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# Neurosteroid replacement therapy using tiagabine and zuranolone restores cerebellar neurodevelopment and reduces hyperactive behaviour following preterm birth

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**Original Article**

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## Abstract

Preterm birth exposes the neonate to hypoxic-ischaemic and excitotoxic insults that impair neurodevelopment and are magnified by the premature loss of placentally supplied, inhibitory neurosteroids. The cerebellum is a neuronally dense brain region, which undergoes critical periods of development during late gestation, when preterm births frequently occur. We propose that neurosteroid replacement therapy using tiagabine and zuranolone will protect the cerebellum against preterm-associated insults. Guinea pig dams received c-section surgery preterm (gestational age (GA) 64) or at term (GA70) with preterm pups administered tiagabine (2.5 mg/kg/day), zuranolone (1 mg/kg/day) or vehicle (15% β-cyclodextrin) until term equivalent age (GA70). Behavioural testing was performed at corrected postnatal day 8 (PND8) and PND41 with tissue collection occurring at PND42. Neurodevelopmental markers (MBP, OLIG2 and NeuN) were assessed within the cerebellum by immunohistochemistry, whilst GABAergic and glutamatergic pathway expression was quantified using high throughput RT-PCR. Zuranolone and, to a lesser extent, tiagabine were able to protect against hyperactive behaviour at PND8 in males, whilst in females, a less marked hyperactive phenotype was present with neither treatment impacting behaviour further. Both treatments improved MBP staining, whilst tiagabine was found to restore oligodendrocyte maturation in females only. GABAergic and glutamatergic pathway expression was found to be restored by both treatments in females. Overall, this study demonstrates the neuroprotective attributes of neurosteroid replacement therapy using tiagabine and zuranolone, thereby demonstrating their potential to mitigate long-term neurodevelopmental impairments. Furthermore, the sexually dimorphic effects observed suggest future investigations may show increased benefit by using sex-specific treatment regimes.

## Introduction

Preterm birth is known to result in numerous complications for the neonate with the degree of prematurity directly related to severity of outcomes.[1] Moderate-late preterm birth represents 80% of all preterm births (32–37 completed weeks gestation), and despite incurring typically less severe pathology, preterm births in this period are still at greatly increased risk of immediate and long-term complications.[2,3] Neurodevelopmental impairments are the most prevalent long-term complications experienced by individuals born in the moderate-late preterm period with many of these disorders spanning through childhood and into adult life.[4–6] Preterm birth arises from numerous aetiologies, and as such, there are limited preventative approaches available.[7] Approximately 15 million preterm births occur globally each year, and with contemporary perinatal care, the vast majority of these neonates will survive.[8] The low mortality of moderate-late preterm birth emphasises the importance of investigating therapies, which will prevent the onset of neurodevelopmental disorders.[9,10]

The cerebellum, a brain region typically known for its role in movement disorders, is being increasingly recognised for its roles in cognitive and social functions.[11,12] Despite being less characterised with respect to preterm birth-associated insults, there is growing evidence of cerebellar vulnerability to these insults and the exposure of the developing neonate to an adverse perinatal environment.[13,14] Preterm birth results in a premature separation of the materno-placental unit from the neonate and therefore results in an untimely loss of placentally supplied progesterone and its neuroprotective derivative, allopregnanolone. The preterm neonate is therefore forced to undergo critical periods of neurodevelopment in an adverse perinatal environment without the trophic effects of allopregnanolone. Allopregnanolone is a positive allosteric modulator of the $GABA_A$ receptor which increases inhibitory tone through

modulation of extrasynaptic receptors, promoting myelination by oligodendrocytes.[15] Pharmacologically decreased fetal allopregnanolone levels in the guinea pig have been found to reduce myelination within the brain, mirroring results found within the hippocampus following preterm birth.[16,17] It is important to note that in humans and other long gestation species, such as the guinea pig, the switch in GABAergic activity from excitatory to inhibitory happens in mid-gestation, as opposed to other rodent species where it occurs postnatally.[18–20] The decreased inhibitory GABAergic tone caused by the loss of placentally supplied allopregnanolone exposes the preterm neonatal brain to an increased risk for disrupted excitatory/inhibitory balance and therefore excitotoxic damage.[21] Repeated bouts of hypoxic-ischaemic insult are common following preterm birth due to reduced respiratory drive, structural and functional immaturity of the lungs, vascular fragility and apnoea of prematurity.[22] These hypoxic stressors are known to potentiate excitotoxic damage, which is specifically deleterious in the highly excitatory dense cerebellar environment.

Cerebellar granule cells exist in high volumes within the cerebellum. These excitatory, glutamatergic neurons comprise over 90% of all neurons in the brain despite being restricted to a single brain region.[23,24] Additionally, the cerebellar cortex relies solely upon the myelinated axons of GABAergic Purkinje cells for its output to the cerebellar nuclei, and therefore, these inhibitory axons are crucial for appropriate communication with other brain regions.[25] The reciprocal connectivity of the cerebellum through these Purkinje cell axons emphasises the dependence this region has on optimal myelination. Disrupted myelination is known to impair cognitive and emotional processes and has been found across multiple brain regions in studies of adults with both attention deficit hyperactivity disorder (ADHD) and anxiety.[26–28] Additionally, significantly reduced cerebellar white matter volumes have also been found in individuals with panic disorders, further implicating disrupted cerebellar myelination in the aetiology of neurobehavioral disorders.[29–31] Late-developing lobes of the posterior cerebellum, such as lobules IX and X, are known to be particularly vulnerable to insults during late gestation and early neonatal life, such as preterm birth.[31,32] Due to their interconnectedness with executive functioning areas of the brain such as the prefrontal cortex, association cortices and limbic system, disruptions to the development of lobules IX and X can have major impacts on the functioning of these regions to which they project.[33,34]

Currently, there are only limited treatment options that address neurodevelopmental impairment following moderate-late preterm birth despite clear evidence demonstrating increased risks of developing pathologies. Induced therapeutic hypothermia has proven to be effective in reducing neonatal death and severe disability following a hypoxic-ischaemic insult.[35] Therapeutic hypothermia, however, can only be utilised in neonates born near term when strict criteria are met as this therapy is fraught with risks.[36] Magnesium sulphate therapy has also been shown to improve neurodevelopmental outcomes following preterm birth.[37] Unfortunately, identification of a high risk of preterm birth is required for the use of magnesium sulphate as it is administered antenatally. As many preterm births occur sporadically and progress quickly, the potential for the use of antenatal magnesium sulphate is greatly reduced.[38] The absence of effective, postnatal treatments which address hypoxic-ischaemic insults following preterm birth thereby presents an opportunity for a targeted therapy that addresses neurodevelopment following preterm birth insults.

The premature loss of allopregnanolone has been shown to contribute to neural impairment following preterm birth, and this gives rise to the potential for neurosteroid replacement therapies. Allopregnanolone treatment has been shown to improve learning and memory whilst reducing neuronal apoptosis following traumatic brain injuries in a rat model.[39] In guinea pigs, the use of a synthetic analogue of allopregnanolone, ganaxolone, has also improved hippocampal myelination and resulted in positive behavioural changes following a preterm birth insult.[40] The development of synthetic analogues of allopregnanolone provides a greater avenue for neurosteroid replacement than was traditionally available. This is due to improved half-lives and structural changes that mean they are not readily metabolised into other antagonistic steroids or those with reduced affinity for the $GABA_A$ receptor.[41] Zuranolone is one such allopregnanolone analogue that has shown promise in clinical use for anxiety disorders, parkinsonian tremors and has received FDA approval for the treatment of post-partum depression.[42–44]

The modulation of inhibitory/excitatory tone within the brain has also been proposed as an avenue with the potential for addressing impaired neurodevelopment following preterm birth. It is well established that hypoxic/ischaemic insults cause unbalanced excitation which leads to excitotoxicity and subsequently to neurodevelopmental impairments. Increasing GABAergic tone, a key function of allopregnanolone, may therefore improve neurodevelopment and ultimately improve outcomes following preterm birth. Tiagabine is a small molecule inhibitor of the GAT1 GABA transporter which when administered increases the synaptic concentrations of GABA, therefore increasing GABAergic tone.[45] Tiagabine has received FDA approval for use in treatment-resistant epilepsy; however, it has also been investigated for potential use in bipolar disorder, generalised anxiety disorder and post-traumatic stress disorder.[46–49] Importantly, tiagabine has also been shown to improve oligodendrocyte maturation and myelination in a mouse model of hypoxia-induced diffuse white matter injury, thereby presenting a clear mechanism through which it could act to improve cerebellar neurodevelopment following preterm birth.[50]

We propose that allopregnanolone replacement therapies (zuranolone and tiagabine) in the immediate postnatal period have the potential to prevent behavioural impairments and disrupted myelination as well as preterm associated changes in gene expression of GABAergic/glutamatergic pathway components. Therefore, this study aims to investigate the impact of preterm birth and the administration of neurosteroid replacement therapies zuranolone and tiagabine upon behaviour in the clinically relevant, long gestation, guinea pig model of preterm birth. This study also aims to determine what mechanistic changes occur at the gene expression and protein abundance level for oligodendrocytes, neurones and the GABAergic/glutamatergic pathways within the cerebellums of these animals.

## Methods

All chemicals and reagents supplied by Sigma unless otherwise specified.

### *Animals*

All animal experiments and protocols in this study were conducted in accordance with the National Health and Scientific Research Council Australia Code of Practice for the Care and Use of Animals

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for Scientific Purposes and were approved by the University of Newcastle Animal Care and Ethics Committee (protocol A-2021-102). Tri-coloured, outbred, guinea pig dams were obtained from the University of Newcastle Animal Services Unit, housed indoors under 12 h light-dark cycle and supplied with commercial guinea pig pellets, fresh fruit/vegetables and hay. Time-mated pregnant dams were then allocated to undergo a caesarean section (c-section) at term (gestational age 70; GA70) or preterm (GA64).

### Caesarean section delivery and care of dam

Dams assigned to preterm delivery received betamethasone (1 mg/kg subcutaneous; Celestone Chondrose, Merck Sharp & Dohme, Macquarie Park, Australia) at GA62 and GA63 to accelerate fetal lung development. On the day of surgery, dams underwent fasting for 2 h (GA64 $n = 17$; GA70 $n = 8$). Dams were administered atropine sulphate (0.05 mg/kg subcutaneous; Atrosite, Troy Lab, Glendenning, Australia) and buprenorphine (0.05 mg/kg subcutaneous; Temvet, Troy Lab) 30 min prior to c-section. Anaesthesia was achieved using isoflurane (1%–3%) by chamber inhalation until unconscious. The dam was then moved onto a heating pad placed on the surgical table with anaesthesia maintained by mask inhalation. The abdomen was shaved and cleaned thoroughly using a povidone-iodine surgical scrub (7.5%; Betadine, Atlantis Health Australia, Macquarie Park, Australia). Prior to the first incision, the intended incision line was injected with lidocaine (2 mg/kg; lignocaine, Troy Lab) and bupivacaine (1 mg/kg; Marcaine, AstraZeneca, Macquarie Park, Australia) to provide local anaesthesia. A small, lateral incision (~6 cm) in the skin was made before an incision in the abdominal wall, corresponding with the linea alba. An incision was then made through the uterine lining, and each fetus was removed. Following the removal of all fetal units, each layer was sutured closed. Once consciousness and posture were regained, the dam was returned to a home cage with a heating pad. The dam was then administered meloxicam (0.1 mg/kg oral; Apex Meloxicam, Dechra, Australia) at 1 h and 24 h and buprenorphine (0.05 mg/kg subcutaneous) at 10 h and 24 h post-operation. Dams typically returned to eating and moving around their cage 6–8 h post-operation with lactation occurring within 4 d of the procedure. Any remaining superficial sutures were removed 10 d post-procedure.

### Neonate care

Resuscitation and respiratory support of neonatal pups were performed as previously described.[16,51] In short, respiratory support was provided for at least 3mins for all preterm-born pups using continuous positive airway pressure at 5 cm $H_2O$ through a Neopuff, *T*-piece, infant resuscitator (Fisher and Paykel, Auckland, New Zealand) with blended oxygen delivered at 5 L/min. Curosurf (poractant alfa, 240 mg surfactant/3 mL) was administered nasally immediately post-birth and at 3 h. Once stable respiration was maintained, pups were housed with their corresponding littermates in a warm, humidified incubator (Bird Brooder ICU, Bell South, Australia) for 48–72 h prior to being returned to their dam's cage. Term pups were returned to home cages within 24 h.

Preterm pups were fed 0.2–0.4 ml of Impact Guinea Pig Colostrum replacement (Wombaroo Food Products, Adelaide, Australia) via syringe every 2 h until 12 h old. Subsequently, pups were fed 0.5–1 ml of Impact Guinea Pig Milk replacement (Wombaroo Food Products) every 2 h or as required to supplement independent suckling. Pups allocated to the preterm treatment groups received tiagabine (2.5 mg/kg/day) or zuranolone (1 mg/kg/day), split into two doses, orally in 1 µl/µg 15% β-cyclodextrin to increase solubility and improve the bioavailability of the compounds, whilst pups in the preterm vehicle group received an equivalent volume of 15% β-cyclodextrin. All treatments continued until term equivalent age. Additionally, a well-being score based on respiration, posture and movement was recorded every two hours.[52] Survival rates for term-born pups were 90% (males) and 100% (females), preterm-born vehicle-treated pups 57% (males) and 77% (females), preterm-born tiagabine-treated pups 57% (males) and 80% (females) and preterm-born zuranolone-treated pups 43% (males) and 77% (females).

### Tissue collection

Pups were euthanised via $CO_2$ asphyxiation at corrected PND42.[16] Each cerebellum was sectioned in the sagittal plane with the left side being fixed in 10% neutral buffered formalin solution and the right snap frozen in liquid nitrogen.

### Behavioural test

Pups underwent behavioural testing on PND8 and PND41.[40,53] Open field (OF) and elevated plus maze (EPM) tests were recorded and analysed using ANY-maze tracking software v4.7 (Stoelting Co., Wood Dale, USA). All testing instruments were cleaned using 70% ethanol between each animal.

### Open field arena

Pups were placed into and allowed to freely explore the OF arena (50 × 50 cm) for 5 min with their activity recorded. The recorded footage was divided into 5 × 5 cm grids with an inner zone designated. Parameters such as distance travelled and number of entries into the inner zone were recorded.

### Elevated plus maze

Pups were placed into an arena consisting of two open arms (10 cm width) and two high-walled closed arms (50 cm height, 10 cm width) arranged in a '+' shape situated 80 cm off the ground. Pups were placed in the maze and allowed to freely roam for 5 min. Parameters such as distance travelled in the closed and open arms and time spent in each arm were recorded.

### Immunohistochemistry

Fixed cerebellar tissue, embedded in paraffin and sectioned into 8 µm thick sections using the Leica RM2154 Microtome (Leica Microsystems PTY LTD, North Ryde, Australia), was stained using antibodies for MBP (myelin), Olig2 (oligodendrocytes), NeuN (neurons) and Calbindin (GABAergic interneurons) (Table 1). Staining was performed on lobes IX, X, and the deep white matter (DWM) of the cerebellum as previously described.[54,55] Briefly, dewaxing and rehydration of the tissue were performed using xylene and ethanol incubations followed by antigen retrieval. All slides then underwent a 15 min cooling period before undergoing washes in 0.1M PBS (pH 7.4) for MBP and NeuN or 0.1M PBST (pH 7.4) for Calbindin and Olig2. Blocking of endogenous peroxidases (20–30 min) and non-specific staining (1 h) was then performed. Primary antibody incubation was performed overnight and followed by PBS washes prior to secondary antibody incubation (1 h). Following a series of PBS washes, tertiary incubation (1 h) was performed using streptavidin-biotin-horseradish peroxidase complex (ab7403, Abcam, Melbourne, Australia) at 1:400 diluted in 0.1M PBS for all antibodies. Incubation in 3,3'-diaminobenzidine tetrahydrochloride solution (metal enhanced DAB substrate kit #34,065; Pierce, ThermoFisher Scientific, Scoresby, Australia) was then performed following a final series of PBS washes to reveal

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**Table 1.** Immunohistochemical methods for each target protein

| Target protein | Antigen retrieval | Endogenous peroxidase block | Non-specific block | Primary antibody | Secondary antibody |
|---|---|---|---|---|---|
| MBP (myelin) | Citrate Buffer (pH 6.0) at 95°C for 25 min | 3% $H_2O_2$ for 20 min | 2% goat serum, 0.4% BSA, 0.3% Triton-X, 0.1M PBS | 1:1000 Rat anti-MBP (M9443, Sigma) in 0.4% BSA, 0.3% Triton-X, 0.1M PBS | 1:300 Goat anti-rat (B7139, Sigma) in 0.4% BSA, 0.3% Triton-X, 0.1M PBS |
| NeuN (neurons) | | | | 1:1000 Mouse anti-NeuN (MAB377, Millipore, Burlington, USA) in 0.4% BSA, 0.3% Triton-X, 0.1M PBS | 1:300 Goat anti-mouse (B6649, Sigma) in 0.3% Triton-X, 0.1M PBS |
| Calbindin (GABAergic interneurons) | | 3% $H_2O_2$ for 30 min | 5% goat serum, 1% BSA and 0.3% Triton-X. 0.1M PBS | 1:1000 Rabbit anti-Calbindin (AB49899, Abcam, Cambridge, UK) in 1% BSA, 0.3% Triton-X. 0.1M PBS | 1:2000 Goat anti-rabbit (B8895, Sigma) in 0.3% Triton-X, 0.1M PBS |
| Olig2 (oligodendrocytes) | Baked at 60°C for 1 h followed by Tris-EDTA buffer (pH 9.0) at 110°C for 25 min | | 5% goat serum, 1% BSA and 0.05% Tween20, 0.1M PBS | 1:500 Rabbit anti-Olig2 (AB9610, Millipore) in 1% BSA, 0.3% Triton-X, 0.1M PBS | |

Specific methods and reagents for immunohistochemical analyses in guinea pig cerebellum tissue.

immunolabelling. Stained slides were cover slipped and imaged using the Aperio Imaging System (Leica Biosystems, North Ryde, Australia). Analysis of slides was performed using ImageJ v1.51 (National Institutes of Health, Bethesda, Maryland), which allowed for analysis of area coverage of positive staining (MBP and NeuN) and for counting cells/mm$^2$ (Olig2 and Calbindin). For MBP and NeuN, three fields of view per lobe from three consecutive sections were analysed and averaged to obtain the area coverage for each lobe, whilst average cell counts within each lobe were obtained for Olig2 and Calbindin. Cell counts were performed within the molecular and granule cell layers of Lobes IX, X and the cerebellar DWM using the polygon selection tool (ImageJ) to ensure the measured area included only the layer being quantified.

### *High throughput RT-PCR using fluidigm methodology*

Frozen cerebellar tissue was homogenised in RLT Plus Buffer (Qiagen PTY LTD, Chadstone, Australia) using the Precellys 24 dual-tissue homogeniser (Bertin Technologies, France), and RNA extraction was then performed using the Qiagen RNeasy Plus Mini Kit (Qiagen) via the protocol supplied with the kit. Total RNA was quantified using a Nanodrop™ One Spectrophotometer (ThermoFisher Scientific) with absorbance ratios at 260/280 nm and 260/230 nm used to assess purity and quality of the extracted RNA and agarose gel electrophoresis used to further confirm sample integrity.

cDNA synthesis was performed using the Superscript IV Reverse Transcription Kit (Invitrogen) on a GeneAmp 9700 PCR machine (Applied Biosystems, Life Technologies PTY LTD, Mulgrave, Australia). cDNA samples then underwent preamplification in a Quantstudio 6 Flex RT-PCR system (Applied Biosystems) using Preamp Master Mix (Fluidigm, San Francisco, USA) as per the manufacturer's instructions. Relative mRNA expression was investigated simultaneously on an integrated, microfluidic chip (Fluidigm) with primer master mix for each primer (0.5pmol/µl) (Fluidigm) and EVAGreen probe (Bio-Rad Laboratories, Hercules, USA) used to detect PCR products for the genes of interest (Table 2). RT-PCR was performed as previously described using the Biomark HD system (Fluidigm) via the comparative CT method of analysis, normalised to two housekeeping genes (*TBP* and *UBE2D2*).[56]

### *Statistical analysis*

Data were analysed using Prism v10.0 (GraphPad Software Inc., La Jolla, USA) and are presented as mean ± SEM with significance considered $p < 0.05$. Body weight and well-being scores were assessed over time by repeated measures two-way ANOVA or mixed effects analysis. Gene expression, protein abundance and individual behaviour parameter data sets were analysed by one-way ANOVA. Tukey's corrections for multiple comparisons were performed when the overall analysis was $p < 0.05$. Hyperactive behavioural composite scores were calculated based on similar previously reported behaviour scoring systems in mice[57]. Briefly, single behavioural test parameters displaying significant or trending ($p < 0.1$) results were $z$-standardised and averaged to establish OF, EPM and overall (combined tests) composite scores so that higher values reflect higher severity of hyperactive/inattentive-like behaviours. Simple linear regression was performed to determine relationships between behaviour composite score and myelin and oligodendrocyte protein staining. When a significant relationship was found, a Pearson correlation was performed to determine the directionality and magnitude of the correlation. All data were split by sex.

## Results

### *Physical characteristics*

At birth, preterm zuranolone-treated males weighed significantly less than term-born males (Table 3, $p = 0.0004$). Additionally, term-born females were heavier at birth than preterm females (vehicle $p = 0.0007$; tiagabine $p = 0.0006$; and zuranolone $p = 0.0006$). Despite this, there were no significant differences in body weight for either sex at post-mortem and although zuranolone-treated females appeared to weigh less than term-born females this did not reach significance ($p = 0.055$). No significant differences in growth rate were identified in males; however, tiagabine-treated females grew significantly more than term-born females ($p = 0.0088$). An increased brain:body weight ratio was identified in preterm-born males that received vehicle compared to term-born males ($p = 0.0128$) although no differences

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**Table 2.** Guinea pig-specific primers for genes of interest

| Gene ID | Protein | Amplicon length | Forward primer/ Reverse primer |
|---|---|---|---|
| *BDNF* | Brain-derived neurotrophic factor | 75 | AATCGGCTGGCGGTTCATAA<br>AGCCACTATCTGCCCCTCTTA |
| *DLG4* | Postsynaptic density protein 95 (PSD-95) | 70 | TATTCCCAGCACCTGGACAA<br>TCATGGCTGTGGGGTAATCA |
| *GAD1* | Glutamic acid decarboxylase | 74 | ACGAGAAAACTGGGGCTGAA<br>ACAAGCCGACTCTTCTCTTCC |
| *GLS1* | Glutaminase | 78 | CACGTTGGTCTTCCTGCAAA<br>GCACATCATGCCCATGACA |
| *GRIN1* | NMDA subunit type 1 | 82 | AGAGCATCCACTTGAGCTTCC<br>TACACGCGCATCATCTCGAA |
| *GRIN2A* | NMDA subunit type 2A | 80 | TCGAGGATGCGAAGACACAA<br>AGCCTCGTCTTTGGAGCAATA |
| *MBP* | Myelin basic protein | 66 | ACCTCCTCCGTCTCAAGGAAA<br>GCTCTGCCTCCATAGCCAAA |
| *OLIG2* | Oligodendrocyte transcription factor 2 | 78 | GCACTCATCCTGGGGACAA<br>CCGACGACGTGGATGATGAA |
| *PVALB* | Parvalbumin | 77 | AAGGATGGGGACGGCAAA<br>GGGTCCATCAGCTCTGCTTA |
| *RBFOX3* | RNA-binding protein fox-1 | 88 | CACAGACAGACAGCCAACCA<br>CGGAAGGGGATGTTGGAGAC |
| *SLC6A1* | GABA transporter 1 | 77 | AGCGCTGCTTCTCCAACTAC<br>ATTGCGCTCCCAAAACTCCA |
| *SLC6A11* | GABA transporter 3 | 82 | ATCATGCTCTGCTGCCTGAA<br>ATCATGCTCTGCTGCCTGAA |
| *SLC17A7* | Vesicular glutamate transporter 1 | 86 | CAGCCTTTTGCGGTTCCTAC<br>AACAGAGCTCCATCCCGAATAC |
| *SLC17A8* | Vesicular glutamate transporter 3 | 81 | GGATGGGCTTCGGTCTTCTA<br>GCACTCATAGGCTTGCAACA |
| *TBP* | TATA-binding protein | 79 | CAAGCGGTTTGCTGCTGTAA<br>CACCATCTTCCCGGAACTGAA |
| *UBE2D2* | Ubiquitin-conjugating enzyme E2 D2 | 77 | CAGTGCTGCGTGTTGTACATA<br>TGCTAGGAGGCAATGTTGGTA |

Primer sequences for detection of genes of interest in the guinea pig cerebellum. Primer sequences are displayed from 5'-3' for forward and reverse primers.

were observed in females. Term-born females had significantly lower adrenal:body weight ratios than preterm-born females that received vehicle ($p = 0.0017$) or zuranolone ($p = 0.0327$). Growth rate, supplemental feeding volume and well-being scores differed as expected between term and preterm groups in the immediate neonatal period; however, importantly, there were no significant differences identified between preterm groups for either sex (data not presented).

## Behaviour

### Early childhood behavioural testing in male offspring

At PND8, in the OF arena, preterm-born males that received vehicle had significantly higher distance travelled than term-born males (Fig. 1*A*; $p = 0.0469$). Preterm-born males that received vehicle treatment also had significantly higher inner-zone (IZ) entries (Fig. 1*B*; $p = 0.0039$), time spent (Fig. 1*C*; $p = 0.0302$), distance travelled (Fig. 1*D*; $p = 0.0049$) and time mobile in inner-zone (Fig. 1*E*; $p = 0.0153$) compared to term-born males. Zuranolone-treated males appeared to have decreased distance travelled overall and in the inner-zone compared to vehicle-treated preterm males; however; neither of these comparisons reached significance (Fig. 1*A*; $p = 0.0753$, and Fig. 1*D*; $p = 0.0518$). These five parameters were *Z*-normalised (Fig. 1*F*), and an OF composite score generated (Fig. 1*G*). Overall, preterm-born males that received vehicle treatment demonstrated a significantly higher OF composite score than term-born males (Fig. 1*G*; $p = 0.0046$) and zuranolone treatment in preterm males appeared to decrease OF composite score compared to vehicle-treated preterm-born males, but it did not reach significance ($p = 0.1013$).

At PND8, in the EPM, preterm-born males that received vehicle treatment exhibited significantly higher overall distance travelled (Fig. 1*H*; $p = 0.0093$) and open arm distance travelled (Fig. 1*J*; $p = 0.0005$). They also appeared to have more time mobile overall and in the open arm compared to term-born males but this did not reach significance (Fig. 1*I*; $p = 0.0648$, and Fig. 1*K*; $p = 0.0921$). Distance travelled in the open arms was found to be significantly reduced in zuranolone-treated preterm males (Fig. 1*J*, $p = 0.0086$)

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**Table 3.** Body weights and organ weight ratios

| Sex | Delivery | Treatment | *N* | Birth Wgt | Post-mortem body Wgt | Body weight growth % | Brain:Body % | Cereb:Brain % | Liver:Body % | Heart:Body % | Kidney:Body % | Adrenals:Body % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Male | Preterm | Vehicle | 4 | 72.3 ± 10.4 | 287.3 ± 24.5 | 427.6 ± 74.0 | 1.07 ± 0.06* | 5.76 ± 0.21 | 4.99 ± 0.32 | 0.50 ± 0.05 | 1.16 ± 0.04 | 0.038 ± 0.005 |
| | | Tiagabine | 4 | 74.9 ± 3.5 | 365.5 ± 7.8 | 490.0 ± 13.4 | 0.86 ± 0.03 | 6.47 ± 0.40 | 4.52 ± 0.15 | 0.58 ± 0.08 | 1.01 ± 0.02 | 0.031 ± 0.002 |
| | | Zuranolone | 3 | 60.8 ± 1.3* | 306.0 ± 44.4 | 501.9 ± 69.0 | 0.97 ± 0.09 | 5.86 ± 0.57 | 4.49 ± 0.33 | 0.48 ± 0.08 | 1.06 ± 0.02 | 0.035 ± 0.002 |
| | Term | N/A | 9 | 85.1 ± 3.6 | 361.8 ± 14.7 | 428.5 ± 17.5 | 0.84 ± 0.03 | 6.57 ± 0.43 | 4.29 ± 0.17 | 0.43 ± 0.02 | 0.91 ± 0.10 | 0.062 ± 0.026 |
| Female | Preterm | Vehicle | 7 | 68.1 ± 4.8* | 311.6 ± 19.1 | 462.2 ± 21.2 | 0.97 ± 0.04 | 6.68 ± 0.56 | 4.17 ± 0.30 | 0.45 ± 0.03 | 1.02 ± 0.03 | 0.052 ± 0.003* |
| | | Tiagabine | 8 | 68.6 ± 3.3* | 335.3 ± 12.4 | 496.5 ± 28.0* | 0.90 ± 0.03 | 6.71 ± 0.44 | 4.66 ± 0.26 | 0.47 ± 0.03 | 0.99 ± 0.03 | 0.047 ± 0.003 |
| | | Zuranolone | 7 | 67.9 ± 3.0* | 306.1 ± 6.2 | 455.5 ± 18.4 | 0.97 ± 0.03 | 7.48 ± 0.69 | 4.23 ± 0.27 | 0.48 ± 0.07 | 1.01 ± 0.04 | 0.049 ± 0.001* |
| | Term | N/A | 11 | 90.2 ± 3.3 | 353.3 ± 10.5 | 396.5 ± 18.2 | 0.87 ± 0.03 | 7.25 ± 0.62 | 4.24 ± 0.16 | 0.45 ± 0.01 | 0.93 ± 0.02 | 0.038 ± 0.002 |

Mean ± SEM for body (in grams) and organ weights (ratio to body or brain weight) recorded upon tissue collection at corrected PND42. * Signifies $p < 0.05$ compared to term within sex. No differences between preterm groups within sex were observed. Wgt = weight, Cereb = cerebellum.

and almost reached significance in tiagabine-treated preterm males ($p = 0.0581$) compared to vehicle-treated preterm males. These parameters were *Z*-normalised (Fig. 1*L*) and an EPM composite score generated (Fig. 1*M*). Preterm-born males that received vehicle had a significantly higher EPM composite score compared to term-born males (Fig. 1*M*; $p = 0.0016$). Zuranolone treatment appeared to reduce this composite score, but it did not reach significance ($p = 0.0717$).

The overall hyperactivity severity score demonstrated that in early childhood preterm-born males that received vehicle had significantly higher hyperactivity severity scores than term-born males (Fig. 1*N*; $p = 0.0003$). Zuranolone-treated preterm-born males had significantly reduced scores ($p = 0.0433$) and tiagabine-treated preterm males' scores appeared to be lower than vehicle-treated preterm males but did not reach significance ($p = 0.0848$).

### *Early childhood behavioural testing in female offspring*

At PND8, in the EPM, preterm-born females that received vehicle had significantly higher distance travelled than term-born females (Fig. 2*A*; $p = 0.0351$). Preterm-born females that received vehicle and tiagabine-treated females were found to have significantly higher entries into the open arm than term-born females (Fig. 2*B*; $p = 0.0098$ and $p = 0.0128$, respectively). Preterm-born females that received vehicle and zuranolone-treated females also had significantly higher distance travelled in the open arm compared to term-born females (Fig. 2*C*; $p = 0.0168$ and $p = 0.0103$, respectively). These parameters were *Z*-normalised (Fig. 2*D*) and the PND8 EPM composite score was found to be significantly lower in term-born females than preterm-born females that had received vehicle (Fig. 2*E*; $p = 0.0050$), tiagabine ($p = 0.0345$) and zuranolone ($p = 0.0436$). Female offspring at PND8 demonstrated no significant differences in the OF arena in any of the parameters examined (data not presented) and hence an overall hyperactivity severity score was not generated.

### *Late childhood behavioural testing*

At PND41, there were no differences identified within male or female OF testing nor between the female offspring in the EPM testing. In the EPM, preterm-born males that received vehicle had significantly higher distance travelled in the open arm compared to term-born males (Fig. 3*A*; $p = 0.0144$), tiagabine-treated males ($p = 0.0092$) and zuranolone-treated males ($p = 0.0274$). Preterm-born males that received vehicle treatment also spent significantly more time in the open arm compared to tiagabine-treated males (Fig. 3*B*; $p = 0.0329$).

### *Mature myelin, oligodendrocyte and neuronal protein expression in the late childhood cerebellum*

In cerebellar lobe IX, tiagabine-treated females appeared to have increased mature myelin compared to preterm-born females that received vehicle but this did not quite reach significance (Fig. 4*A*; $p = 0.0508$). Within cerebellar lobe X, preterm-born males that received vehicle had significantly less mature myelin than term-born males (Fig. 4*B*; $p = 0.0141$) and tiagabine-treated males ($p = 0.0008$). Preterm-born females that received vehicle were also found to have significantly less mature myelin in lobe X than term-born ($p = 0.0059$) and tiagabine-treated females ($p = 0.0001$). Within the cerebellar DWM, there was significantly less myelin in preterm-born males that received vehicle compared to term-born (Fig. 4*C*; $p = 0.0012$), tiagabine-treated ($p = 0.0014$) and zuranolone-treated males ($p = 0.0005$). Similarly, significantly less myelin

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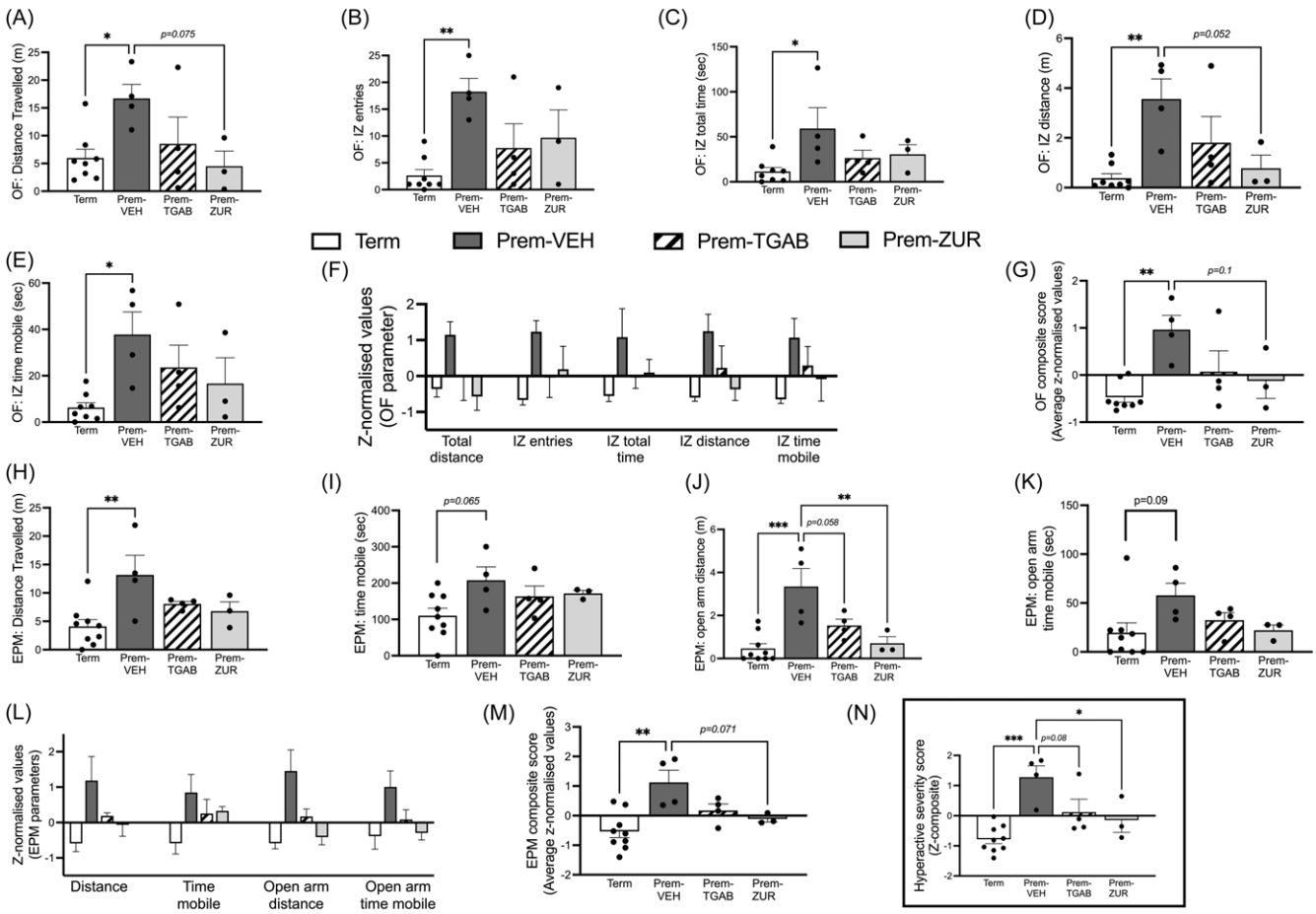


**Figure 1.** Assessment of behavioural testing parameters for the open field (OF) arena and elevated plus maze (EPM) in male PND8 guinea pigs born at term (white bars), preterm with vehicle treatment (Prem-VEH; dark grey bars), tiagabine treatment (Prem-TGAB; hashed bars) or zuranolone treatment (Prem-ZUR; light grey bars). Male offspring underwent open field (A–G) and EPM testing (H-M) on corrected PND8. Individual open field parameters: (A) total distance travelled, (B) entries into the inner zone (IZ), (C) time spent in IZ, (D) distance travelled in IZ and (E) time mobile in IZ. (F) *Z*-normalised values of significant individual OF parameters integrated into the OF composite score (G). Individual EPM parameters: (H) total distance travelled, (I) time spent mobile, (J) distance in the open arms, (K) time mobile in the open arms. (L) *Z*-normalised values of significant individual EPM parameters integrated into the EPM composite score (M). (N) Hyperactivity-like behaviour composite score. Data presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

protein was observed in the DWM of preterm-born females that received vehicle compared to term-born ($p = 0.0020$), tiagabine-treated ($p = 0.0237$) and zuranolone-treated females ($p = 0.0271$). There were no other significant differences identified.

In cerebellar lobe IX, preterm-born females that received vehicle exhibited significantly more OLIG2-positive cells than term-born (Fig. 5*A*; $p = 0.0008$) and tiagabine-treated females ($p = 0.0031$). In the cerebellar DWM, preterm-born females that received vehicle appeared to have increased OLIG2-positive cells compared to term-born females, but this did not reach significance (Fig. 5*C*; $p = 0.0592$). There were no other significant differences identified.

Immunostaining for overall neuronal nuclei (NeuN) and GABAergic interneurons (Calbindin) was also performed; however, there were no significant differences identified for either sex (data not presented). There were also no significant differences identified for total lobe thickness, or area or layer thicknesses of the molecular, granular and white matter tracts for lobes IX or X (Supplementary Table 1).

### *Relationship between early childhood hyperactivity and late childhood myelination in male offspring*

Hyperactivity severity scores in male offspring in early childhood (Fig. 1) were correlated against mature myelin and oligodendrocyte protein abundance in late childhood (Figs. 4 and 5). Hyperactivity severity scores were negatively correlated against mature myelin protein expression in cerebellar lobe X (Fig. 6*A*; $p = 0.039$, $r = 0.52$) and DWM (Fig. 6*B*; $p = 0.017$, $r = 0.541$). Conversely, oligodendrocyte protein expression in lobe X appeared to positively correlate with hyperactivity severity score but did not reach significance (Fig. 6*C*; $p = 0.067$, $r = 0.468$).

### *Relative mRNA expression of oligodendrocyte and neuronal genes in the cerebellum*

Contrary to the immunohistochemistry findings, no significant differences were identified for *OLIG2* (Fig. 7*A*) or *MBP* (Fig. 7*B*) mRNA expression. Expression of *MBP* appeared to be increased following zuranolone treatment in preterm-born males compared to those that received vehicle; however this did not reach significance ($p = 0.0583$). *BDNF* was, however, significantly lower following zuranolone treatment in preterm-born males compared to those that received vehicle (Fig. 7*E*; $p = 0.0241$) and also appeared to be lower than term-born, but this did not reach significance ($p = 0.0515$). *DLG4* was significantly higher in term-born females than in preterm-born females that received vehicle (Fig. 7*F*; $p = 0.0410$) and tiagabine ($p = 0.0205$). There were no

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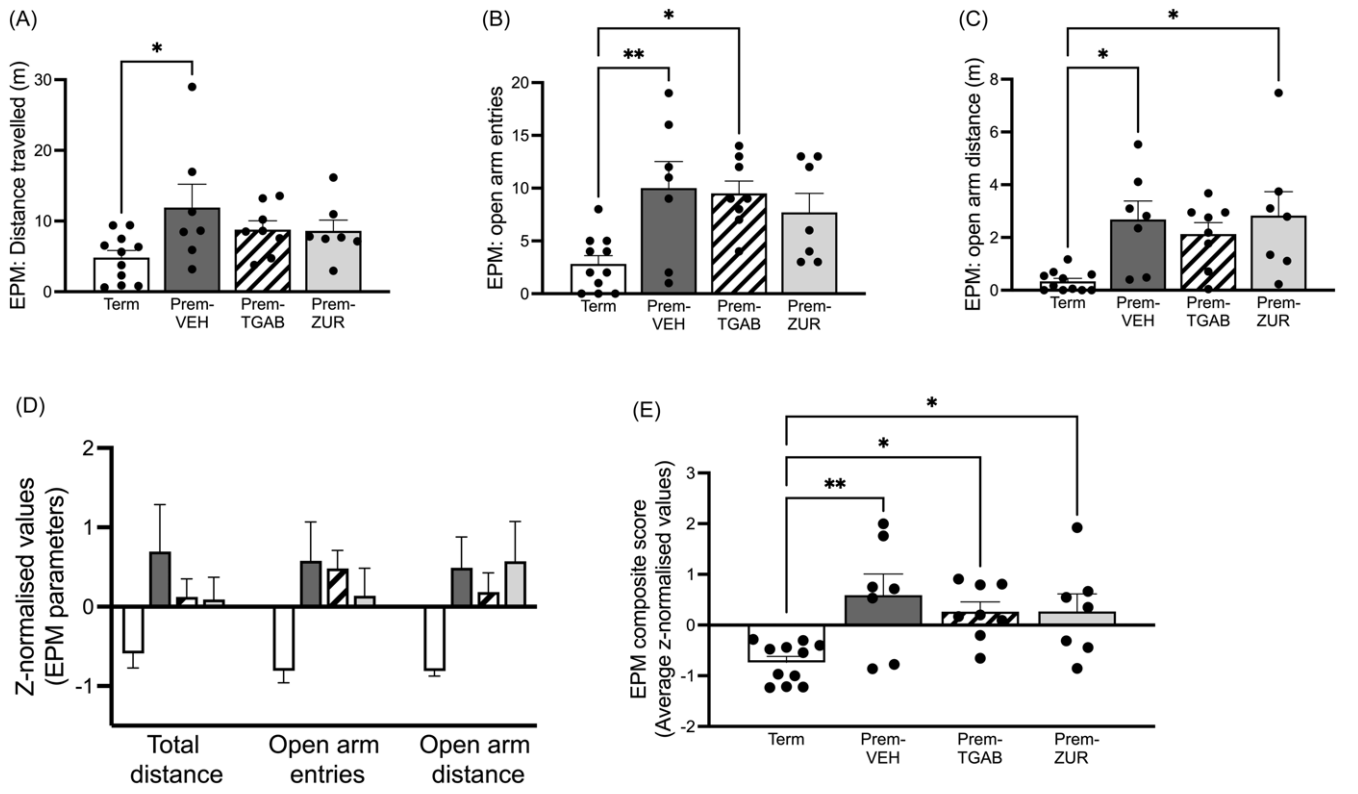


**Figure 2.** Assessment of behavioural testing parameters for the elevated plus maze (EPM) in female PND8 guinea pigs born at term (white bars), preterm with vehicle treatment (Prem-VEH; dark grey bars), tiagabine treatment (Prem-TGAB; hashed bars) or zuranolone treatment (Prem-ZUR; light grey bars). Female offspring underwent EPM testing on corrected PND8. Individual EPM parameters: (A) total distance travelled, (B) open arm entries, (C) distance travelled in the open arms, (D) *Z*-normalised values of significant individual EPM parameters integrated into the EPM composite score (E). Data presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$).

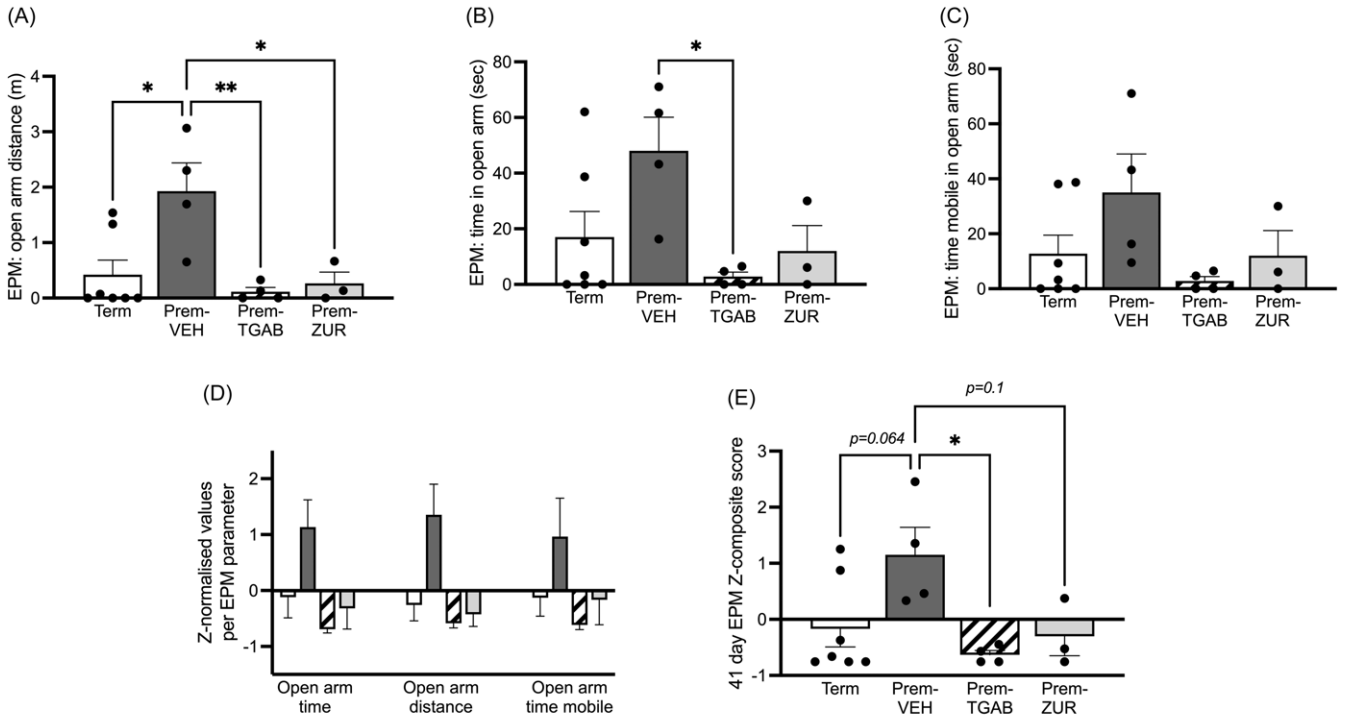


**Figure 3.** Assessment of behavioural testing parameters for the elevated plus maze (EPM) in male PND41 guinea pigs born at term (white bars), preterm with vehicle treatment (Prem-VEH; dark grey bars), tiagabine treatment (Prem-TGAB; hashed bars) or zuranolone treatment (Prem-ZUR; light grey bars). Male offspring underwent EPM testing on corrected PND41. Individual EPM parameters: (A) distance travelled in the open arms, (B) time in the open arms. Data presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$).

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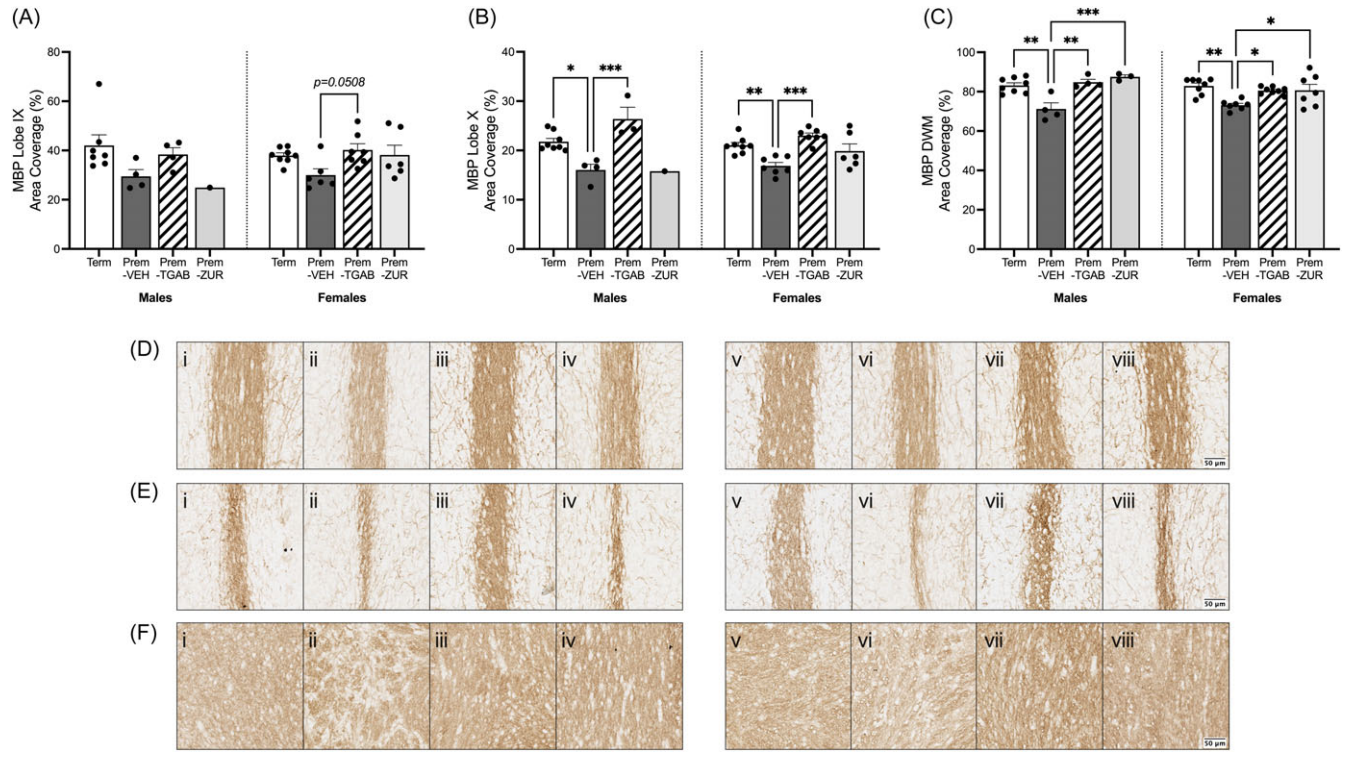


**Figure 4.** Myelin basic protein (MBP) immunostaining area coverage in (A) lobe IX, (B) lobe X and (C) cerebellar deep white matter (DWM) of PND42 guinea pigs born at term (white bars), and preterm with vehicle (Prem-VEH; dark grey bars), tiagabine treatment (Prem-TGAB; hashed bars) or zuranolone treatment (Prem-ZUR; light grey bars). Data presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$). Representative photomicrographs are shown for (D) lobe IX, (E) lobe X and (F) cerebellar DWM with scale bar = 50 µm. Males (i = term, ii = Prem-VEH, iii = Prem-TGAB, iv = Prem-ZUR), and females (v = term, vi = Prem-VEH, vii = Prem-TGAB, viii = Prem-ZUR).

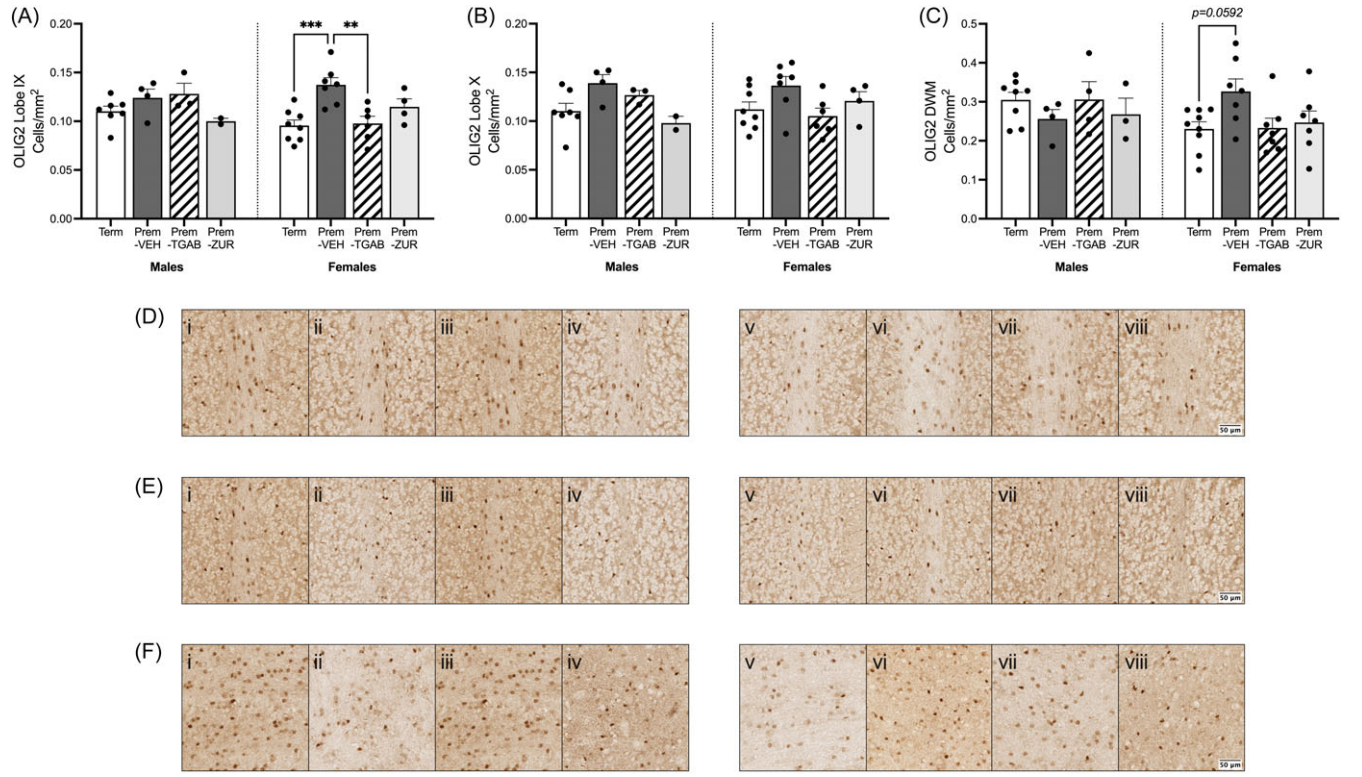


**Figure 5.** OLIG2 positive cells in (A) lobe IX, (B) lobe X and (C) cerebellar deep white matter (DWM) of PND42 guinea pigs born at term (white bars) and preterm with vehicle (Prem-VEH; dark grey bars), tiagabine treatment (Prem-TGAB; hashed bars) or zuranolone treatment (Prem-ZUR; light grey bars). Data presented as mean ± SEM (**$p < 0.01$, ***$p < 0.001$). Representative photomicrographs are shown for (D) lobe IX, (E) lobe X and (F) cerebellar DWM with scale bar = 50 µm. Males (i = term, ii = Prem-VEH, iii = Prem-TGAB, iv = Prem-ZUR) and females (v = term, vi = Prem-VEH, vii = Prem-TGAB, viii = Prem-ZUR).

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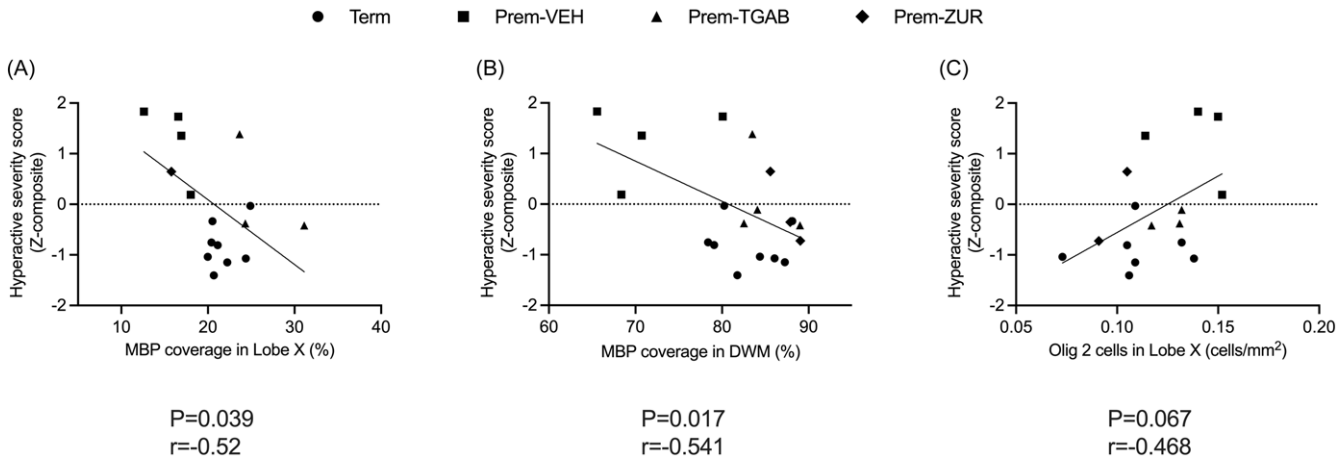


**Figure 6.** Early childhood hyperactivity severity scores in males correlated with myelination in (A) cerebellar lobe X, (B) cerebellar DWM as well as oligodendrocyte positive cell density in (C) cerebellar lobe X (Term = circles, Prem-VEH = squares, Prem-TGAB = triangles, Prem-ZUR = diamonds).

significant differences identified in *RBFOX3* (Fig. 7*C*) or *PVALB* expression (Fig. 7*D*).

### *Relative mRNA expression of excitatory/inhibitory pathway genes in the cerebellum*

*SLC6A1* (GAT1) was significantly higher in term-born females compared to preterm-born females that received vehicle (Fig. 8*C*; $p = 0.0110$) or tiagabine ($p = 0.0224$). *SLC17A7* (VGLUT1) appeared to be increased in term-born females compared to tiagabine-treated preterm-born females; however, this did not reach significance (Fig. 8*E*; $p = 0.0569$), whilst *GRIN1* was significantly higher in term-born females than preterm-born females that received vehicle (Fig. 8*G*; $p = 0.0443$) or tiagabine ($p = 0.0076$). There were no significant differences identified in *GAD1, GLS1, SLC6A11, SLC17A8 or GRIN2A* gene expression.

## Discussion

This study has demonstrated the potential novel neurosteroid replacement therapies have for reducing the adverse impacts of preterm birth. Specifically, the study demonstrated that these treatments reduce hyperactive behaviour, promote oligodendrocyte maturation and restore myelination in the cerebellum. This is consistent with our previous findings that preterm-born guinea pigs display a hyperactive phenotype during childhood.[16,40] There were differences in hyperactivity between the two sexes with a more definitive hyperactive-impulsive phenotype observed in early childhood-age males than was observed in females. This phenotype in males was demonstrated by more overall motor activity (distance travelled and movements between zones/arms entered) and novelty-seeking and risk-taking behaviours (increased exploration of the inner zone and open arms). The literature shows there is a clear bias towards males developing ADHD and even when diagnosed with ADHD, females are understood to typically display symptoms of inattention, whilst males are more prone to hyperactive and impulsive traits including novelty-seeking and risk-taking behaviours.[58] The decreased prevalence and intensity of hyperactive symptomology in females may explain the lesser hyperactive phenotype observed within the female preterm cohort in this study. Despite not displaying a typical hyperactive phenotype at PND41, preterm-born females may still experience ADHD-like symptoms that our methodologies are unable to assess, such as the inattentive symptoms they are more likely to experience. It was also observed that by late childhood, males only displayed hyperactive traits in the EPM with insufficient evidence of a full hyperactive phenotype. Many individuals will "grow out" of their hyperactivity disorders and this may partially explain the reduced hyperactivity metrics in late childhood.[59] Alternatively, the true phenotype of these late childhood-aged pups may be masked by the size of the OF arena. No differences were found by the OF testing, but it has been noted that the OF may not be of appropriate size for the much larger, late childhood-age guinea pigs. A larger OF arena may have, when paired with the EPM results, elucidated a hyperactive phenotype in late childhood-age pups. Importantly, zuranolone and, to a lesser extent, tiagabine were found to reduce hyperactive behaviour in males but not females at PND8.

Myelination is critical in providing insulation to axons and promoting efficient action potential propagation. We observed significantly reduced myelination in cerebellar lobes IX, X and in the DWM in females and reduced myelination in lobe X and cerebellar DWM in males following preterm birth. The cerebellum relies heavily on its myelinated axonal outputs for its communication with extracerebellar brain regions involved in behavioural and cognitive processing.[25] Despite there being no conclusive evidence showing a direct relationship between myelination and hyperactive phenotypes, there is, however, a large body of evidence indicating that myelination deficits and white matter structural abnormalities are present in animal models that exhibit hyperactive phenotypes and in human children, adolescents and adults with ADHD.[60–62] The deficits observed here in cerebellar myelination may underlie the hyperactive phenotypes seen in the preterm-born pups. Importantly, this study also found that tiagabine and, to a lesser extent, zuranolone were able to prevent the decreased myelination in both sexes although, in females, this did not equate to reductions in hyperactivity suggesting that myelination deficits in other brain regions may be responsible for the phenotype in females. The impact of these treatments on cerebellar myelination which persists into late childhood, may underpin a potential mechanism through which these treatments reduce hyperactive phenotypes following preterm birth. Additionally, in males, mature myelin protein expression was negatively correlated against hyperactivity severity, mirroring

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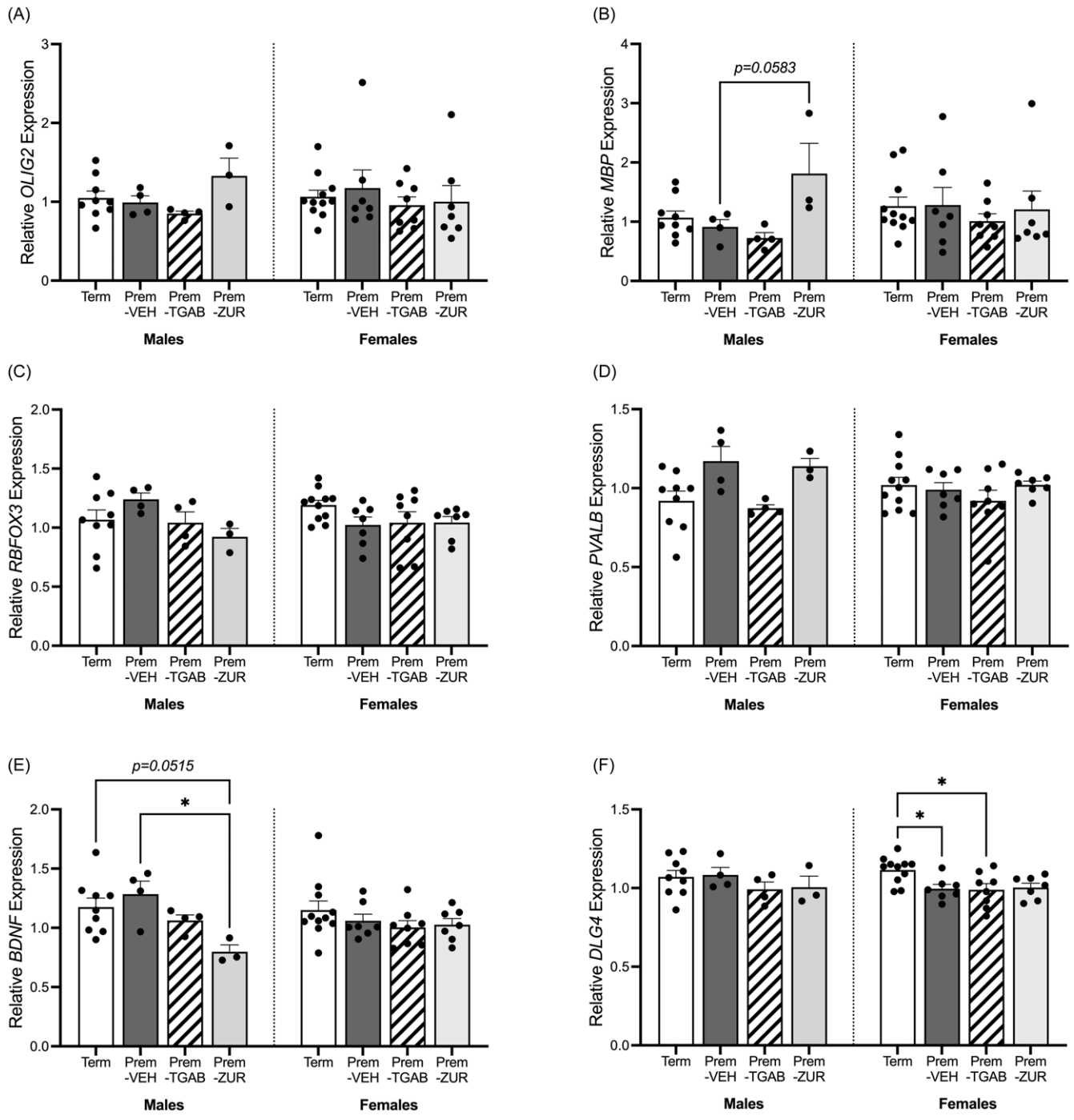


**Figure 7.** Relative mRNA expression of neurone and oligodendrocyte population-related genes (A) *OLIG2*, (B) *MBP*, (C) *RBFOX3*, (D) *PVALB*, (E) *BDNF* and (F) *DLG4* in the cerebellum of PND42 guinea pigs born at term (white bars) and preterm with vehicle (Prem-VEH; dark grey bars), tiagabine treatment (Prem-TGAB; hashed bars) or zuranolone treatment (Prem-ZUR; light grey bars). Data presented as mean ± SEM (*$p < 0.05$).

clinical findings that show impaired cerebellar myelination in ADHD and anxiety disorders.[63–65] This further supports the use of these treatments for reducing the development of hyperactive behaviour through preventing impaired myelination following preterm birth.

Oligodendrocytes are critically important in the production of myelin within the brain and these glial cells are known to mature across a developmental lineage. Olig2, which is known to be expressed by oligodendrocytes at all stages of the lineage, was significantly increased following preterm birth in lobe IX and the cerebellar DWM of females. Increased Olig2 expression within the cerebellum has also been reported in a mouse model of placental allopregnanolone insufficiency, whereby allopregnanolone concentration is prematurely decreased in a similar manner as following preterm birth.[57] Importantly, the increase in Olig2 expression paired with decreased MBP observed here suggests that oligodendrocyte maturation is arrested following preterm birth in females and that the impacts of this arrest continue long after the neonatal period.[66] Furthermore, in males, hyperactivity severity score was positively correlated against Olig2 positive staining in

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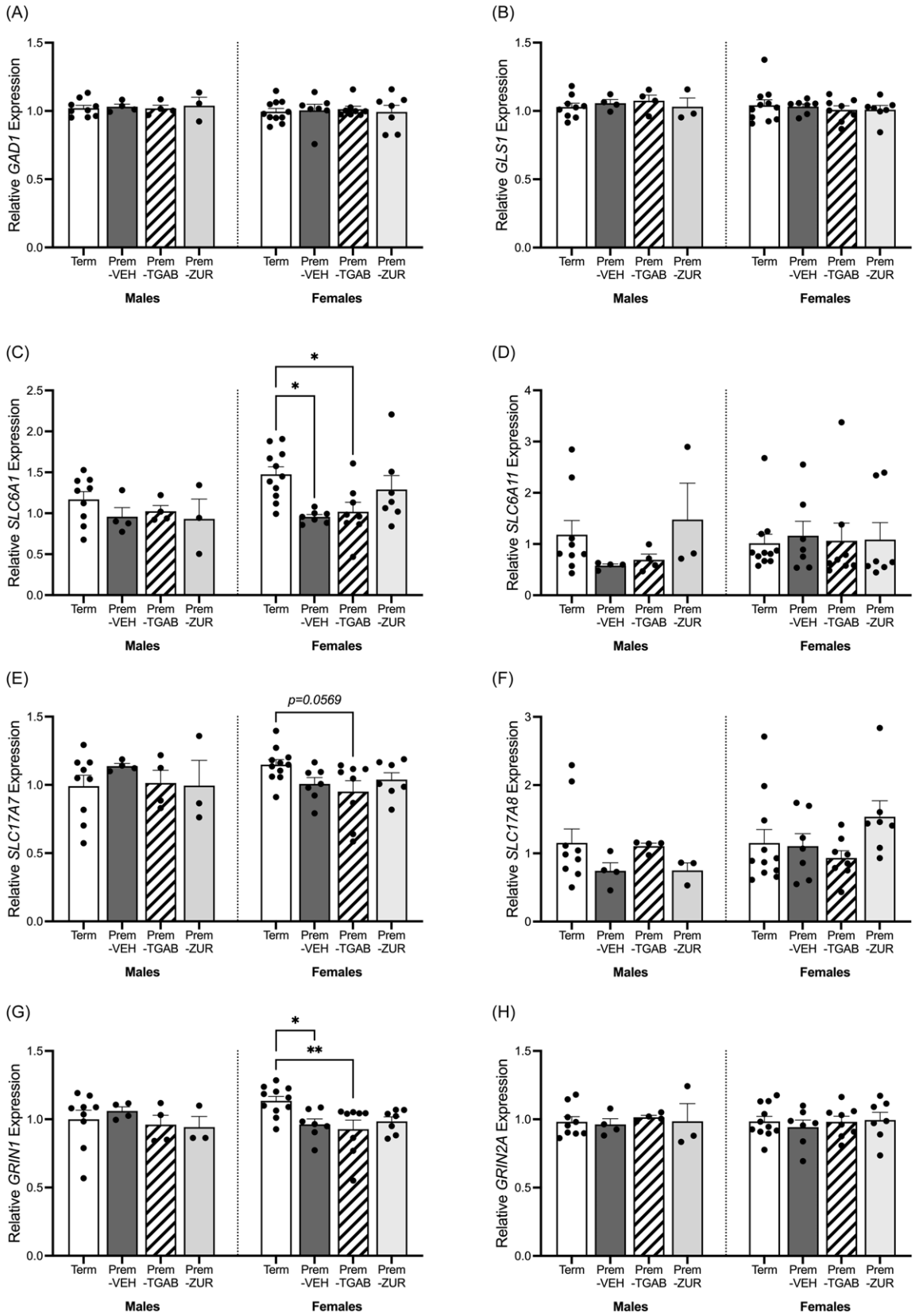


**Figure 8.** Relative mRNA expression of excitatory/inhibitory pathway-related genes (A) *GAD1*, (B) *GLS1*, (C) *SLC6A1*, (D) *SLC6A11*, (E) *SLC17A7*, (F) *SLC17A8*, (G) *GRIN1* and (H) *GRIN2A* in the cerebellum of PND42 guinea pigs born at term (white bars) and preterm with vehicle (Prem-VEH; dark grey bars), tiagabine treatment (Prem-TGAB; hashed bars) or zuranolone treatment (Prem-ZUR; light grey bars). Data presented as mean ± SEM (*$p < 0.05$, **$p < 0.01$).

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cerebellar lobe X. $GABA_A$ receptor activation is a known negative modulator of oligodendrocyte progenitor cell (OPC) proliferation, whilst preterm birth is known to cause decreased GABAergic tone due to decreased allopregnanolone concentrations.[67] Both tiagabine and zuranolone act to increase GABAergic tone, and this likely explains their ability to restore OPC proliferation, improve oligodendrocyte maturation and thus reduce hyperactive behaviour following preterm birth. In contrast, no significant differences were observed in the mRNA expression of *OLIG2* or *MBP*, despite the alterations in MBP and Olig2 protein. The mismatch in mRNA to protein expression may be explained by developmental programming, the basis of the developmental origins of health and disease hypothesis.[68] Indeed, developmental programming of the oligodendrocyte population, maturation and thereby myelination has been demonstrated previously.[69,70] Thus, it is probable that *OLIG2* and *MBP* mRNA expression would have shown significant differences in response to treatment if measured at an earlier timepoint prior to translation into protein.

Of note, components of the inhibitory/excitatory pathways in the cerebellum appeared to be impacted in preterm-born females but not males. Clearance of GABA from the extracellular space is critical in the regulation of excitatory/inhibitory balance and tiagabine works via this mechanism by blocking GAT1 (*SLC6A1*), the principal transporter for GABA reuptake by neurones and glia throughout the brain.[71,72] Interestingly, *SLC6A1* expression has been shown to decrease under reduced extracellular calcium concentrations.[73] Preterm birth-associated insults are known to result in aberrant excitation, which increases calcium influx and therefore reduces extracellular calcium. This reduced extracellular calcium may therefore be programming a decrease in *SLC6A1* which was observed in this study to persist, in female pups, into late childhood.[74] Additionally, tiagabine did not further influence *SLC6A1*. It has been shown that chronic GABA exposure, as would occur following tiagabine treatment, downregulates GAT1 expression, potentially explaining the continued decreased expression of *SLC6A1* following tiagabine treatment.[75] *GRIN1* (GLUN1) is critically important for glutamatergic signalling due to its presence in all NMDA receptors.[76] Interestingly, preterm-born female pups, including those treated with tiagabine, displayed decreases in *GRIN1*. As NMDA receptors are the primary glutamate receptor responsible for calcium signalling, this suggests that preterm birth-related insults result in glutamatergic dysfunction that persists into late childhood. *DLG4* encodes a post-synaptic density protein (PSD-95) which is responsible for the regulation and post-synaptic anchoring of NMDA and AMPA glutamate receptors.[77] Interestingly, preterm-born females (including tiagabine-treated) also had persisting, decreased expression of *DLG4* in a similar manner to *GRIN1*. As *GRIN1* and *DLG4* are critical regulators of excitation, this suggests that these females undergo a compensatory downregulation of the excitatory pathway, which is unable to be modulated by the treatments used in this study. The sexually dimorphic nature of these findings emphasises the importance of considering sexual differences in the mechanisms of preterm birth-associated neurodevelopmental impacts. Whilst we acknowledge that the male cohort is potentially underpowered ($n = 3$–9), future investigation of targeted treatments for males and females is warranted based on the differences observed here and the well-established notion that males experience increased vulnerability to preterm birth complications.[78]

In conclusion, this study has demonstrated that neurosteroid replacement therapy in the immediate neonatal period has the potential to reduce hyperactive behaviour in early childhood and prevent impairments in cerebellar oligodendrocyte maturation and myelination in late childhood. Additionally, changes in gene expression were observed, which suggest preterm birth impairs excitatory/inhibitory balance although we postulate that additional mRNA expression changes would have occurred at an earlier age, thus programming the changes in protein and behaviour observed in this study. Overall, these findings suggest that excitation-modulating, neurosteroid replacement treatments used in the immediate neonatal period following preterm birth have the potential to mitigate long-term neurodevelopmental impairments.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S2040174424000394.

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**Competing interests.** The authors confirm that there are no financial ties to products or conflicts of interest to disclose.

**Ethical standard.** 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 Scientific Research Council Australia 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).

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