Journal of Developmental Origins of Health and Disease (2015), **6**(4), 350–361.

doi:10.1017/S2040174415001075
Themed Issue: Preterm Birth

ORIGINAL ARTICLE

Effect of postnatal progesterone therapy following preterm birth on neurosteroid concentrations and cerebellar myelination in guinea pigs

H. K. Palliser[1,2]*, M. A. Kelleher[3], M. Tolcos[4], D. W. Walker[4] and J. J. Hirst[1,2]

[1]*Mothers and Babies Research Centre, Hunter Medical Research Institute, Newcastle, NSW, Australia*
[2]*School of Biomedical Science, University of Newcastle, NSW, Australia*
[3]*Oregon National Primate Research Centre, Oregon, USA*
[4]*Department of Obstetrics and Gynaecology, The Ritchie Centre, MIMR-PHI Institute of Medical Research, Monash University, Clayton, Victoria, Australia*

Allopregnanolone protects the fetal brain and promotes normal development including myelination. Preterm birth results in the early separation of the infant from the placenta and consequently a decline in blood and brain allopregnanolone concentrations. Progesterone therapy may increase allopregnanolone and lead to improved oligodendrocyte maturation. The objectives of this study were to examine the efficacy of progesterone replacement in augmenting allopregnanolone concentrations during the postnatal period and to assess the effect on cerebellar myelination – a region with significant postnatal development. Preterm guinea pig neonates delivered at 62 days of gestation by caesarean section received daily s.c. injections of vehicle (2-Hydroxypropyl-β-cyclodextrin) or progesterone (16 mg/kg) for 8 days until term-equivalent age (TEA). Term delivered controls (PND1) received vehicle. Neonatal condition/wellbeing was scored, and salivary progesterone was sampled over the postnatal period. Brain and plasma allopregnanolone concentrations were measured by radioimmunoassay; cortisol and progesterone concentrations were determined by enzyme immunoassay; and myelin basic protein (MBP), proteolipid protein (PLP), oligodendroctye transcription factor 2 (OLIG2) and platelet-derived growth factor receptor-α (PDGFRα) were quantified by immunohistochemistry and western blot. Brain allopregnanolone concentrations were increased in progesterone-treated neonates. Plasma progesterone and cortisol concentrations were elevated in progesterone-treated male neonates. Progesterone treatment decreased MBP and PLP in lobule X of the cerebellum and total cerebellar OLIG2 and PDGFRα in males but not females at TEA compared with term animals. We conclude that progesterone treatment increases brain allopregnanolone concentrations, but also increases cortisol levels in males, which may disrupt developmental processes. Consideration should be given to the use of non-metabolizable neurosteroid agonists.

Received 28 October 2014; Revised 12 March 2015; Accepted 17 March 2015; First published online 24 April 2015

Key words: allopregnanolone, cortisol, neonate, oligodendrocyte, premature

Introduction

A recent systematic review of the literature identified that at 6 months of age, up to 30% of all preterm infants have some degree of neurodevelopmental impairment.[1] The severity and incidence of neurodevelopmental disorders increases with decreasing gestational age at birth.[2] However, even late preterm infants, born between 34 and 36 weeks gestation, are over three times more likely to develop cerebral palsy and other neurodevelopmental deficits.[3] The increased incidence of neurodevelopmental disorders that become evident in childhood such as behavioral, attention and learning deficits underscore the impact of preterm birth even at these later stages.[4] Structural and functional deficits have been observed in preterm infants after reaching term-equivalent age (TEA) when compared with infants delivered at term.[5,6]

*Address for correspondence: H. Palliser, Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, Callaghan, NSW 2308, Australia.
(Email Hannah.palliser@newcastle.edu.au)

With the advent of functional magnetic resonance imaging, the cerebellum has emerged as a region important for cognitive and complex behavior development, in addition to its accepted role in motor co-ordination.[7,8] The cerebellum undergoes major growth and maturation during mid to late gestation and throughout the 1st year of life.[9,10] Perinatal insults that occur during the period of rapid cerebellar growth may result in marked disruption of development, such as reductions in cerebellar hemispheric volumes, loss of white matter and changes to cerebellar morphology including external granular layer thickness.[11–13] Cerebellar injury following preterm birth is increasingly being recognized as a potential cause of a number of long-term neurodevelopmental deficits.[14]

Despite the advance in diagnostic techniques and therapeutic interventions that have improved the survival of even extremely preterm infants, the development of effective therapies to treat preterm brain injury and improve developmental outcomes remains limited. Progesterone, largely responsible for maintaining uterine quiescence during pregnancy, is produced in human pregnancy by the placenta in increased amounts with

advancing gestation, with plasma concentrations reaching over 600 nmol/l,[15–17] and with both progesterone and its direct metabolites reaching both the maternal and fetal circulations. One of the most important of these metabolites is allopregnanolone, a neuroactive pregnane steroid, which is a potent positive allosteric modulator of γ-aminobutyric acid type A ($GABA_A$) receptor function. Allopregnanolone, therefore, has the effect of increasing GABAergic inhibition, decreasing central nervous system excitability, producing anxiolytic, sedative and anticonvulsant effects, which in the context of hypoxia–ischemia are neuroprotective effects.[18] As a result of placental steroidogenesis, maternal plasma concentrations of allopregnanolone increase nine-fold between 10 and 36 weeks gestation, and concentrations of >40 nmol/l have been reported, but they fall abruptly to <2 nmol/l at delivery,[17] indicating the critical importance of the placental supply of progesterone metabolites during pregnancy itself.

The fetal and neonatal brain express the enzymes and have the capacity for the synthesis of allopregnanolone from progesterone.[19–21] 5α-reductases, the rate-limiting enzymes in the conversion of progesterone to allopregnanolone, are strongly expressed in Purkinje and other cerebellar cells, and allopregnanolone levels are high in the fetal cerebellum during late gestation.[22–25] This '*in situ*' production of allopregnanolone during fetal life may also depend on placentally derived precursors, and therefore levels of this important neuroactive steroid in this (and other) brain region are likely to fall following preterm birth. Progesterone has positive effects on myelination and oligodendrocyte development,[26] and is known to promote dendritic growth of Purkinje cells in neonatal rat slice cultures.[27] *In vivo* studies have demonstrated that progesterone administered into the reticulospinal fluid around the posterior vermal lobule of the cerebellum of 3- to 7-day-old rat pups increased dendrite area, differentiation, synapse density and the size (perimeter) of Purkinje cells.[27] These effects provide evidence for the growth-promoting action of allopregnanolone and progesterone within the fetal brain, and suggest that neuroactive steroids may provide important regulatory, neuroprotective and developmental signals in the immature brain. Therefore, experimental reduction of allopregnanolone concentrations is associated with increased cell death in the fetal sheep cerebellum.[28]

Thus, the loss of the important neurotrophic effects of progesterone and allopregnanolone with preterm birth may contribute to the poor developmental outcomes associated with the early loss of the placenta. Specifically, we hypothesize that the premature loss of progesterone and allopregnanolone contributes to the disruption of cerebellar maturation, in particular maturation of cerebellar white matter. The objective of this study was to assess whether cerebellar white matter maturity is altered in the neonatal guinea pig after preterm delivery, and subsequently to determine whether progesterone replacement in these preterm neonates increases allopregnanolone concentrations in the brain and improves cerebellar development.

Method

Animals

All animal procedures were carried out with approval from the University of Newcastle Animal Care and Ethics Committee according to guidelines from the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Pregnant, outbred tricolor guinea pigs were supplied by the University of Newcastle Research Support Unit and were housed indoors with a 12-h light/dark cycle, with commercial guinea pig pellets and water (fortified with ascorbic acid) provided *ad libitum*.

Neonates were delivered by caesarean section at 62 (preterm) or 69 (term) days of gestation and maintained until TEA (70 days of gestation –i.e, for 8 days or 1 day, respectively). C-section delivery was performed following maternal anesthesia with 1–3% isoflurane and 24 h after maternal administration of 1 mg/kg (s.c) betamethasone sodium phosphate/betamethasone acetate. The uterus was exposed via a midline abdominal incision, fetuses were removed from the fetal membranes and the umbilical cord was tied and cut. The maternal guinea pig was then euthanized using intracardiac sodium pentobarbital (200 mg/kg). Neonatal guinea pigs received surfactant treatment (50 µl Curosurf, 80 mg/ml Poractant Alfa, Douglas Pharmaceuticals) and continuous positive airway pressure (CPAP) ventilation (7 mmH_2O positive end-expiratory pressure and 20 mmH_2O peak inspiratory pressure) via a Neopuff Infant T-Piece Resuscitator (Fisher & Paykel Healthcare, Australia) fitted with a modified small animal mask. Once stabilized, neonates were transferred to a small animal incubator and fed at 2-h intervals using commercial milk formula (Wombaroo Food Products, Adelaide, Australia) and then with mixed pellet food after postnatal day 4–5. Saliva was collected from the neonates before each feed at 8 am, 4 pm and midnight, by allowing them to chew on a cotton bud for 3 min to collect passive salivary secretions. At these times, pups were also monitored for wellbeing as previously described.[19] In brief, this involved assessment and scoring of respiration, posture and alertness out of a maximum of 12 points, with low scores indicating poor wellbeing.

Preterm neonates were randomly assigned to progesterone treatment (16 mg/kg in vehicle) or vehicle [control, 22.5% w/v (2-Hydroxypropyl)-β-cyclodextrin]. Neonates received an initial i.p. injection at 1 h after delivery, followed by a second s.c injection at 6 h and daily s.c injections until TEA (8 days postnatal). Neonates were euthanized at TEA and cerebellar tissue and plasma were collected. Each cerebellum was divided in half along the sagittal plane, and one half was immersion-fixed in 4% paraformaldehyde before paraffin embedding, sectioning and immunostaining, and the other half was snap-frozen in liquid N_2 for steroid or protein extraction. To prevent pregnancy bias, one neonate of each sex per pregnancy was included in the study. Additional whole brains were frozen for steroid analyses.

Steroid analyses

Salivary progesterone concentrations were measured over the postnatal period using Salivary Progesterone enzyme

https://doi.org/10.1017/S2040174415001075 Published online by Cambridge University Press

immunoassay (EIA) kits (Salimetrics LLC, State College, PA, USA). Assays were performed according to the manufacturers' instructions, with samples diluted in assay buffer as needed. The intra-assay coefficient of variance of this assay was 16.5%.

Allopregnanolone concentrations were measured in the neonatal brain (total brain homogenate) and plasma by radioimmunoassay, as described in detail previously.[21,29] In brief, plasma and brain steroids were isolated using solid-phase extraction with graded, acidified, methanol washes. To reduce cross-reactivity, the extracts were then treated with potassium permanganate to oxidize the excess progesterone. Plasma assays were performed using a polyclonal anti-allopregnanolone antibody supplied by Dr R.H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA). All the brain assays of allopregnanolone were performed using an egg-yolk polyclonal anti-allopregnanolone antibody supplied by Agrisera (Vannas, Sweden) and raised against the same antigen (3α-hydroxy-20-oxo-5α-pregnan-11-yl carboxymethyl ether) coupled with bovine serum albumin.[30] The Agrisera antibody had previously been validated against the Purdy antibody for use following steroid extraction.[31] The average extraction recovery was 53.4 ± 1.4% for plasma samples, with an intra-assay coefficient of variance of 15.6%. Brain extractions had 60.3 ± 1.3% average recovery and an intra-assay coefficient of 8.1%. Owing to the large amount of brain tissue required to perform the steroid extraction, only the brains (one hemisphere) of five animals per group (two to three of each sex) could be quantified, and thus brain allopregnanolone data are not split by sex.

Plasma progesterone and cortisol concentrations were assayed in neonatal guinea pig plasma, collected at the termination of the experiments, by EIA carried out by Hunter Area Pathology Service (John Hunter Hospital, Newcastle, NSW, Australia) using the Beckman Coulter UniCel Dx1800 Access Immunoassay system. The intra-assay coefficients of variance were 7.9% for progesterone and 4.3% for cortisol assays.

Immunoblot analysis

Frozen cerebellum tissue was crushed and the proteins were extracted in protein extraction buffer (50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulfate with protease and phosphatase inhibitors). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using Novex 12% Bis/Tris gels (Invitrogen), followed by western transfer to polyvinylidene fluoride membranes. Expressions of oligodendrocyte transcription factor 2 (OLIG2, a marker of oligodendrocytes at all stages of their lineage)[32] and platelet-derived growth factor receptor-α (PDGFRα), a marker of oligodendrocyte progenitor cells) were determined in the cerebellar protein extract by immunodetection with polyclonal primary antibodies (OLIG2, AB9610, Millipore; PDGFRα, AF1062, R&D Systems) and detected using horseradish peroxidase (HRP)-conjugated secondary antibody (anti rabbit polyclonal, RPN1004; GE Healthcare and anti-goat polyclonal, P0449; DakoCytomation, respectively) and chemiluminescence, quantified using Multigauge v2.4 software (Fuji), normalized to β-actin and internal controls.

Immunohistochemistry

Immunohistochemistry was performed on cerebellar sections from formalin-fixed, paraffin-embedded brains. Sections (8 μm) cut using a rotary microtome were de-waxed, antigen retrieval was carried out using RevealIt Solutions (ImmunoSolution Pty Ltd) and the sections were incubated in primary antibody overnight (Calbindin, ab9481, Abcam; MBP, M9434, Sigma-Aldrich; PLP, MAB388, Millipore). Sections were subsequently incubated in biotinylated secondary antibodies, streptavidin–biotin–HRP complex (RPN1051V; Amersham), and immunostaining was detected using diaminobenzidine tetrahydrochloride chromagen. Myelin basic protein (MBP) and proteolipid protein (PLP) immunostaining (for the quantification of mature, myelin-producing oligodendrocytes and axonal myelination) were quantified in lobules VIII and X of the cerebellum; these lobules were chosen because of the time (lobule X early; lobule VIII late) of their major development and maturation during gestation.[33] The area of positive staining was analyzed using ImageJ software for three fields of view per region, on two serial sections per animal, at 40× magnification. Purkinje cells in mid-sagittal cerebellar sections were stained for calbindin for morphometric analysis and cell counting in lobules VIII and X. In captured images taken from lobules VIII and X (photomicrographs at 20× magnification taken from two serial sections), Purkinje cells were counted manually, and the length of the Purkinje cell layer was measured using ImageJ software to determine overall number and linear density (cells/mm) of cells within the Purkinje cell layer. The soma area of Purkinje cells (with a clearly defined cell body) and the cell perimeter (six cells per animal) were also calculated using ImageJ software analysis on photomicrographs at 40× magnification. Photomicrographs were coded and researchers were blinded to treatment groups until the analysis was completed.

Statistical analyses

All data are presented as the mean ± S.E.M. for each treatment group. Normal distribution was assessed by the Q-Q plot, and data were log or square root transformed if necessary. In order to identify sex differences, data were first analyzed using two-way ANOVA by neonatal sex and treatment group. *Post-hoc* Bonferroni analysis was then performed when $P < 0.05$ by two-way ANOVA. Where data were not normally distributed, non-parametric Kruskal–Wallis analysis was performed with *post-hoc* multiple comparison using Dunn's test. Salivary progesterone and neonatal scoring data were analyzed by repeated measures two-way ANOVA. Survival data were analyzed using Fisher's exact test. $P < 0.05$ was considered statistically significant and indicated on graphs by asterisks. All the analyses were performed using Prism v6.0f for Mac OSX (GraphPad Software Inc., La Jolla, CA, USA).

Results

Survival, physical characteristics and wellbeing scoring

There were 3.31 ± 0.17 pups/litter with 1.32 males for every female pup. Of all the neonates delivered preterm in this study, 56.8% of the vehicle-treated and 47.6% of the progesterone-treated neonates survived until postmortem age at PND8. Overall survival of male neonates was 45.7% (52.4%, vehicle-treated; 40%, progesterone-treated), and for female neonates it was 60.6% (62.5%, vehicle-treated; 58.8%, progesterone-treated). Of the offspring that did not survive, the period of greatest loss was the immediate neonatal period, the first 8 h after birth, due to respiratory failure. Neonates showing signs of respiratory distress during this period were administered further CPAP. There was no difference between the number of male and female pups who required further CPAP, nor in the duration of this respiratory aid. There were no significant differences in survival rates between preterm male and female neonates or between vehicle- and progesterone-treated groups. All term neonates survived the postnatal period.

The mean body weights and organ weights for term, preterm neonates at TEA and progesterone-treated neonates at TEA are presented in Table 1. The relative weight loss was similar from delivery to postmortem (% weight change) for all the groups. As expected, placental weight at birth as well as the body, brain and kidney weights at postmortem were significantly lower in preterm neonates compared with neonates delivered at term ($P < 0.05$). These differences were also present when data were analyzed by sex. When brain and kidney weights were assessed as a ratio to body weight, however, no differences were found except in the female TEA neonates who demonstrated significantly higher brain:body weight ratio compared with female neonates born at term. There were no differences in body, organ weights or ratios at TEA between the preterm groups that received the vehicle or progesterone treatment. Liver weights were not different between groups, but for progesterone-treated preterm males liver weight was lower at TEA compared with term male neonates. No differences were identified in male body:liver weight ratio; however, female TEA neonates exhibited higher body:liver weight ratio compared with term female neonates. Brain-to-liver ratio was not found to differ between any of the neonatal groups. Adrenal weight did not differ apart from progesterone-treated TEA males who had greater adrenal weight and adrenal:body ratio than the other male groups. Although vehicle-treated preterm female neonates had significantly lower birth weights compared with term females, the progesterone-treated female preterm neonates that survived the postnatal period had birth weights that did not differ from female neonates delivered at term ($P = 0.063$).

The average daily wellbeing scores (1 being very poor, 12 being excellent) are presented in Fig. 1. As expected, term neonates received higher scores than neonates delivered preterm, whose scores remained low until postnatal day 4. There were no differences in average daily scores between female vehicle- and progesterone-treated preterm neonates at any time point until TEA. Male progesterone-treated neonates demonstrated overall lower scores than male vehicle-treated neonates ($P = 0.008$). No sex differences were observed.

Salivary progesterone concentrations during the postnatal period

As expected, neonatal salivary progesterone concentrations were markedly higher in the progesterone-treated group

Table 1. *Physical characteristics of term, vehicle-treated preterm (TEA) and progesterone-treated preterm (TEA + Prog) neonates at term-equivalent age*

	Male			Female		
	Term ($n = 6$)	TEA ($n = 5$)	TEA + Prog ($n = 5$)	Term ($n = 6$)	TEA ($n = 5$)	TEA + Prog ($n = 5$)
Birth weight (g)	86.21 ± 4.17	69.95 ± 9.05*	65.76 ± 2.83*	88.76 ± 3.33	66.18 ± 4.58*	72.19 ± 2.53
PM weight (g)	80.70 ± 3.99	66.3 ± 3.32*	62.23 ± 3.81*	85.16 ± 3.50	63.28 ± 3.45*	65.31 ± 4.05*
% change body weight	− 6.73 ± 1.4	− 4.61 ±3.7	− 5.34 ± 4.3	− 4.13 ± 1.1	− 3.66 ± 4.2	− 9.67 ± 3.6
Placental weight (g)	3.85 ± 0.30	2.16 ± 0.51*	1.25 ± 0.10*	4.06 ± 0.22	1.70 ± 0.61*	1.67 ± 0.49*
Brain weight (g)	2.31 ± 0.04	2.05 ± 0.07*	2.06 ± 0.07*	2.301 ± 0.04	2.08 ± 0.06*	2.09 ± 0.05*
Brain:body weight (%)	2.92 ± 0.12	3.12 ± 0.09	3.34 ± 0.20	2.74 ± 0.11	3.33 ± 0.13*	3.24 ± 0.15*
Liver weight (g)	3.69 ± 0.26	3.00 ± 0.20	2.80 ± 0.20*	3.59 ± 0.21	3.09 ± 0.25	3.03 ± 0.19
Liver:body weight (%)	4.54 ± 0.19	4.41 ± 0.16	4.49 ± 0.08	4.20 ± 0.13	4.87 ± 0.23*	4.63 ± 0.09
Kidney weight (g)	0.903 ± 0.05	0.513 ± 0.17*	0.379 ± 0.04*	0.967 ± 0.04	0.562 ± 0.16*	0.522 ± 0.10*
Kidney:body weight (%)	1.08 ± 0.04	0.74 ± 0.24	0.60 ± 0.03	1.08 ± 0.02	0.88 ± 0.23	0.82 ± 0.18
Adrenal weight (g)	0.0311 ± 0.0034	0.0352 ± 0.0015	0.0528 ± 0.0148*	0.0379 ± 0.0016	0.0363 ± 0.0030	0.0318 ± 0.0029
Adrenal:body weight (%)	0.040 ± 0.005	0.052 ± 0.003	0.084 ± 0.022* +	0.043 ± 0.003	0.057 ± 0.003	0.048 ± 0.004
BLR	0.655 ± 0.043	0.717 ± 0.041	0.745 ± 0.046	0.659 ± 0.031	0.688 ± 0.05	0.702 ± 0.035

BLR, brain-to-liver ratio; PM, postmortem; TEA, preterm born, vehicle-treated neonates at term-equivalent age (PND8); TEA + Prog, preterm born, progesterone-treated neonates at term-equivalent age; term, term born neonates.

*$P < 0.05$ compared with term neonates within sexes.

https://doi.org/10.1017/S2040174415001075 Published online by Cambridge University Press

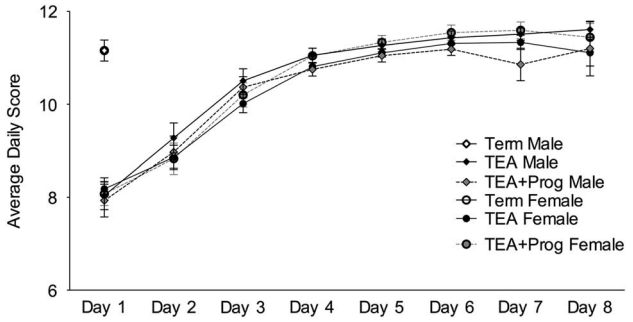

Fig. 1. Mean daily condition scores in term (open; postnatal day 1), vehicle-treated preterm [term-equivalent age (TEA), black] and progesterone-treated preterm neonates (TEA + Prog, gray) at TEA (male, diamond; female, circle symbols). Neonates were scored on a 4 hourly basis on respiration, activity and posture, with a maximum score of 12 indicating very good condition. Preterm neonatal scores were lower than term neonatal scores and increased significantly over the postnatal period ($P < 0.05$). Progesterone-treated TEA males had lower scores than vehicle-treated TEA male neonates ($P = 0.008$). Each point represents daily group mean ± S.E.M.

compared with the vehicle-treated neonates during the postnatal period (Fig. 2a). However, when pooled daily salivary progesterone concentrations were assessed, a significant difference was only found on postnatal day 3 and 6 in the male neonates and on postnatal day 1 in the female neonates. Salivary progesterone concentrations at term were lower at delivery and throughout the following 24-h period compared with both preterm groups ($P < 0.05$) in both sexes. When preterm neonatal salivary progesterone concentrations were assessed based on the time of collection (8 am, 4 pm and 12 am), male progesterone-treated neonates demonstrated higher salivary progesterone concentrations at all time points ($P < 0.01$, $P < 0.05$ and $P < 0.01$, respectively; average PND1–8 values; Fig. 2b), whereas female progesterone-treated neonates demonstrated higher salivary progesterone concentrations at 4 pm ($P < 0.05$). Interestingly, although salivary progesterone was always low for the vehicle-treated preterm group at TEA, there were time-related differences in progesterone concentrations with significantly higher concentrations present at 4 pm compared with 8 am ($P < 0.05$) in the female neonates. In addition, a reduction in progesterone concentrations was found between 4 pm and 12 am in the progesterone-treated female neonates ($P < 0.05$).

Plasma steroid concentrations

Plasma progesterone concentrations in the progesterone-treated male neonates were significantly elevated compared with term and vehicle-treated preterm neonates ($P < 0.01$ and $P < 0.001$, respectively, Fig. 3a), but no such differences were identified in female neonates, suggesting rapid breakdown of progesterone in the circulation. Plasma allopregnanolone concentrations (Fig. 3b) were not different between male term or preterm (both vehicle-treated and progesterone-treated) neonates. However, in the preterm female neonates, progesterone treatment resulted in markedly higher plasma allopregnanolone concentrations at TEA ($P < 0.01$) compared with term neonates. Circulating cortisol concentrations were found to be significantly higher in the progesterone-treated male neonates than in the vehicle-treated neonates at TEA ($P < 0.05$; Fig. 3c). There were no differences in circulating cortisol concentrations between the female neonates.

Brain allopregnanolone concentrations

Brain allopregnanolone concentrations were significantly higher in the progesterone-treated neonates compared with vehicle-treated neonates at TEA ($P < 0.05$; Fig. 4). Despite a trend for allopregnanolone concentrations to be lower in the brain of vehicle-treated preterm neonates at TEA compared with the full-term brain, this difference was not significant.

Purkinje cell morphology

There were no differences in the cell number or linear density of Purkinje cells in lobules VIII or X between any of the groups (data not shown). In lobule X only, Purkinje cell area and perimeter were significantly reduced in the preterm cerebellum of both vehicle- and progesterone-treated neonates at TEA compared with the term cerebellum ($P < 0.05$; Fig. 5).

Myelination and oligodendrocytes

MBP expression in lobule X was lower in progesterone-treated preterm male neonates compared with term male neonates ($P < 0.05$; Fig. 6a and 6c). Despite a trend for lower MBP expression in lobule VIII in vehicle- and progesterone-treated male neonates compared with term neonates, no statistical significance was identified ($P = 0.07$ and 0.07, respectively, Fig. 6b). As for MBP, PLP area coverage was lower in lobule X in progesterone-treated preterm male neonates compared with term male neonates ($P < 0.01$; Fig. 7a and 7c). PLP expression in lobule VIII of progesterone-treated preterm male neonates tended to be lower, although again no statistical significance was found ($P = 0.08$ and $P = 0.14$, respectively; Fig. 7b). No differences in MBP or PLP expressions were seen in the cerebella of female neonates from any birth or treatment group nor between the male vehicle- or progesterone-treated preterm neonates.

OLIG2 protein expression was significantly lower in the cerebellum of both vehicle- and progesterone-treated preterm male neonates compared with male term neonates (Fig. 8; $P < 0.01$). PDGFRα protein expression was significantly lower in progesterone-treated preterm male neonates compared with term male neonates (Fig. 9, $P < 0.05$). The trend for lower expression in vehicle-treated preterm male cerebella compared with term males did not reach statistical significance ($P = 0.07$). No differences in cerebellar OLIG2 or PDGFRα protein expressions were found between female neonates.

https://doi.org/10.1017/S2040174415001075 Published online by Cambridge University Press

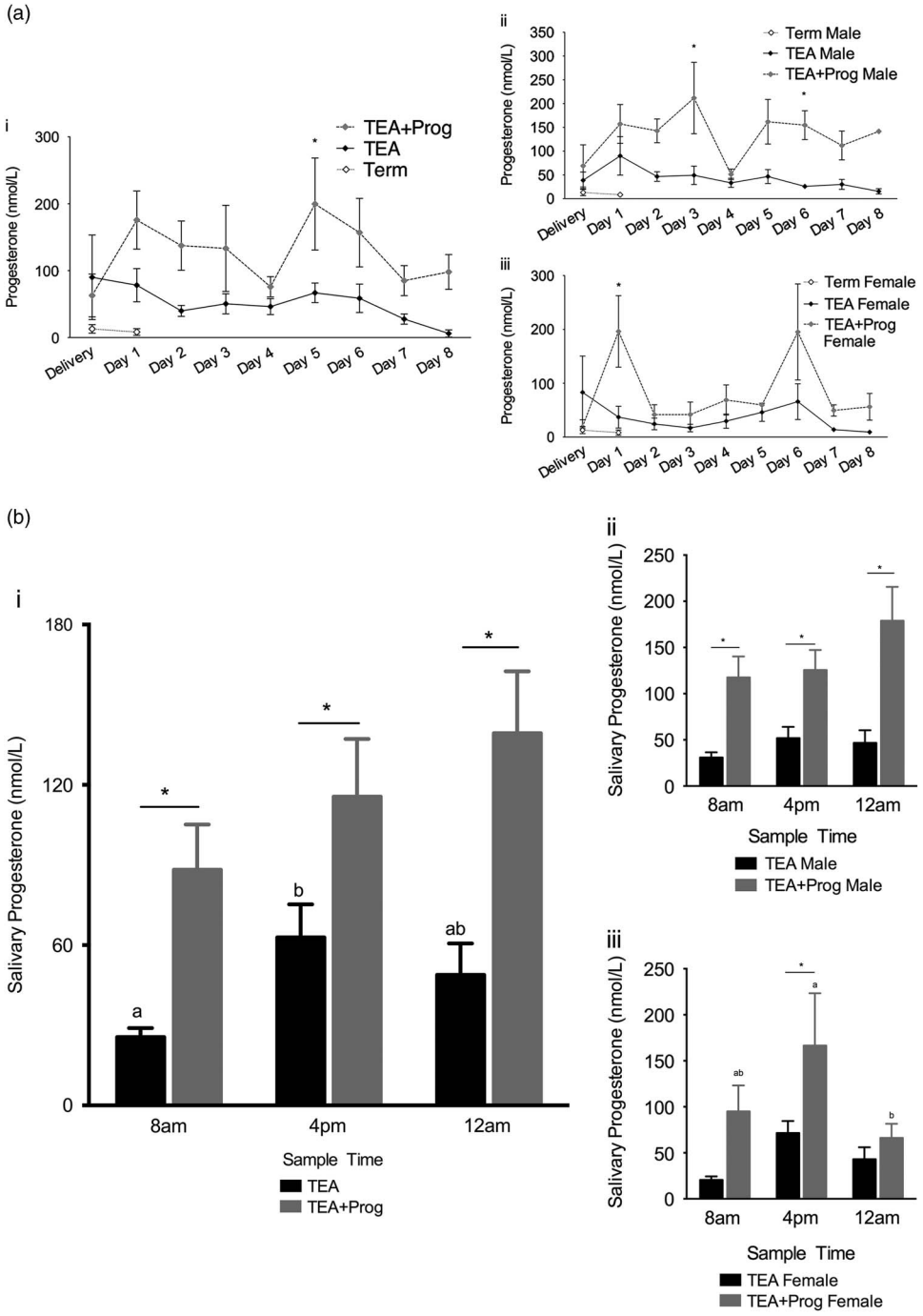

Fig. 2. Mean daily salivary progesterone concentrations (*a*) in term (open), vehicle-treated preterm [term-equivalent age (TEA), black] and progesterone-treated preterm (TEA + Prog, gray) neonates at TEA (2*aii*, male, diamond; 2*aiii*, female, circle symbols). Preterm neonates were studied until TEA (postnatal day 8). The mean salivary progesterone concentrations over the postnatal period (*b*) were significantly elevated at each of the three sampling times in TEA + Prog (gray) compared with TEA (black) neonates. Each bar/symbol represents mean ± S.E.M. $^*P < 0.05$ between TEA and TEA + Prog groups. Letters indicate significant difference between samples within the vehicle-treated or the progesterone-treated preterm groups ($P < 0.05$).

Discussion

A key finding of this study was the ability of progesterone treatment to raise allopregnanolone concentrations in the neonatal brain. The dose of progesterone used raised salivary progesterone levels throughout the postnatal period until TEA, and the results suggest that this dose also increased allopregnanolone synthesis in the brain. However, the elevation of plasma progesterone in the treated preterm males, but not females, at the time of postmortem, suggests that there are

https://doi.org/10.1017/S2040174415001075 Published online by Cambridge University Press

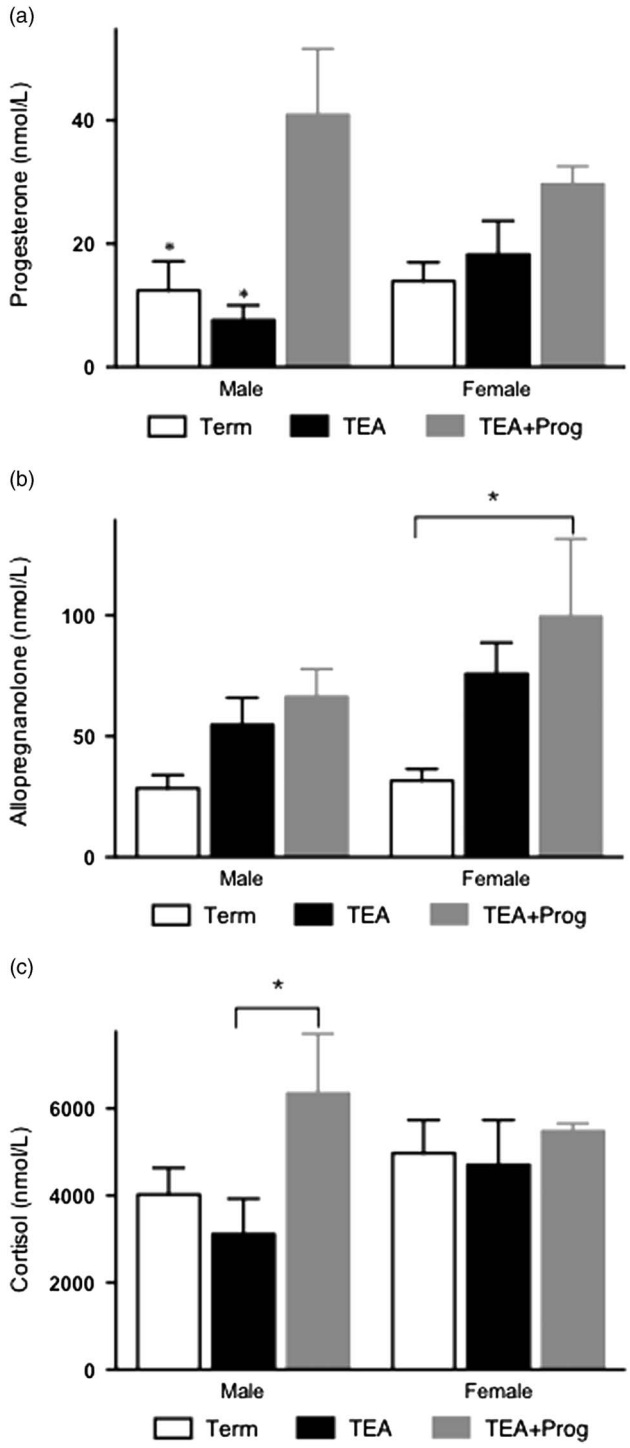

Fig. 3. Circulating progesterone (*a*), allopregnanolone (*b*) and cortisol (*c*) concentrations in term (open bars), vehicle-treated preterm [term-equivalent age (TEA), black bars] and progesterone-treated preterm (TEA + Prog, gray bars) male and female neonates at term-equivalent age. $^{*}P < 0.05$.

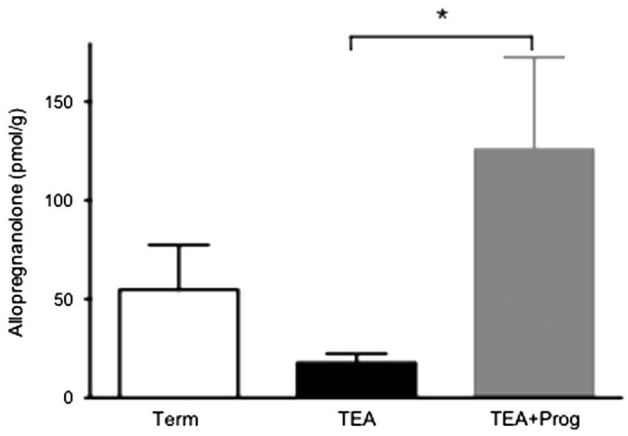

Fig. 4. Brain allopregnanolone concentrations for term (open bars, $n = 6$), vehicle-treated preterm [term-equivalent age (TEA), black bars, $n = 6$) and progesterone-treated preterm (TEA + Prog, gray bars, $n = 6$) neonates at term-equivalent age. $^{*}P < 0.05$.

significant differences in progesterone metabolism between the sexes. This suggestion of a fundamental sex difference in steroid metabolism was also supported by the increased plasma cortisol concentrations in the progesterone-treated male neonates compared with vehicle-treated males and increased plasma allopregnanolone concentrations in the preterm female neonates compared with term female neonates. The elevated circulating cortisol concentrations observed in progesterone-treated preterm male neonates may have some negative effects on neurodevelopment, similar to those produced by chronic stress, reversing the neuroprotective benefits conferred by progesterone or its metabolites such as allopregnanolone.[34] In support of this contention, we found that expression of markers spanning the whole oligodendroctye lineage remained markedly lower in the cerebellum of preterm males at TEA following progesterone treatment. Oligodendroctye markers in the vehicle-treated TEA neonates, although appearing lower, were not statistically different, suggesting that the increased circulating cortisol in the progesterone-treated neonates could be perturbing myelination. A study in rat neonates found that cortisol treatment caused a significant reduction in the amount of myelin and the number of myelinated axons (but not total number of axons).[35] These investigators found no evidence of myelin debris or degeneration of myelin sheaths, suggesting that cortisol blocked the formation of myelin rather than causing degeneration. Our data demonstrate a reduction in all oligodendrocyte markers measured in the male preterm progesterone-treated cerebellum, further supporting the role of cortisol in preventing myelination.

The male vehicle-treated TEA neonates demonstrated delayed cerebellar development, with lower expression of the oligodendroctye markers, although only OLIG2 expression was statistically lower, and smaller Purkinje cells. Interestingly, unlike male TEA neonates, female neonates at TEA had greater brain:body weight ratio than term neonates, suggesting different *ex utero* growth. This brain sparing fits with clinical data in pregnancy, suggesting that female fetuses are able to alter their growth and protect their brain when faced with adverse conditions.[36] There was no difference in

https://doi.org/10.1017/S2040174415001075 Published online by Cambridge University Press

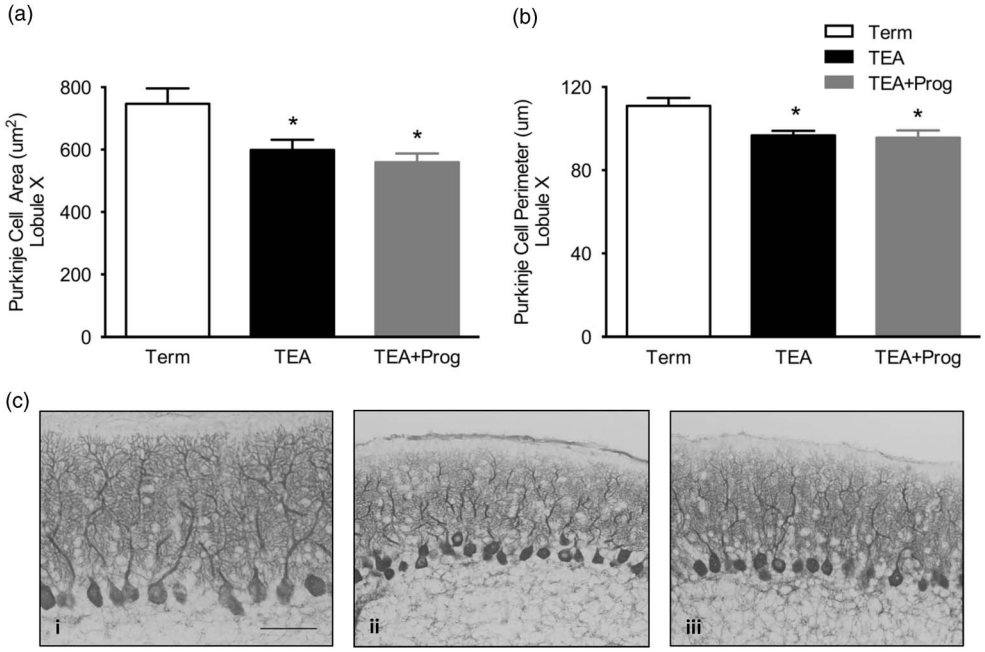

Fig. 5. Average Purkinje cell perimeter (*a*), area (*b*) and representative images in lobule X (*c*) of the cerebellum in term ($n = 6$, open bars, *i*), vehicle-treated preterm [$n = 6$, term-equivalent age (TEA), black bars, *ii*] and progesterone-treated preterm ($n = 6$, TEA + Prog, gray bars, *iii*) male and female neonates at term-equivalent age. Scale bar = 100 μM; $^*P < 0.05$.

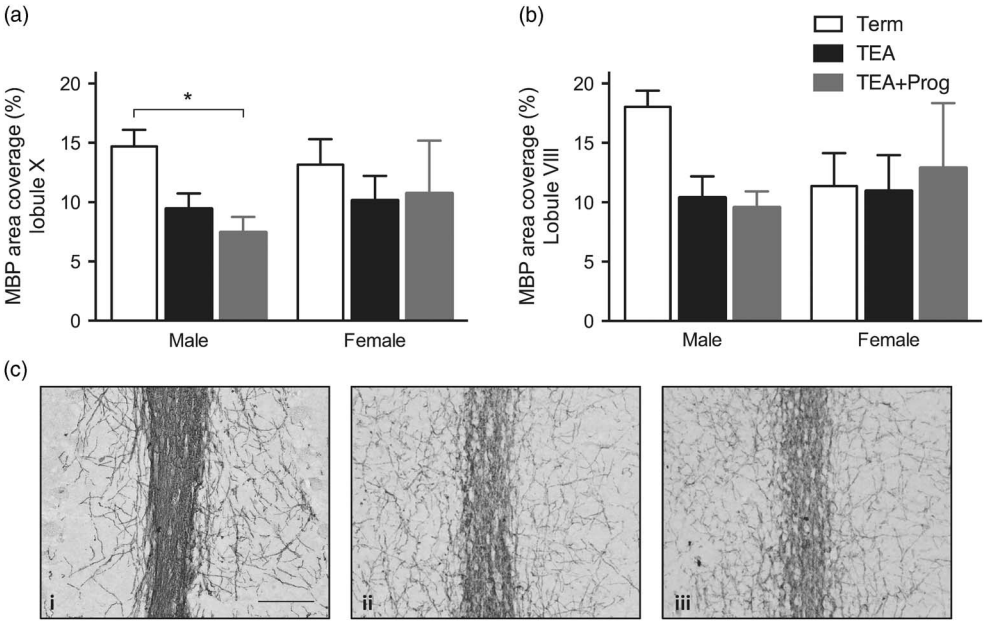

Fig. 6. Myelin basic protein (MBP) expression in the cerebellum of term (open bars, *i*), vehicle-treated preterm [term-equivalent age (TEA), black bars, *ii*] and progesterone-treated preterm (TEA + Prog, gray bars, *iii*) neonates at term-equivalent age. Average MBP area coverage is shown in male and female neonates in lobule X (*a*) and VIII (*b*) and representative images (*c*) of lobule X cerebellum of male neonates. Scale bar = 100 μM; $^*P < 0.05$.

oligodendrocyte expression in the cerebellum of preterm female neonates compared with term born females at TEA, indicating a difference in 'catch up' development between the sexes and which is consistent with clinical findings that preterm male neonates have poorer outcomes.[37] Further investigation is required to assess this developmental sexual dimorphism, including the examination of cell proliferation, apoptosis and astrocyte and microglial influence on myelination.

We have previously reported that treatment of preterm guinea pigs over 24 h with progesterone, as used in this study, markedly

https://doi.org/10.1017/S2040174415001075 Published online by Cambridge University Press

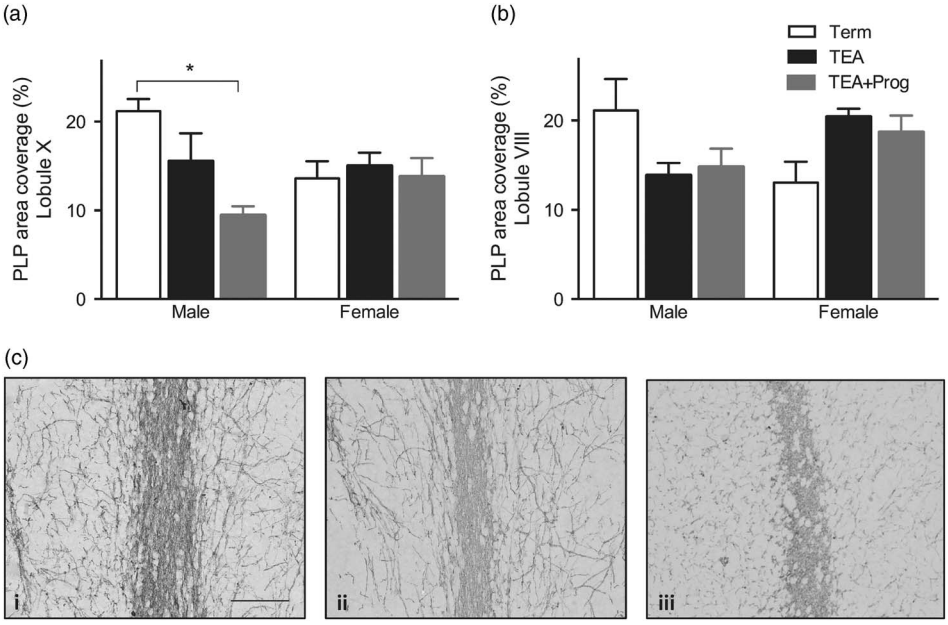

Fig. 7. Proteolipid protein (PLP) expression in the cerebellum of term (open bars, *i*), vehicle-treated preterm [term-equivalent age (TEA), black bars, *ii*] and progesterone-treated preterm (TEA + Prog, gray bars, *iii*) neonates at term-equivalent age. Average PLP area coverage is shown in male and female neonates in lobule X (*a*) and VIII (*b*) and representative images (*c*) of lobule X cerebellum of male neonates. Scale bar = 100 μM; $^*P < 0.05$.

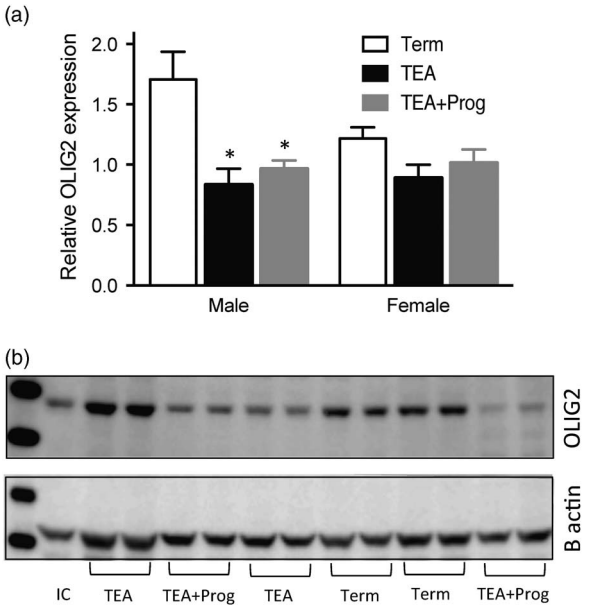

Fig. 8. Oligodendroctye transcription factor 2 (OLIG2) expression in the cerebellum of term (open bars), vehicle-treated preterm [term-equivalent age (TEA), black bars] and progesterone-treated preterm (TEA + Prog, gray bars) neonates at term-equivalent age. Average OLIG2 expression in male and female neonates (*a*) and representative male OLIG2 and β actin western blots (*b*). IC, internal control. $^*P < 0.05$.

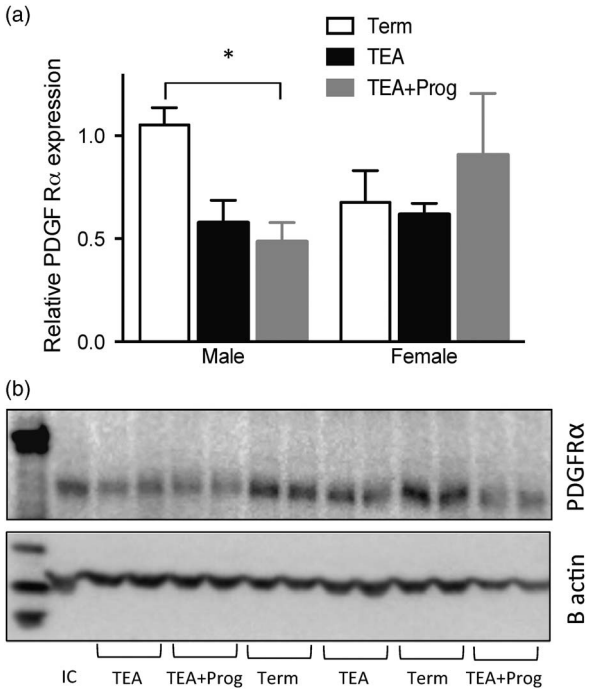

Fig. 9. Platelet-derived growth factor receptor-α (PDGFRα) expression in the cerebellum of term (open bars), vehicle-treated preterm [term-equivalent age (TEA), black bars] and progesterone-treated preterm (TEA + Prog, gray bars) neonates at term-equivalent age. Average PDGFRα expression in male and female neonates (*a*) and representative male PDGFRα and β actin western blots (*b*). IC, internal control. $^*P < 0.05$.

https://doi.org/10.1017/S2040174415001075 Published online by Cambridge University Press

increased both plasma and brain allopregnanolone levels.[19] The increase of allopregnanolone 24 h after progesterone treatment[18] was larger than those seen in the present study, suggesting that more prolonged progesterone treatment induces other changes in progesterone metabolism, reducing the amount of allopregnanolone that can be produced. Evidence for induction of alternative metabolic pathways with chronic treatment is suggested by the increased cortisol production in male neonates; elevated levels of progesterone and metabolites may render redundant (i.e., bypass) the rate-limiting step of cholesterol availability for steroid synthesis by mitochondrial and 11-βHSD activities.[38] These findings are also consistent with lower plasma levels of progesterone that were achieved in the present study with longer exposure.[19] The finding that increased cortisol was only observed in progesterone-treated males is noteworthy. Although plasma cortisol levels in the females were already somewhat higher than in the males, the greater stress arising from preterm birth that typically occurs in males[37] may have induced the sympathoadrenal pathways for cortisol production in the early postnatal period, with the consequence of greater conversion of progesterone metabolites to cortisol. Thus, preterm birth in females may allow more allopregnanolone to be produced, whereas cortisol production is increased in males.

As the postnatal period is important for cerebellar development,[39] the immaturity or abnormal development of this neurosteroid endocrine system may explain some of the postnatal morbidity associated with preterm birth. Models of *in utero* asphyxia in fetal sheep have demonstrated the vulnerability of the immature cerebellum, with lipid peroxidation products and pyknosis increased in Purkinje cells and other regions of the cerebellum as soon as 24 h after the asphyxial (umbilical cord occlusion) event.[40] Clinical studies have also identified the vulnerability of the immature preterm cerebellum in the *ex utero* environment.[11,13] In the present study, somal area and perimeters of the Purkinje cells in lobule X were reduced in both preterm groups compared with the neonates born at term, suggesting an effect of postnatal development on Purkinje cell growth and development in the preterm guinea pig, with no 'catch up' growth by TEA. As somal size has been correlated with dendritic arborisation in Purkinje cells and other neurons,[41–43] the reduction in somal size observed in the present study may have important implications on cerebellar function and signaling. The delay in Purkinje cell development following preterm birth may also be significant for postnatal neuroactive steroidogenesis, as the Purkinje cell is an important site of progesterone and allopregnanolone synthesis.[25,44,45] The contribution of altered cerebellar development in the etiology of lifelong cognitive and sensorimotor deficits is also gaining more clinical attention.[46] The cerebellum has traditionally been reported to process sensory data and regulate motor movements with lobule X having roles in balance and gait (the execution of motor activity to maintain and adjust body posture) and stabilization of the eye on a moving object.[47] There is increasing recognition that the cerebellum contributes to cognitive processing and emotional control in addition to its role in motor co-ordination; however, further exploration of cerebellar function and structure in this area is required.[8] The development of the myelin sheath enables the rapid and synchronized information transfer required for co-ordinated movement, decision-making and other higher order cognitive, behavioral and emotive functions; therefore any delay or perturbation of myelination can have detrimental functional outcomes.[48] Further functional assessment of the male TEA and TEA + Prog neonates is required to assess any balance, gait and possible cognitive deficits.

Studies in slice cultures of the neonatal rat cerebellum have shown increases in MBP expression mediated by both progesterone and allopregnanolone signaling at the progesterone receptor and $GABA_A$ receptor, respectively.[49] However, this was not confirmed by the present *in vivo* study of the preterm cerebellum at TEA. The marked rise in plasma cortisol in male neonates following 8 days of postnatal progesterone therapy raises the possibility that exposure to chronically elevated cortisol may have had a negative impact on the ability of the developing cerebellum to respond to such progesterone treatment, although the increased cortisol levels were not sufficient to impact on neonatal growth (weight) or other indixes of neonatal wellbeing. Exogenous glucocorticoid treatment and increases in perinatal cortisol concentrations have been associated with altered stress responses, neurobehavioral changes, programming of the HPA axis and increased risk for metabolic diseases in later life.[50] In addition, exposure of guinea pig fetuses to prenatal maternal stress, when endogenous glucocorticoid secretion is significantly increased, is associated with reduced myelination and reduced transfer of neuroactive steroids to the fetal brain.[51] Although we cannot exclude a direct action of progesterone in addition to an effect of cortisol, this seems less likely, as effects were restricted to males, whereas progesterone was elevated in both sexes.

Progesterone rather than allopregnanolone was selected for postnatal treatment due to its apparent safety profile and widespread use, even at high doses, in clinical practice.[52] The rationale was that the treatment with progesterone would provide the substrate for augmentation of allopregnanolone synthesis over the time normally covered by placental production of progesterone, and which is lost in the event of preterm birth. Furthermore, earlier studies about potential benefits of progesterone and estradiol replacement in preterm infants have provided promising findings with respect to neonatal respiratory function,[53] bone mineralization[54] and neurodevelopmental outcomes.[55] Importantly, no adverse effects were identified in these trials of postnatal steroid replacement therapy, although effects on cerebellar and brain development in general have not been investigated. The present findings, although supporting a role for progesterone therapy in maintaining neurosteroid synthesis after preterm birth, highlights the potential for negative wide-reaching endocrine effects in the male preterm neonates following progesterone treatment. Therefore, the use of progesterone in some high-risk pregnancies may have significant, and sex-dependent, adverse effects on neuroactive and adrenal steroid concentrations in the perinatal brain.

The findings of this study suggest that chronic progesterone treatment in early postnatal life, although raising neurosteroid levels toward gestation levels, may affect other steroidogenic pathways. The evaluation of a less metabolizable allopregnanolone analog may have greater efficacy in replacing allopregnanolone action and promoting development compared with progesterone, and such strategies following preterm birth warrant investigation.

Acknowledgments

The authors thank Britt Saxby for her assistance with the allopregnanolone measurements and Poonam Rani for her assistance with the oligodendrocyte analyses.

Financial Support

This work was supported by the NHMRC of Australia (grant number 1003517).

Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the Australian code for the care and use of animals for scientific purposes (8th edn, 2013), and has been approved by the institutional committee (University of Newcastle Animal Care and Ethics Committee).

References

1. Mwaniki M, Atieno M, Lawn J, Newton C. Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review. *Lancet*. 2012; 379, 445–452.
2. Behrman RE, Butler AS. eds. *Preterm Birth: Causes, Consequences and Prevention*. 2007. National Academy of Sciences: Washington.
3. Petrini J, Dias T, McCormick M, *et al*. Increased risk of adverse neurological development for late preterm infants. *J Pediatr*. 2009; 154, 169–176.
4. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet*. 2008; 371, 261–269.
5. Huppi P, Schuknecht B, Boesch C, *et al*. Structural and neurobehavioural delay in postnatal brain development of preterm infants. *Pediatr Res*. 1996; 39, 895–901.
6. Inder TE, Warfield SK, Wang H, Huppi PS, Volpe JJ. Abnormal cerebral structure is present at term in premature infants. *Pediatrics*. 2005; 115, 286–294.
7. Altman J. Autoradiographic and histological studies of postnatal neurogenesis. III. Dating the time of production and onset of differentiation of cerebellar microneurons in rats. *J Comp Neurol*. 1969; 136, 269–293.
8. Buckner Randy L. The cerebellum and cognitive function: 25 years of insight from anatomy and neuroimaging. *Neuron*. 2013; 80, 807–815.
9. Abraham H, Tornoczky T, Kosztolanyi G, Seress L. Cell formation in the cortical layers of the developing human cerebellum. *Int J Dev Neurosci*. 2001; 19, 53–62.
10. Knickmeyer RC, Gouttard S, Kang C, *et al*. A structural MRI study of human brain development from birth to 2 years. *J Neurosci*. 2008; 28, 12176–12182.
11. Limperopoulos C, Soul JS, Gauvreau K, *et al*. Late gestation cerebellar growth is rapid and impeded by premature birth. *Pediatrics*. 2005; 115, 688–695.
12. Messerschmidt A, Brugger P, Bolthauser E, *et al*. Disruption of cerebellar development: potential complications of extreme prematurity. *Am J Neuroradiol*. 2005; 26, 1659–1667.
13. Haldipur P, Bharti U, Alberti C, *et al*. Preterm delivery disrupts the developmental program of the cerebellum. *PLoS One*. 2011; 6, e23449.
14. Biran V, Verney C, Ferriero DM. Perinatal cerebellar injury in human and animal models. *Neurol Res Int*. 2012; 2012, 858929.
15. Davis M, Plotz E. Progesterone, the pregnancy hormone. *Fertil Steril*. 1957; 8, 603–618.
16. Challis J, Lockwood C, Myatt L, *et al*. Inflammation and pregnancy. *Repro Sci*. 2009; 16, 206–215.
17. Gilbert Evans SE, Ross LE, Sellers EM, Purdy RH, Romach MK. 3alpha-reduced neuroactive steroids and their precursors during pregnancy and the postpartum period. *Gynecol Endocrinol*. 2005; 21, 268–279.
18. Paul SM, Purdy RH. Neuroactive steroids. *FASEB J*. 1992; 6, 2311–2322.
19. Kelleher MA, Hirst JJ, Palliser HK. Changes in neuroactive steroid concentrations after preterm delivery in the guinea pig. *Reprod Sci*. 2013; 20, 1365–1375.
20. Nguyen PN, Billiards SS, Walker DW, Hirst JJ. Changes in 5a-pregnane steroids and neurosteroidogenic enzyme expression in the perinatal sheep. *Pediatr Res*. 2003; 53, 956–964.
21. Kelleher MA, Palliser HK, Walker DW, Hirst JJ. Sex-dependent effect of a low neurosteroid environment and intrauterine growth restriction on fetal guinea pig brain development. *J Endocrinol*. 2011; 208, 1–9.
22. Mellon SH, Deschepper CF. Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. *Brain Res*. 1993; 629, 283–292.
23. Ukena K, Usui M, Kohchi C, Tsutsui K. Cytochrome P450 side-chain cleavage enzyme in the cerebellar Purkinje neuron and its neonatal change in rats. *Endocrinology*. 1998; 139, 137–147.
24. Petratos S, Hirst JJ, Mendis S, Anikijenko P, Walker DW. Localization of p450scc and 5alpha-reductase type-2 in the cerebellum of fetal and newborn sheep. *Brain Res Dev Brain Res*. 2000; 123, 81–86.
25. Tsutsui K. Biosynthesis and organizing action of neurosteroids in the developing Purkinje cell. *Cerebellum*. 2006; 5, 89–96.
26. Ghoumari A, Baulieu E, Schumacher M. Progesterone increases oligodendroglial cell proliferation in rat cerebellar slice cultures. *Neuroscience*. 2005; 135, 47–58.
27. Sakamoto H, Ukena K, Tsutsui K. Effects of progesterone synthesized *de novo* in the developing Purkinje cell on its dendritic growth and synaptogenesis. *J Neurosci*. 2001; 21, 6.
28. Yawno T, Hirst JJ, Castillo-Melendez M, Walker DW. Role of neurosteroids in regulating cell death and proliferation in the late gestation fetal brain. *Neuroscience*. 2009; 163, 838–847.
29. McKendry A, Palliser H, Yates D, Walker D, Hirst J. The effect of betamethasone treatment on neuroactive steroid synthesis in a

foetal guinea pig model of growth restriction. *J Neuroendocrinol*. 2010; 22, 166–174.
30. Purdy R, Moore P, Rao P, *et al*. Radioimmunassay of 3 alpha-hydroxy-5 alpha-pregnan-20-one in rat and human plasma. *Steroids*. 1990; 55, 290–296.
31. Timby E, Balgard M, Nyberg S, *et al*. Pharmacokinetic and behavioral effects of allopregnanolone in healthy women. *Psychopharmacology (Berl)*. 2006; 186, 414–424.
32. Jakovcevski I, Filipovic R, Mo Z, Rakic S, Zecevic N. Oligodendrocyte development and the onset of myelination in the human fetal brain. *Front Neuroanat*. 2009; 3, 5. doi:10.3389/neuro.05.005.2009.
33. Tolcos M, Bateman E, O'Dowd R, *et al*. Intrauterine growth restriction affects the maturation of myelin. *Exp Neurol*. 2011; 232, 53–65.
34. Huang WL, Harper CG, Evans SF, Newnham JP, Dunlop SA. Repeated prenatal corticosteroid administration delays myelination of the corpus callosum in fetal sheep. *Int J Dev Neurosci*. 2001; 19, 415–425.
35. Bohn MC, Friedrich VL Jr. Recovery of myelination in rat optic nerve after developmental retardation by cortisol. *J Neurosci*. 1982; 2, 1292–1298.
36. Clifton VL. Review: sex and the human placenta: mediating differential strategies of fetal growth and survival. *Placenta*. 2010; 31(Suppl.), S33–S39.
37. Abdel-Latif ME, Kecskes Z, Bajuk B. Actuarial day-by-day survival rates of preterm infants admitted to neonatal intensive care in New South Wales and the Australian Capital Territory. *Arch Dis Child Fetal Neonatal Ed*. 2011; 98, F212–F217.
38. Miller WL, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev*. 2011; 32, 81–151.
39. Knickmeyer R, Gouttard S, Kang C, *et al*. A structural MRI study of human brain development from brith to 2 years. *J Neurosci*. 2008; 28, 12176–12182.
40. Castillo-Melendez M, Chow JA, Walker DW. Lipid peroxidation, caspase-3 immunoreactivity, and pyknosis in late-gestation fetal sheep brain after umbilical cord occlusion. *Pediatr Res*. 2004; 55, 864–871.
41. Andrews T, Li D, Halliwell J, Cowen T. The effect of age on dendrites in the rat superior cervical ganglion. *J Anat*. 1994; 184, 111–117.
42. Jacobs B, Driscoll L, Schall M. Life-span dendritic and spine changes in areas 10 and 18 of human cortex: a quantitative Golgi study. *J Comp Neurol*. 1997; 386, 661–680.
43. Rees S, Loeliger M, Munro K, *et al*. Cerebellar development in a baboon model of preterm delivery: impact of specific ventilatory regimes. *J Neuropathol Exp Neurol*. 2009; 68, 605–615.
44. Agis-Balboa RC, Pinna G, Zhubi A, *et al*. Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. *Proc Natl Acad Sci U S A*. 2006; 103, 14602–14607.
45. Bixo M, Andersson A, Winblad B, Purdy R, Backstrom T. Progesterone, 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnane-20-one in specific regions of the human female brain in different endocrine states. *Brain Res*. 1997; 764, 173–178.
46. Alin M, Matsumoto H, Santhouse A, *et al*. Cognitive and motor function and the size of the cerebellum in adolescents born very pre-term. *Brain*. 2001; 124, 60–66.
47. Stoodley CJ, Schmahmann JD. Evidence for topographic organization in the cerebellum of motor control versus cognitive and affective processing. *Cortex*. 2010; 46, 831–844.
48. Fields RD. White matter in learning, cognition and psychiatric disorders. *Trends Neurosci*. 2008; 31, 361–370.
49. Ghoumari A, Ibanez C, El-Etr M, *et al*. Progesterone and its metabolites increase myelin basic protein expression in organotypic slice cultures of rat cerebellum. *J Neurochem*. 2003; 86, 848–859.
50. Matthews S. Dynamic changes in glucocorticoid and mineralocorticoid receptor mRNA in the developing guinea pig brain. *Dev Brain Res*. 1998; 107, 123–132.
51. Bennett GA, Palliser HK, Saxby B, Walker DW, Hirst JJ. Effects of prenatal stress on fetal neurodevelopment and responses to maternal neurosteroid treatment in guinea pigs. *Dev Neurosci*. 2013; 35, 416–426.
52. Norwitz ER, Caughey AB. Progesterone supplementation and the prevention of preterm birth. *Rev Obstet Gynecol*. 2011; 4, 60–72.
53. Dodd J, Crowther C, McPhee A, Flenady V, Robinson J. Progesterone after previous preterm birth for prevention of neonatal respiratory distress syndrome (PROGRESS): a randomised controlled trial. *BMC Pregnancy Childbirth*. 2009; 9, 6–12.
54. Trotter A, Maier L, Grill HJ, *et al*. Effects of postnatal estradiol and progesterone replacement in extremely preterm infants. *J Clin Endocrinol Metab*. 1999; 84, 4531–4535.
55. Trotter A, Steinmacher J, Kron M, Pohlandt F. Neurodevelopmental follow-up at five years corrected age of extremely low birth weight infants after postnatal replacement of 17β-estradiol and progesterone. *J Clin Endocrinol Metab*. 2012; 97, 1041–1047.