Pediatric RESEARCH

BASIC SCIENCE ARTICLE

Neurosteroid replacement therapy using the allopregnanolone-analogue ganaxolone following preterm birth in male guinea pigs

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BACKGROUND: Children born preterm, especially boys, are at increased risk of developing attention deficit hyperactivity disorder (ADHD) and learning difficulties. We propose that neurosteroid-replacement therapy with ganaxolone (GNX) following preterm birth may mitigate preterm-associated neurodevelopmental impairment.

METHODS: Time-mated sows were delivered preterm (d62) or at term (d69). Male preterm pups were randomized to ganaxolone (Prem-GNX; 2.5 mg/kg subcutaneously twice daily until term equivalence), or preterm control (Prem-CON). Surviving male juvenile pups underwent behavioural testing at d25-corrected postnatal age (CPNA). Brain tissue was collected at CPNA28 and mature myelinating oligodendrocytes of the hippocampus and subcortical white matter were quantified by immunostaining of myelin basic protein (MBP).

RESULTS: Ganaxolone treatment returned the hyperactive behavioural phenotype of preterm-born juvenile males to a term-born phenotype. Deficits in MBP immunostaining of the preterm hippocampus and subcortical white matter were also ameliorated in animals receiving ganaxolone. However, during the treatment period weight gain was poor, and pups were sedated, ultimately increasing the neonatal mortality rate.

CONCLUSION: Ganaxolone improved neurobehavioural outcomes in males suggesting that neonatal treatment may be an option for reducing preterm-associated neurodevelopmental impairment. However, dosing studies are required to reduce the burden of unwanted side effects.

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INTRODUCTION

Preterm birth comprises ~10% of births each year but accounts for up to 70% of neonatal deaths, and ~50% of survivors develop a long-term neurodevelopmental disability.[1–3] Preterm infants are more likely than term-born children to develop learning disorders, anxiety and hyperactive behaviours that become apparent at school age.[4,5] There are no targeted therapies to prevent these late-developing effects, or targeted treatments for children that address the unique pathophysiology of preterm-associated developmental dysfunction. The resultant burden on the individual, their family and the wider socio-economic environment is considerable.

The brain is especially vulnerable following preterm birth as it is not only prematurely removed from the placental supply of neurosteroids and other placentally supplied nutrients, but also prematurely exposed to a stimulating environment, increased glutamate exposure and associated excitotoxic damage. The neurosteroid 3α-hydroxy,5α-pregnane-20-one (allopregnanolone) is found in very high concentrations in the fetal plasma and brain compared to concentrations following birth.[6,7] This elevated concentration of allopregnanolone provides neuroprotection for the developing fetal brain by increasing neuronal inhibitory tone. Conversely a reduction in the normal fetal neurosteroid environment is associated with adverse outcomes related to excessive excitatory tone, including the occurrence of seizures, which can lead to permanent neurodevelopmental damage.[8,9] Allopregnanolone specifically enhances gamma-aminobutyric acid A ($GABA_A$) receptor-mediated inhibition via agonist action on extra-synaptic $GABA_A$ receptors,[10] which are highly expressed throughout the fetal brain from mid-gestation onwards in the sheep and human.[11,12] Following term birth, and associated loss of the placenta, allopregnanolone concentrations are markedly reduced. Birth-associated loss of allopregnanolone also occurs following preterm birth, thus the brains of preterm infants have significantly lower exposure to allopregnanolone than that of a fetus at the same post-conceptional age.[6] In our clinically relevant guinea pig model,[13] we have shown premature loss of allopregnanolone significantly decreases myelination in the hippocampus, subcortical white matter and cerebellum, key areas involved in memory and learning, and that these deficits are maintained until juvenility.[6,14–17] Interestingly, the deficits observed are associated with disturbances in behaviour, supporting a role for reduced allopregnanolone in the behavioural changes seen in this model.[16,17]

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Neurosteroids have been shown to exert neuroprotective effects following damage to neurons and glia by preventing apoptosis and inflammation, and increasing re-myelination and regenerative mechanisms.[18,19] Rat studies of traumatic brain injury have demonstrated the therapeutic benefit of neurosteroid supplementation with progesterone (pre-cursor of allopregnanolone) reducing neuronal loss.[20] In humans, patients administered progesterone following traumatic brain injury had a lower 30-day mortality risk and were more likely to have a good outcome than those receiving placebo.[21] Allopregnanolone administration has been shown to reduce memory deficits and loss of neurons in the frontal cortex of rats following bilateral injury.[22] Previously we have examined the use of progesterone in an attempt to restore preterm neurosteroid levels to that experienced by a fetus at equivalent post-conceptional age. We showed that high doses of progesterone (16 mg/kg) administered to neonatal preterm guinea pigs from birth until term equivalence increased plasma allopregnanolone in females.[15] In males however, allopregnanolone concentrations were unchanged whereas cortisol concentrations were elevated.[15] Excessive cortisol exposure may result in a burden of adverse side effects including disruption of myelination and glial cell proliferation, which can lead to poor neurodevelopmental and behavioural outcomes,[15,23,24] highlighting the potential of progesterone to be metabolized to steroids with adverse effects on development. This suggests that males and females need to be considered as separate populations with different responses to therapeutic options. The use of allopregnanolone itself is also clinically challenging: the half-life of allopregnanolone (~2 h in adult humans)[25] means that it may not be sufficient to achieve a sustained action at $GABA_A$ receptors within the brain. Additionally, enzymes present in the brain can metabolize allopregnanolone to its immediate precursor (5α-dihydroprogesterone) which has very low affinity for the $GABA_A$ receptor,[26] and furthermore, to potent neurosteroid-sensitive $GABA_A$ receptor antagonists, such as isopregnanolone.[27]

Thus, in the current proof of concept study we have piloted postnatal therapy with ganaxolone, a β-methylated analogue of allopregnanolone with a methyl group that prevents metabolism into other active steroids.[28] Ganaxolone binds to the neurosteroid-binding site of $GABA_A$ receptors with similar affinity and efficacy to allopregnanolone, but a much longer half-life of ~12–20 h in adult humans.[29] Ganaxolone is currently being used in phase 2 trials for infantile spasms and epilepsy, due to its actions in increasing activation of the $GABA_A$ receptor mediated pathways.[30] We hypothesized that postnatal neurosteroid-based therapy using ganaxolone would ameliorate the adverse effects of a premature loss of allopregnanolone on neurodevelopment and behaviour in a guinea pig model of preterm birth. Based on our previous findings in the juvenile guinea pig, we hypothesize that males will gain the greatest benefit and therefore have performed a male-only pilot study.

METHODS

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

Animals

Mature breeding Dunkin Hartley female guinea pigs were obtained from the University of Otago Wellington Biomedical Research Unit. Guinea pigs were housed indoors under a 12 h light/dark cycle and supplied with standard guinea pig pellets, Specialty Feeds (Glen Forrest, Australia), hay, fresh vegetables, and drinking water supplemented with Vitamin C. Pregnant sows were bred via 11 males and randomly allocated to either preterm ($n = 14$) or term ($n = 12$) delivery. Term delivery sows received no further intervention, with pups delivered spontaneously and receiving no additional support (Term; $n = 14$). Preterm (d62 of 69-day pregnancy) pups were born following induction of labour.[16,17,31] Average litter size was 3.5 pups (51% males), and a maximum of two pups/pregnancy were allocated to this study to ensure there was no litter bias.

Sows received betamethasone 1 mg/kg subcutaneously (Celestone Chronodose; Merck, Sharp & Dohme, Auckland, New Zealand) 48 and 24 h prior to preterm delivery to accelerate fetal lung maturation and surfactant production. Aglepristone 10 mg/kg (Provet, Auckland, New Zealand) was administered subcutaneously 24 h prior to, and on the morning of delivery to inhibit progesterone-based continuance of pregnancy. Intramuscular oxytocin 3IU/kg (Provet) was administered to stimulate uterine contractions 1 h after the second Aglepristone dose and repeated until all pups and placentas were delivered.

Resuscitation and respiratory support of pups occurred as previously described.[16,17,31] Respiratory support was provided for at least 3 min to all preterm pups by continuous positive airway pressure (CPAP) support at 5 cm H_2O using the "Neopuff" t-piece infant resuscitator (Fisher & Paykel, Auckland, New Zealand) with blended oxygen delivered at 5 l/min. If spontaneous respiration was not achieved or sustained, positive pressure ventilation at 60 breaths/min with an inflation pressure of 12 cm H_2O and expiratory pressure of 5 cm H_2O was provided until spontaneous respiration was observed. All preterm pups were also given an initial fractional inspired oxygen concentration of 30% that was adjusted based on the colour, heart rate, and respiratory activity of the pup. Once stable respiration was maintained, pups were housed with their mothers and littermates in a warm humidified human infant incubator (Dräger 8000 IC; Drägerwerk AG & Co., Lübeck, Germany); ambient temperature 33 °C (titrated down to 28 °C by 24 h), and 60% humidity (titrated down to 35% by 12 h).

Preterm pups received 0.3–0.5 ml of Impact guinea pig colostrum replacement (Wombaroo Food Products, Adelaide, Australia) orally within the first hour after birth and then every 3 h until 24 h old. Between postnatal days 1 and 7 the pups were fed 0.5–2.0 ml of Impact guinea pig milk replacement (Wombaroo Food Products) every 3 h or as needed to supplement independent suckling. Pups assigned to neurosteroid-replacement therapy received subcutaneous 2.5 mg/kg ganaxolone in 1.25 µl/g of 45% β-cyclodextrin (Prem-GNX; $n = 4$) twice daily from birth until term equivalence. Control pups used for brain tissue studies received subcutaneous saline injections as part of routine preterm care but did not receive vehicle (Prem-CON, $n = 10$). Further controls that received vehicle ($n = 5$) were included for behavioural studies (Prem-CON, $n = 15$). Subcutaneous administration of 45% β-cyclodextrin (vehicle) did not affect weight gain or any of the other physical parameters measured and reported.

On postnatal day 6 (1 day prior term equivalence), pups were transferred with their mothers from the infant incubator into standard single cages, and on postnatal day 7 into the nursery pen with other sows and pups. All neonates remained with their mother in the nursery pen until weaning at CPNA21, after which they were placed into same-sex floor pens.

Behavioural tests

Juveniles underwent behavioural testing at CPNA25 as previously described.[16] A pre-test saliva sample was obtained from each animal immediately prior the first test by allowing the pup to chew a cotton bud, and a post-test sample following the completion of the environment exploration testing. ANY-maze tracking software v4.7 (Stoelting Co., Wood Dale, Illinois) was used to analyse the videos obtained from each test. All salivary sampling and behavioural testing was performed by staff familiar to the animals, in a designated space free from the sight, sound, or scent of other animals. Assessments were made with the observer blinded to the treatment group.

Open field and environment exploration test

The open field test is a measure of anxiety, hyperactivity and locomotion.[16,23,32] Guinea pigs were allowed to explore the arena (40 cm × 40 cm) for 10 min, with entries into the inner zone (20 cm × 20 cm) recorded. The environment exploration test was used to measure the animals' exploratory behaviour and anxiety.[16] Immediately following the open field test the animal was removed from the arena and two objects were placed in the center. The animal was then placed back into the arena and allowed to investigate the objects (quantified by sniffing and/or touching the objects) for 10 min.

Familiar social test

The familiar social test was used to measure social interactions towards a familiar animal.[16,33,34] The test animal was placed into the arena (same as above) with an animal of the same sex, age, and home pen for 5 min. Parameters measured included approaching (quantified by an active change in orientation and/or movement of the test animal towards the familiar animal), and having an affectionate or agonistic interaction with the familiar animal.

Tissue collection

Juveniles were euthanized at CPNA28 by sodium pentobarbitone (300 mg/kg intracardiac injection; Pentabarb, Provet), and organ weights recorded. Each brain was sectioned in the sagittal plane to separate the hemispheres. Each left hemisphere was fixed in 4% paraformaldehyde, whilst the right hemisphere was further dissected and frozen in liquid nitrogen.

Cortisol ELISA

A commercially available salivary cortisol assay (Salimetrics Inc., State College, Pennsylvania) was used to measure the concentration of cortisol in saliva.[16] The sensitivity of the assay is 0.012–3.0 µg/dl, and the assays performed in our laboratory had inter- and intra-assay coefficients of variances of 10.89% and 2.58%.

Immunohistochemistry

Mature myelinating oligodendrocyte expression was quantified in the CA1 region of the dorsal hippocampus, overlying subcortical white matter and adjacent cingulum. Neuronal dendrite coverage was quantified in the CA1 region of the dorsal hippocampus, the overlying subcortical white matter, and the dentate gyrus. Immunohistochemistry was performed on 8 µm sections of paraffin-embedded brains cut using a Leica RM2145 Microtome (Leica Microsystems Pty Ltd, North Ryde, Australia).[8,14,16,17] Tissues were dewaxed, incubated in citrate buffer (pH 6.0) and PBS containing 3% hydrogen peroxide, and blocked for 1 h at room temperature in BSA and serum Blocking Solution (0.4% BSA, 2% normal goat serum, 0.3% TritonX in 0.1 M PBS). Incubation in primary (myelin basic protein [MBP] M9434; microtubule associated protein-2 [MAP2] M9942) and secondary antibodies (biotinylated anti-rat IgG B7139; biotinylated anti-mouse IgG B6649) were performed before tertiary incubation in streptavidin-biotin-horseradish peroxidase complex. Incubation in 3,3′-diaminobenzidine tetrahydrochloride solution (Metal Enhanced DAB Substrate Kit; ThermoFisher Scientific, Scoresby, Australia) revealed immunolabelling.

Slides were imaged using the Aperio imaging system (Leica Biosystems, North Ryde, Australia). ImageJ v1.47 (National Institutes of Health, Bethesda, Maryland) was used to calculate percent area coverage by conversion to gray-scale and then binary, and manually adjusting threshold based on the original stained image.[14,16,17] Overall average of staining was calculated by taking the average of four images captured from two consecutive sections per animal; i.e. a total of eight images/region/animal.

Real-time PCR

Frozen hippocampal tissue was homogenized in RLT Plus Buffer (Qiagen, Chadstone Centre, Australia) using a Precellys 24 dual tissue homogenizer (Bertin Technologies, Provence, France). RNA was extracted using the Qiagen RNeasy Plus Mini Kit (Qiagen).[14,16,17] Samples with poor RNA A260/280 ratios and integrity were not used for further analysis.

Superscript III Reverse Transcription kit (Invitrogen, Carlsbad, California) was used to synthesize cDNA on a GeneAmp 9700 (Applied Biosystems, Life Technologies Pty Ltd, Mulgrave, Australia). RT-PCR was performed using a 7500 ABI real-time machine (Applied Biosystems) for primer pairs (α4, α5 and δ), and Alien (Agilent, Santa Clara, California) as a control (see Table 1 for sequences and efficiencies).[14,16,17] Samples were run in duplicate along with an associated negative control sample (treated the same throughout but in absence of reverse transcriptase). Products were detected using SYBR Green (Applied Biosystems). Results were analysed by Sequence Detection Software v2.01 (Applied Biosystems) and relative fold change calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Controls included Alien and a calibrator, which consisted of pooled brain samples and was used across all plates. Consistent Ct values were obtained for Alien across the term/Prem-CON/Prem-GNX samples.

Statistical analyses

Data were analysed using Prism v7.0 (Graphpad Software Inc., La Jolla, California) and presented as mean ± SEM for each group with significance considered $p < 0.05$. In order to identify differences between groups the data was first analysed by one-way ANOVA. Post-hoc tests with Tukey corrections for multiple comparisons were performed when the ANOVA was $p < 0.05$. When data were not normally distributed, non-parametric Kruskal–Wallis test with post hoc tests using Dunn's correction for multiple comparisons were performed. Fractional weight gain and supplemental feeding data were analysed by repeated measures two-way ANOVA, with corrections for multiple comparisons by Bonferroni. Survival data were analysed using a Kaplan–Meier survival curve comparison.

Table 1. $GABA_A$ receptor subunit primer pair sequences and efficiencies

Gene of interest	Forward primer sequence	Reverse primer sequence	Efficiency
$GABA_AR$ α4 subunit	TGG GCA AAC AGT GTC AAG TG	GAC ACT TTG GGC AGA GAA TG	106.17%
$GABA_AR$ α5 subunit	CAC GGG CGA ATA CAC GAT TA	CAA TCA GAG CAG AGA ACA CGA	101.18%
$GABA_AR$ δ subunit	GCG TCT ACA TCA TCC AGT CC	AAT GGG CAA AGG CAT ACT CC	104.11%
Agilent Alien Primer			106.68%

Primer sequences designed for guinea pig γ-amino-butyric-acid A receptor ($GABA_AR$) α4, α5 and δ subunit gene expression, and their respective PCR primer efficiencies. Primer sequences are displayed from 5′-3′ for forward and reverse primers. The efficiency for the housekeeping gene (Alien; Agilent) is also provided

Table 2. Body and organ weights

Group	*n*	Birth weight	TEA weight	CPNA28 weight	Fractional growth rate	Brain-body weight	Hippocampus-brain weight	Liver-body weight	Adrenal-body weight	Heart-body weight	Kidney-body weight	Sub. fat-body weight	Vis. fat-body weight
Term	14	96.8 ± 3.4	96.8 ± 3.4	284.6 ± 10.4	195.9 ± 10.2	1.2 ± 0.04	3.6 ± 0.1	4.3 ± 0.2	0.031 ± 0.003	0.36 ± 0.02	0.84 ± 0.02	0.97 ± 0.07	0.34 ± 0.05
Prem-CON	15	76.2 ± 1.7*	95.7 ± 2.8	302.7 ± 8.7	217.0 ± 10.7	1.1 ± 0.03	3.8 ± 0.2	4.4 ± 0.2	0.035 ± 0.002	0.41 ± 0.02	0.91 ± 0.02*	0.88 ± 0.11	0.29 ± 0.05
Prem-GNX	4	80.1 ± 5.3*	86.2 ± 2.9	258.2 ± 9.2	199.9 ± 9.1	1.3 ± 0.05	3.8 ± 0.2	3.7 ± 0.1	0.033 ± 0.003	0.39 ± 0.03	0.93 ± 0.02	0.73 ± 0.07	0.11 ± 0.02*

Values presented are mean ± SEM and are calculated for animal numbers with birth, term equivalence age (TEA) and corrected postnatal age (CPNA) 28 weights in grams. Fractional growth rate from TEA to CPNA28 is expressed as a percentage. All data for organ weights are from CPNA28 and displayed as organ-to-body weight ratio percentages. * denotes $p < 0.05$ compared to term-born males

Table 3. Body measurements

Group	*n*	Nose-rump length (birth)	Nose-rump length (TEA)	Nose-rump length	Head length	Hind limb length	Hock-toe length	Head circum.	Neck circum.	Chest circum	Abdominal circum
Term	14	12.3 ± 0.2	12.3 ± 0.2	23.0 ± 0.35	6.62 ± 0.14	4.91 ± 0.14	4.00 ± 0.03	10.79 ± 0.29	9.76 ± 0.42	13.7 ± 0.34	17.02 ± 0.45
Prem-CON	15	10.9 ± 0.1*	13.0 ± 0.1	21.6 ± 0.44*	6.86 ± 0.15	4.85 ± 0.12	4.02 ± 0.12	11.83 ± 0.23*	9.0 ± 0.36	14.04 ± 0.26	16.98 ± 0.37
Prem-GNX	4	11.1 ± 0.4*	12.5 ± 0.2	21.6 ± 0.37	6.22 ± 0.06	4.5 ± 0.04	3.92 ± 0.08	11.38 ± 0.19	9.7 ± 0.23	13.03 ± 0.19	16.15 ± 0.49

Values presented are mean ± SEM and are calculated for animal numbers with all measurements in centimetres. Unless otherwise stated, measurements are obtained at corrected postnatal age (CPNA) 28. *TEA* term equivalence age. * denotes $p < 0.05$ compared to term-born males.

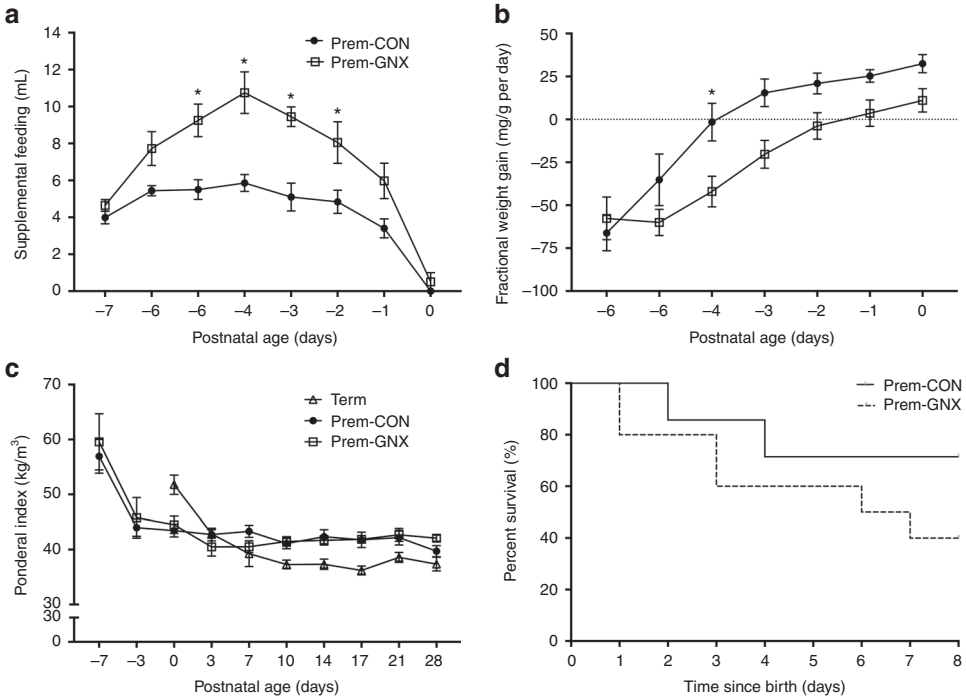

Fig. 1 **a** Supplemental feeding until TEA, **b** fractional weight gain until term equivalence, **c** ponderal index and **d** Kaplan–Meier survival curve of control preterm male guinea pigs (Prem-CON; solid circles and solid line $n = 5$ and $n = 7$), or ganaxolone therapy (Prem-GNX; open squares $n = 4$ and dashed line $n = 10$), and term male guinea pigs (Term; open triangles $n = 7$). Mean ± SEM, * indicates significance $p < 0.05$

RESULTS

Physical characteristics

Animal weights and organ-body weight ratios are detailed in Table 2, and body measurements are detailed in Table 3. Prem-CON (62.0 ± 0 days of gestation) and Prem-GNX animals (62.0 ± 0) were born significantly earlier than term animals (68.9 ± 0.3, $p < 0.0001$) and, as expected, all preterm animals weighed significantly less at birth than term-born animals (Prem-CON $p < 0.0001$ and Prem-GNX $p = 0.02$). Despite this, by term equivalence age (corrected postnatal age 0 days; TEA) and post mortem (corrected postnatal age 28 days; CPNA28) there were no significant differences in body weights across the three groups, and no significant difference in fractional growth rate from TEA to CPNA28. At birth, nose-rump lengths were similar for the Prem-CON and Prem-GNX cohorts, however they were shorter than term pups ($p < 0.0001$ and $p = 0.002$). Furthermore, at CPNA28, nose-rump length was significantly shorter for Prem-CON animals relative to term animals ($p = 0.02$), whilst head circumference was larger ($p = 0.02$). There were no other significant differences in body measurements at birth, TEA or CPNA28.

At CPNA28, kidney–body weight ratio was increased in Prem-CON animals compared to term ($p = 0.03$), this also appeared to be increased in the Prem-GNX animals compared to the term but did not reach significance. Visceral fat-body weight ratio was significantly lower in Prem-GNX animals compared to term ($p = 0.05$) suggesting Prem-GNX animals were leaner; this was not observed in Prem-CON animals. There were no other significant differences in organ-body weight ratios.

Nutritional support for preterm animals, weight gain and survival

Nutritional support in the week leading up to term equivalence for Prem-CON and Prem-GNX animals was recorded and daily averages are depicted in Fig. 1a. On CPNA-7 (birth), CPNA-6, CPNA-1 and CPNA0 (term equivalence) there were no significant differences in additional feeding amounts required. Prem-GNX animals, however, required significantly more nutritional support than Prem-CON animals on CPNA-5 ($p = 0.001$), CPNA-4 ($p < 0.0001$), CPNA-3 ($p = 0.0001$) and CPNA-2 ($p = 0.008$), but this was not reflected in fractional weight gain where Prem-GNX animals gained significantly less weight at CPNA-4 compared to Prem-CON animals (Fig. 1b, $p = 0.02$). Despite this, there was no significant difference in ponderal index between the treatment groups at any postnatal age (Fig. 1c).

Survival from birth until term-equivalent age was higher for the Prem-CON animals, with 71.4% surviving compared to just 40% of the Prem-GNX animals (Fig. 1d). The increased mortality rate in the Prem-GNX animals was largely a result of sedation resulting in increased episodes of apnoea, respiratory depression and hypotonia leading to an increased risk of aspiration of milk, and an inability to reposition themselves when being overlain by the sow. Importantly, this sedation was not seen >24 h after the final ganaxolone dose at TEA.

Behavioural outcomes and associated cortisol levels at juvenility

Results of open field testing are shown in Fig. 2, foreign object exploration in Fig. 3a, b, and social interaction test in Fig. 3c–f. In the open field, Prem-CON animals had significantly higher activity compared to term animals, measured by increased distances travelled in the total arena (Fig. 2a, $p = 0.01$) and inner zone (Fig. 2c, $p = 0.002$), and more time spent mobile in both the total arena (Fig. 2b, $p = 0.02$) and inner zone (Fig. 2d, $p = 0.005$). There was no difference in the number of entries between any of the groups (Fig. 2e), however the Prem-CON animals spent significantly more time in the inner-zone compared to term (Fig. 2f, $p = 0.04$). The percentage of overall time spent in the inner zone (Fig. 2g), and duration of the maximum visit to the inner zone (Fig. 2h) were not significantly different between the groups. Prem-GNX animals showed no differences compared to the term or Prem-CON animals in any of the parameters measured.

In the environment exploration test, the Prem-CON animals had significantly more interactions with the objects (Fig. 3a) and spent significantly longer interacting with them (Fig. 3b) compared to

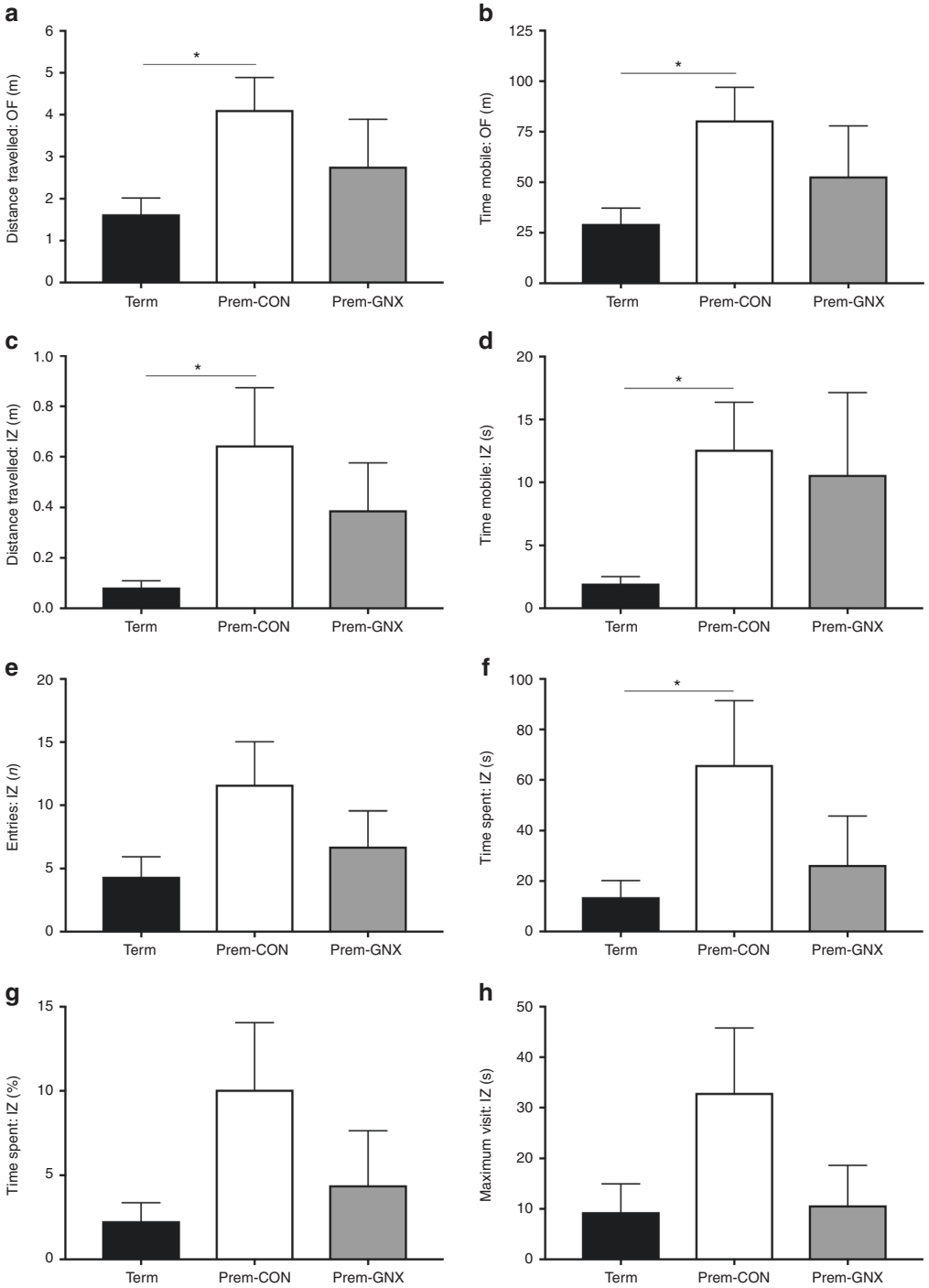

Fig. 2 Open field exploration outcomes for term control (black bars, $n = 14$), control preterm guinea pigs (Prem-CON; white bars, $n = 11$), and preterm receiving ganaxolone therapy (Prem-GNX; grey bars, $n = 4$) at corrected postnatal age 25. Parameters measured included **a** distance travelled and **b** time mobile in the open field arena, **c** distance travelled and **d** time mobile in the inner zone, **e** number of entries and **f** time spent in the inner zone, and **g** the percentage of overall time spent in the inner zone, in addition to **h** the duration of the maximum visit. Mean ± SEM, * indicates significance $p < 0.05$

term animals ($p = 0.02$ and $p = 0.04$). The number of interactions with the objects by the Prem-CON animals was also significantly higher than the Prem-GNX animals ($p = 0.05$). There was no difference in behaviour between the Prem-GNX and term animals.

Prem-CON animals approached the familiar animal (Fig. 3c) and exhibited a higher frequency of interactions with the familiar animal (Fig. 3d) when compared to term ($p = 0.02$ and $p = 0.02$) and Prem-GNX animals ($p = 0.04$ and $p = 0.03$) in the social interaction test. Furthermore, the number of times the Prem-CON animals sniffed the familiar animal (Fig. 3e), as well as the time spent sniffing (Fig. 3f), was significantly more than the Prem-GNX animals ($p = 0.03$ and $p = 0.03$, respectively). Behaviour exhibited by Prem-GNX animals was not significantly different to term animals in any of the parameters.

There were no significant differences in salivary cortisol concentrations of the animals before or after behavioural testing

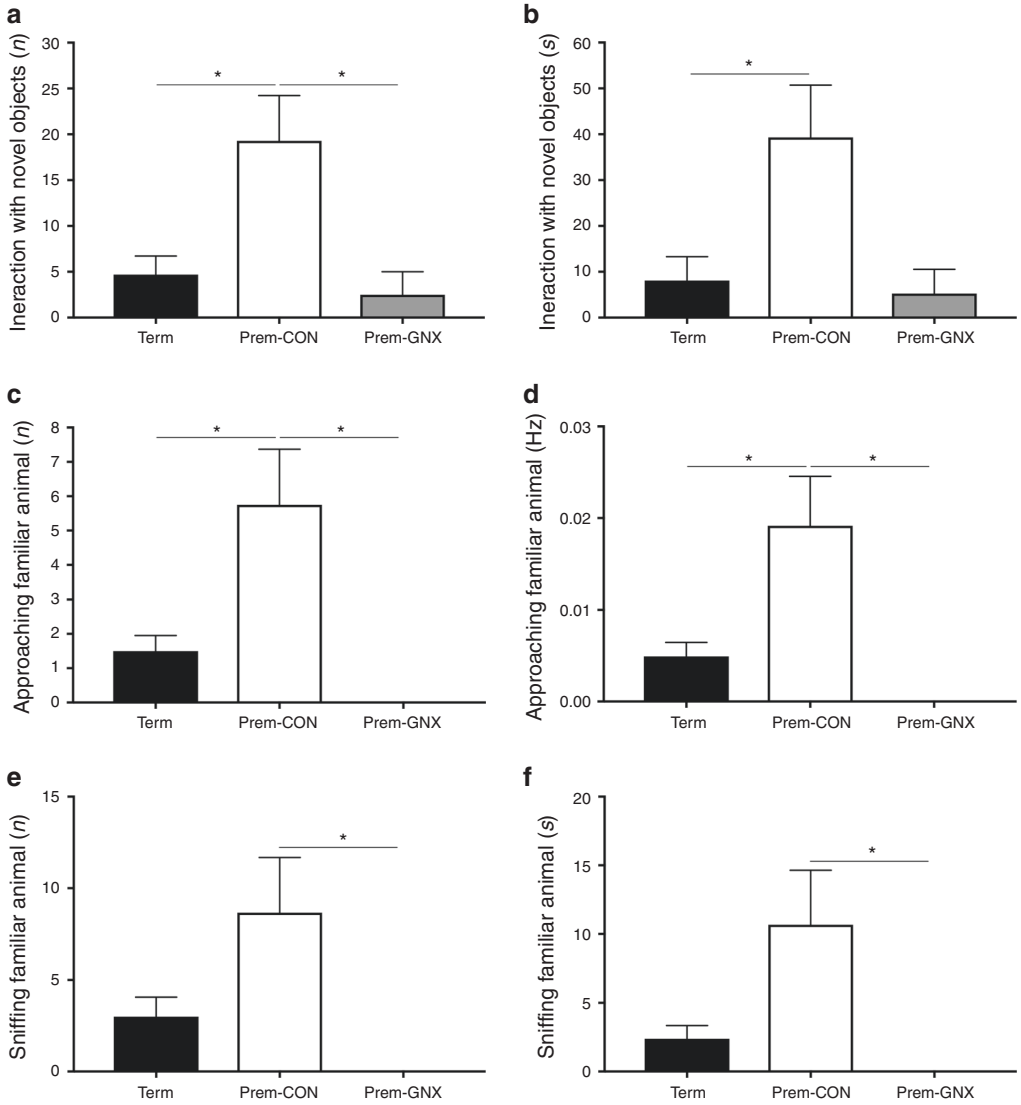

Fig. 3 Foreign object investigation (**a**, **b**) and social interaction (**c**–**f**) by term control (black bar, $n = 10$), preterm control (Prem-CON; white bar, $n = 10$), and preterm guinea pigs receiving ganaxolone therapy (Prem-GNX; grey bar, $n = 4$) at corrected postnatal age 25. In the foreign object investigation behavioural test the parameters measured were **a** the number of interactions with a foreign object, and **b** the time spent interacting with the objects. In the social interaction test **c** the number of approach actions and **d** the frequency of actions was recorded, in addition to **e** the number of times the familiar animal was sniffed, and **f** the time spent sniffing the familiar animal. Mean ± SEM, * indicates significance $p < 0.05$

between any of the groups. Furthermore, there were no significant differences identified in the change in concentration following the foreign environment exposure measured as a percentage of baseline concentration (supplementary figure S1).

$GABA_A$ receptor subunit expression in the hippocampus at juvenility

There were no significant differences identified between the three groups for either the α4, α5 or δ subunits (supplementary figure S2).

Myelination in guinea pig brains at juvenility

Prem-CON animals had significantly less immunostaining in the CA1 region of the hippocampus compared to both term (Fig. 4a, $p = 0.003$) and Prem-GNX animals ($p < 0.0001$), furthermore Prem-GNX animals had significantly more immunostaining compared to term animals ($p = 0.002$). Similarly, in the subcortical white matter Prem-CON animals also had significantly less immunostaining compared to term (Fig. 4b, $p = 0.02$) and Prem-GNX animals ($p = 0.04$), and there was no difference in immunostaining between the term and Prem-GNX animals. There were no significant differences identified in the cingulum (Fig. 4c).

Neuronal dendrite expression in juvenile guinea pig brains

There were no significant differences identified between any of the three groups for neuronal dendrite area coverage in the CA1 region of the hippocampus (Fig. 5a), the overlying subcortical white matter (Fig. 5b), or the dentate gyrus (Fig. 5c).

DISCUSSION

The key finding of this paper was that neurosteroid replacement therapy with ganaxolone reversed some of the effects observed when animals were prematurely exposed to the *ex utero* environment. As we have previously shown,[16] male juvenile preterm born guinea pigs exhibit hyperactive behaviour signified by greater distances travelled in the foreign arena, more time

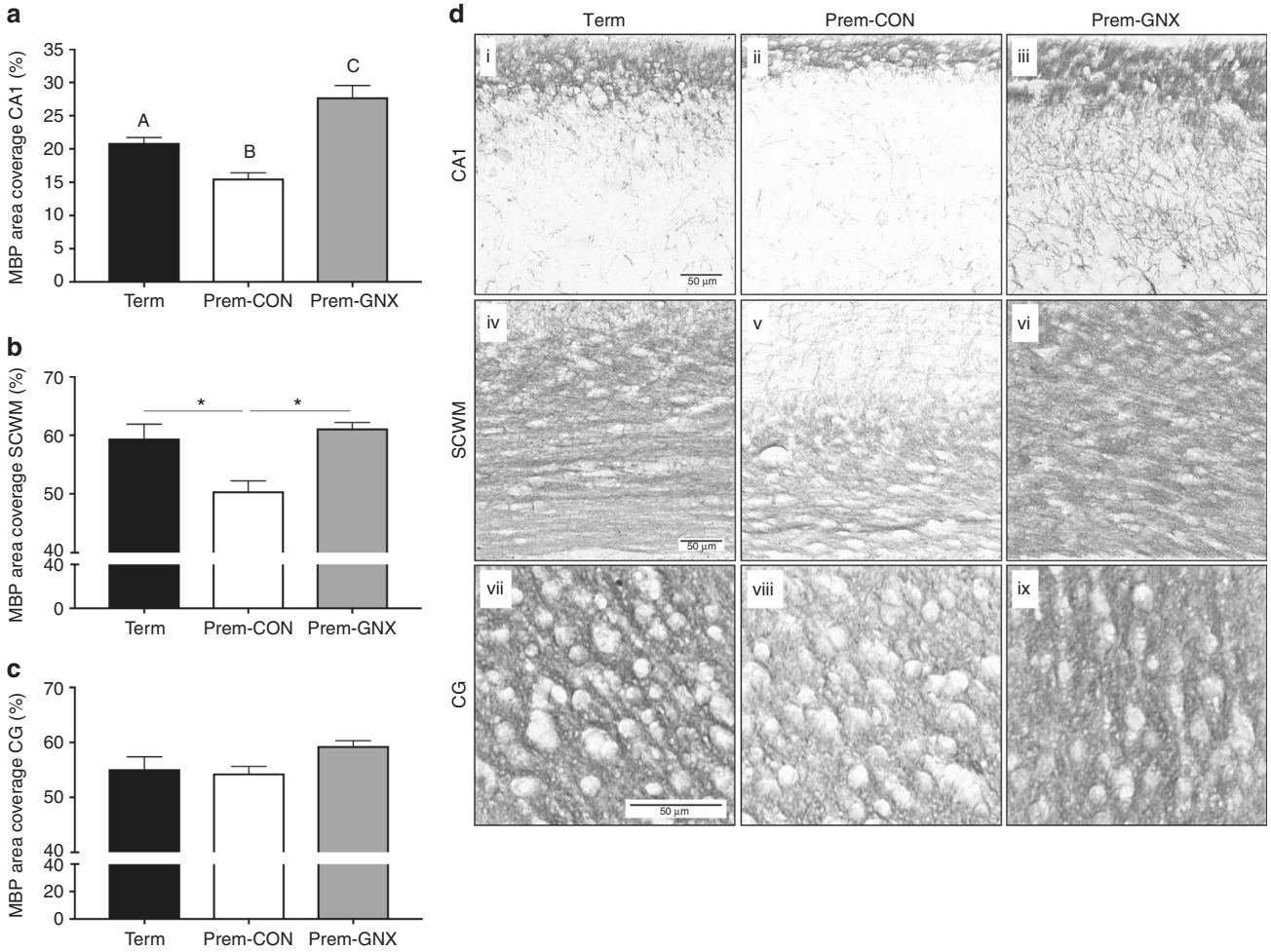

Fig. 4 Myelin basic protein (MBP) immunostaining was quantified in the brain of term control (black bars, $n = 7$), control preterm guinea pigs (Prem-CON; white bars, $n = 8$), and preterm receiving ganaxolone therapy (Prem-GNX; grey bars, $n = 4$) in **a** the CA1 region of the hippocampus, **b** the subcortical white matter, and **c**) the cingulum. Mean ± SEM, different letters or * indicates significance $p < 0.05$. **d** Representative photomicrographs of MBP immunostaining of the CA1 region (i = Term, ii = Prem-CON, iii = Prem-GNX), subcortical white matter (iv = Term, v = Prem-CON, vi = Prem-GNX), and cingulum (vii = Term, viii = Prem-CON, ix = Prem-GNX). Scale bar = 50 µm

spent in an open environment, and more investigation of foreign objects as opposed to 'safer' behaviour such as staying closer to the walls of the arena. In addition to this hyperactive phenotype the ex-preterm males have a disinhibited social response, i.e. the persistent approaching to the other animal. This hyperactive behavioural phenotype in the preterm guinea pig juvenile is similar to hyperactivity exhibited by children with ADHD[5,35,36] and suggests the deficits we observe after preterm birth may contribute to the increased risk of the development of this disorder. Importantly we observed a reversal of these phenotypes in the preterm animals treated with ganaxolone. The effects of ganaxolone, a synthetic analogue of allopregnanolone, on GABAergic activity and behaviour have been previously reported with ganaxolone shown to increase inhibitory action of GABAgeric pathways in a similar manner to allopregnanolone.[37]

Fetal sheep studies involving the inhibition of allopregnanolone synthesis show an increase in arousal and seizure-like activity in utero compared to when allopregnanolone supply is restored.[38] We have previously shown that suppression of allopregnanolone concentrations has detrimental effects on neurodevelopment and that this has adverse effects on long–term behaviour, including increased anxiety in female guinea pigs.[39] The anti-anxiolytic effects of ganaxolone have been demonstrated in socially isolated mice where anxiety-like behaviour was successfully reduced following treatment.[37] The anti-seizure/anti-convulsion properties of ganaxolone have also been verified in a neonatal rat model of infantile spams where treatment with doses of 25 and 50 mg/kg both decreased the occurrence and delayed the onset of spasms.[40]

The dose used in the current study was markedly lower than those used in the anti-seizure anti-convulsive studies, however, the preterm pups receiving ganaxolone appeared heavily sedated compared to their preterm-control counterparts which indicated that the dose used was excessive. There was also decreased independent suckling from the dam, which resulted in the need for additional nutritional support. Despite this support the weight gain in the period leading up to term equivalence was reduced for those receiving ganaxolone, perhaps due to the effects of ganaxolone on the immature gastrointestinal tract, combined with the lack of active suckling. The adverse side effects, including respiratory depression, that were induced by the high level of sedation mean that further studies investigating lower doses are required to achieve neurological benefit without negative impacts on overall health. However, the present study does provide the proof of principal that neurosteroid-replacement therapy between birth and term equivalence may improve brain function following

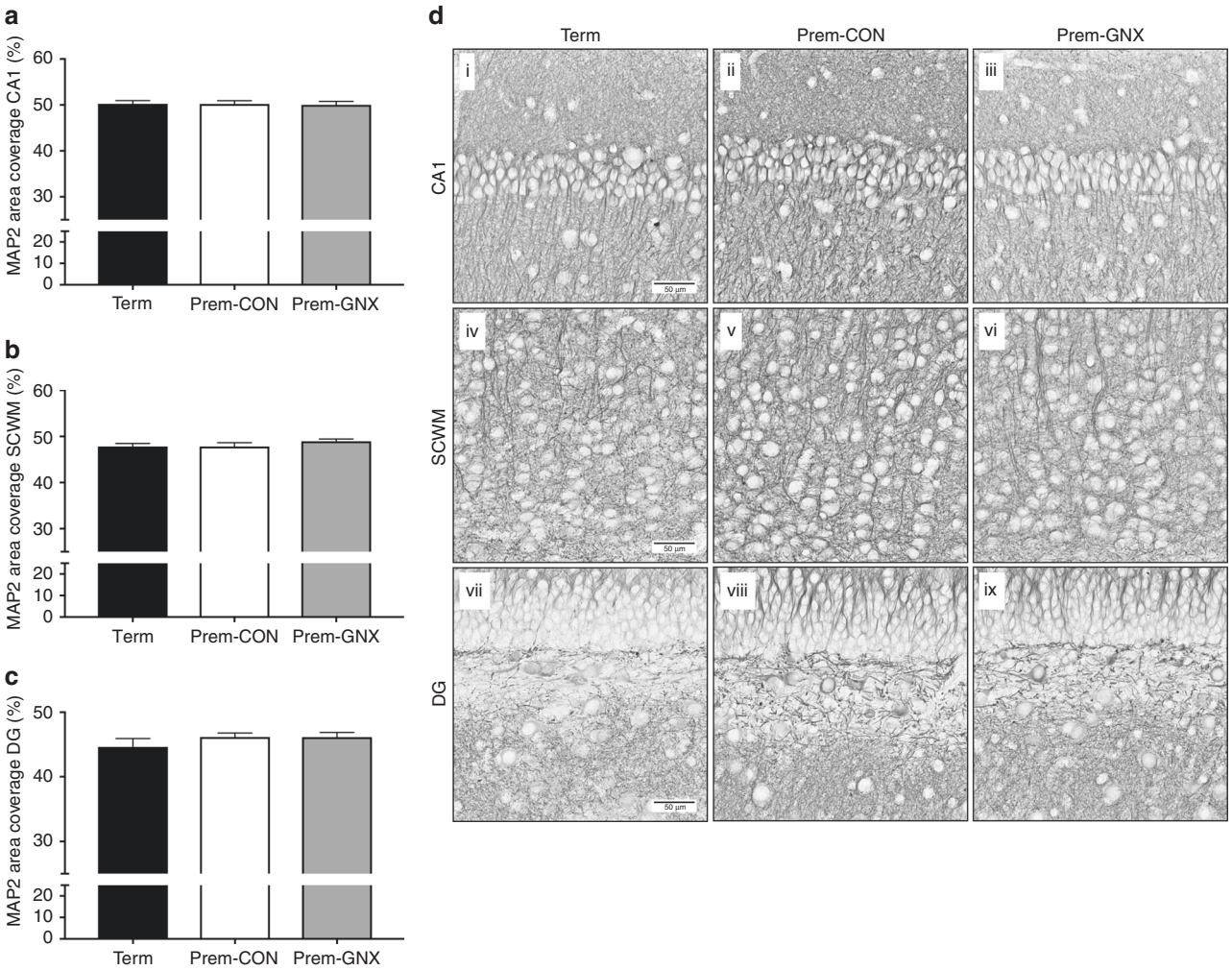

Fig. 5 Microtubule associated protein-2 (MAP2) immunostaining was quantified in the brain of term control (black bars, $n = 7$), control preterm guinea pigs (Prem-CON; white bars, $n = 8$), and preterm receiving ganaxolone therapy (Prem-GNX; grey bars, $n = 4$) in **a** the CA1 region of the hippocampus, **b** the subcortical white matter, and **c** the dentate gyrus. Mean ± SEM, * indicates significance $p < 0.05$. **d** Representative photomicrographs of MAP2 immunostaining of the CA1 region (i = Term, ii = Prem-CON, iii = Prem-GNX), subcortical white matter (iv = Term, v = Prem-CON, vi = Prem-GNX), and dentate gyrus (vii = Term, viii = Prem-CON, ix = Prem-GNX). Scale bar = 50 µm

preterm birth. It also highlights the imperative to test new therapeutic adjuncts in a suitable animal model, as we have previously treated term-born pups with ganaxolone without any adverse side effects or increase in mortality. These disparities indicate there are considerable differences in metabolism and dose requirements between term and preterm born animals, indicating the need for further animal studies of optimal dosing after preterm birth.

Whilst neuronal proliferation is complete by this stage of gestation, as evidenced by no change in the density of microtubular associated protein-2 (MAP2) at the time of birth,[6] and no changes in neuronal density in this study, we have previously identified lasting deficits in mature myelinating oligodendrocytes of the ex-preterm male juvenile hippocampus and subcortical white matter.[16] The hippocampus and overlying subcortical white matter are known to be particularly vulnerable to damage and delays following exposure to the ex utero environment, and in the absence of the pro-myelinating neurosteroid allopregnanolone.[6,8] The finding that ganaxolone therapy successfully restored myelination levels to that of term-born animals in both of these areas is consistent with the trophic action of allopregnanolone on brain development during late gestation. This action involves upregulation of $GABA_A$ receptor signalling, and therefore, the challenge will be to find an optimal dose suitable for chronic supplementation therapy following preterm birth, which maintains $GABA_A$ receptor-mediated tropic actions and yet minimizes sedative effects.

Allopregnanolone exerts its pro-myelinating effects via the $GABA_A$ receptors of the central nervous system. The sensitivity of these receptors to different ligands depends on the makeup of subunits. Neurosteroid-sensitive $GABA_A$ receptors within the hippocampus predominantly feature the α4, α5 and particularly the δ subunits. We did not observe any changes in the expression of the α4, α5 or δ subunits in the preterm control animals, or those receiving ganaxolone. However, it is possible that short-term alterations were present in the immediate neonatal period, contributing to the loss of neurosteroid action during this time. We have previously shown subunit levels are reduced in preterm neonates,[14] and short-term studies are required to determine if ganaxolone therapy increased these levels to promote myelination.

Despite the substantial evidence that neurosteroids, in particular allopregnanolone, are integral to fetal neurodevelopment and are subsequently lost prematurely in the event

of preterm birth,[6] this is the first study to replace an analogue of allopregnanolone and demonstrate therapeutic benefits. The advantages of using the synthetic analogue ganaxolone include low metabolism to other steroid metabolites and the longer half-life of ~12-20 h in adult humans,[29] allowing for sustained stimulation of $GABA_A$ receptors, with convenient dosing intervals, which is likely to be important for trophic processes.

Overall, the present results show that neurosteroid-replacement therapy using ganaxolone resulted in myelination levels and patterns of behaviour closer to those seen in term-born juveniles. In order for ganaxolone to represent a targeted therapy to prevent against the long-term neurodevelopmental sequelae of preterm birth, further studies are required to establish optimal doses, and determine whether the potential long-term benefits of ganaxolone therapy can be obtained without excess short-term morbidity and mortality.

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ADDITIONAL INFORMATION

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethical standardsThe authors assert that all animal work complies with the ethical standards of the relevant national guides on the care and use of laboratory animals (National Animal Ethics Advisory Committee of New Zealand, and the 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 committees (University of Otago, Wellington Animal Ethics Committee, and the University of Newcastle Animal Care and Ethics Committee).

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