

Sex-dependent effect of a low neurosteroid environment and intrauterine growth restriction on foetal guinea pig brain development

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Abstract

Progesterone and its neuroactive metabolite, allopregnanolone, are present in high concentrations during pregnancy, but drop significantly following birth. Allopregnanolone influences foetal arousal and enhances cognitive and behavioural recovery following traumatic brain injury. Inhibition of allopregnanolone synthesis increases cell death in foetal animal brains with experimental hypoxia. We hypothesised that complications during pregnancy, such as early or preterm loss of placental steroids and intrauterine growth restriction (IUGR), would disrupt the foetal neurosteroid system, contributing to poor neurodevelopmental outcomes. This study aimed to investigate the effects of chronic inhibition of allopregnanolone synthesis before term and IUGR on developmental processes in the foetal brain. Guinea pig foetuses were experimentally growth restricted at mid-gestation and treated with finasteride, an inhibitor of allopregnanolone synthesis. Finasteride treatment reduced

foetal brain allopregnanolone concentrations by up to 75% and was associated with a reduction in myelin basic protein (MBP) ($P=0.001$) and an increase in glial fibrillary acidic protein expression in the subcortical white matter brain region ($P<0.001$). IUGR resulted in decreased MBP expression ($P<0.01$) and was associated with a reduction in the expression of steroidogenic enzyme 5 α -reductase (5 α R) type 2 in the foetal brain ($P=0.061$). Brain levels of 5 α R1 were higher in male foetuses ($P=0.008$). Both IUGR and reduced foetal brain concentrations of allopregnanolone were associated with altered expression of myelination and glial cell markers within the developing foetal brain. The potential role of neurosteroids in protecting and regulating neurodevelopmental processes in the foetal brain may provide new directions for treatment of neurodevelopmental disorders in infants who are exposed to perinatal insults and pathologies. *Journal of Endocrinology* (2011) **208**, 301–309

Introduction

Foetuses that are born small for gestational age due to intrauterine growth restriction (IUGR) are at higher risk for perinatal morbidity, mortality and long-term disability (Larroque *et al.* 2001). Abnormal foetal growth is associated with a high risk of foetal brain injury, leading to postnatal motor disorders, neurodevelopmental delay and long-term cognitive impairments. The risk of preterm birth is also higher in infants who are growth restricted or small for gestational age (Lackman *et al.* 2001). Placental insufficiency and IUGR have many implications for foetal brain development. Along with clinical observations, animal studies have also revealed the morphological changes and neurological impairments associated with foetal IUGR. In foetal sheep, experimental chronic placental insufficiency late in gestation impairs neural development and results in white matter damage and a reduction in the density of pyramidal cells within the hippocampus (Rees *et al.* 1998). In pregnant guinea pigs, IUGR induced by experimentally limiting

uterine blood flow to the placenta results in reduced hippocampal volume and fewer hippocampal pyramidal neurons in the foetal brain at the end of gestation (Mallard *et al.* 2000).

Progesterone is present in high concentrations throughout pregnancy, and plasma concentrations increase in both the maternal and foetal circulations during late gestation in humans, nonhuman primates and guinea pigs (Gilbert Evans *et al.* 2005, Mitchell & Taggart 2009). Levels of allopregnanolone, a major metabolite of progesterone, increase concurrently with progesterone over the course of gestation in both tissues and plasma (Gilbert Evans *et al.* 2005). In the brain, allopregnanolone has positive modulatory actions at the γ -aminobutyric acid type A (GABA_A) receptor, causing neuronal hyperpolarisation and inhibition of neural activity, resulting in anxiolytic (de Brito Faturi *et al.* 2006), sedative (Paul & Purdy 1992) and anticonvulsant (Kokate *et al.* 1999) effects. Allopregnanolone has also been shown to influence foetal arousal and sleep–wake activity *in utero* (Nicol *et al.* 1997), demonstrating that synthesis and release of this steroid

has a physiological and regulatory role within the central nervous system (CNS).

Allopregnanolone is converted from progesterone by the enzymes 5 α -reductase types 1 and 2 (5 α R1; 5 α R2) and 3 α -hydroxysteroid oxidoreductase (3 α -HSOR; Compagnone & Mellon 2000). These enzymes are present in the adrenal gland, ovary, brain and placenta. *De novo* synthesis of allopregnanolone from cholesterol wholly within the CNS is also possible (Poletti *et al.* 1998).

Endogenous neurosteroids, such as allopregnanolone, have been implicated in the regulation of normal brain development and may be particularly important to the foetal brain exposed to suboptimal or adverse conditions *in utero*, including pregnancies complicated by placental insufficiency and IUGR. For example, in a foetal sheep model of chronic placental insufficiency, expression of 5 α R2 increased in many regions of the foetal brain following late gestation umbilico-placental embolisation, a procedure that reduces the area of the placental vasculature available for nutrient exchange (Nguyen *et al.* 2003).

There is increasing evidence that allopregnanolone has a neuroprotective function in both the adult (Djebaili *et al.* 2004, He *et al.* 2004, Schumacher *et al.* 2007) and foetal brains (Yawno *et al.* 2007, 2009). Inhibition of allopregnanolone synthesis, using finasteride (a 5 α R inhibitor), in foetal sheep increased the number of cells undergoing programmed cell death within the brain, and accentuated the cell death caused by asphyxia arising from transient umbilical cord occlusion (Yawno *et al.* 2007). Inhibition of 5 α R also appears to influence cell proliferation in the foetal sheep brain, with an increase in proliferating astrocytes observed in the hippocampus and cerebellum after finasteride treatment (Yawno *et al.* 2009).

We have previously shown that 5 α R1 and 5 α R2 mRNA levels are altered within the foetal guinea pig brain in a sex-specific manner in response to uterine artery ablation, a procedure that produces IUGR in this species (McKendry *et al.* 2010). This disruption of neurosteroid synthesis in pregnancies with perturbed placental function may result in vulnerability to foetal brain injury. In human pregnancies, IUGR often occurs in association with preterm birth (Lackman *et al.* 2001), and prematurity is itself a risk factor for perinatal brain damage (Saigal & Doyle 2008). It is also known that foetal sex affects susceptibility to brain injury, with perinatal brain injury occurring more frequently in male infants (Di Renzo *et al.* 2007). A reduction in foetal allopregnanolone concentrations in late gestation may mimic the removal of progesterone and neurosteroid support as occurs at birth, either at term or with preterm delivery. We hypothesised that complications during pregnancy, such as IUGR, would disrupt the foetal neurosteroid system, contributing to poor neurodevelopmental outcomes in fetuses with these pregnancy complications. This study aimed to investigate the effects of IUGR, the chronic inhibition of allopregnanolone synthesis and the combination of these insults on late developmental processes within the foetal brain.

Materials and Methods

Animals

All animal procedures were approved by the University of Newcastle Animal Care and ethics committee and carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Time-mated outbred tricolour guinea pigs, supplied by the University of Newcastle Research Support Unit, were housed indoors under a 12 h light:12 h darkness cycle. Animals were provided with commercial guinea pig pellets and water fortified with ascorbic acid was made available *ad libitum*.

Surgery was performed under strict aseptic conditions between 33 and 35 days gestation to induce placental insufficiency and foetal IUGR. Food was removed 4 h prior to the surgery and the pregnant guinea pig dams then received 0.5 mg/kg Temgesic (324 μ g buprenorphine HCl and glucose anhydrous/ml, Reckitt Benckiser Healthcare Ltd, Hull, UK) prior to induction of general anaesthesia with 4% isoflurane in oxygen and maintenance of anaesthesia with 2% isoflurane in oxygen. A midline abdominal incision was made, the uterus was exposed and ~50% of the radial arteries supplying each placenta were ablated using diathermy (Turner & Trudinger 2009). The uterus was then replaced and the incision closed. Animals were returned to individual cages and received a second dose of Temgesic at 8 h post surgery. A control group of animals received sham surgery in which the same procedures were followed but no ablation of radial arteries was performed.

Animals were divided into groups based on surgery (sham/normally grown or IUGR) and daily drug treatment (vehicle or finasteride). This resulted in four foetal groups: control (sham, vehicle treated), IUGR (growth restricted, vehicle treated), FIN (sham, finasteride treated) and IUGR + FIN (growth restricted, finasteride treated). The pregnant dams received daily s.c. injections of vehicle (600 μ l/kg; 16% v/v ethanol in peanut oil) or finasteride (25 mg/kg; Steraloids, New York, NY, USA) from day 55 until they were killed at 65 days of gestation by carbon dioxide inhalation. Term in this colony of guinea pigs is 71 ± 0.5 days.

Tissue collection

Foetuses were removed from the uterus and foetal sex, body weight and organ weights were recorded. The foetal brain was removed from the skull within 2–3 min of maternal death before being weighed, hemisected and divided coronally into rostral, middle and caudal blocks. Blocks from the right hemisphere were snap frozen in liquid nitrogen and stored at -80 °C before being finely crushed for protein and steroid extraction. Tissues from the left hemisphere were fixed by immersion in paraformaldehyde (PFA) (4% w/v PFA in 0.1 M phosphate buffer). The blocks used in this study (middle block) contained the cerebral cortex, subcortical white matter, corpus callosum, thalamus and hippocampus.

RIA

Allopregnanolone was extracted from brain tissue and measured by RIA using previously described methods (McKendry *et al.* 2010). Briefly, crushed frozen brain tissue was treated with 50% methanol with 1% acetic acid and added to Sep-Pak C₁₈ cartridges (Waters, Milford, MA, USA) for separation of steroid components using methanol gradients. Residual methanol was removed by vacuum drying. In order to minimise cross-reactivity, the concentration of progesterone was reduced by treating the samples with potassium permanganate to oxidise non-saturated steroids. Recovery was measured by the addition of tritium-labelled allopregnanolone (1000–1500 c.p.m., 5 α -[9, 11, 12, ³H(N)]; PerkinElmer Life and Analytical Sciences, Boston, MA, USA) to each sample prior to extraction. Each sample was corrected for its extraction loss in the final calculation of allopregnanolone concentrations. The average recovery was 57.8 \pm 2.6%. Allopregnanolone was quantified by RIA using a polyclonal antibody supplied by Dr R H Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, USA). The limit of detection for allopregnanolone was 35.0 \pm 2.5 pg/tube. Intra-assay coefficient was 10.9%.

Western blot analysis

The protein expression of the neurosteroidogenic enzymes, 5 α R1 and 5 α R2, was determined by western blot analyses. Brain tissue was homogenised in RIPA protein extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with Complete Protease Inhibitor Cocktail (Roche Diagnostics Australia Pty Ltd) and PhosStop Phosphatase Inhibitor Cocktail Protein (Roche Diagnostics)). The protein concentration of each sample was then determined using BCA Protein Assay kit (Pierce, Rockford, IL, USA). Proteins (20 μ g total protein) were separated by electrophoresis on precast NuPAGE Novex 12% Bis/Tris gels (Invitrogen) before transfer to Hybond-P PVDF membrane (GE Healthcare, Sydney, NSW, Australia). Membranes were blocked in BSA Blocking Solution (5% w/v BSA, 5% w/v skim milk in 1 \times TBS-T (25 mM Tris-HCl, 15 mM NaCl, 0.1% v/v Tween-20)) for 1 h before incubation overnight in primary antibody. Goat polyclonal antibody against 5 α R1 (NB100-1491; Novus Biologicals, Littleton, CO, USA) and goat polyclonal antibody to 5 α R2 (Ab27469; AbCam, Cambridge, UK) were used at a dilution of 1:1000. The immunoreactive protein was detected using the ECL Western Blotting Detection kit (GE Healthcare) and LAS-3000 Imaging System (Fuji Photo Film, Tokyo, Japan) following incubation with anti-goat secondary antibody conjugated to HRP (P0449; DakoCytomation, Glostrup, Denmark) at 1:3000 dilution for 1 h at room temperature. The relative amount of 5 α R1 (~26 kDa) and 5 α R2 (~29 kDa) protein was quantified using Multigauge v2.4 software (Fuji Photo Film), adjusted to β -actin loading control (ab8227-50; Abcam)

and an internal control sample (human placental protein extract) present on each gel. Control membranes in which the 5 α R1 primary antibody was omitted and where the primary antibody was pre-incubated with the blocking peptide (NB100-1491PEP; Novus Biologicals) confirmed the specificity of the 26 kDa band. For 5 α R2, control membranes in which the primary antibody was omitted and replaced by goat IgG (sc-2028; Santa Cruz Biotechnology, Santa Cruz, CA, USA) confirmed specificity of the 29 kDa band.

Immunohistochemistry

In all, 8 μ m coronal sections were cut from paraffin-embedded brain tissue blocks using a rotary microtome. Sections were dewaxed in xylene and rehydrated through a graded series of ethanol/water washes. Endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide in methanol. Antigen recovery was performed with Reveal It Solution (ImmunoSolution Pty Ltd, Everton Park, Qld, Australia) as per the manufacturer's instructions, before being blocked with BSA in PBS (0.1 M PBS, pH 7.2 with 0.5% w/v BSA, 0.05% w/v saponin and 0.05% v/v sodium azide). Sections were then incubated in 1:5000 dilution of either glial fibrillary acidic protein (GFAP) (monoclonal anti-GFAP antibody (G3893; Sigma-Aldrich)), myelin basic protein (MBP) (monoclonal anti-MBP antibody (M9434; Sigma-Aldrich)) or activated caspase-3 (anti-h/m caspase-3 active (AF835; R&D Systems, Minneapolis, MN, USA)) primary antibodies for 3 days. Sections were washed in PBS, followed by incubation in the appropriate biotinylated secondary antibodies: polyclonal rabbit anti-mouse (E0354; DakoCytomation), anti-rat IgG (B7139; Sigma-Aldrich) and anti-rabbit IgG (BA1000; Vector Labs, Burlingame CA, USA) respectively. Sections were then washed and incubated overnight in streptavidin-biotin-HRP complex (RPN1051V; Amersham). Labelling was revealed using 3,3'-diaminobenzidine tetrahydrochloride solution as a chromagen. Sections were mounted with DEPX (Merck) and examined using a Zeiss Axioskop Microscope. Images were acquired using a SPOT RT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). GFAP, MBP and caspase-3 immunostaining were examined in subcortical white matter and hippocampal CA1; additionally, GFAP-stained sections from the dentate region of the hippocampus were also analysed. The GFAP and MBP immunoreactivities were analysed by densitometry using ImageJ 1.40 (National Institutes of Health, Bethesda, MD, USA) and made binary by adjusting the threshold manually, with the per cent area of coverage recorded for four fields of view per region on two sections per animal. Cells positive for activated caspase-3 that showed apoptotic morphology were counted in four fields of view per region (cortex and hippocampal CA1) on two sections per animal. Controls for specificity of primary antibodies were run using the appropriate IgG substituted for each primary antibody.

Statistical analysis

Statistical analysis was carried out using PASW Statistics Package v18.0 for Mac (SPSS, Inc., Chicago, IL, USA). Multiple regression was used to model the measured and continuous outcome variables against the three categorical predictors: finasteride, IUGR and sex, using backwards stepwise mode. Two-way interactions were tested. A two-tailed significance level of 5% was used throughout. The dependent variables were transformed as required in order to satisfy model assumptions. Subgroup analysis of means was carried out using two-tailed Student's *t*-test where appropriate. Significance levels of $P < 0.05$ are represented with asterisks in Figs 1–4, where applicable.

Results

Foetal characteristics

The mean body and organ weights for foetal guinea pigs are shown in Table 1. A brain to liver weight ratio (BLR) of $> 90\%$ was used to define asymmetric growth restriction in foetuses that received IUGR surgery. Animals that received sham surgeries had BLRs of $\sim 55\%$, both with and without finasteride treatment.

Regression analyses found that foetal guinea pigs with IUGR had significantly reduced placenta weight ($P < 0.001$), liver weight ($P < 0.001$) and BLRs ($P < 0.001$). Finasteride treatment was not associated with foetal body weight change. Following adjustment for other variables, foetal body weight was significantly reduced in IUGR foetuses ($P < 0.001$), with the overall body weight of female foetuses, across all treatment groups, reduced in comparison to male animals ($P = 0.040$). Mean foetal brain weight was significantly reduced in IUGR foetuses ($P = 0.005$) and a marginally significant reduction in brain weights was observed in female foetuses compared with those in males ($P = 0.051$). Finasteride treatment alone was not associated with foetal body weight change. Interestingly, however, there was a significant positive interaction effect ($P = 0.020$) between finasteride treatment and IUGR.

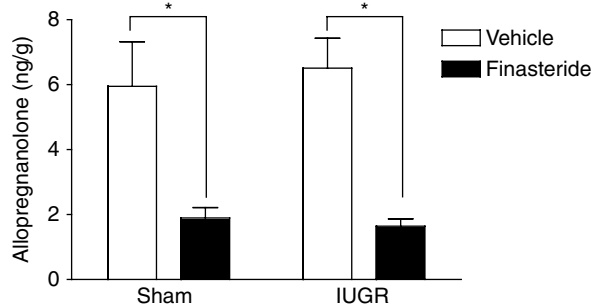


Figure 1 Foetal brain allopregnanolone concentrations with finasteride treatment and IUGR. Relative expression measured by RIA and expressed as mean allopregnanolone (ng/g of wet weight tissue) concentrations \pm S.E.M. ($n = 8-10$). *Significant difference between vehicle- and finasteride-treated animals, $P < 0.05$ (Student's *t*-test).

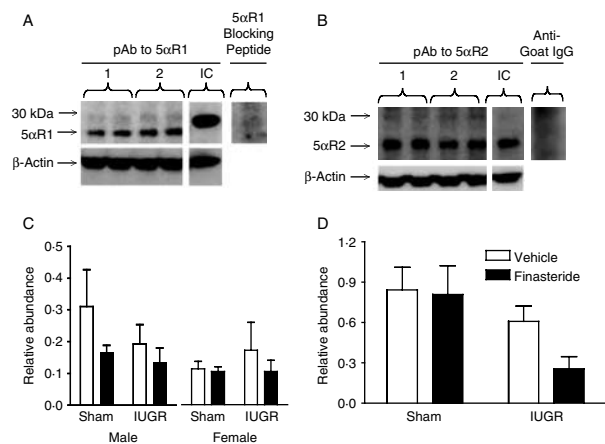


Figure 2 Effect of IUGR and finasteride treatment on the expression of 5αR1 and 5αR2 enzymes in the foetal guinea pig brain.

Representative western blots showing two paired guinea pig brain tissue samples and human placenta internal control (20 μg total protein per lane) and corresponding β-actin loading controls for 5αR1 (A; 26 kDa band) and 5αR2 (B; 29 kDa band). Pre-incubation of primary 5αR1 antibody with blocking peptide blocked specific binding at 26 kDa in pooled guinea pig brain sample. Goat normal IgG showed no binding at 29 kDa in pooled guinea pig brain sample. The expressions of 5αR1 for male and female foetuses (C; $n = 4-6$) and 5αR2 (D; $n = 8-10$) with IUGR and finasteride treatment are expressed as mean relative abundance \pm S.E.M.

Foetal brain allopregnanolone concentrations

Figure 1 presents the mean foetal brain concentrations of allopregnanolone with and without IUGR and finasteride treatment. Control animals (sham + vehicle) had average brain allopregnanolone concentrations of 11.63 ± 2.33 ng/g. Overall, finasteride treatment significantly reduced brain allopregnanolone concentrations ($P = 0.001$). Normally grown animals that received finasteride in late gestation had reduced average brain allopregnanolone concentrations of 4.20 ± 0.51 ng/g. IUGR alone did not significantly affect allopregnanolone concentrations in the foetal brain, with a mean allopregnanolone brain concentration of 7.72 ± 1.43 ng/g. Brain allopregnanolone was reduced by up to 75% in IUGR foetuses treated with finasteride (3.09 ± 0.57 ng/g) compared to control.

Brain 5αR enzyme expression

The mean abundances of 5αR1 and 5αR2 enzymes, expressed relative to internal control and β-actin loading control, are shown in Fig. 2C and D respectively. Representative western blots for both isoforms (Fig. 2A and B) with results for negative controls are shown for blots incubated in the presence of 5αR1 antibody blocking peptide and anti-goat pre-immune serum for 5αR2, demonstrating specificity of antibody staining. Foetal sex was significantly associated with the expression of the 5αR1 enzyme isoform ($P = 0.008$), with female foetuses having

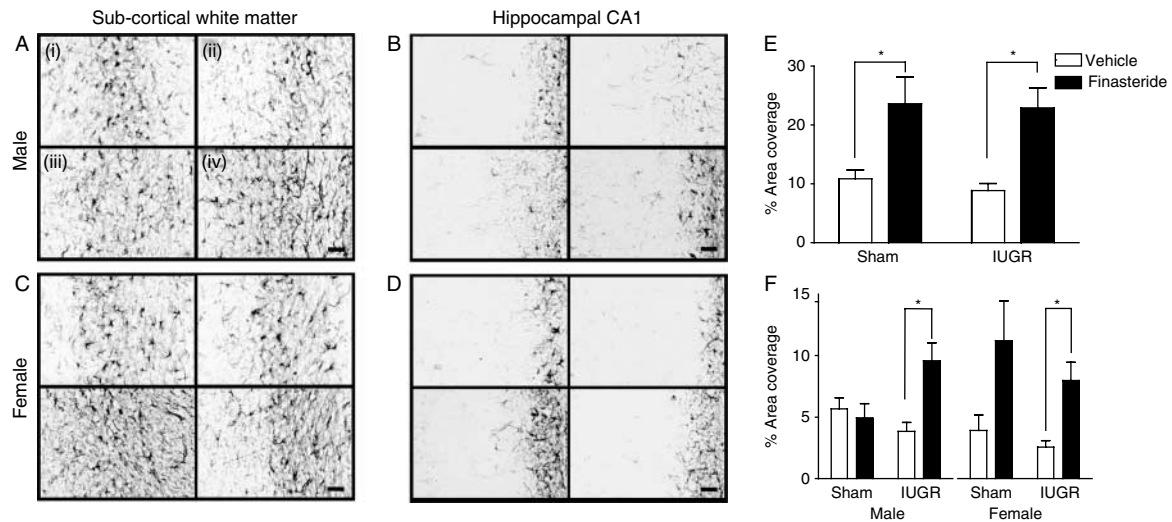


Figure 3 Effect of IUGR and finasteride treatment on glial fibrillary acidic protein (GFAP) immunostaining in the subcortical white matter (A, C and E) and the hippocampal CA1 region (B, D and F). Representative micrographs show staining in male (A and B) and female (C and D) foetal guinea pig brain regions. Staining is expressed as mean percentage coverage of total area \pm S.E.M. (E and F). Foetuses were normally grown, with vehicle (i; sham+veh) or finasteride treatment (iii; sham+fin) and growth restricted with vehicle (ii; IUGR+veh) or finasteride treatment (iv; IUGR+fin). In E ($n=10-12$) and F ($n=6-7$ males, $n=5-6$ female foetuses) * indicates significant difference between vehicle and finasteride treatment for male and female foetuses with IUGR ($P<0.05$; Student's *t*-test). Horizontal scale bars represent 0.1 mm.

lower expression than males. Finasteride and IUGR had no significant effect on the $5\alpha R1$ expression. IUGR was marginally associated with a reduction in the expression of $5\alpha R2$ in the foetal brain ($P=0.060$, Fig. 2B and D). Finasteride and foetal sex were not associated with changes in the $5\alpha R2$ expression.

GFAP expression

Figure 3A and C show GFAP immunostaining in the subcortical white matter in male and female foetuses respectively with foetuses that received sham or IUGR surgery and subsequently administered with vehicle or finasteride. The greatest density of GFAP staining appeared to be present in finasteride-treated IUGR male foetuses and finasteride-treated females (Fig. 3A(iii) and C(iii)), however, no significant effect of foetal sex was identified. Animals that were vehicle treated and received either IUGR or sham surgery showed no qualitative increase in GFAP staining in this region. The mean densities of GFAP staining, by proportion of area coverage, for treatment groups with sham and IUGR surgery and vehicle and finasteride treatments are shown in Fig. 3E. Data for the dentate region are not shown.

Regression analysis of GFAP coverage showed that finasteride treatment was associated with a significant increase in GFAP expression in the subcortical white matter ($P<0.001$) and dentate ($P=0.009$) regions. No significant interactions with or effects of IUGR or sex were observed in these regions. This was also confirmed by subgroup analysis

of means, which indicated that in both sham and IUGR subgroups finasteride-treated foetuses had a significant increase in GFAP expression (Fig. 3E, $P<0.05$).

In the CA1 region of the hippocampus, male foetuses with finasteride treatment (Fig. 3B(iv)) and female IUGR foetuses with finasteride treatment (Fig. 3D(iii)) appeared to have the highest degrees of GFAP expression. As an effect of foetal sex was identified (see regression model below), Fig. 3F presents the mean proportion of GFAP staining in the CA1 region divided by treatment groups and foetal sex. Regression analysis of density of GFAP staining in the hippocampal CA1 region found that IUGR was not associated with a change in GFAP expression. A marginally significant increase in overall GFAP expression in this region was observed in female guinea pigs compared with males ($P=0.051$) and while finasteride treatment alone did not have a significant effect on GFAP in the CA1 ($P=0.625$), there was a significant positive interaction between finasteride treatment and sex ($P=0.021$). A subgroup analysis of means indicated that in the CA1 region, both male and female IUGR foetuses significantly increased GFAP expression compared to IUGR vehicle foetuses of the same sex (Fig. 3F, $P<0.05$).

MBP expression

In both the subcortical white matter (Fig. 4A and C) and CA1 region of the hippocampus (Fig. 4B and D), MBP expression was highest in control male (i) and control female foetuses (i). There appeared to be a large reduction in MBP immunostaining in animals that were both growth restricted and

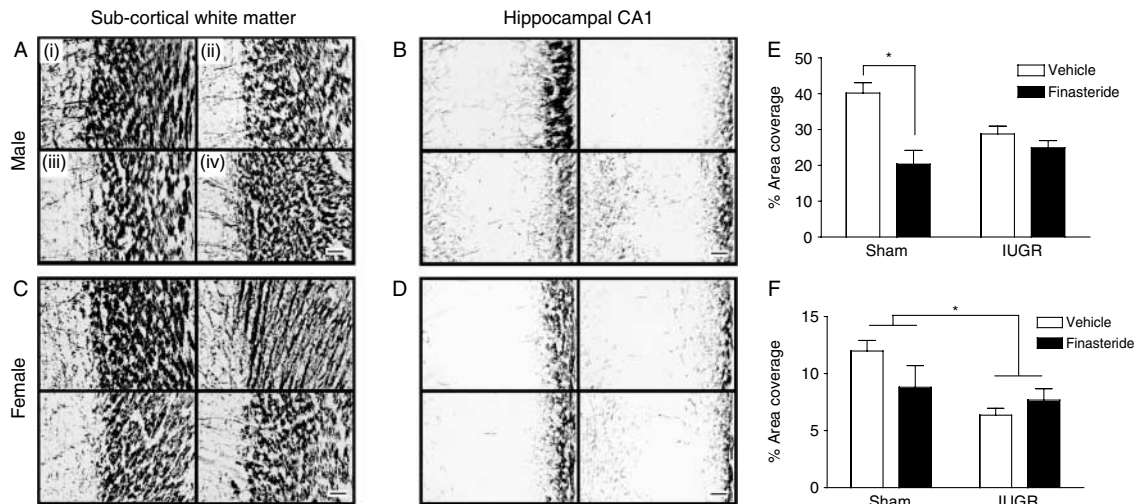


Figure 4 Effect of IUGR and finasteride treatment on myelin basic protein (MBP) immunostaining in the subcortical white matter (A, C and E) and the hippocampal CA1 region (B, D and E). Representative micrographs show staining in male (A and B) and female (C and D) foetal guinea pig brain regions. Staining is expressed as mean percentage coverage of total area \pm s.e.m. (E and F; $n=10-12$). Foetuses were normally grown, with vehicle (i; sham+veh) or finasteride treatment (iii; sham+fin) and growth restricted with vehicle (ii; IUGR+veh) or finasteride treatment (iv; IUGR+fin). (E) * Indicates significant difference between vehicle- and finasteride-treated sham foetuses. (F) * Indicates significant difference between sham and IUGR foetuses ($P<0.05$; Student's *t*-test). Horizontal scale bars represent 0.1 mm.

received finasteride treatment. The mean per cent coverage of MBP for each treatment group is presented in Fig. 4E and F.

Multiple regression analysis identified a significant reduction in MBP expression in the subcortical white matter associated with finasteride treatment ($P=0.001$) and IUGR ($P=0.003$). A significant positive interaction between finasteride and IUGR was also identified ($P=0.044$). Additional subgroup analysis of means showed a significant reduction in MBP expression between sham vehicle- and finasteride-treated foetuses (Fig. 4E, $P<0.05$) but not in IUGR foetuses with the different drug treatments, indicating a mediation of the effect of finasteride in the presence of IUGR. Foetal sex did not affect MBP expression in this region.

Multiple regression and subgroup analysis identified an association between IUGR and reduced MBP expression in the CA1 region of the hippocampus ($P<0.001$). The different pattern of expression of vehicle and finasteride animals between the sham and IUGR groups (Fig. 4F) suggests an interplay between finasteride treatment and IUGR, which was supported by the regression model showing a significant interaction effect between IUGR and finasteride treatment ($P=0.016$), despite finasteride treatment alone not having a significant effect ($P=0.116$). Foetal sex did not have a significant effect on MBP expression in this region.

Activated caspase-3 expression

There was no significant effect of finasteride treatment, IUGR or foetal sex on the number of activated caspase-3-positive cells in the CA1 region of the hippocampus of the foetal guinea pigs in this study (data not shown).

Discussion

The neurosteroid allopregnanolone has many associated neuroprotective, developmental and regulatory effects. This study examined late gestation brain development in foetal guinea pigs with the chronic gestational insult, IUGR, in the presence and absence of high late gestation allopregnanolone concentrations. The current model involves chronic placental insufficiency resulting in significantly lower birth weights, with relative brain sparing, typical of IUGR pregnancies. IUGR can be thought of as a chronic adaptation in the foetus that becomes maladaptive, potentially disrupting the neurodevelopmental actions of neurosteroids and leading to strong associations between IUGR, poor neonatal outcome and perinatal brain injury.

In this study, the chronic administration of finasteride during late gestation was successful in markedly reducing allopregnanolone concentrations in the foetal guinea pig brain. It has been proposed that this late gestation reduction in foetal brain allopregnanolone may mimic the change in brain neurosteroid concentration that occurs when a foetus is born preterm and the placenta, as a major source of progesterone, an important precursor of allopregnanolone, is prematurely removed (Hirst *et al.* 2006). The ability of the preterm infant to synthesise important neurosteroids independently of placentally derived precursors may be limited and the effect of this decline in endogenous steroids on preterm *ex utero* brain development may influence the vulnerability of the preterm neonatal brain to injury.

Overall, male foetuses were found to have higher levels of expression of $5\alpha R1$ than female foetuses. This may be related to the action of $5\alpha R2$ in androgen and testosterone synthesis

Table 1 Body and organ weight characteristics of foetal guinea pigs. Values are expressed as mean \pm S.E.M.

	<i>n</i>	Body Wgt	Placenta Wgt	Brain Wgt	Liver Wgt	BLR
Control						
Female	6	85.9 \pm 3.3	6.3 \pm 0.2	2.3 \pm 0.04	4.1 \pm 0.3	0.57 \pm 0.04
Male	9	91.1 \pm 2.6	6.0 \pm 0.3	2.4 \pm 0.02	4.6 \pm 0.2	0.54 \pm 0.03
Total	15	89.0 \pm 2.1	6.1 \pm 0.2	2.4 \pm 0.03	4.5 \pm 0.2	0.55 \pm 0.03
IUGR						
Female	8	57.9 \pm 4.2	4.3 \pm 0.7	2.2 \pm 0.03	2.5 \pm 0.3	1.06 \pm 0.22
Male	8	59.5 \pm 5.5	4.4 \pm 0.5	2.3 \pm 0.08	2.5 \pm 0.3	1.03 \pm 0.17
Total	16	58.7 \pm 3.4	4.3 \pm 0.4	2.3 \pm 0.04	2.5 \pm 0.2	1.04 \pm 0.13
Fin						
Female	4	84.3 \pm 7.0	5.0 \pm 0.3	2.3 \pm 0.03	4.1 \pm 0.6	0.60 \pm 0.08
Male	7	90.4 \pm 3.1	6.2 \pm 0.5	2.3 \pm 0.04	4.6 \pm 0.4	0.52 \pm 0.05
Total	12	87.4 \pm 3.0	5.8 \pm 0.4	2.3 \pm 0.03	4.4 \pm 0.3	0.56 \pm 0.04
IUGR+Fin						
Female	8	53.4 \pm 3.5	3.2 \pm 0.5	2.0 \pm 0.29	2.4 \pm 0.1	0.95 \pm 0.08
Male	3	68.0 \pm 6.4	4.1 \pm 0.3	2.4 \pm 0.08	2.8 \pm 0.2	0.86 \pm 0.04
Total	17	57.1 \pm 2.4	3.6 \pm 0.2	2.2 \pm 0.12	2.5 \pm 0.1	0.93 \pm 0.05

Wgt, weight (g); BLR, brain to liver ratio; control represents sham, vehicle-treated animals; IUGR, intrauterine growth restriction; Fin, finasteride.

as, along with allopregnanolone, 5 α R action is also involved in the synthetic pathways of other steroid hormones. In this study, IUGR did not affect foetal brain concentrations of allopregnanolone. By subgroup analysis, IUGR foetuses had a reduction in the type 2 5 α R isoform of the enzyme. A previous study carried out by members of our laboratory showed an upregulation of 5 α R2 in the foetal sheep brain following late gestation chronic umbilico-placental embolisation (Nguyen *et al.* 2003). This may be explained by the different modes of chronic insults used in the two models. Both models showed no change in brain allopregnanolone concentrations despite the changes noted in enzyme expression. Local reductions in allopregnanolone may have been present at the initiation of the chronic-type insults, however, this may not persist to the end of gestation. Acute or transient hypoxic insults during pregnancy have been shown to cause an increase in foetal brain concentrations of allopregnanolone, for a period immediately following the insult (Nguyen *et al.* 2004). This raises the relative importance of the placenta and other glandular sources for the supply of allopregnanolone and its precursors and the ability for this system to compensate for chronic disruptions of supply, particularly when, as often occurs with IUGR, placental growth or function is compromised. The finding of reduced 5 α R2 expression in the brain of growth-restricted foetuses suggests that IUGR may further limit their neurosteroid synthetic capacity following birth. Such a disruption of neurosteroid synthesis or supply potentially predisposes the vulnerability of the IUGR foetus to neurodevelopmental disorders. The relative importance or otherwise of the various sources of neurosteroids is still unclear and the elucidation of these mechanisms may be useful for the understanding of neurosteroid function in both normal pregnancies and when chronic pathological gestational changes are present.

A key finding of this study is the effect of finasteride treatment and the resultant low concentrations of

allopregnanolone on myelination. Correct myelination is essential for the conduction of brain signals and disruption of brain myelination processes during development can lead to lasting neurological, cognitive and motor effects. In the subcortical white matter region, finasteride was associated with significantly reduced myelination and in addition, a significant interaction was also identified between finasteride treatment and IUGR in both regions examined. This interaction indicates that the effect of chronically low foetal brain allopregnanolone concentrations on myelination is altered in foetuses with a chronic perturbation of growth. This is suggestive of a protective mechanism acting in the presence of chronic IUGR, however the mechanism is unclear. These processes may involve the IUGR-induced reduction in 5 α R2 enzyme and consequently a chronic reduction in the conversion of progesterone into other steroids, prior to finasteride treatment. Progesterone has been shown to have positive effects on myelination in the developing CNS, potentially by the enhancement of maturation processes of oligodendrocyte precursors into myelinating oligodendrocytes (Ghousari *et al.* 2003, 2005). Therefore, whilst allopregnanolone has been shown to have positive effects on MBP expression, progesterone appears to have a more potent effect. The future investigation of direct progesterone–progesterone receptor effects in this guinea pig model of IUGR may therefore help to explain the changes observed in foetal brain MBP expression. Together the present observations of changes in MBP expression are consistent with the studies that have previously suggested a trophic effect of neurosteroids on the late gestation myelination (Schumacher *et al.* 2000) and support the concept that the low neurosteroid levels may negatively impact myelination when endogenous neurosteroid supply is disrupted due to impaired placental function or preterm birth.

The finding, in this study, that GFAP expression increased when allopregnanolone synthesis was inhibited supports a key regulatory role of this steroid. GFAP is expressed in astrocytes throughout development and when activated by pathological processes (Eng *et al.* 2000). The increase in GFAP expression may be a consequence of the loss of allopregnanolone-mediated inhibition of neural excitability and hence an increase in excitotoxic cellular processes, cell death and damage. This study identified no significant associations between IUGR and GFAP expression. This is consistent with a previous study that demonstrated no marked difference in GFAP-positive cells in growth-restricted foetal guinea pig brains (Nitsos & Rees 1990). *In vitro* studies in organotypic brain slice cultures have previously shown that pretreatment with allopregnanolone reduces astrogliosis following a hypoxic insult (Kruse *et al.* 2009). Studies of traumatic brain injury in adult rats have also shown that both progesterone and allopregnanolone administration reduces the size of GFAP-positive astrocytes (Djebaili *et al.* 2005). Despite the increase in GFAP expression when allopregnanolone synthesis was inhibited, qualitative analysis identified no overt areas of glial scarring or obvious signs of damage related to GFAP staining.

We have previously demonstrated the neuroprotective role of allopregnanolone in the foetal sheep brain, when allopregnanolone synthesis was inhibited transiently and acutely. The suppression of allopregnanolone synthesis increases markers of cell proliferation, an effect that is ameliorated when the synthetic neurosteroid, alfaxalone, is used to replace the loss of the endogenous neurosteroid (Yawno *et al.* 2009). In adult rat models of traumatic brain injury, allopregnanolone administration has also reduced the size of GFAP-positive astrocytes at the site of the experimental lesions (Djebaili *et al.* 2005), and there is increasing evidence for the effectiveness of allopregnanolone in reducing lesion size and enhancing functional recovery in animal models of traumatic brain injury (Djebaili *et al.* 2004, 2005, Schumacher *et al.* 2007). In addition to the previously reported neuroprotective actions of allopregnanolone, this study provides evidence of delayed neurodevelopment in the presence of a low neurosteroid environment and suggests an important role of allopregnanolone in the regulation of developmental processes in the late gestation foetal brain. These findings support the concept that the loss of endogenous neurosteroids and long-term physiological stressors *in utero* may contribute to the vulnerability of the prematurely born IUGR neonate to abnormal brain development and function. We have also shown that the neurosteroid response of the foetus to an *in utero* insult is highly dependent on the type and length of insult. The potentially important role of neurosteroids in the regulation of developmental processes and neuroprotection in the foetal brain may provide new directions for the prevention and treatment of neurological disorders that are associated with perinatal insults and pathologies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Health and Medical Research Council of Australia (project grant ID#455527).

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Received in final form 16 November 2010

Accepted 13 December 2010

Made available online as an Accepted Preprint

13 December 2010