K, Fu Z, Yu D, Huang H, Zang X, et al. Brain development and Akt signaling: the crossroads of signaling pathway and neurodevelopmental diseases. J Mol Neurosci. 2017;61(3):379–84.

83. Iroegbu JD, Ijomone OK, Femi-Akinlosotu OM, Ijomone OM. ERK/MAPK signalling in the developing brain: Perturbations and consequences. Neurosci Biobehav Rev. 2021;131:792–805.

84. Giese KP, Friedman E, Telliez J-B, Fedorov NB, Wines M, Feig LA, et al. Hippocampus-dependent learning and memory is impaired in mice lacking the Ras-guanine-nucleotide releasing factor 1 (Ras-GRF1). Neuropharmacology. 2001;41(6):791–800.

85. Nicolas CS, Peineau S, Amici M, Csaba Z, Fafouri A, Javalet C, et al. The Jak/STAT pathway is involved in synaptic plasticity. Neuron. 2012;73(2):374–90.

86. Chiba T, Yamada M, Aiso S. Targeting the JAK2/STAT3 axis in Alzheimer's disease. Expert Opin Ther Targets. 2009;13(10):1155–67.

87. Dresselhaus EC, Meffert MK. Cellular Specificity of NF-κB Function in the Nervous System. Front Immunol. 2019;10:1043.

88. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002;30(1):207–10.

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