Journal of Developmental Origins of Health and Disease (2015), **6**(3), 250–260.

doi:10.1017/S2040174415000069
Themed Issue: Preterm Birth

ORIGINAL ARTICLE

Preterm birth affects $GABA_A$ receptor subunit mRNA levels during the foetal-to-neonatal transition in guinea pigs

J. C. Shaw[1,2]*, H. K. Palliser[1,2], D. W. Walker[3] and J. J. Hirst[1,2]

[1]*School of Biomedical Sciences and Pharmacy, University of Newcastle, Newcastle, Australia*
[2]*Hunter Medical Research Institute, Mother and Babies Research Centre*
[3]*Monash Institute of Medical Research, Ritchie Centre*

Modulation of gamma-aminobutyric acid A ($GABA_A$) receptor signalling by the neurosteroid allopregnanolone has a major role in late gestation neurodevelopment. The objective of this study was to characterize the mRNA levels of $GABA_A$ receptor subunits (α4, α5, α6 and δ) that are key to neurosteroid binding in the brain, following preterm birth. Myelination, measured by the myelin basic protein immunostaining, was used to assess maturity of the preterm brains. Foetal guinea pig brains were obtained at 62 days' gestational age (GA, preterm) or at term (69 days). Neonates were delivered by caesarean section, at 62 days GA and term, and maintained until tissue collection at 24 h of age. Subunit mRNA levels were quantified by RT-PCR in the hippocampus and cerebellum of foetal and neonatal brains. Levels of the α6 and δ subunits were markedly lower in the cerebellum of preterm guinea pigs compared with term animals. Importantly, there was an increase in mRNA levels of these subunits during the foetal-to-neonatal transition at term, which was not seen following preterm birth. Myelination was lower in preterm neonatal brains, consistent with marked immaturity. Salivary cortisol concentrations, measured by EIA, were also higher for the preterm neonates, suggesting greater stress. We conclude that there is an adaptive increase in the levels of mRNA of the key $GABA_A$ receptor subunits involved in neurosteroid action after term birth, which may compensate for declining allopregnanolone levels. The lower levels of these subunits in preterm neonates may heighten the adverse effect of the premature decline in neurosteroid exposure.

Received 3 July 2014; Revised 7 January 2015; Accepted 12 January 2015; First published online 9 February 2015

Key words: $GABA_A$ receptors, neurosteroids, preterm birth

Introduction

Preterm birth is the leading cause of death and neurodevelopment-related disability in neonates, accounting for up to 70% of neonatal deaths, and with ∼50% of the survivors developing a long-term neurodevelopmental disability.[1–3] Of the 133 million births each year, roughly 10% are preterm.[4] There is a growing body of evidence suggesting that late preterm infants are more likely to develop neurodevelopmental morbidities and exhibit poor school performance compared with term infants.[5–7] Furthermore, mothers and teachers of children who are born moderately to late preterm indicate higher rates of anxious and depressed behaviour in conjunction with learning difficulties at primary school age.[8] Aiding postnatal development of vulnerable brain regions following preterm birth remains a therapeutic target for the prevention of these neurodevelopmental disorders. Two regions of the brain that are particularly vulnerable to damage and developmental delay following preterm birth are the hippocampus and the cerebellum.[9,10] Development and maturation of the cerebellum, which is responsible for the regulation and co-ordination of movement with additional roles in attention and language, continues throughout late gestation until after birth in the guinea pig and in humans.[11] The same is true for the hippocampus, which has a major role in learning, memory formation and spatial recognition.[12]

Myelination, which occurs during late gestation, is reduced in neonates born preterm and may be the result of a reduced number of mature oligodendrocytes at the time of birth.[13,14] Appropriate levels of excitability are essential for normal neurodevelopment, including myelination,[13,15–17] and during foetal life they are regulated by the suppressive action of the neurosteroid allopregnanolone in the foetal brain.[18,19] Throughout gestation, the precursors required for allopregnanolone synthesis are supplied in high concentrations by the placenta and are, therefore, lost at birth. Thus, allopregnanolone levels decline rapidly after removal of the placenta and reach levels observed in neonates within 24 h after birth.[20] The resultant decrease in neurosteroid exposure may contribute to the continued delay in myelination following preterm birth, in addition to reducing inhibitory tone and exposing these immature brains to damaging excitotoxicity. Neurosteroids, including allopregnanolone, exert their suppressive action by increasing GABAergic inhibition. These effects of allopregnanolone on central nervous system (CNS) activity are due to agonist actions at the gamma-aminobutyric acid A ($GABA_A$) receptors, specifically to enhance $GABA_A$ receptor-mediated inhibition.[19,21] Steroid-sensitive $GABA_A$ receptors are highly

*Address for correspondence: J. C. Shaw, Mothers and Babies Research Centre, Hunter Medical Research Institute, University of Newcastle, Callaghan, NSW 2308, Australia.
(Email julia.shaw@uon.edu.au)

expressed throughout the foetal brain from mid-gestation, including on oligodendrocytes.[22] Compared with the receptors found in the adult brain, those found in the foetal brain are more sensitive to modulation by allopregnanolone and are strongly activated by the concentrations of allopregnanolone found in foetal brain extracts.[23] The premature loss of allopregnanolone supply following preterm birth, therefore, may have consequences on $GABA_A$ receptor expression and ultimately in the reduction of inhibitory tone in the developing brain. Excessive excitation is damaging during the second half of gestation, and in long-gestation species such as the guinea pig and the sheep the $GABA_A$-mediated inhibitory pathway is active in the foetal brain from mid-gestation onwards.[22,24,25] Animal studies have shown that reducing allopregnanolone levels in the foetus *in utero* leads to increased cell death within the brain and a reduction in myelination.[24,26,27] Interestingly, there is evidence demonstrating the plastic nature of $GABA_A$ receptors, as neurosteroid withdrawal increases the expression of the α4 subunit.[28] This highlights the adaptive nature of $GABA_A$ receptors and the potential of replacement therapies that increase neonatal allopregnanolone concentrations to prevent excitotoxicity and associated cell death in premature brains.

$GABA_A$ receptors exist in a pentameric form comprising five subunits. The subunit composition of receptors varies greatly. Extra-synaptic subunits, which have a major role in tonic inhibition, commonly comprise α4, α5, α6 and δ subunits, with the expressions of α4 and α5 subunits high in the hippocampus and that of α6 and δ subunits predominant in the cerebellum.[29] Receptors containing these four subunits are highly sensitive to modulation by neurosteroids with consequent suppression of foetal CNS excitation,[30] whereas reduced expression of these receptor subunit types may lead to excitotoxic brain injury and suboptimal development. Previous studies have shown that $GABA_A$ receptor subunit expression is influenced by changes in glucocorticoid exposure. Specifically, acute stress has been linked with the increased expression of α5 and δ subunits along with raising baseline tonic conductance, which may be a compensatory mechanism to cope with the stress and prime for re-exposure.[31,32] Alternatively, chronic stress has been found to have the opposite effect, lowering expressions and overall inhibitory functions of the $GABA_A$ receptors.[33] Early exposure of the foetus to the *ex utero* environment is a potentially highly stressful situation; however, it is unclear whether this exposure may have a further effect on the expressions of $GABA_A$ receptor subunits in the newborn brain. The potential alterations to the $GABA_A$ receptor subunit expression profile following preterm birth warrants investigation, as this appears to be one component involved in the susceptibility of preterm brains to *ex utero* vulnerability and subsequent development of long-term neurodevelopmental disorders, and will, therefore, aid in developing effective prevention strategies. The objective of the present study was to characterize the mRNA levels of key $GABA_A$ receptor subunits in the hippocampus and cerebellum of preterm and term guinea pig foetuses and 1-day-old neonates. In addition, myelination and cortisol concentrations were assessed in these neonates to ascertain neurodevelopmental immaturity and *ex utero* stress exposure. We hypothesize that preterm animals will have lower $GABA_A$ receptor subunit mRNA levels. Furthermore, we suggest that this immaturity will be associated with reduced myelination within the hippocampus and cerebellum, and with high salivary cortisol levels, once exposed to the *ex utero* environment.

Method

Unless specified otherwise, all basic reagents and chemicals were supplied by Sigma Aldrich (Castle Hill, NSW, Australia).

Animals

Before beginning this work, approval for all the animal experiments and procedures carried out throughout this study was obtained from the University of Newcastle Animal Care and Ethics Committee. In addition, all experiments and procedures were carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Time-mated outbred tricolour guinea pigs were obtained from the University of Newcastle Research Support Unit. Guinea pigs were housed indoors with a 12-h light/dark cycle, and were supplied with a diet consisting of commercial guinea pig pellets, Lucerne hay and water supplemented with ascorbic acid. The following animal groups were studied: term and preterm foetuses, and term and preterm neonates at 24 h of age. One male and one female foetus or neonate was used from each dam to prevent pregnancy bias. Term (GA69) and preterm (GA62) neonates were delivered by caesarean section as previously described.[20] After vigorously rubbing and quickly inverting each pup to remove fluid from the airways and to stimulate respiration, a 50 µl dose of surfactant (Curosurf, 80 mg/ml Poractant alfa, Douglas Pharmaceuticals, Baulkham Hills, NSW, Australia) was administered into the oropharynx. To encourage respiration, continuous positive airway pressure (CPAP) was administered using a small animal anaesthesia mask (Harvard apparatus, Holliston, MA, USA) attached to a Neopuff infant T-piece resuscitator (Fisher and Paykel Healthcare, Melbourne, VIC, Australia). Positive end expiratory pressure of 7 mm H_2O and peak inspiratory pressure (PIP) of 20 mm H_2O were applied at a flow rate of 8 l/min. Medical oxygen and air were adjusted to give a fraction of inspired O_2 of 60% during CPAP. An initial sustained PIP of 20 s was administered. CPAP and PIP were performed again when respiration became unstable, and an additional dose of 50-µl surfactant was administered at 3 h. Neonates were then placed in a humidified incubator (small animal intensive care incubator, Thermocare, Incline Village, NV, USA) at 34°C.

Pups were monitored for well-being, fed using a gastric tube and their saliva was collected every 2 h, up until 24 h.[20] The monitoring for well-being involved scoring respiration, posture

and alertness out of a maximum of 12 points at every 2-h interval. Each category was assigned a score from 0 to 4 (with 0 being the poorest and 4 being the optimal score); scores were then added together to give a total out of 12. Total scores between 0 and 3 indicated very poor well-being, 4–6 indicated poor well-being, 7–9 indicated good well-being and 10–12 indicated very good well-being.

The feeding regime used was commercial guinea pig milk-replacement formula (Wombaroo Food Products, Adelaide, SA, Australia), at 100 µl/g/24 h, made up in 50% v/v water and glucose solution (5% glucose solution, Baxter Healthcare). Following the fourth feed, glucose solution was no longer added to the milk formula.

Immediately after the 24-h cortisol sample collection, the neonates were euthanized and tissues were collected. Term and preterm foetal tissues were also obtained from pups at the time of caesarean section. At the time of tissue collection, body and organ weights were recorded. Each brain was sectioned down the midline in the sagittal plane to separate the two hemispheres. The left hemisphere was fixed for immunohistochemistry, whereas the right hemisphere was further dissected and frozen in liquid nitrogen and used for further processing, including RT-PCR.

Immunohistochemistry

Immunodectection of myelin basic protein (MBP), a protein that is present in mature myelinating oligodendrocytes, was used to assess myelination within the neonatal brains. Hippocampal CA1, sub-cortical white matter and cerebellum sections were immunostained for MBP as previously described.[27] In brief, for each region, 7-µm sections were cut using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, NSW, Australia). Before immunostaining, each slide was de-waxed and incubated in methanol containing 3% hydrogen peroxide. Antigen retrieval was performed using Reveal-It solution (ImmunoSolution Pty Ltd, QLD, Australia), according to the manufacturer's instructions. Blocking for 30 min occurred at room temperature in bovine serum albumin (BSA) Blocking Solution (0.5% w/v BSA, 0.05% w/v Saponin, 0.05% v/v Sodium Azide in 0.1 M PBS). Primary antibody (rat monoclonal anti-MBP antibody, M9434; Sigma-Aldrich) diluted to 1:4000 in BSA Blocking Solution was incubated overnight at room temperature. The secondary antibody, biotinylated anti-rat IgG (B7139; Sigma-Aldrich) diluted to 1:300 in BSA Blocking Solution, was incubated for 2 h at room temperature. Finally, incubation in the tertiary reagent, streptavidin–biotin–horseradish peroxidase complex (RPN1051V; Amersham), diluted to 1:300 in blocking solution (instead of Sodium Azide, 0.05% v/v thimerasol was added), was incubated for 2 h at room temperature. To reveal the immunolabelling, incubation in 3,3′-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; Pierce) was carried out. The samples were stained with cresyl violet and coverslipped using DEPX (Merck) the following day.

Stained slides were imaged using a Nikon upright microscope eclipse Ni-U with a Nikon DS-Ri1 camera attached (Nikon Instruments Inc., New York, USA) and NIS-Elements Advanced Research software (Nikon Instruments Inc.). Four consecutive images of the CA1 region of the hippocampus, sub-cortical white matter, lobe VIII, lobe X and deep white matter of the cerebellum from two sections per region were used for each animal. To quantify MBP expression, ImageJ (version 1.47, National Institutes of Health, USA) was used to calculate the area coverage of MBP expression. This was carried out by, first, removing the background and converting the image to 8-bit greyscale. The threshold of the image was then adjusted until all processes were visible, enabling the programme to calculate the total area coverage percentage of the stained regions. An overall average of MBP area coverage was then calculated for each neonate by taking the average of the four images captured per section.

Real-time PCR

Real-time PCR was performed as previously described.[34] Frozen cerebellum and hippocampus samples were homogenized in RLT Plus Buffer (obtained from the Qiagen RNeasy Plus Mini Kit, Qiagen Pty Ltd, VIC, Australia) using Precellys 24 dual-tissue homogenizer (Bertin Technologies, France). Extraction of RNA from the homogenate was carried out using the Qiagen RNeasy Plus Mini Kit by following the manufacturer's instructions. The NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to quantify the RNA in each sample. RNA purity was also confirmed using the NanoDrop, with A260/A280 ratios of 2.1–2.19 obtained for each sample. The RNA was then run on 1% agarose gel to confirm quality and integrity. The gels were imaged using a UVP benchtop UV transilluminator chamber (BioDoc-It Imaging System, Upland, CA, USA), and were examined for bands at 18S and 28S at a ratio of 1:2.

cDNA was synthesized on the GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, VIC, Australia) using the Superscript III Reverse Transcription kit (Invitrogen), according to manufacturer's instructions. Five primer pairs (α4, α5, α6 and δ $GABA_A$ receptor subunits, as well as the housekeeping gene β-actin) were designed and optimized within our laboratory for detection in the guinea pig and are detailed in Table 1. For each primer, a primer master mix concentration of 5 pmole/µl was used. The SYBR Green (Applied Biosystems) DNA-binding dye method of RT-PCR was used to detect the PCR products of each gene examined. Master mixes containing SYBR green, forward and reverse primers and MilliQ water (Millipore system treatment includes RNase and DNase filtration) were made up to final primer concentrations of 100 nM for the $GABA_A$ receptor α4 subunit, 600 nM for the α6 subunit and 400 nM for the α5 subunit, δ subunit and for β-actin. RT-PCR was performed using a 7500 ABI real-time machine

Table 1. *Guinea pig-specific primer sequences*

Gene of interest	Forward primer sequence	Reverse primer sequence
$GABA_AR$ α4 subunit	TGG GCA AAC AGT GTC AAG TG	GAC ACT TTG GGC AGA GAA TG
$GABA_AR$ α5 subunit	CAC GGG CGA ATA CAC GAT TA	CAA TCA GAG CAG AGA ACA CGA
$GABA_AR$ α6 subunit	ATA AGG AGT CAG TCC CAG CA	ACG AAA GCA AAG CAT ACA GC
$GABA_AR$ δ subunit	GCG TCT ACA TCA TCC AGT CC	AAT GGG CAA AGG CAT ACT CC
β-actin	TGC GTT ACA CCC TTT CTT GAC A	ACA AAG CCA TGC CAA TCT CAT

Primer sequences designed for Guinea pig $GABA_A$ receptor α4, α5, α6, δ subunits and β-actin gene expression. Primer sequences are displayed from 5′–3′ for forward and reverse primer.

(Applied Biosystems), and the results were analysed by Sequence Detection Software v2.01 (Applied Biosystems). The comparative C_t method ($2^{-\Delta\Delta C_t}$) was used to calculate relative fold changes in the mRNA levels of each gene. A house-keeping gene (β-actin) and a calibrator sample were used as controls in the comparative C_t method of analysis. Consistent C_t values were obtained for β-actin across the term/preterm, male/female and foetal/neonatal samples. The calibrator was a pooled sample of hippocampal and cerebellar brain samples, and was used as a control on every PCR plate.

Cortisol EIA

The Salimetrics Salivary Cortisol Assay competitive immunoassay kit (Salimetrics Inc., State College, PA, USA) was used to measure the concentration of cortisol in guinea pig saliva samples, and was performed by following the manufacturer's instructions. Saliva samples were obtained from term and preterm neonates by encouraging the neonates to chew on a cotton bud. For each animal, samples collected at 2 and 4 h were pooled together to create a 2–4-h sample, and the same was followed to obtain a 22–24-h sample. As previously described, the sensitivity of the assay was 0.012–3.0 μg/dl, with the inter- and intra-assay coefficients of variance being 6.89% and 5.52%, respectively.[35]

Statistical analysis

Data were analysed by two-way ANOVA using Graphpad Prism software (version 6.01, Graphpad Software Inc., La Jolla, CA, USA). When a significant difference was found, Tukey's *post-hoc* tests and corrections for multiple comparisons were performed. Unless otherwise stated, all data were expressed as mean ± S.E.M., and significance was considered at $P < 0.05$.

Results

Foetal and neonatal physical characteristics

Table 2 depicts the mean birth weights and organ-to-body weight ratios for foetuses collected at term and preterm. Within each sex, preterm foetuses had lower body weight ($P < 0.0001$) than their term counterparts. In addition, brain-to-body weight ratios (males $P = 0.0023$ and females $P = 0.0094$) and placenta-to-body weight ratios were higher for preterm foetuses (males $P = 0.015$ and females $P = 0.0029$), as well as heart-to-body weight ratios (males $P = 0.019$, and females $P = 0.04$). Brain-to-liver ratios were not significantly different, nor did they indicate growth restriction. Adrenal-to-body weight ratio was not significantly different between groups. No differences were found between the sexes in either the preterm or term foetal groups.

Body weights and organ-to-body weight ratios for term and preterm neonates at 24 h of age are shown in Table 3. Birth weights ($P = 0.011$) and postmortem weights ($P = 0.018$) of the preterm male neonates were significantly lower than the term male neonates. However, no differences were identified between preterm and term female neonates. Placenta-to-body weight ratios were significantly higher for the preterm neonates (males $P = 0.001$ and females $P = 0.039$). No other organ-to-body weight ratios were significantly different between sexes or gestational ages (GAs), nor was brain-to-liver ratio.

Neonates were also assessed for well-being during the 24-h period using a set of criteria that included resuscitation requirements, posture, alertness and movement. Average scores (average of the scores taken every 2 h over the 24-h period) were significantly lower for the preterm neonates compared with the term neonates ($P < 0.0001$), the same was found for final scores (the final score measured at 24 h; $P < 0.0001$; Table 3). The preterm neonates also had periods of apnea, forelimb spasticity and irregular respiration.[20] No differences were found between the sexes in either the preterm or term foetal groups.

Confirmation of neonatal prematurity

MBP immunostaining in the CA1 region of the hippocampus was significantly lower in the preterm female neonates compared with the term female neonates ($P = 0.014$, Fig. 1b). Area coverage in the hippocampus did not differ between preterm and term male neonates. In the sub-cortical white matter and lobe X of the cerebellum, MBP coverage was significantly reduced in preterm neonates compared with term neonates ($P = 0.048$, Fig. 1d, and $P = 0.043$, Fig. 1f, respectively). There were no differences in MBP immunostaining in lobe VIII and the deep white matter of the cerebellum observed

https://doi.org/10.1017/S2040174415000069 Published online by Cambridge University Press

Table 2. *Foetal physical characteristics*

Sex	Group	*n*	Body weight (g)	Brain-to-body weight	Placenta-to-body weight	Heart-to-body weight	Liver-to-body weight	Adrenal gland-to-body weight	BLR
Male	Preterm	8	62.39 ± 2.94*	3.29 ± 0.13*	6.17 ± 0.23*	0.65 ± 0.03*	4.95 ± 0.2	0.04 ± 0.002	0.68 ± 0.04
	Term	11	93.44 ± 3.86	2.53 ± 0.11	4.95 ± 0.31	0.52 ± 0.03	4.81 ± 0.15	0.03 ± 0.002	0.53 ± 0.03
Female	Preterm	8	60.14 ± 3.35*	3.40 ± 0.20*	6.61 ± 0.24*	0.62 ± 0.04*	5.48 ± 0.45	0.04 ± 0.002	0.65 ± 0.06
	Term	11	89.57 ± 4.34	2.71 ± 0.13	5.10 ± 0.24	0.50 ± 0.02	5.11 ± 0.16	0.04 ± 0.003	0.54 ± 0.03

All values are represented as a percentage of body weight at the time of postmortem with the exception of BLR, which is a ratio value of brain weight to liver weight. The BLR value is indicative of growth restriction and brain sparing, whereby a value of >0.9 is used to classify growth-restricted foetuses. Values are expressed as the mean percentage + S.E.M. and are calculated for animal numbers.

*Denotes a significant effect of age within a sex group ($P < 0.05$).

Table 3. *Neonatal physical characteristics*

Sex	Group	*n*	Birth Weight (g)	Postmortem weight (g)	Brain-to-body weight	Placenta-to-body weight	Heart-to-body weight	Liver-to-body weight	Adrenal gland-to-body weight	BLR	Average score	Final score
Male	Preterm	9	72.17 ± 1.83*	66.81 ± 1.57*	3.19 ± 0.08	6.37 ± 0.28*	0.63 ± 0.03	4.49 ± 0.22	0.05 ± 0.006	0.73 ± 0.04	8.05 ± 0.28*	8.31 ± 0.49*
	Term	9	89.12 ± 5.23	84.13 ± 5.36	2.94 ± 0.15	4.82 ± 0.30	0.63 ± 0.02	4.04 ± 0.17	0.04 ± 0.007	0.76 ± 0.04	11.63 ± 0.19	11.89 ± 0.11
Female	Preterm	11	70.26 ± 3.32	66.3 ±3.14	3.20 ± 0.12	5.73 ± 0.27*	0.62 ± 0.03	4.64 ± 0.18	0.05 ± 0.004	0.70 ± 0.04	7.85 ± 0.46*	8.61 ± 0.63*
	Term	12	78.58 ± 3.80	73.83 ± 3.75	3.078 ± 0.22	4.79 ± 0.15	0.60 ± 0.03	4.08 ± 0.12	0.05 ± 0.01	0.76 ± 0.08	11.64 ± 0.11	11.96 ± 0.04

All values are represented as a percentage of body weight at the time of postmortem with the exception of BLR, which is a ratio value of brain weight to liver weight, and the average and last scores. The BLR value is indicative of growth restriction and brain sparing, whereby a value of >0.9 is used to classify growth-restricted foetuses. Average and final scores are indicative of well-being and are scored out of a maximum 12, whereby 12 indicates good well-being. Values are expressed as the mean percentage + S.E.M. and are calculated for animal numbers.

*Denotes a significant effect of age within a sex group ($P < 0.05$).

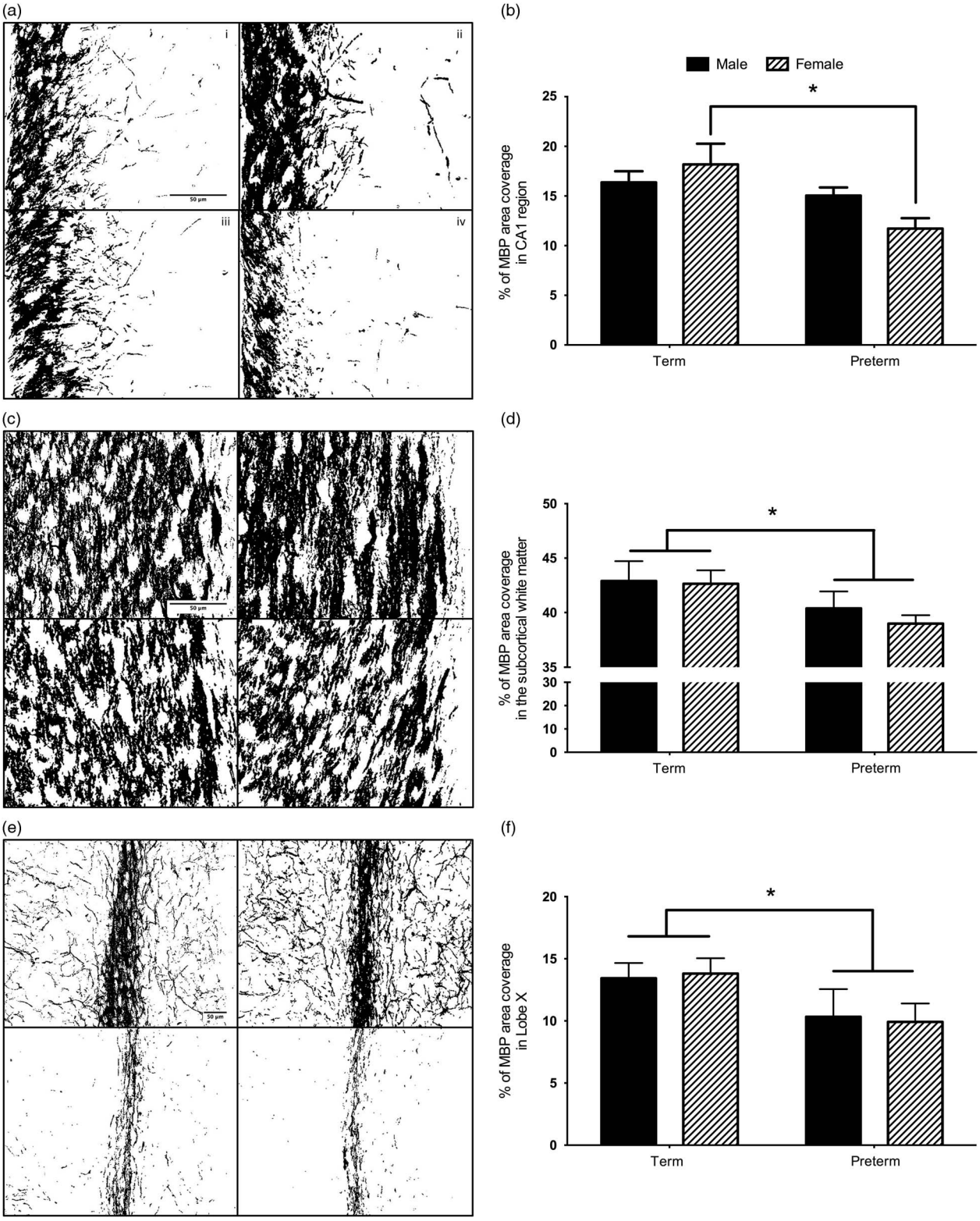

Fig. 1. Representative photomicrographs of myelin basic protein (MBP) immunolabelling and percent coverage in the neonatal Guinea pig external capsule adjacent to the CA1 region of the hippocampus (a and b), the sub-cortical white matter (c and d) and lobe X of the cerebellum (e and f). Scale bar = 50 μm; (i) male term, (ii) female term, (iii) male preterm and (iv) female preterm for all photomicrographs. Mean ± S.E.M. Males = black bars, females = hashed bars, *indicates $P < 0.05$, all groups are $n = 4–6$.

https://doi.org/10.1017/S2040174415000069 Published online by Cambridge University Press

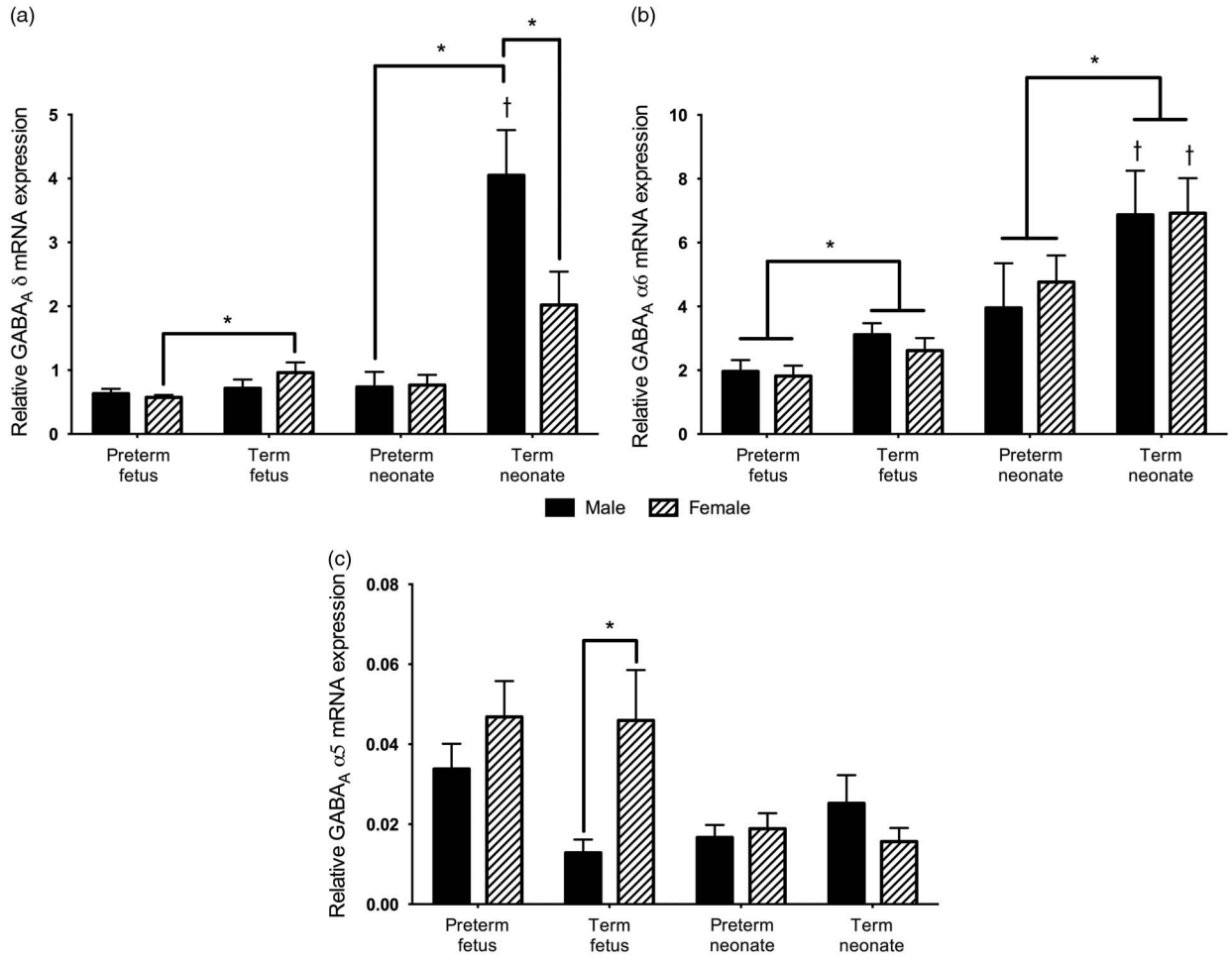

Fig. 2. Relative $GABA_A$ receptor (a) δ, (b) α6 and (c) α5 subunit mRNA levels in the cerebellum. Values are for preterm (62 day) and term (69 day) foetuses, and preterm and term neonates at 24 h of age for males (filled bars) and females (hashed bars). All groups are $n = 5$–8, * indicates $P < 0.05$ within gestational age or sex, † indicates $P < 0.05$ between foetal and neonatal groups within gestational age and sex groups.

between preterm and term neonates (data not shown), nor were any sex differences observed in any of the regions examined.

$GABA_A$ receptor subunit mRNA levels

In cerebellar tissues, foetal mRNA levels of the δ subunit were significantly higher in term females compared with the preterm females ($P = 0.047$; Fig. 2a), but did not reach significance in the neonates ($P = 0.28$). Conversely, term male neonates exhibited significantly higher δ subunit mRNA levels compared with preterm male neonates ($P = 0.0003$) and term female neonates ($P = 0.026$). In addition, cerebellar δ subunit mRNA levels in the term male neonates were significantly higher than in the term male foetuses ($P < 0.0001$), and approached significance for higher levels in the term female neonates compared with term female foetuses ($P = 0.065$). Cerebellar δ subunit mRNA levels between the preterm foetal and neonatal populations did not change. Preterm foetuses and neonates showed significantly lower cerebellar mRNA levels of the α6 subunit compared with the term foetuses ($P = 0.011$) and neonates ($P = 0.044$; Fig. 2b). In addition, the mRNA levels of both the male and female term neonates were significantly higher than levels of the male and female term foetuses ($P = 0.017$ and $P = 0.0073$, respectively). Cerebellar α5 subunit mRNA levels were significantly lower in term male foetuses compared with term female foetuses ($P = 0.047$; Fig. 2c) in the foetal cohort. By 24 h, however, levels of α5 mRNA were similar between term female and male neonates. No significant difference was identified in the levels of α4 subunit mRNA in either the foetal or neonatal cerebellum (data not shown).

In the hippocampal tissues, there were no significant differences in relative mRNA levels identified for the α4, α5, α6 and δ subunits within either the foetal cohort or the neonatal cohort, between sexes or between GAs (data not shown).

Neonatal salivary cortisol

Salivary cortisol levels were assessed in neonates at 2–4 and 22–24 h. Overall, preterm neonates were found to have

https://doi.org/10.1017/S2040174415000069 Published online by Cambridge University Press

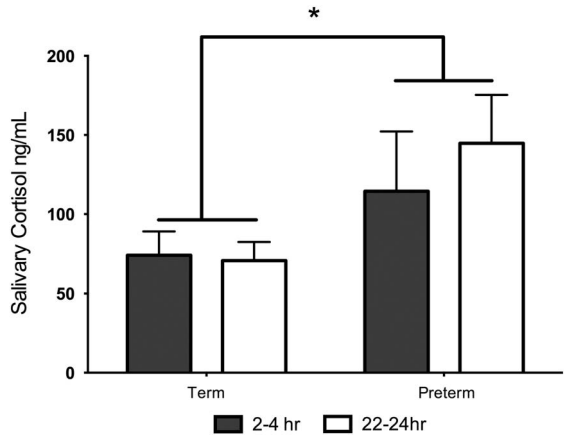

Fig. 3. Comparison of salivary cortisol between term and preterm neonates. Male and female neonates are combined to create overall term and preterm groups at 2–4 h (filled bars) and 22–24 h of life (open bars). *Indicates $P < 0.05$, all groups are $n = 10$.

significantly higher levels of salivary cortisol compared with the term neonates ($P = 0.035$; Fig. 3). Furthermore, the concentrations did not increase from birth to 24 h within the term or preterm neonatal group. No differences were observed between males and females; therefore, these data were combined.

Discussion

The major finding of this study shows that, for the first time using the established guinea pig model, neonates that are born preterm have disrupted GABA$_A$ receptor subunit mRNA levels. Interestingly, these changes were region specific, as levels in the cerebellum were markedly affected, whereas no changes in the subunits examined were found in the hippocampus. Specifically, preterm neonates have lower mRNA levels of the α6 and δ (male only) subunits in the cerebellum compared with neonates delivered at term. Interestingly, term neonates exhibited higher α6 (male and female) and δ (male only) subunit mRNA levels than term foetuses. As this increase was not present in the preterm neonatal cohort, these results indicate that birth at this earlier stage in gestation not only leaves these neonates with an immature mRNA profile for α6 and δ subunits, but also, unlike term neonates, no further increase occurs during the 24 h following birth. Although the regulatory mechanism remains to be determined, the increase in subunit mRNA levels in term males and females over this initial 24-h period may be an adaptation for the transition to the neonatal environment, which is not available to preterm neonates. Separation of the foetus from the placenta at the time of birth results in a loss of supply of progesterone required for allopregnanolone synthesis.[20] Therefore, after birth, allopregnanolone levels decline rapidly, such that markedly lower neonatal levels are observed by 24 h after delivery. This dramatic drop highlights the importance of the increased mRNA levels of the α6 and δ subunits following birth in the term neonates, which may compensate for the dramatic reduction in the supply of allopregnanolone, and therefore maximize allopregnanolone action at the GABA$_A$ receptors. The results of this study suggest that preterm neonates are incapable of increasing their α6 and δ mRNA levels, which would lead to further vulnerability to excitation-induced brain injury and suboptimal development following preterm birth.

Although term male foetuses had lower cerebellar α5 subunit mRNA levels compared with term female foetuses, a finding not present in the neonatal population, levels for this subunit and that for α4 were very low, suggesting that the role of these subunits in the cerebellum may not be important in determining receptor-binding affinities. The observation that there were no differences in the mRNA levels of GABA$_A$ receptor subunits in the hippocampus, in both the foetal and neonatal cohorts, suggests that this region has matured at least in terms of GABA$_A$ receptor development before the time of preterm delivery in our model (62 days GA). As mentioned, sex differences were identified in this study, with the expression of the α5 subunit in term foetuses and the δ subunit in term neonates differing between males and females. These differences between sexes were only identified in the term populations, suggesting that they are physiologically normal differences in neurodevelopment between males and females. Interestingly, the same differences were not observed in the preterm populations, providing further evidence for a deregulated transition to the *ex utero* environment. Specifically, the large sex difference present in the term neonate population, with the males expressing higher levels of the δ subunit, is not apparent in the preterm population. This may be an indicator of vulnerability in the immediate neonatal period for preterm males, but seemingly the levels for preterm females reached term equivalent.

These findings suggest that the action of neurosteroids in the preterm brain may differ due to the marked disparity in GABA$_A$ receptor subunit composition and mRNA levels compared with the term brain, therefore leading to major differences in neurosteroid sensitivity after birth. The implications of these alterations, however, requires further investigation, including long-term animal studies to determine whether these mRNA profiles 'catch up' to those present in term neonates or whether the differences are permanent and whether they are associated with changes in behaviour or susceptibility to hyperactivity states, as has been demonstrated in other rodent models of GABA$_A$ receptor subunit knockouts. Behaviour following a knockout of the δ subunit (which is known to commonly group with the α6 subunit) has been extensively studied and linked with development of multiple neurodevelopmental conditions, a cause for concern if the low mRNA levels in preterm neonates identified in this study are permanent. Various knockout studies in mice and rats have demonstrated an increase in behaviour common to preterm infants, such as anxiety-like and pro-epileptic behaviour in the absence of the δ subunit, which may be a result of reduced sensitivity to neurosteroids.[36–38] Studies in humans have

https://doi.org/10.1017/S2040174415000069 Published online by Cambridge University Press

shown that preterm infants have smaller cerebellar volume and white matter, and at school age they are described as 'clumsier' and with worse fine motor control, such as hand dexterity, than those born at term age.[39–41] Preterm guinea pig neonates in this study also demonstrated seizure-like behaviour and ataxia in the initial 24-h period, suggesting that motor function of the cerebellum may be affected. Although there is limited information concerning $GABA_A$ receptors and motor function, one study has examined subunit mRNA levels in a mutant mouse strain that presented ataxia and head tossing. This study showed that mRNA levels of extrasynaptic receptors containing both the α6 and δ subunits were significantly decreased in the cerebellum compared with wild-type mice, providing evidence for the role of $GABA_A$ receptors in this region.[42] In the case of preterm birth, the initial effect of the deficit in mRNA levels would be compounded by the low allopregnanolone concentrations seen after delivery. As the δ subunit has a major role in controlling the steroid sensitivity of extrasynaptic $GABA_A$ receptors, and that neurosteroids have a role in regulating tonic levels of excitability, differences in the levels of this subunit could influence overall levels of excitability in a period when inhibitory action over the brain is crucial for neurodevelopment. Recent studies support previously unidentified roles of the cerebellum in cognitive functions. Functional imaging and clinical studies have shown that the cerebellum is involved in language processing and reading, working memory and associative learning.[43–45] It is also now well documented that children born preterm function more poorly in school, with learning difficulties and lower IQs compared with term children.[6,8] Future behavioural studies with a cohort of preterm neonates may further support the role of the $GABA_A$ receptor cerebellar deficits, observed here, in these poor neurodevelopmental and motor outcomes that are common in preterm children.

A key finding was that salivary cortisol concentrations of preterm neonates over the initial 24-h period were significantly higher than term neonates, suggesting increased stress exposure. In human premature newborns, studies looking at cortisol concentrations are contradictory due to interactions with prenatal glucocorticoid treatment. Despite this, however, one study found that as both GA and birth weight decreased, circulating cortisol concentrations increased.[46] Furthermore, cortisol levels have been shown to influence $GABA_A$ receptor subunit levels.[31–33] However, this does not appear to account for the differences found between the term and preterm neonates here, as similar $GABA_A$ receptor mRNA levels were evident in the foetal preterm population before the exposure to the *ex utero* environment, and therefore the associated stress. The lower mRNA levels of the α6 and δ subunits in the preterm neonates in this case instead appear to be due to immaturity at the time of birth and lack of a birth-associated rise as opposed to being induced by high cortisol exposure over the 24-h period.

Children and adolescents born preterm have higher incidences of a range of behavioural, cognitive and motor disorders including anxiety, depression, internalizing behaviour and hyperactivity disorders.[8,47,48] Reduced $GABA_A$ receptor-related inhibition might have a contributory role in these conditions. There have been suggestions that differing $GABA_A$ subunit expression and actions of neurosteroids may be involved in controlling cortisol release and the response to stress.[49,50] $GABA_A$ receptors possessing the δ subunit are highly expressed in the paraventricular nucleus, where the corticotropin-releasing hormone (CRH) neurons involved in the stress response are located.[49] Interestingly, knockout of the δ subunit reduces the sensitivity of these CRH neurons to the stress-induced neurosteroid tetrahydrodeoxycorticosterone, ultimately affecting the response to stress.[49] Further long-term studies in juvenility, adolescence and adulthood are required to determine the long-term consequences of the differences in subunit mRNA levels that we observed, particularly for the development of anxiety-like behaviour in the preterm neonates. This would determine whether preterm neonates permanently experience higher levels of cortisol and differences in mRNA levels of the $GABA_A$ receptor subunits implicated in the stress response, and whether this, in part, contributes to the higher incidences of neurobehavioural illnesses and delays that preterm neonates develop later in childhood and adolescence.

The development of neurobehavioural delays in children born preterm are suggested to be partly attributed to a decreased white matter volume at the time of birth.[13] A decrease in the area coverage of myelination in several regions of the hippocampus and cerebellum was identified in the preterm neonates in our study, confirming previous findings within our laboratory and the prematurity of these neonates.[20] The differences identified in the sub-cortical white matter and lobe X of the cerebellum appear to be quite small; however, imaging studies in preterm humans show that white matter injuries in the immediate postnatal period persist long-term and are correlated with poor cognitive outcome and motor impairment.[39,51] Furthermore, when the difference between total percentages covered (as opposed to area coverage) is compared between the term and preterm neonates in lobe X, for example, the reduction in total myelination is quite substantial. Future analysis of these regions may also focus on axon length and markers of oligodendrocyte progenitors and apoptosis to explore the differences in more detail, as the analysis used in this study focused on mature oligodendrocytes only. The most striking decrease in mature oligodendrocyte expression was observed in the CA1 region of the female preterm neonates. This is further evidence for the differing neurodevelopmental trajectories of males and females.

Previous studies within our laboratory have shown that allopregnanolone levels following preterm birth are significantly lower compared with foetal levels.[14] These lower concentrations may potentially disrupt the long-term development of myelin within these vulnerable brains. Other studies have identified the importance of allopregnanolone in the formation of myelin. This has been demonstrated in rat brain slice cultures, where administration of allopregnanolone enhanced

myelination.[52] Similarly, in a mouse model of neurodegeneration, allopregnanolone treatment delayed damage to myelin and increased survival.[53] Our studies have also shown that inhibition of allopregnanolone synthesis using finasteride reduces myelin maturation.[27] Furthermore, changes in receptor expression have been identified in human brain tissues in Alzheimer's and Parkinson's disease, but their role in disease progression is unknown.[54] Based on this knowledge that neurosteroids promote myelination, and that their primary site of action is $GABA_A$ receptors, it is feasible that catch-up myelination in preterm neonates may be further impaired due to the lack of the α6 and δ subunits. Depending on the results of future long-term studies, there is the potential of neurosteroid replacement therapy to act on the limited levels of neurosteroid-sensitive subunits in the preterm brain and mimic *in utero* conditions to encourage correct neurodevelopment of preterm brains, increasing receptor expression to term equivalent levels, and thus decreasing damaging excitation in this vulnerable window for neurodevelopment, ultimately improving outcomes for these neonates.

Overall, this study has highlighted key differences in $GABA_A$ receptor subunit mRNA levels in the cerebellum of neonates born prematurely and has identified an additional vulnerability that preterm neonates face. The lack of a birth-related adaptive increase in the mRNA levels of cerebellar α6 and δ $GABA_A$ receptor subunits after birth potentially reduces the effect of allopregnanolone postnatally and may contribute to neurodevelopmental disability in preterm neonates by exposing the immature brain to damaging excitotoxicity. This finding provides a basis for investigation in long-term studies of behavioural outcome.

Acknowledgements

The authors acknowledge Meredith Kelleher, Rebecca Dyson and Greer Bennett for performing the animal work.

Financial Support

This work was funded by the NHMRC (grant number APP1003517).

Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (National Health and Medical Research Council Australian Code of Practice for the Care and Use of Animals for Scientific Purposes) and has been approved by the institutional committee (University of Newcastle Animal Care and Ethics Committee).

References

1. Goldenberg RL, Culhane J, Iams J, Romero R. Epidemiology and causes of preterm birth. *Lancet*. 2008; 371, 75–84.
2. Mathews T, Menacker F, MacDorman MF. Infant mortality statistics from the 2002 period linked birth/infant death data set. *Natl Vital Stat Rep*. 2004; 53, 1–32.
3. Ananth CV, Vintzileos AM. Epidemiology of preterm birth and its clinical subtypes. *J Matern Fetal Neonatal Med*. 2006; 19, 773–782.
4. Beck S, Wojdyla D, Say L, *et al*. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ*. 2010; 88, 31–38.
5. Cheong JL, Doyle LW. Increasing rates of prematurity and epidemiology of late preterm birth. *J Paediatr Child Health*. 2012; 48, 784–788.
6. Chyi LJ, Lee HC, Hintz SR, Gould JB, Sutcliffe TL. School outcomes of late preterm infants: special needs and challenges for infants born at 32 to 36 weeks gestation. *J Pediatr*. 2008; 153, 25–31.
7. Moster D, Lie RT, Markestad T. Long-term medical and social consequences of preterm birth. *N Engl J Med*. 2008; 359, 262–273.
8. van Baar AL, Vermaas J, Knots E, de Kleine MJ, Soons P. Functioning at school age of moderately preterm children born at 32 to 36 weeks' gestational age. *Pediatrics*. 2009; 124, 251–257.
9. Rees S, Harding R, Walker D. An adverse intrauterine environment: implications for injury and altered development of the brain. *Int J Dev Neurosci*. 2008; 26, 3–11.
10. Rivkin MJ. Hypoxic-ischemic brain injury in the term newborn. Neuropathology, clinical aspects, and neuroimaging. *Clin Perinatol*. 1997; 24, 607–625.
11. de Graaf-Peters VB, Hadders-Algra M. Ontogeny of the human central nervous system: what is happening when? *Early Hum Dev*. 2006; 82, 257–266.
12. Rice D, Barone S Jr. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environ Health Perspect*. 2000; 108, 511–533.
13. Rees S, Inder T. Fetal and neonatal origins of altered brain development. *Early Hum Dev*. 2005; 81, 753–761.
14. Kelleher MA, Palliser HK, Hirst JJ. Neurosteroid replacement therapy in the preterm neonate. In *The 38th Annual Meeting Fetal and Neonatal Physiological Society*. 2011. Australia.
15. Nicol M, Hirst J, Walker D. Effect of pregnane steroids on electrocortical activity and somatosensory evoked potentials in fetal sheep. *Neurosci Lett*. 1998; 253, 111–114.
16. Nguyen PN, Billiards SS, Walker DW, Hirst JJ. Changes in 5 alpha-pregnane steroids and neurosteroidogenic enzyme expression in fetal sheep with umbilicoplacental embolization. *Pediatr Res*. 2003; 54, 840–847.
17. Nosarti C, Al-Asady MH, Frangou S, *et al*. Adolescents who were born very preterm have decreased brain volumes. *Brain*. 2002; 125, 1616–1623.
18. Yawno T, Yan E, Walker D, Hirst J. Inhibition of neurosteroid synthesis increases asphyxia-induced brain injury in the late gestation fetal sheep. *Neuroscience*. 2007; 146, 1726–1733.
19. 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.
20. Kelleher MA, Hirst JJ, Palliser HK. Changes in neuroactive steroid concentrations after preterm delivery in the Guinea pig. *Reprod Sci*. 2013; 20, 1365–1375.

21. Belelli D, Lambert JJ. Neurosteroids: endogenous regulators of the GABAA receptor. *Nat Rev Neurosci*. 2005; 6, 565–575.
22. Crossley KJ, Nitsos I, Walker DW, *et al*. Steroid-sensitive GABAA receptors in the fetal sheep brain. *Neuropharmacology*. 2003; 45, 461–472.
23. Crossley KJ, Walker DW, Beart PM, Hirst JJ. Characterisation of GABAA receptors in fetal, neonatal and adult ovine brain: region and age related changes and the effects of allopregnanolone. *Neuropharmacology*. 2000; 39, 1514–1522.
24. Nicol MB, Hirst JJ, Walker DW. Effect of finasteride on behavioural arousal and somatosensory evoked potentials in fetal sheep. *Neurosci Lett*. 2001; 306, 13–16.
25. Coleman H, Hirst JJ, Parkington HC. *The GABAA excitatory-to-inhibitory switch in the hippocampus of perinatal Guinea-pigs.* In *The 40th Annual Meeting Fetal and Neonatal Physiological Society*. 2013. Chile.
26. Mirmiran M. The function of fetal/neonatal rapid eye movement sleep. *Behav Brain Res*. 1995; 69, 13–22.
27. Kelleher MA, Palliser HK, Walker DW, Hirst JJ. Sex-dependent effect of a low neurosteroid environment and intrauterine growth restriction on foetal Guinea pig brain development. *J Endocrinol*. 2011; 208, 301–309.
28. Gulinello M, Gong Q, Smith S. Progesterone withdrawal increases the α4 subunit of the GABAA receptor in male rats in association with anxiety and altered pharmacology – a comparison with female rats. *Neuropharmacology*. 2002; 43, 701–714.
29. Burgard EC, Tietz EI, Neelands TR, Macdonald RL. Properties of recombinant gamma-aminobutyric acid A receptor isoforms containing the alpha 5 subunit subtype. *Mol Pharmacol*. 1996; 50, 119–127.
30. Belelli D, Harrison NL, Maguire J, *et al*. Extrasynaptic GABAA receptors: form, pharmacology, and function. *J Neurosci*. 2009; 29, 12757–12763.
31. Maguire J, Mody I. Neurosteroid synthesis-mediated regulation of GABA(A) receptors: relevance to the ovarian cycle and stress. *J Neurosci*. 2007; 27, 2155–2162.
32. Jacobson-Pick S, Audet MC, McQuaid RJ, Kalvapalle R, Anisman H. Stressor exposure of male and female juvenile mice influences later responses to stressors: modulation of GABAA receptor subunit mRNA expression. *Neuroscience*. 2012; 215, 114–126.
33. Serra M, Pisu MG, Littera M, *et al*. Social isolation-induced decreases in both the abundance of neuroactive steroids and GABA(A) receptor function in rat brain. *J Neurochem*. 2000; 75, 732–740.
34. 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*. 2009; 22, 166–174.
35. 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.
36. Mihalek RM, Banerjee PK, Korpi ER, *et al*. Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad Sci*. 1999; 96, 12905–12910.
37. Spigelman I, Li Z, Banerjee PK, *et al*. Behavior and physiology of mice lacking the GABAA-receptor delta subunit. *Epilepsia*. 2002; Suppl. 5, 3–8.
38. Spigelman I, Li Z, Liang J, *et al*. Reduced inhibition and sensitivity to neurosteroids in hippocampus of mice lacking the GABA(A) receptor delta subunit. *J Neurophysiol*. 2003; 90, 903–910.
39. Spittle AJ, Cheong J, Doyle LW, *et al*. Neonatal white matter abnormality predicts childhood motor impairment in very preterm children. *Dev Med Child Neurol*. 2011; 53, 1000–1006.
40. Allin M, Matsumoto H, Santhouse AM, *et al*. Cognitive and motor function and the size of the cerebellum in adolescents born very pre-term. *Brain*. 2001; 124, 60–66.
41. Pitcher JB, Schneider LA, Burns NR, *et al*. Reduced corticomotor excitability and motor skills development in children born preterm. *J Physiol*. 2012; 590, 5827–5844.
42. Payne HL, Connelly WM, Ives JH, *et al*. GABAA alpha6-containing receptors are selectively compromised in cerebellar granule cells of the ataxic mouse, stargazer. *J Biol Chem*. 2007; 282, 29130–29143.
43. Stoodley CJ. The cerebellum and cognition: evidence from functional imaging studies. *Cerebellum*. 2012; 11, 352–365.
44. Buckner RL. The cerebellum and cognitive function: 25 years of insight from anatomy and neuroimaging. *Neuron*. 2013; 80, 807–815.
45. Timmann D, Drepper J, Frings M, *et al*. The human cerebellum contributes to motor, emotional and cognitive associative learning. A review. *Cortex*. 2010; 46, 845–857.
46. Kajantie E, Phillips DI, Andersson S, *et al*. Size at birth, gestational age and cortisol secretion in adult life: foetal programming of both hyper- and hypocortisolism? *Clin Endocrinol (Oxf)*. 2002; 57, 635–641.
47. Potijk MR, de Winter AF, Bos AF, Kerstjens JM, Reijneveld SA. Higher rates of behavioural and emotional problems at preschool age in children born moderately preterm. *Arch Dis Child*. 2012; 97, 112–117.
48. Loe IM, Lee ES, Luna B, Feldman HM. Behavior problems of 9-16 year old preterm children: biological, sociodemographic, and intellectual contributions. *Early Hum Dev*. 2011; 87, 247–252.
49. Sarkar J, Wakefield S, MacKenzie G, Moss SJ, Maguire J. Neurosteroidogenesis is required for the physiological response to stress: role of neurosteroid-sensitive GABAA receptors. *J Neurosci*. 2011; 31, 18198–18210.
50. Herman JP, Mueller NK, Figueiredo H. Role of GABA and glutamate circuitry in hypothalamo-pituitary-adrenocortical stress integration. *Ann N Y Acad Sci*. 2004; 1018, 35–45.
51. Limperopoulos C, Bassan H, Gauvreau K, *et al*. Does cerebellar injury in premature infants contribute to the high prevalence of long-term cognitive, learning, and behavioral disability in survivors? *Pediatrics*. 2007; 120, 584–593.
52. 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.
53. Liao G, Cheung S, Galeano J, *et al*. Allopregnanolone treatment delays cholesterol accumulation and reduces autophagic/lysosomal dysfunction and inflammation in Npc1-/- mouse brain. *Brain Res*. 2009; 1270, 140–151.
54. Luchetti S, Huitinga I, Swaab DF. Neurosteroid and GABA-A receptor alterations in Alzheimer's disease, Parkinson's disease and multiple sclerosis. *Neuroscience*. 2011; 191, 6–21.