Journal of Developmental Origins of Health and Disease (2014), **5**(2), 74–78.

doi:10.1017/S2040174413000573

BRIEF REPORT

Increased placental neurosteroidogenic gene expression precedes poor outcome in the preterm guinea pig

A. L. Cumberland[1,2]*, H. K. Palliser[1,2] and J. J. Hirst[1,2]

[1]*School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, New South Wales, Australia*
[2]*Hunter Medical Research Institute, Mothers and Babies Research Centre, Newcastle, New South Wales, Australia*

Placental 5α-reductase (5αR) is influenced by *in utero* compromises and has a role in regulating neuroactive steroid concentrations in the fetus. The objective of this study was to determine if changes in placental 5αR were associated with neonatal outcome after birth. Guinea pigs were delivered by cesarean section at term (GA69, $n = 22$) or preterm (GA62, $n = 36$) and the placenta collected. Preterm neonates were maintained for 24 h unless their condition deteriorated before this time. Enzyme mRNA expression of 5αR type-1 and 5αR type-2 were determined using real-time PCR. All preterm neonates had significantly higher 5αR2 expression in their placenta compared with placentae from term neonates ($P < 0.0001$). Expression was also markedly higher in the placentae from neonates that did not survive until 24 h, compared with surviving preterm neonates ($P = 0.04$). These findings suggest differences of *in utero* neurosteroidogenic capacity between surviving and non-surviving preterm guinea pig neonates. The increased 5αR2 mRNA expression in the placenta of non-survivors suggests an induction of the neurosteroid pathway due to prior exposure to an *in utero* compromise, with such exposure possibly a predisposing factor that contributed to their poor *ex utero* outcome.

Received 12 September 2013; Revised 17 December 2013; Accepted 18 December 2013; First published online 10 January 2014

Key words: allopregnanolone, placenta, preterm, survival, 5α-reductase

Introduction

Placental steroidogenesis has a key role in regulating levels of neuroactive steroid concentrations in the fetus during late pregnancy.[1] These steroids include the progesterone derivative 3α-hydroxy-5α-pregnan-20-one (allopregnanolone). Allopregnanolone is a potent modulator of gamma aminobutyric acid type A ($GABA_A$) receptors and markedly influences their inhibitory activity. This action suppresses central nervous system activity and maintains the generally low levels of excitability that typifies fetal life. Neuroactive steroids also inhibit apoptotic pathways, promote synaptogenesis and stimulate proper glial cell activity and myelin formation by oligodendrocytes,[2–4] all of which are critical for optimal fetal brain development.

Progesterone, supplied by the placenta, is essential for the maintenance of pregnancy with levels increasing steadily in the maternal circulation as gestation progresses.[5] The placenta also makes a major contribution to circulating concentrations of allopregnanolone, with levels in maternal and fetal plasma also increasing towards term.[6] 5α-reductases type 1 and 2 (5αR1 and 5αR2) catalyse the rate-limiting step in the metabolism of progesterone into intermediate hormone 5α-dihydroprogesterone. The enzyme 3α-hydroxysteroid dehydrogenase then reversibly converts this precursor to allopregnanolone. These isoforms have low homology and their activity differs with development. The type 1 isoform is found in many tissues at relatively stable levels, whereas type 2 is found mostly within the sex organs, the brain and the placenta during pregnancy and shows greater developmental regulation.[7,8] Both 5αR isoforms are expressed in the human placenta with expression increasing with advancing gestation.[9] After birth, and subsequent removal of the placental supply of progesterone and its metabolites, concentrations of neuroactive steroids fall dramatically in maternal as well as neonatal plasma of humans and sheep.[5,10]

Pharmacological inhibition of neuroactive steroid production by finasteride, a 5αR inhibitor, during pregnancy reduces allopregnanolone concentrations resulting in marked reductions in myelination, and an increase in cell death within fetal sheep and guinea pig brains.[11,12] Acute and chronic *in utero* stressors, including hypoxic episodes and intrauterine growth restriction (IUGR), are associated with poor neonatal outcome and adverse effects on long-term health.[13,14] Previous studies in sheep and rats have shown that these insults raise fetal brain 5αR enzyme protein expression, as well as central and circulating neuroactive steroid concentrations.[15,16] These findings suggest an upregulation of 5αR enzyme expression following compromise, leading to higher levels of protective neuroactive steroids acting to minimize adverse effects in the fetus.

Preterm birth is a leading cause of infant morbidity and mortality[17] and may lead to neurological and behavioral deficits.[18] However, these complications may not arise until later in neonatal life or childhood. In addition, chronic *in utero* stressors that often precede preterm birth may potentiate adverse outcomes after premature delivery.[19]

These stressors may raise neuroactive steroid production in the placenta; with a consequent reduction in the risk of developing neurological deficits, as occurs in the brain.[20] Therefore, elevated

*Address for correspondence: A. L. Cumberland, School of Biomedical Sciences and Pharmacy, University of Newcastle 2308, Australia.
(Email Angela.Cumberland@uon.edu.au)

placental 5αR expression may indicate prior *in utero* fetal stress and thus provide a marker of *in utero* compromise and subsequently potential problems in neonatal or juvenile life. The objective of this study was to determine 5αR mRNA expression in placenta from term and preterm guinea pig neonates and compare expression with their outcome during the immediate 24 h after delivery, and to elucidate whether elevated expression was seen in neonates with poor outcome.

Methods

Time-mated, tri-color, outbred pregnant dams were obtained from the University of Newcastle Research Support Unit. Dams were randomly allocated to either a term delivery (GA69) or preterm delivery (GA62) group. Neonates were delivered no more than 24 h apart within the term and preterm groups (68–69 and 62–63 days, respectively). Term deliveries were performed as close to spontaneous term labour (~71 days for our colony) as possible. Animals that displayed signs of cervical change predictive of delivery (assessed by pubic symphysis separation) were delivered at GA68 to avoid using animals in active labour.

Previous pilot studies from our group have seen a maximum survival of 5% of preterm guinea pig neonates delivered at GA62 without maternal betamethasone administration. Thus, dams received 1 mg/kg of betamethasone (Celestone Chronodose, Schering-Plough, North Ryde, NSW, Australia) subcutaneously 24 and 12 h before delivery, a dosing regime that improves survivability as is seen in humans. Anesthesia was induced in dams in a chamber with 4% isoflurane in 6 l medical grade oxygen. Anesthesia was maintained via mask inhalation of 2% isoflurane in 2 l oxygen, while pups were delivered via cesarean section. Following delivery dams were euthanized, and the placenta from each pup was weighed and snap frozen in liquid nitrogen.

All neonates had their airways cleared of fluid and received 50 μl of surfactant (Curosurf 80 mg/ml Poractant alfa, Douglas Pharmaceuticals, Baulkam Hills, NSW, Australia). Neonates received thermal support by heat lamps and pads and received a short period of continuous positive airway pressure to aid in establishing functional residual capacity. Once stable breathing was achieved all guinea pig neonates were weighed and sexed before being housed in a humidified incubator and monitored continuously. Neonates received 24 h care, and were scored every 2 h for their ability to maintain respiration, posture and activity. The scoring system was based on a scale of 1 to 4 for each criterion, with 1 being poor and 4 being good. A total score out of 12 was given to each neonate at the time of scoring. Preterm neonatal guinea pigs that did not survive to 24 h, due to cessation of respiration, formed a preterm non-survivor experimental group. Neonates who died within the first 2 h after delivery were excluded from this study (term $n = 0$, preterm $n = 8$). All remaining neonates were euthanized at the 24-h end point, and one male and one female neonate per litter used in this study, resulting in three study groups – term ($n = 22$; male $n = 12$, female $n = 10$), preterm surviving ($n = 21$; male $n = 10$, female $n = 11$) and preterm non-surviving ($n = 15$; male = 8, female $n = 7$) neonates.

Frozen placental tissue was crushed on dry ice and RNA was extracted using a commercial kit (RNeasy Plus Mini Kit, Qiagen Pty Ltd, Chadstone, VIC, Australia) and quality tested before undergoing reverse transcription. Placental RNA (1 μg) was reversed transcribed to cDNA using Superscript III First Strand Synthesis Reverse Transcription kit (Invitrogen, Life Technologies Pty Ltd, Mulgrave, VIC, Australia). Real-time PCR, using SYBR green detection, was performed using primer sequences previously designed and validated by our group to detect guinea pig 5αR1 and 5αR2 sequences.[21] Relative fold changes in expression were determined using the $-2^{\Delta\Delta Ct}$ method, with expression data normalized to β-actin for each sample.

Statistical significance was set at $P = 0.05$. A Fisher's exact test was used to assess survival data from the entire cohort of animals. Differences between means were analyzed by one-way ANOVA with subsequent Tukey multiple comparisons test to determine statistically significant differences between term, preterm survivor and preterm non-survivor placenta samples. All data are presented as mean ± S.E.M.

Results

No statistically significant sex differences were found between neonates within the term and preterm groups and hence male and female data have been merged. Term pregnancies were delivered at 68.3 ± 0.2 days while preterm neonates were delivered at 62.2 ± 0.1 days. The gestational age at delivery of those neonates who did not survive the immediate neonatal period was not significantly different to those preterm neonates who did survive (62.4 ± 0.2 *v.* 62.0 ± 0.2 days, respectively, $P = 0.30$). The range of survival for non-surviving neonates was 3–22 h, with a mean survival time of 8.16 ± 1.24 h. Although the survival rates showed no significance between males and females, sex ratios of surviving *v.* non-surviving neonates were 1:1.5 for males and 1:1 for females for the overall cohort. Survival rates of the neonates were independent of the litter, with no patterns of reduced survival within litters.

No differences were found among placental weights collected from term and preterm guinea pigs. As expected, body weight (post mortem) of term guinea pig neonates (83.1 ± 2.9 g) was significantly more than preterm survivors and non-survivors (64.0 ± 1.9 and 62.9 ± 1.2 g, respectively, $P < 0.0001$). Body weight of the neonates did not differ significantly from time of delivery to the time of post mortem. Non-surviving preterm neonates had a reduced ability to maintain respiration, posture and activity than surviving preterm neonates (average scores 6.2 ± 0.5 *v.* 8.1 ± 0.3, respectively, $P < 0.0002$). Both preterm groups expectedly performed worse in the 24-h time period than neonates born at term (average score 11.5 ± 0.1, $P < 0.0001$).

No significant differences were seen in placental 5αR1 expression at time of delivery between the term, preterm surviving and non-surviving groups (Fig. 1a). In contrast, 5αR2 mRNA expression was markedly lower in placenta collected at term compared with levels in the placenta from surviving and

https://doi.org/10.1017/S2040174413000573 Published online by Cambridge University Press

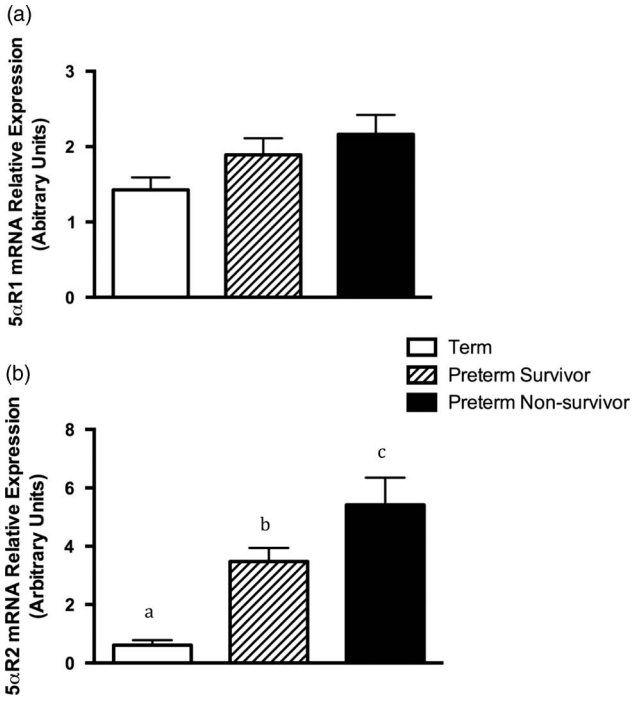

Fig. 1. Placental 5α-reductase type 1 (*a*) and type 2 (*b*) mRNA expression from term (open bars), preterm surviving (striped bars) and preterm non-surviving (closed bars) guinea pig neonates. Relative expression presented as arbitrary units. Data presented as mean ± S.E.M. Bars with different lower case letters are significantly different ($P < 0.05$).

non-surviving prematurely delivered neonates ($P = 0.0004$ and $P < 0.0001$, respectively, Fig. 1b). Interestingly, expression in placenta from preterm non-surviving neonates was significantly greater than placenta from preterm neonates that did survive to 24 h ($P = 0.04$). As mentioned above, no sex differences were found and when split by sex placental 5αR mRNA expressions followed the same patterns.

Discussion

Our findings are the first to show that placental neurosteroidogenic capacity is elevated in the placenta of non-surviving preterm guinea pig neonates. *In utero* compromises are often major contributors to adverse perinatal outcomes and underlying non-detected insufficiencies have been suggested to potentiate hypoxic/ischemic injury at birth.[19] Guinea pigs occasionally have fetuses that suffer *in utero* death, which may be due to placental insufficiencies similar to that seen in human pregnancies. This suggests that some fetuses that do not die *in utero* may be exposed to periods of less severe hypoxia, which may be related to placental implantation. The present findings suggest elevated 5αR2 expression is indicative of insufficiency, potentially due to a hypoxic episode or multiple episodes, and underdevelopment that may have contributed to the reduced survival of the immature pups. The relative contribution of 5αR2 in the placenta and brain to central allopregnanolone concentrations is unclear, however, the placenta is likely to not only supply the allopregnanolone precursor progesterone but also contribute to the supply of allopregnanolone itself. During fetal life, 5αR2 has the key role in producing allopregnanolone, with expression developmentally regulated. After birth expression of 5αR2 in the brain declines with advancing postnatal age and neurosteroid production becomes more dependent on 5αR1.[22]

Previous studies by us investigating the effects of betamethasone on 5α-reductase expression showed a marked reduction in 5αR2 mRNA in the placenta of betamethasone-treated fetuses.[21] Hence, the current study administered betamethasone to dams before both term and preterm delivery to reduce variability among experimental groups. However, the McKendry study employed multiple administrations of betamethasone over a prolonged period. This chronic exposure could have potentially caused the significant reductions in placental 5αR2. Our study used fewer administrations over a shorter time period, mimicking protocols undertaken in human pregnancies at high risk of preterm delivery, to aid in lung maturation and increase the survival of the preterm guinea pig neonates. Studies using human placenta found no differences in preterm placental 5αR1 and 5αR2 expression with and without prior betamethasone exposure.[9] Additionally, although it is known that betamethasone can be metabolized by the enzyme 11β-hydroxysteroid dehydrogenase type 2, this is slow compared with endogenous glucocorticoids.[23] Therefore, levels of exposure between the term and preterm groups are likely to have been similar.

Allopregnanolone concentrations decline rapidly in sheep neonatal plasma and brain after birth, demonstrating the critical role of the placenta in producing allopregnanolone and its precursors during late gestation.[10] Concentrations in the brain, including the brainstem, also decline after birth, which increases excitability and may increase respiratory activity.[10] In this study the neonates that did not survive died of respiratory failure, often following repeated apnoeic episodes. Allopregnanolone levels in the neonatal rat brain are increased by chronic exposure to stress,[24] albeit from a lower base, but these changes required repeated exposure and are unlikely to influence neonatal survival over the first 24 h of life. This supports the contention that *in utero* stress and consequent effects on development may have a major influence on outcome. The increased levels of 5αR2 gene expression observed in the placenta of non-surviving preterm neonates is consistent with the induction of the protective neurosteroid pathway, which may potentially be due to an *in utero* stressor, which was not sufficient to influence fetal weight.

Previous observations in sheep models suggest that moderate IUGR causes an increase in 5αR2 protein expression in the brain.[20] Although there were no differences in birth weight between this study's preterm groups, less severe compromise toward the time of delivery may have raised 5αR2 transcription and lead to sub-optimal development. As brain to body weight ratio was not available at delivery, evaluation of asymmetric

growth was not possible. Furthermore, the upregulation of neuroactive steroid pathways and an increase in neurosteroid-mediated trophic actions may have improved brain development but not other organ systems. This type of *in utero* stressor may have predisposed these non-surviving neonates to their poor outcome in the first 24 h after delivery, particularly when faced with premature delivery.

Despite the known contribution of the placenta to fetal neurosteroidogenesis, recent studies have focussed on 5αR expression within the fetal brain in response to gestational challenges such as IUGR and hypoxia, with the recent human placental studies characterizing 5αR 1 and 2 protein expression within uncomplicated term and preterm placentae.[9] As such, this the first study to suggest that alterations in placental neuroactive steroid synthesis may be associated with an intrauterine compromise and a consequent poor outcome. Previous studies have shown that male preterm neonates are at a greater risk of morbidity and mortality than females.[25] This may potentially be reflected in their neuroactive enzyme protein levels and activity; and potentially steroid concentration profiles in times of compromise. Although allopregnanolone is the most well-characterized neurosteroid of pregnancy, we cannot rule out the influence of other neurosteroids such as cortisol-derived tetrahydro-deoxycorticosterone and testosterone-derived 3α-androstanediol, and how these may influence the sexes differentially. Future studies could also examine if sex differences are present in brain 5α-reductase protein levels and neuroactive steroid concentrations at birth or in the neonatal period. In conclusion, the finding of markedly higher 5αR2 mRNA expression in the placenta of neonates with poorer outcomes support the measurement of expression of this enzyme as a potential marker of *in utero* compromise that may have ongoing influences on postnatal development.

Acknowledgments

The authors thank Dr Meredith Kelleher and Dr Rebecca Dyson for their contribution to the animal studies.

Financial Support

This research was supported by the National Health and Medical Research Council grant 1044846.

Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the National Health and Scientific Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and have been approved by the University of Newcastle Animal Care and Ethics Committee.

References

1. Hirst JJ, Palliser HK, Yates DM, Yawno T, Walker DW. Neurosteroids in the fetus and neonate: potential protective role in compromised pregnancies. *Neurochem Int*. 2008; 52, 602–610.
2. Ghoumari AM, 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.
3. He J, Hoffman SW, Stein DG. Allopregnanolone, a progesterone metabolite, enhances behavioral recovery and decreases neuronal loss after traumatic brain injury. *Restor Neurol Neurosci*. 2004; 22, 19–31.
4. Purdy RH, Morrow AL, Blinn JR, Paul SM. Synthesis, metabolism, and pharmacological activity of 3 alpha-hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptoneurosomes. *J Med Chem*. 1990; 33, 1572–1581.
5. Gilbert Evans SE, Ross LE, Sellers EM, Purdy RH, Romach MK. 3α-reduced neuroactive steroids and their precursors during pregnancy and the postpartum period. *Gynecol Endocrinol*. 2005; 21, 268–279.
6. Bicikova M, Klak J, Hill M, *et al*. Two neuroactive steroids in midpregnancy as measured in maternal and fetal sera and in amniotic fluid. *Steroids*. 2002; 67, 399–402.
7. Normington K, Russell DW. Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. *J Biol Chem*. 1992; 267, 19548–19554.
8. Poletti A, Celotti F, Rumio C, Rabuffetti M, Martini L. Identification of type 1 5α-reductase in myelin membranes of male and female rat brain. *Mol Cell Endocrinol*. 1997; 129, 181–190.
9. Vu TT, Hirst JJ, Stark M, *et al*. Changes in human placental 5α-reductase isoenzyme expression with advancing gestation: effects of fetal sex and glucocorticoid exposure. *Reprod Fertil Dev*. 2009; 21, 599–607.
10. Nguyen PN, Billiards SS, Walker DW, Hirst JJ. Changes in 5 alpha-pregnane steroids and neurosteroidogenic enzyme expression in the perinatal sheep. *Pediatr Res*. 2003; 53, 956–964.
11. Yawno T, Yan EB, Walker DW, Hirst JJ. Inhibition of neurosteroid synthesis increases asphyxia-induced brain injury in the late gestation fetal sheep. *Neuroscience*. 2007; 146, 1726–1733.
12. 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, 301–309.
13. Gadisseux M, Gressens P, editors. *Hypoxia Opportunism During Brain Development. Acute Perinatal Asphyxia in Term Infants: Report of the Workshop*. 1997. DIANE Publishing: National Institute of Health, Bethesda, Maryland, USA.
14. Breeze ACG, Lees CC. Prediction and perinatal outcomes of fetal growth restriction. *Seminars in Fetal and Neonatal Medicine*. 2007; 12, 383–397.
15. Nguyen PN, Yan EB, Castillo-Melendez M, Walker DW, Hirst JJ. Increased allopregnanolone levels in the fetal sheep brain following umbilical cord occlusion. *J Physiol*. 2004; 560, 593–602.
16. Westcott KT, Hirst JJ, Ciurej I, Walker DW, Wlodek ME. Brain allopregnanolone in the fetal and postnatal rat in response to

uteroplacental insufficiency. *Neuroendocrinology*. 2008; 88, 287–292.
17. World Health Organization; March of Dimes; The Partnership for Maternal NCHStC. Born Too Soon: the Global Action Report on Preterm Birth 2012.
18. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from infancy to adulthood. *Lancet*. 2008; 371, 261–269.
19. Garite TJ, Clark R, Thorp JA. Intrauterine growth restriction increases morbidity and mortality among premature neonates. *Am J Obstet Gynecol*. 2004; 191, 481–487.
20. Nguyen PN, Billiards SS, Walker DW, Hirst JJ. Changes in 5alpha-pregnane steroids and neurosteroidogenic enzyme expression in fetal sheep with umbilicoplacental embolization. *Pediatr Res*. 2003; 54, 840–847.
21. McKendry AA, Palliser HK, Yates DM, Walker DW, Hirst JJ. The effect of betamethasone treatment on neuroactive steroid synthesis in a fetal guinea pig model of growth restriction. *J Neuroendocrinol*. 2010; 22, 166–174.
22. Poletti A, Negri-Cesi P, Rabuffetti M, *et al*. Transient expression of the 5α-reductase type 2 isozyme in the rat brain in late fetal and early postnatal life. *Endocrinology*. 1998; 139, 2171–2178.
23. Murphy VE, Fittock RJ, Zarzycki PK, *et al*. Metabolism of synthetic steroids by the human placenta. *Placenta*. [Comparative Study Research Support, Non-U.S. Gov't]. 2007; 28, 39–46.
24. Kehoe P, Mallinson K, McCormick CM, Frye CA. Central allopregnanolone is increased in rat pups in response to repeated, short episodes of neonatal isolation. *Dev Brain Res*. 2000; 124, 133–136.
25. Shapiro-Mendoza CK, Tomashek KM, Kotelchuck M, *et al*. Risk factors for neonatal morbidity and mortality among healthy late preterm newborns. *Semin Perinatol*. 2006; 30, 54–60.