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Preterm birth affects GABA\textsubscript{A} receptor subunit mRNA levels during the fetal to neonatal transition in guinea pigs

Short Title: GABA\textsubscript{A} receptors and the preterm neonate

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Abstract

Modulation of GABA<sub>A</sub> receptor signalling by the neurosteroid allopregnanolone has a major role in late gestation neurodevelopment. The objective of this study was to characterise the mRNA levels of GABA<sub>A</sub> receptor subunits (α4, α5, α6, and δ) that are key to neurosteroid binding in the brain, following preterm birth. Myelination, measured by myelin basic protein (MBP) immunostaining, was used to assess maturity of the preterm brains. Fetal 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 hours of age. Subunit mRNA levels were quantified by RT-PCR in the hippocampus and cerebellum of fetal and neonatal brains. Levels of the α6 and δ subunits were markedly lower in the cerebellum of preterm guinea pigs compared to terms. Importantly, there was an increase in mRNA levels of these subunits during the fetal 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 there is an adaptive increase in levels of mRNA of the key GABA<sub>A</sub> 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.

Key words: preterm birth, neurosteroids, GABA<sub>A</sub> receptors.
Introduction

Preterm birth is the leading cause of death and neurodevelopmental related disability in neonates, accounting for up to 70% of neonatal deaths and with approximately 50% of 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 to term infants \[^{5-7}\]. Furthermore, mothers and teachers of children that 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 of the cerebellum, which is responsible for regulation and coordination of movement with additional roles in attention and language, continues to mature throughout late gestation until after birth in the guinea pig and human \[^{11}\]. 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 fetal life are regulated by
the suppressive action of the neurosteroid allopregnanolone in the fetal brain \[18, 19\]. Throughout gestation the precursors required for allopregnanolone synthesis are supplied in high concentrations from the placenta and are therefore lost at birth. Hence, allopregnanolone levels decline rapidly after removal of the placenta and reach levels observed in neonates by 24 hours 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 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 expressed throughout the fetal brain from mid gestation, including on oligodendrocytes \[22\]. Compared to the receptors found in the adult brain, those found in the fetal brain are more sensitive to modulation by allopregnanolone and are strongly activated by the concentrations of allopregnanolone found in fetal brain extracts \[23\]. The premature loss of allopregnanolone supply following preterm birth therefore may have consequences on GABA\(_A\) receptor expression and ultimately reduce 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 fetal brain from mid-gestation onwards \[22, 24, 25\]. Animal studies have shown that reducing allopregnanolone levels in the fetus \textit{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<sub>A</sub> receptors, as neurosteroid withdrawal increases the expression of the α4 subunit<sup>[28]</sup>. This highlights the adaptive nature of GABA<sub>A</sub> receptors and the potential of replacement therapies that increase neonatal allopregnanolone concentrations to prevent excitotoxicity and associated cell death in premature brains.

GABA<sub>A</sub> receptors exist in a pentameric formation of 5 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 expression of α4 and α5 subunits high in the hippocampus, and α6 and δ subunits predominating in the cerebellum<sup>[29]</sup>. Receptors containing these four subunits are highly sensitive to modulation by neurosteroids with consequent suppression of fetal CNS excitation<sup>[30]</sup>, whereas reduced expression of these receptor subunit types may lead to excitotoxic brain injury and suboptimal development. Previous studies have shown that GABA<sub>A</sub> receptor subunit expression is influenced by changes in glucocorticoid exposure. Specifically, acute stress has been linked with an increase in expression of the 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<sup>[31, 32]</sup>. Alternatively, chronic stress has been found to have the opposite effect, lowering expression and overall inhibitory function of the GABA<sub>A</sub> receptors<sup>[33]</sup>. Early exposure of the fetus to the ex utero environment is a potentially highly stressful situation, however it is unclear if this exposure may have a further effect on the expression of GABA<sub>A</sub> receptor subunits in the newborn brain. The potential alterations to the GABA<sub>A</sub> 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 current study was to characterise the mRNA levels of key GABA_A receptor subunits in the hippocampus and cerebellum of preterm and term guinea pig fetuses and one day old neonates. Additionally, 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 this immaturity will be associated with reduced myelination within the hippocampus and cerebellum, and high salivary cortisol 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

Prior to beginning this work, approval for all of the animal experiments and procedures carried out throughout the 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 hour light/dark cycle and were supplied with a diet consisting of commercial guinea pig pellets, Lucerne hay, and water supplemented with ascorbic acid. Four animal groups were studied: term and preterm fetuses, and term and preterm neonates at 24 hours of age. One male and one female fetus 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 50μL dose of surfactant (Curosurf, 80mg/mL Poractant alfa, Douglas Pharmaceuticals,
Baulkham Hills, NSW, Australia) was administered into the oropharynx. To encourage respiration, continuous positive airway pressure (CPAP) was administered by 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 7mm H₂O and peak inspiratory pressure (PIP) of 20mm H₂O was applied at a flow rate of 8L/min. Medical oxygen and air were adjusted to give a fraction of inspired O₂ of 60% during CPAP. An initial sustained PIP of 20 seconds was administered. CPAP and PIP were performed again when respiration became unstable, and an additional dose of 50μL surfactant was administered at 3 hours. 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 wellbeing, fed by gastric tube, and had saliva collected at 2 hourly intervals, up until 24 hours \[^{20}\]. The monitoring for wellbeing involved scoring respiration, posture, and alertness out of a maximum of 12 points at each 2 hourly interval. Each category was assigned a score from 0 – 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 – 3 indicated very poor wellbeing, and 4 – 6 indicated poor wellbeing, whereas scores of 7 – 9 indicated good wellbeing, and 10 – 12 indicated very good wellbeing.

The feeding regime used was commercial guinea pig milk replacement formula (Wombaroo Food Products, Adelaide, SA, Australia), at 100μL/g/24 hour, 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 hour cortisol sample the neonates were euthanized and tissues collected. Term and preterm fetal tissues were also obtained from pups collected 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. Each left hemisphere was fixed for immunohistochemistry, whilst the right hemisphere was further dissected and frozen in liquid nitrogen and used for further processing, including RT-PCR.

**Immunohistochemistry**

Myelin basic protein (MBP), a protein that is present in mature myelinating oligodendrocytes, immunodetection was used to assess myelination within the neonatal brains. Hippocampal CA1, subcortical white matter and cerebellum sections were immunostained for MBP as previously described [27]. Briefly, for each region, 7μm sections were cut using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, NSW, Australia). Prior to immunostaining, each slide was dewaxed and incubated in methanol containing 3% hydrogen peroxide. Antigen retrieval was performed using Reveal-It solution (ImmunoSolution Pty Ltd, QLD, Australia) according to the manufacturers instructions. Blocking for 30 minutes 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 0.1M PBS). Primary antibody (rat monoclonal anti-myelin basic protein 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 hours 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 for 2 hours at room temperature. To reveal the immunolabelling, incubation in 3,3’-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; Pierce) occurred. Staining with cresyl violet and coverslipping using DEPX (Merck) was carried out 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., New York, USA). 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 area coverage of MBP expression. This was done by firstly 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 program 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**

The real-time PCR was performed as previously described [34]. Frozen cerebellum and hippocampus were homogenised in RLT Plus Buffer (obtained from the Qiagen RNeasy Plus Mini Kit, Qiagen Pty Ltd, VIC, Australia) using a Precellys 24 dual tissue homogeniser (Bertin Technologies, France). Extraction of RNA from the homogenate was performed using the Qiagen RNeasy Plus Mini Kit by following the manufacturer's instructions. The NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DEM, USA) was used to quantify the RNA in each sample. RNA purity was also confirmed using the NanoDrop, with A$_{260}$/A$_{280}$ ratios of 2.1 – 2.19 obtained for each sample. The RNA was then run on a 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 examined for bands at 18S and 28S at a ratio of 1:2.

cDNA was synthesised 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 manufacturers instructions. Five primer pairs (α4, α5, α6, and δ GABA$_A$ receptor subunits, as well as the housekeeping gene β-actin) were designed and optimised within our laboratory for detection in the guinea pig and are detailed in Table 1. For each primer, a primer master mix concentration of 5pmole/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 100nM for the GABA<sub>A</sub> receptor α4 subunit, 600nM for the α6 subunit, and 400nM for the α5 subunit, δ subunit, and for β-actin. RT-PCR was performed using a 7500 ABI real-time machine (Applied Biosystems), and the results were analysed by Sequence Detection Software v2.01 (Applied Biosystems). The comparative Ct method (2<sup>-ΔΔCt</sup>) was used to calculate relative fold change in the mRNA levels of each gene. A house-keeping gene (β-actin) and a calibrator sample were used as controls in the comparative Ct method of analysis. Consistent Ct values were obtained for β-actin across the term/preterm, male/female and fetal/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 hours were pooled together to create a 2-4 hour sample, the same occurred for a 22-24 hour sample. As previously described, the sensitivity of the assay is 0.012 – 3.0µg/dl, with the inter- and intra-assay coefficients of variance 6.89% and 5.52% respectively. [35]
Statistical Analysis

Data was 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 post-hoc tests and corrections for multiple comparisons were performed. Unless otherwise stated, all data is expressed as mean ± SEM and significance considered as $p < 0.05$. 
Results

Fetal and neonatal physical characteristics

Table 2 depicts the mean birth weights and organ-to-body weight ratios for fetuses collected at term and preterm. Within each sex, preterm fetuses had lower body weight \( (p<0.0001) \), than their term counterparts. Additionally, brain-to-body weight ratios (males \( p=0.0023 \), and females \( p=0.0094 \)) and placenta-to-body weight ratios were higher for preterm fetuses (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 fetal groups.

Body weights and organ-to-body weight ratios for term and preterm neonates at 24 hours of age are shown in Table 3. Birth weights \( (p=0.011) \) and post mortem 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, nor was brain to liver ratio.

Neonates were also assessed for wellbeing during the 24 hour period using a set of criteria which included resuscitation requirements, posture, alertness, and movement. Average scores (average of the scores taken every two hours over the 24
hour period) were significantly lower for the preterm neonates compared to the terms (p<0.0001), the same was found for final scores (the final score measured at 24 hours) (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 fetal groups.

Confirmation of neonatal prematurity

MBP immunostaining in the CA1 region of the hippocampus was significantly lower in the preterm female neonates compared to 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 subcortical white matter and Lobe X of the cerebellum, MBP coverage was significantly reduced in preterm neonates compared to 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 between preterm and term neonates (data not shown), nor were any sex differences observed in any of the regions examined.

GABA<sub>A</sub> receptor subunit mRNA levels

In cerebellar tissues, fetal mRNA levels of the δ subunit were significantly higher in term females compared to 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 than preterm male neonates (p=0.0003), and term female neonates (p=0.026). Additionally, cerebellar δ subunit mRNA levels in the term male neonates was significantly higher than in the term male fetuses (p<0.0001), and approached significance for higher levels in the term female neonates compared to term female fetuses (p=0.065). Cerebellar δ subunit mRNA levels between the preterm fetal and neonatal populations did not change. Preterm fetuses and neonates showed significantly lower cerebellar mRNA levels of the α6 subunit compared to the term fetuses (p=0.011) and neonates (p=0.044) (Fig 2b). Additionally, the mRNA levels of both the male and female term neonates were significantly higher than levels of the male and female term fetuses (p=0.017, and p=0.0073 respectively). Cerebellar α5 subunit mRNA levels were significantly lower in term male fetuses compared to term female fetuses (p=0.047) (Fig 2c) in the fetal cohort. By 24 hours 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 fetal 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 fetal cohort, or the neonatal cohort, between sexes or between gestational ages (data not shown).

**Neonatal salivary cortisol**

Salivary cortisol was assessed in neonates at 2-4 and 22-24 hours. Preterm neonates were found to have significantly higher levels of salivary cortisol compared to
the term neonates overall (p=0.035) (Fig 3). Furthermore, the concentrations did not increase from birth to 24 hours 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 for the first time, using the established guinea pig model, that 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 whilst 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 to neonates delivered at term. Interestingly, term neonates exhibited higher α6 (male and female) and δ (male only) subunit mRNA levels than term fetuses. 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 unlike term neonates, no further increase occurs during the 24 hours following birth. While the regulatory mechanism remains to be determined, the increase in subunit mRNA levels in the term males and females over this initial 24 hour period, may be an adaptation for the transition to the neonatal environment, which is not available to preterm neonates. Separation of the fetus from the placenta at the time of birth results in a loss of the supply of progesterone required for allopregnanolone synthesis [20]. Therefore following birth allopregnanolone levels decline rapidly such that markedly lower neonatal levels are observed by 24 hours 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 maximise allopregnanolone action at the GABA_A receptors. The present
results 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. 

Whilst, term male fetuses had lower cerebellar α5 subunit mRNA levels compared to term female fetuses, a finding not present in the neonatal population, levels for this subunit and that of α4 were very low, suggesting 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<sub>A</sub> receptor subunits in the hippocampus, in both the fetal and neonatal cohorts, suggests this region has matured at least in terms of GABA<sub>A</sub> receptor development prior to 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 fetuses 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 have reached term equivalent.
These present findings suggest the action of neurosteroids in the preterm brain may differ due to the marked disparity in GABA<sub>A</sub> receptor subunit composition and mRNA levels compared to term delivery, 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 if these mRNA profiles ‘catch up’ to those present in term neonates, or if the differences are permanent, whether they are associated with changes in behaviour or susceptibility to hyperactivity states as has been demonstrated in other rodent models of GABA<sub>A</sub> 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 neurodevelopment 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 proepileptic behaviour in the absence of the δ subunit, which may be a result of reduced sensitivity to neurosteroids. Studies in humans have shown that preterm infants have smaller cerebellar volume and white matter, and at school age are described as ‘clumsier’ and with worse fine motor control, such as hand dexterity, than those born at term age. The preterm guinea pig neonates in this study also demonstrated seizure-like behaviour and ataxia in the initial 24 hour period, suggesting that motor function of the cerebellum may be affected. Whilst there is limited information concerning GABA<sub>A</sub> receptors and motor function, one study has examined subunit mRNA levels in a mutant mouse strain that presents with ataxia and head tossing. This study showed that mRNA levels of extrasynaptic receptors containing both the α6 and
δ subunits was significantly decreased in the cerebellum compared to wild type mice, providing evidence for the role of GABA<sub>δ</sub> receptors in this region. 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<sub>δ</sub> 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. It is also now well documented that children born preterm function more poorly in school, with learning difficulties and lower IQs compared to term children. Future behavioural studies with a cohort of preterm neonates may further support the role of the GABA<sub>δ</sub> receptor cerebellar deficits observed here with these poor neurodevelopmental and motor outcomes common in preterm children.

A key finding was that salivary cortisol concentrations of preterm neonates over the initial 24 hour period are 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 gestational age and birth weight decreased, circulating cortisol concentration increased. Furthermore, cortisol levels have been shown to influence GABA<sub>δ</sub> receptor subunit levels. This does not appear to account for the differences found between the term and preterm
neonates here however as similar GABA\textsubscript{A} receptor mRNA levels were evident in the fetal preterm population before the exposure to the \textit{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 appears 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 hour period.

Children and adolescents that were born preterm have higher incidences of a range of behavioural, cognitive, and motor disorders including anxiety, depression, internalising behaviour, and hyperactivity disorders \cite{8, 47, 48}. Reduced GABA\textsubscript{A} receptor related inhibition might have a contributory role in these conditions. There has been suggestions that differing GABA\textsubscript{A} subunit expression and actions of neurosteroids may be involved in controlling cortisol release and the response to stress \cite{49, 50}. GABA\textsubscript{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 \cite{49}. Interestingly, knockout of the δ subunit reduces the sensitivity of these CRH neurons to the stress-induced neurosteroid tetrahydrodeoxycorticosterone (THDOC), ultimately affecting the response to stress \cite{49}. Further long-term studies into juvenility, adolescence, and adulthood, are required to determine the long term consequences of the differences in subunit mRNA levels 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\textsubscript{A} receptor subunits implicated in the stress response and whether this, in part, contributes to the higher
incidences of neurobehavioral 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 were identified in the preterm neonates here, confirming previous findings within our laboratory and the prematurity of these neonates [20]. The differences identified in the subcortical white matter and lobe X of the cerebellum appear to be quite small, however imaging studies in preterm humans show that white matter injury 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 to fetal 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 formation of myelin. This has been demonstrated in rat brain slice cultures, where administration of allopregnanolone enhanced myelination \cite{52}. Similarly, in a mouse model of neurodegeneration, allopregnanolone treatment delayed damage to myelin and increased survival \cite{53}. Our studies have also shown that inhibition of allopregnanolone synthesis using finasteride reduces myelin maturation \cite{27}. Furthermore, changes to receptor expression have been identified in human brain tissue in Alzheimer’s and Parkinsons disease, but their role in disease progression is unknown \cite{54}. Based on this knowledge that neurosteroids promote myelination, and that their primary site of action is GABA\textsubscript{A} receptors, it is feasible that catch up myelination in preterm neonates may be further impaired due to a 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 \textit{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\textsubscript{A} receptor subunit mRNA levels in the cerebellum of neonates born prematurely and 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\textsubscript{A} receptor subunits after birth is potentially reducing 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.
### Table 1. Guinea Pig Specific Primer Sequences.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
</tr>
</thead>
<tbody>
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<td>$\text{GABA}_A\text{R }\alpha_4$ subunit</td>
<td>TGG GCA AAC AGT GTC AAG TG</td>
<td>GAC ACT TTG GGC AGA GAA TG</td>
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<tr>
<td>$\text{GABA}_A\text{R }\alpha_5$ subunit</td>
<td>CAC GGG CGA ATA CAC GAT TA</td>
<td>CAA TCA GAG CAG AGA ACA CGA</td>
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<tr>
<td>$\text{GABA}_A\text{R }\alpha_6$ subunit</td>
<td>ATA AGG AGT CAG TCC CAG CA</td>
<td>ACG AAA GCA AAG CAT ACA GC</td>
</tr>
<tr>
<td>$\text{GABA}_A\text{R }\delta$ subunit</td>
<td>GCG TCT ACA TCA TCC AGT CC</td>
<td>AAT GGG CAA AGG CAT ACT CC</td>
</tr>
<tr>
<td>$\beta$-actin</td>
<td>TGC GTT ACA CCC TTT CTT GAC A</td>
<td>ACA AAG CCA TGC CAA TCT CAT</td>
</tr>
</tbody>
</table>

Primer sequences designed for guinea pig $\text{GABA}_A\text{R}$ receptor $\alpha_4$, $\alpha_5$, $\alpha_6$, $\delta$ subunits and $\beta$-actin gene expression. Primer sequences are displayed from 5’-3’ for forward and reverse primer.
### Table 2. Fetal Physical Characteristics.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Preterm</td>
<td>8</td>
<td>62.39 ± 2.94*</td>
<td>3.29 ± 0.13*</td>
<td>6.17 ± 0.23*</td>
<td>0.65 ± 0.03*</td>
<td>4.95 ± 0.2</td>
<td>0.04 ± 0.002</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Term</td>
<td>11</td>
<td>93.44 ± 3.86</td>
<td>2.53 ± 0.11</td>
<td>4.95 ± 0.31</td>
<td>0.52 ± 0.03</td>
<td>4.81 ± 0.15</td>
<td>0.03 ± 0.002</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>Female</td>
<td>Preterm</td>
<td>8</td>
<td>60.14 ± 3.35*</td>
<td>3.40 ± 0.20*</td>
<td>6.61 ± 0.24*</td>
<td>0.62 ± 0.04*</td>
<td>5.48 ± 0.45</td>
<td>0.04 ± 0.002</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Term</td>
<td>11</td>
<td>89.57 ± 4.34</td>
<td>2.71 ± 0.13</td>
<td>5.10 ± 0.24</td>
<td>0.50 ± 0.02</td>
<td>5.11 ± 0.16</td>
<td>0.04 ± 0.003</td>
<td>0.54 ± 0.03</td>
</tr>
</tbody>
</table>

All values are represented as a percentage of body weight at the time of post-mortem 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 fetuses. Values are expressed as the mean percentage ± SEM and are calculated for animal numbers. * denotes a significant effect of age within a sex group (p < 0.05).
### Table 3. Neonatal Physical Characteristics.

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

All values are represented as a percentage of body weight at the time of post-mortem 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 fetuses. Average

and final scores are indicative of wellbeing and are scored out of a maximum 12, whereby 12 indicates good wellbeing. Values are expressed as the mean percentage + SEM

and are calculated for animal numbers. * denotes a significant effect of age within a sex group (p < 0.05).
Figure 1. Representative photomicrographs of MBP immunolabelling and percent coverage in the neonatal guinea pig external capsule adjacent to the CA1 region of the hippocampus (a and b), the subcortical white matter (c and d) and Lobe X of the cerebellum (e and f). Scale bar = 50µm; i male term, ii female term, iii male preterm, and iv female preterm for all photomicrographs. Mean ± SEM. Males = black bars, females = hashed bars, * indicates p < 0.05, all groups are n = 4-6.
Figure 2. Relative GABA<sub>A</sub> receptor a) δ, b) α6, and c) α5 subunit mRNA levels in the cerebellum. Values are for preterm (62 day) and term (68 day) fetuses, and preterm and term neonates at 24 hours 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 fetal and neonatal groups within gestational age and sex groups.
Figure 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 hours (filled bars) and 22-24 hours of life (open bars). * indicates $p < 0.05$, all groups are $n = 10$. 
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We would like to acknowledge Meredith Kelleher, Rebecca Dyson, and Greer Bennett for performing the animal work.

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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


