Neurodevelopmental effects of placental restriction in sheep

by

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Abstract

Intrauterine growth restriction (IUGR) and poor perinatal growth in humans are associated with poorer cognition and memory and altered functional lateralisation. Altered brain morphology and neurodevelopment following IUGR appears responsible, and may be ameliorated by neonatal catch-up growth, however assessing relative effects of prenatal and postnatal growth on cognition in humans is difficult due to environmental confounders. Experimental placental restriction (PR) in sheep, via surgical removal of uterine epithelial attachment sites prior to mating, restricts intrauterine growth and is followed by catch-up growth. Cognitive consequences have not been examined in this model. Effects of sex, age and prior learning on cognition were therefore characterised in control (CON) sheep, then effects of PR on learning, memory, cognition, functional and morphological lateralisation were investigated.

Size at birth and neonatal fractional growth rates during the first 16 days of life (ie. neonatal catch-up growth) were measured for CON and PR offspring. Behavioural testing occurred at 18 and 40 weeks old. In maze tasks, trials and time per task, bleats and arm entries were recorded for initial learning (L), memory (M1, M2) and reversal (R1, R2) tasks. Behavioural lateralisation was recorded using obstacle avoidance and maze exit preference tasks, and structural lateralisation were measured in the prefrontal cortex brain region at 52 weeks of age.

In CON sheep, naive sheep aged 18 or 40 weeks required longer to complete task R1 than 40 week olds retested after learning the task at 18 weeks old, indicating prior learning was recalled at later ages. The exit route used for earlier learning tasks also predicted speed required to solve task R1 in 40N females.

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Body weight and skull size at birth did not differ between CON and PR lambs utilised for behavioural testing. At 18 weeks, placentally restricted male lambs took more trials to solve the initial learning task, but required less time to complete task R1 than control males. Trials and time required to solve task M1 in 40 week old males correlated negatively with neonatal growth. Bleat frequency during task R1 in 18 week old females correlated positively with birth weight and neonatal fractional growth rate.

In 40 week old females, PR were more strongly lateralised in the maze exit preference task lateralisation than CON. Lateralisation direction was consistent between ages in PR females only, and was more consistent between tasks at 18 weeks in PR than CON females. Behavioural lateralisation did not correlate with perinatal growth, and brain morphology at 52 weeks did not differ between treatments. Correlations between perinatal growth and adult brain morphology were largely limited to males, whereas correlations between behaviour and brain morphology existed largely in females.

In conclusion, effects of age, sex and experience on cognitive and behavioural outcomes must be taken into account when evaluating these outcomes in sheep. Effects of PR on cognition and behavioural lateralisation were limited but suggested sex-specific programming of postnatal neurodevelopment. Neonatal growth rate correlated with memory performance in males, suggesting interventions during this period may improve outcomes.

Statement of Originality

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Damien S. Hunter

Acknowledgements

Abbreviation list

18N – 18 week-old sheep naive to cognitive and behavioural tests

40E – 40 week olds retested after learning the task at 18 weeks old

40N - 40 week-old sheep naive to cognitive and behavioural tests

AGA – born at a birth weight appropriate for gestational age

ACTH – adrenocorticotrophic hormone

APIB - Assessment of Preterm Infant Behaviour

BSID - Bayley Scale of Infant Development

BW – birth weight

CA1-4 – cornu ammonis fields 1-4

CC - corpus callosum

CON – controls

CX – carunclectomy model

DG – dentate gyrus

DTI – diffusion tensor imaging

EQ – energy quotient (energy intake/kg body weight per day)

FA – functional anisotropy

FGR – fractional growth rate

fMRI – functional magnetic resonance imaging

GA – gestational age

GW – gestational week

HPA – hypothalamic-pituitary-adrenal axis

IQ – intelligence quotient

IUGR – intrauterine growth restriction

LBW – low birth weight

MRI – magnetic resonance imaging

NBAS - Neonatal Behavioural Assessment Scale

PR – placentally restricted

PVH - paraventricular hypothalamic nucleus

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SES – socioeconomic status

SGA – born at a birth weight small for gestational age

Task L – initial learning task

Task M1 – first memory task

Task R1 – first reversal task

Task M2 – second memory task

Task R2 – second reversal task

THROM - thromboxane A_2 analogue (STA$_2$) administration

UN – maternal gestational undernutrition

UPE - uteroplacental vessel bed embolisation

UPL - uteroplacental vessel ligation

VMH - ventromedial hypothalamic nucleus

Outputs arising from thesis

Publications directly arising from thesis

1. **Hunter, D. S.**, Hazel, S.J., Kind, K.L., Liu, H., Marini, D., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2015). "Do I turn left or right? Effects of sex, age, experience and exit route on maze test performance in sheep." <u>Physiology and Behavior</u> 139: 244–253.

2. **Hunter, D. S.**, Hazel, S.J., Kind, K.L., Liu, H., Marini, D. Giles, L.C., De Blasio, M. J., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2015). "Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behaviour in sheep in an age- and sex-specific manner." <u>Physiology and Behavior</u> 152(Pt A): 1-10.

3. **Hunter, D.S.**, Hazel, S.J., Kind, K.L., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2016). "Programming the brain: Common outcomes and gaps in knowledge from animal studies of IUGR." <u>Physiology & Behavior</u> 164, Part A: 233-248.

4. **Hunter, D. S.**, Hazel, S.J., Kind, K.L., Liu, H., Marini, D. Giles, L.C., De Blasio, M. J., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2016). "Effects of induced placental and fetal growth restriction, size at birth and early neonatal growth on behavioural and brain structural lateralization in sheep." <u>Laterality</u> (Accepted paper, 28/9/16)

Collaborative publications related to this thesis

5. Wooldridge, A. L., Bischof, R.J., Meeusen, E.N., Liu, H., Heinemann, G.K., **Hunter, D.S.**, Giles, L.C., Kind, K.L., Owens, J.A., Clifton, V.L., Gatford, K.L. (2014). "Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep." <u>American Journal of Physiology - Regulatory, Integrative and Comparative Physiology</u> 306(7): R441-446.

6. Liu, H., Schultz, C.G., De Blasio, M.J., Peura, A.M., Heinemann, G.K., Harryanto, H., **Hunter, D.S.**, Wooldridge, A.L., Kind, K.L., Giles, L.C., Simmons, R.A., Owens, J.A., Gatford, K.L. (2015). "Effect of placental restriction and neonatal exendin-4 treatment on postnatal growth, adult body composition, and in vivo glucose metabolism in the sheep." American Journal of Physiology - Endocrinology and Metabolism 309(6): E589-E600.

Conference oral presentations

7. **D Hunter**, H Liu, KL Gatford, JA Owens, KL Kind, J Pitcher, S Hazel 2012 Maternal dietary methyl supplementation normalises brain structure in the placentally-restricted sheep. *26th National Workshop on Fetal and Neonatal Physiology, Port Stephens, Australia, March 2012*

8. **D Hunter**, KL Gatford, KL Kind, H Liu, M De Blasio, JA Owens, J Pitcher, S Hazel 2013 Placental restriction of fetal growth induces sex-specific changes to learning in maze tasks in adolescent and young adult sheep. *27th National Workshop on Fetal and Neonatal Physiology, Barossa Valley, Australia, April 2013*

9. **DS Hunter**, S Hazel, KL Kind, H Liu, D Marini, L Giles, JA Owens, J Pitcher1, KL Gatford 2014 Low birth-weight and poor postnatal growth correlate with poorer memory and cognitive flexibility in male IUGR sheep in maze tasks. *27th National Workshop on Fetal and Neonatal Physiology, Yanchep, Australia, April 2014*

Chapter 1 – Literature review

1.1. Preamble

Chapter 1 opens with descriptions of fetal development in the healthy human pregnancy before progressing into a review of the human IUGR literature. I discuss the morphological and cognitive consequences of IUGR, as well as confounding factors that necessitate use of animal models. I then review the animal models of IUGR in which neurodevelopmental outcomes have been assessed, and compare developmental and cognitive outcomes between the various species, models and timing of restriction. Unless otherwise specified, all references to IUGR made are referring to term-born individuals.

Section 1.7 of this chapter has been published in Physiology and Behavior [1/, Appendix 3], of which I was the first author and wrote all drafts. As this work is published, section 1.7 has been reproduced verbatim from the manuscript, with only formatting, section and figure numbering changed, as per University of Adelaide guidelines.

Hunter, D. S**.**, Hazel, S.J., Kind, K.L., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2016). "Programming the brain: Common outcomes and gaps in knowledge from animal studies of IUGR." <u>Physiology & Behavior</u> 164, Part A: 233-248.

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Principal Author

Name of Principal Author (Candidate)	Damien Hunter		
Contribution to the Paper	Wrote the first draft of the manuscript, wrote all subsequent drafts, and produced all figures.		
Overall percentage (%)	70.00%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	1/9/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Susan J. Hazel		
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Contribution to the Paper	Assisted in planning review scope and structure, revised and edited manuscript and figures		
Signature		Date	12/9/16

1.3. Normal human development

1.3.1. Placental and fetal development

The placenta is a vital temporary organ that sustains the fetus during gestation. This well vascularised organ contains the maternally derived basal plate, the fetally derived chorionic plate, and the intervillous space between these, the main functional unit of the placenta, in which the majority of maternal-fetal substrate transfer takes place [2]. As pregnancy progresses, the placenta adapts to increasing fetal substrate demands by increasing exchange surface area, efficiency and blood flow [2-5]. Nevertheless in the third trimester of pregnancy the rate of fetal growth is much greater than that of the placenta (Fig. 1-1) [6]. Placental capacity therefore becomes rate-limiting for fetal growth, such that placental weight and birth weight are correlated in the human [7]. Differences between the fetal and adult circulatory systems are one of the mechanisms that maximise supply of nutrients from the placenta to critical organ systems during fetal development.

1.3.2. Fetal circulation

Adequate supply of substrates to the fetus is vital for its development, and blood flow is carefully managed during fetal life, permitting extraction of oxygen at low saturation, particularly in late pregnancy, when rapid fetal growth reduces the ratio of blood flow relative to fetus size [8, 9]. Although the brain receives a comparatively small volume of absolute cardiac output, it has proportionately high blood flow for organ size [10], vital for its rapid growth. Three shunting systems are important for this preferential supply of blood and nutrients to the brain during gestation. The ductus venosus, a vein in the fetal liver, plays a vital role in shunting blood from the umbilical vein directly to the inferior vena cava, preventing its passage through the liver and increasing the proportion of oxygen and nutrient-

rich blood delivered directly to the brain and heart [9-11]. Once in the right atrium,

oxygenated blood is preferentially shunted to the left atrium via the foramen ovale, which

connects the two chambers, and thus reduces the proportion of nutrient and oxygen-rich

blood entering the pulmonary circulation [9, 10, 12].
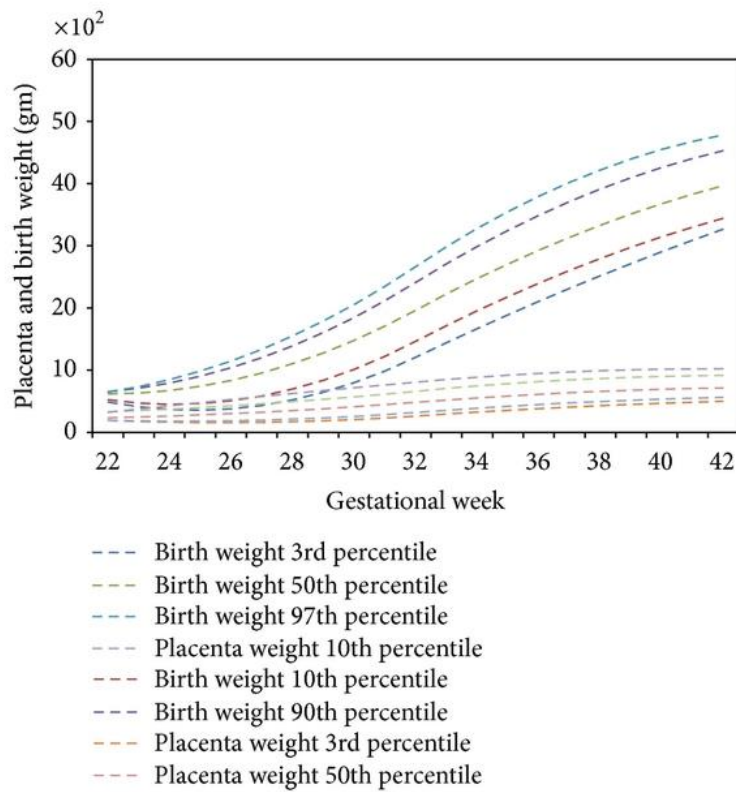
Fig. 1-1. Placental and birth weight percentiles by age in London-born male babies.

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Finally, a proportion of the blood that initially enters the pulmonary circulation from the right atrium re-enters the descending aorta from the pulmonary artery via the ductus arteriosus [10, 12]. In healthy human pregnancies generally >30% of blood from the umbilical vein is shunted through the ductus venosus directly to the heart, with the proportion of shunted blood decreasing with fetal age [8, 9, 13]. In both sheep and human studies proportionate blood flow through the ductus venosus compared to the hepatic portal vein increases following adverse pregnancy exposures such as hypoxemia, when 55-60% of umbilical blood is shunted through the ductus venosus; and IUGR, particularly in severe cases with reduced umbilical vein blood flow [12, 13]. Compared with adult circulation, these shunts in the fetal circulation increases coronary and cerebral blood flow, and results in these organs receiving blood with 80-85% oxygen saturation, compared to the 25-30% saturation in blood from the vena cava [12, 13].

1.3.3. The fetal brain

Fig. 1-2 gives an overview of the timing of fetal neurodevelopment. Neurons are derived from the ectodermal layer of embryonic cells, with a population of the ectoderm differentiating into the neuroectodermal cells, which are the neural progenitor cells [14]. In humans, the neural tube forms between embryonic days 20 to 27 (E20-27), segments into three brain vesicles that will become the forebrain, midbrain and hindbrain, and closes at E30 [14, 15]. The majority of cell divisions of neural precursor cells occur in the ventricular zone of the developing brain, which contains the proliferative germinal matrix [14, 15]. From approximately E33 to E42 post-mitotic neurons begin to migrate from the ventricular zone of the brain, to become predecessor neurons, with a subventricular layer of neurons forming
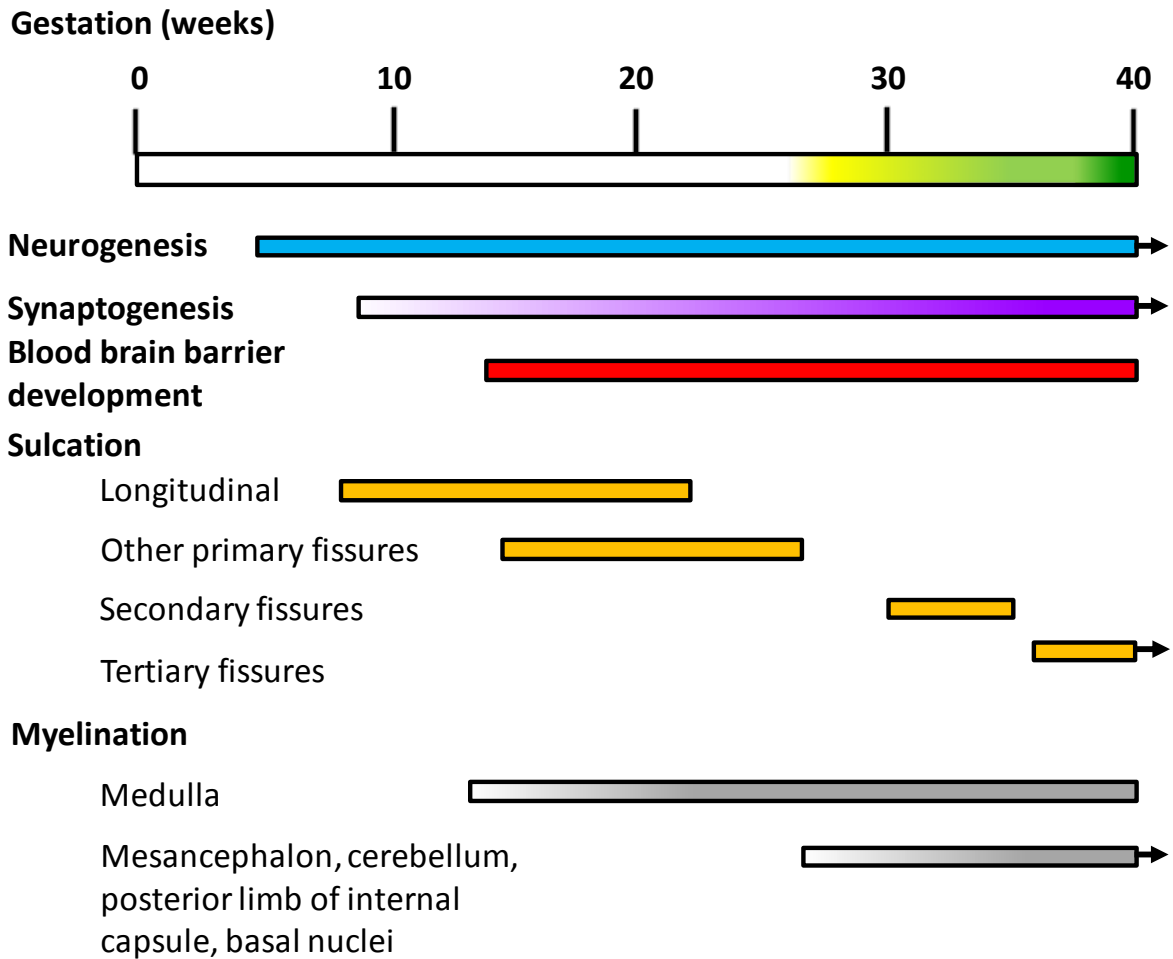
Fig. 1-2. An overview of the timing of fetal neurodevelopment in humans.

Gestation bar indicates timing of delivery as follows: yellow shading – preterm birth, including very to extremely preterm (>32 weeks), light green shading - moderate to late preterm (32-37 weeks) as previously defined [16]. Dark green shading – term birth, including early (37-39 weeks, gradient) and full term (39-40, solid shading), as previously defined [17]. Blue bar = period of neurogenesis [15, 18]. Purple bar = period of synaptogenesis, which increases with age to reach a peak from gestational week 34 into early postnatal life [18, 19]. Red bar = blood brain barrier development [18]. Orange bar = sulcation [14]. Grey shading = myelination [18, 20]. Black arrows indicate processes that continue postnatally.

from E40-41, and the cortical plate evident by E50-51 [14, 15]. The layers of cortex develop in an inside-out fashion, with the earliest neurons forming layer 6, and the youngest layer 2 [15], and sulcation starts with the longitudinal fissure from gestational week 8 (GW8; Fig. 1-2), as the cortex expands [14]. Cortical neurogenesis generally concludes by E108 [14], but the timing of neurogenesis in other regions varies considerably. For example, granule cells first appear in the dentate gyrus at GW13-14, and term-born neonates have 75-80% of the adult number of these cells [18], indicating that neurogenesis in this region continues postnatally.

Synaptogenesis begins in GW9-10 (Fig. 1-2), and proceeds at an increasing rate as gestation progresses, and continues into postnatal life [19]. Peak synaptogenesis occurs at GW34, such that the term-born neonate's striatum has >60% of the number of synapses of the fully developed adult [18]. Myelination begins in the brainstem from approximately the third month of gestation, with myelination progressing through to the rest of the brain in a craniocaudal direction [20]. While myelination of regions such as the medulla, mesencephalon (including the colliculi and lemnisci), and the cerebellum begin prenatally (Fig. 1-1), myelination of many other regions such as the corpus callosum, corticospinal tract, olfactory tract, optic radiations, fornix and anterior limb of the internal capsule, only commence postnatally [18, 20].

Angiogenesis in the brain mirrors and supports the process of neurodevelopment, with rate of blood flow in the brain increasing with gestational age [21-23]. The majority of blood vessels early in gestation are radial, and penetrate into the brain from the pia mater to form distinct vascular layers, penetrating the layers of cortex and deep brain structures as these develop [24, 25]. Areas of high cellular density, such as grey matter and developing nuclei, are more

heavily vascularised than areas such as white matter [26, 27]. The germinal matrix is the most highly vascularised region of the fetal brain, with close to double the blood vessel area compared to grey matter [26, 27]. The rate of maturation of blood vessel morphology differs dependent on region, with vascular smooth muscle layers developing in regions such as the leptomeningeal vascular bed by mid-gestation [24]. The majority of blood vessels within the germinal tissue and extrastrial vascular beds, however, do not contain a tunica media or adventitia, and instead are composed of a single layer of endothelium [24], and thus blood flow to these regions cannot be regulated by these vessels.

1.4. Lateralisation

Cerebral lateralisation is indicative of specialisation of regions within the left and right hemispheres, and is a trait that evolved quite early in evolution, given its presence in fish, amphibians, reptiles, avians and mammals [28]. While cerebral asymmetry and outcomes such as handedness are highly heritable [29, 30], even in twins, environment predicts 10-15% of variance in asymmetry [30]. IUGR disrupts the ordinary trajectory of pre- and postnatal neurodevelopment, and may in turn affect cerebral and functional lateralisation. This review section describes the pattern of cerebral asymmetry observed in healthy individuals, and the associated functional lateralisation. It then summarises the limited knowledge regarding lateralisation following human IUGR, and identifies the gaps in knowledge requiring additional research.

1.4.1. Cerebral asymmetry

Cerebral asymmetry is evident *in utero* and in preterm infants, with motor and temporal lobe network development occurring more rapidly in the left hemisphere than right [31] At birth, healthy neonates have a larger left hemisphere than right [32], but in adulthood, the overall and grey matter volume is larger in the right cerebellum, frontal, inferior parietal and superior temporal lobes than in left, whereas the left caudate nucleus, occipital, posterior parietal and temporal lobes are larger than the right [33-35]. Longitudinal studies indicate that cerebral asymmetry of cortical thickness also changes with age. The medial occipital region, orbitofrontal and inferior frontal gyri are thicker in the left hemisphere than the right hemisphere of the cortex in childhood, but these regions of the right cortex are thicker than those in the left hemisphere in late adolescence [36]. The reverse pattern exists for the thicknesses of the middle occipital and angular gyri, with a gain of leftwards-favouring asymmetry with age [36].

Asymmetry of white matter is also evident from neonatal life, with leftwards asymmetry in the corticospinal tract observed in neonates, adolescents and adults [31]. Connectomic studies indicate that from two weeks of age, adolescence, and into adulthood, there is right-side favouring asymmetry in global efficiency (eg. speed of information processing), interconnectivity within each hemisphere, and betweenness (eg. importance of each region within the hemisphere to information flow) [37-40]. Cerebral asymmetry of white matter decreases between adolescence to young adulthood [40], resulting in the adult pattern of asymmetry, with greater white matter volume in the right compared to the left prefrontal cortex, inferior corona radiata, and anterior internal capsule [35, 41, 42]. Conversely in adults, there is greater white matter in the left supplementary motor area, caudate, occipital and frontal lobes, superior and middle corona radiata, corticospinal tract and cingulum than in

the corresponding regions of the right hemisphere [35, 37, 39, 41, 42]. Changes in the underlying grey and white matter translate into topological asymmetry between the two hemispheres [43], with magnetic resonance imagining (MRI) studies indicating increasing perisulcal asymmetry with age during childhood [43, 44], although once maturation is reached, the positive relationship between age and degree of asymmetry ceases to continue in adults [45].

Boys and girls differ in age-related gains in asymmetry, with boys favouring right hemisphere gain, whereas girls favour left hemisphere gain [36]. As adults, men and women differ greatly in cerebral asymmetry. Men have rightwards asymmetry of central sulcus depth, whereas women are symmetrical in this measure [46], and men have a leftward asymmetry of grey matter, whereas women have a greater rightwards asymmetry of grey matter [35].

1.4.2. Functional lateralisation and its relationship to cerebral lateralisation

Whereas cerebral asymmetry is evident throughout development, and is part of brain architecture, functional lateralisation develops postnatally. Twin studies suggest functional lateralisation is far less heritable than cortical lateralisation [31]. This section briefly describes functional outputs that preferentially utilise regions within one hemisphere more than the other hemisphere (Fig. 1-3). It also describes side bias (eg. handedness, spatial learning), and then discusses consistency of functional and spatial lateralisation.

Left lateralised

Frontal lobe
Complex movement
Detour navigation
Visual attention
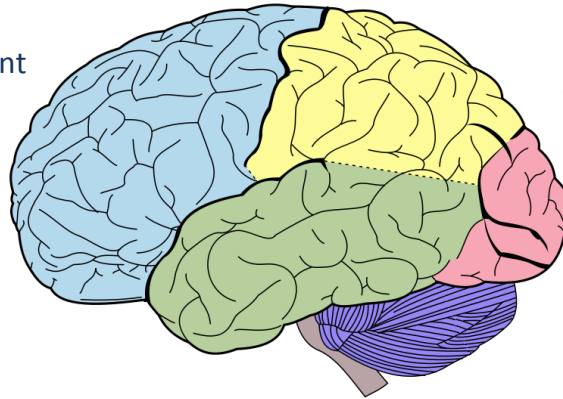
Parietal lobe
Object identification
Visual attention

Temporal lobe
Language

Right lateralised

Parietal lobe
Visual search
Spatial attention

Frontal lobe
Spatial working memory
Visual search

Occipital lobe
Spatial attention

Fig. 1-3. Functional asymmetry within each lobe of the human brain.

There is greater use of the left hemisphere for ipsilateral side movements than right, particularly of complex movement [47-49], and for object identification [50] and visual attention [51]. Speech is also lateralised to the left temporal lobe in adults [52-57]. The right hemisphere is dominant in spatial learning [31, 54-59], spatial attention [50], and visual search tasks [53, 57, 60]. The figure above was created by modifying an image from Wiki Commons (https://commons.wikimedia.org/wiki/File:Brain_diagram_without_text.svg), and is based on an image from the 20th U.S. edition of Gray's Anatomy of the Human Body, originally published in 1918, with copyright now lapsed into the public domain.

1.4.2.1. Motor lateralisation and handedness

Motor lateralisation is one of the most studied aspects of functional lateralisation, but is complex, with effects both of handedness and of specialisation of function within each hemisphere. Right handers are more proficient in motor tasks using their right hand, whereas left handers only show superior left hand skill for simple tasks, but equal proficiency for more complex tasks [49]. While both ipsi- and contralateral cortices are activated in unilateral hand movements [49], there are greater modulating effects of direct stimulation of the left primary motor cortex in right handers than left [61]. Morphologically, right handed individuals have more prominent left-favouring asymmetry of primary motor cortex sulcal depth [46], and right-favouring asymmetry of hippocampal and amygdalar volume [62] and small-world white matter networks [39]. Left-handers, by contrast, show fewer asymmetries on any of these measures [39, 46, 62]. In terms of hemispheric specialisation, there is activation of both contralateral and ipsilateral hemispheres for unilateral hand movements. There is greater left than right side activation for movements made in the ipsilateral hand [47-49], particularly in right handers, [47], which appears to be due to the role of the left motor cortex in the execution of complex movements (Fig. 1-2) [49]. Cerebellar connectivity to and inhibition of the motor cortex is also highly lateralised [63, 64], with the right cerebellum exerting more inhibition over the primary motor cortex than left [64].

1.4.2.2. Language

Another well-known example of functional lateralisation is in the development of language. Neonates and young children utilise both hemispheres when listening to spoken language, and with age progress towards the left-lateralised pattern, particularly of the temporal lobe, observed in older children and adults (Fig. 1-3) [52-57]. This is associated with functional

benefits, as strength of leftwards lateralisation of the temporal lobe correlates positively with verbal IQ in children [53]. Rate of development of left-side lateralisation during childhood and adolescence differs between the regions utilised in language processes, such that bilateral or lateralised use of the brain for language tasks differs between articulation and comprehension tasks [31], although there is no relationship between laterality of brain utilised and age in verbal memory tasks [54]. Utilisation of the frontal lobe in language tasks is sex-specific during adulthood, with functional MRI (fMRI) evidence indicating left-side lateralisation in males, whereas activation of both the left and right inferior frontal gyri is observed in

females [65].

1.4.2.3. Perceptual lateralisation

In humans, perceptual asymmetry is quite task-specific (Fig. 1-3). Visual attention tasks utilise the bilateral fronto-parietal network, with more activation in the left frontal and parietal lobe than right, and left dorsolateral prefrontal cortex and interparietal sulcus dependent on the nature of the attention task [51]. Visual search tasks are right-side lateralised, however, utilising the right occipital, parietal and frontal lobes (Fig. 1-3) [53, 57, 60]. Lesions in the left parietal lobe due to stroke are associated with impaired object identification, whereas lesions in the right parietal lobe impair spatial attention [50]. These specific asymmetries develop during childhood, with functional benefit, as strength of brain lateralisation for visual search correlates positively with visuomotor and visuospatial scores in IQ tests [53].

1.4.2.4. Spatial lateralisation and side preference

Lateralisation of spatial learning is one of the more poorly understood forms of functional lateralisation. Visuospatial skills appear to be largely lateralised to the right hemisphere [31, 54-59]. Working memory and allocentric navigation strategies during searching tasks – in which external visual cues are used for navigation - utilise the right hippocampus, caudate, temporal, parietal and frontal lobes more than the left [59]. Damage to the right dorsolateral prefrontal cortex results in greater deficits in spatial working memory tasks than damage to the left [58]. The left frontal cortex is utilised in navigation around detours however [66]. Adults have superior spatial working memory than children, and the degree of lateralisation in spatial tasks does not differ between children and adults [54]. Rat lesion studies indicate that the side preference favoured in spatial learning seems related directly to caudate nucleus function. Lesions in the caudate ipsilateral to side-preference increased strength of lateralisation, whereas lesions to the contralateral caudate either decreased strength of lateralisation or even reversed it [67]. There are also sex differences, with female rats exhibiting weaker behavioural lateralisation than males in T-maze tasks [68, 69].

1.4.2.5. Do different kinds of lateralisation interact? Determinants of lateralisation

Consistency of functional lateralisation between tasks is highly variable. Side preference in spatial tasks has limited correlation with motor lateralisation in mice [70], and strength of lateralisation of visuospatial and language functions to each hemisphere are independent in humans [56, 57]. Observations in fish and chicks led to the suggestion that cerebral lateralisation evolved to permit parallel processing of different information within each hemisphere [71], but this does not appear to occur in humans, possibly because mammals possess a corpus callosum [56]. Lateralisation of language skills to the left hemisphere is not

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related to measures of spatial skills during tasks that assess both outcomes, or vice versa; these functions remain independent [56]. Nevertheless, the strength of left-side lateralisation for language correlates negatively with fractional anisotropy of the corpus callosum, and incidence of atypical patterns of spatial lateralisation are associated with increased functional anisotropy of the corpus callosum [55]. This suggests that pruning of corpus callosal fibres is important for the development of lateralisation for both language and spatial function [55].

In contrast, handedness appears to be correlated with other measures of cerebral asymmetry. Individuals with language lateralised to the hemisphere ipsilateral to that of motor dominance – eg. left hemisphere for right-handed individuals - have stronger lateralisation than those with the opposite pattern [29, 55]. Strongly left-handed individuals have a 27% incidence of right hemisphere dominance on language tasks, sevenfold the incidence found in strongly right-handed individuals [29]. It is difficult to explain this observation, as the determinants of handedness are unclear, with numerous genetic and epigenetic theories not yet entirely able to explain the non-Mendelian inheritance of handedness from parents [reviewed in 72]. From GW15, the majority of fetuses show a right side preference in thumb sucking [73], but only 57% of 7-13 month old infants show any kind of side preference in reaching tasks [74], and frequency of unilateral, bilateral, right and left hand use varies dramatically month by month in early life [75].

1.5. Intrauterine growth restriction in humans

1.5.1. Fetal environment, redistribution of blood flow and growth

Intrauterine growth restriction (IUGR) occurs the fetus is prevented from meeting its genetic potential for growth due to a restricted environment [76], which may be the result of maternal or placental factors. Maternal factors, such as undernutrition and age under 16 years of age, are less common causes of IUGR in developed countries, although these contribute significantly to IUGR in underdeveloped countries [76]. It is difficult to determine to what extent impaired placental growth or function contributes to IUGR in developed countries due to poor prediction factors and the complex, multifactorial contributors to IUGR; however many of the clinical risk factors for IUGR are indicators of poor placentation [77]. In IUGR pregnancies blood vessel resistance in the placental bed is higher than in normal pregnancies, particularly later in pregnancy, which greatly reduces placental blood flow. This in turn leads to decreased substrate transfer from mother to fetus, with lower fetal circulating concentrations of oxygen and nutrients, including essential and non-essential amino acids [78-80]. Doppler ultrasound is a valuable tool for examining the resistance of the umbilical artery, umbilical vein and middle cerebral artery and thus determining whether placental restriction is present. This tool is also used to calculate relative risk of adverse perinatal outcomes [78, 81, 82], allowing for confirmation of IUGR status, including of infants that are not ultimately born at low birth weight. In many cases of IUGR placental dysfunction is progressive, and Doppler examination reveals deterioration with time, with a trajectory reflecting severity of the placental restriction [78, 83]. Although spiral artery resistance and blood flow does not differ between IUGR and non-IUGR in early pregnancy (11-24 weeks gestation) [84], abnormally high spiral artery resistance in later pregnancy (18-41 weeks gestation) occurs in 44% of complicated pregnancies, the majority being IUGR, and is

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predictive of adverse perinatal outcomes [79]. Accordingly, substrate deficiency in IUGR pregnancies is greatest during the third trimester, which also corresponds with maximal *in utero* rates of neurodevelopment, as peak brain growth velocity in humans occurs around term [18].

Fetal responses to restricted nutrient supply include redistribution of blood flow. IUGR fetuses have greater vascular density in the brain and increased blood flow through these vessels compared to age-matched non-IUGR [21]. Relative blood flow through the ductus venosus is also increased in IUGR fetuses to 90% of fetal blood from the umbilical vein, which increases the flow of more highly oxygenated blood to the brain instead of the liver [13, 85]. Models of fetal circulation indicate that blood flow redistribution is able to maintain stable high levels of cerebral oxygenation in response to small increases in placental blood flow resistance, but that pathological increases in placental resistance decrease cerebral oxygen availability [86]. Increased cerebral flow in some pregnancies also occurs due to brain sparing, in which the brain receives a greater proportion of blood flow and thus oxygen supply than other organs, due to decreased resistance of the fetal middle cerebral artery [87-89]. Brain sparing occurs in ~ 17% of IUGR pregnancies [90], but has 78% incidence in groups with the most severe outcomes, including higher perinatal mortality rates, and abnormal blood flow velocity in fetal and placental venous vessels [91]. Brain sparing results in neonates with proportionately greater head and brain size relative to body weight (cephalisation index) compared to neonates born at a size appropriate for their gestational age (AGA) [92, 93]. The redistribution of blood flow to favour the brain in brain sparing does not appear to be able to restore normal blood supply to the brain in all cases, possibly due to lower cardiac output in children born small for gestational age (SGA) compared to AGA fetuses [94]. In addition, placental insufficiency has been associated with retrograde blood

flow in the aortic isthmus in a proportion of IUGR fetuses, suggesting that blood from the pulmonary artery and descending aorta, which was destined for the placenta and has lower oxygen and nutrient concentration, is also being diverted back to the brain, heightening the risk of cerebral damage [95]. Despite brain sparing, IUGR fetuses still have reduced total and relative brain volume compared to non-IUGR fetuses [96]. Within IUGR neonates, those with brain sparing have reduced birth weight, shorter gestation, and a higher incidence of postnatal morbidity compared to those without brain sparing [91].

1.5.2. Neonatal catch up growth

The majority of SGA and IUGR children undergo accelerated, or catch-up, growth in early postnatal life, with 80-90% of term and preterm children achieving catch-up in weight and/or height to that of AGA peers within the first 2-3 years of postnatal life [97, 98]. Catch-up growth of height and weight in IUGR occurs largely within the first two months of postnatal life [99]. A subset of IUGR children fail to catch-up and the population therefore remains smaller as a whole. At one year of age height and weight of late preterm IUGR children remains one standard deviation lower than preterm AGA controls [100], and at three years of age term-born IUGR children remain on average 3 cm shorter than term AGA peers [92]. Term-born low birth weight (LBW) individuals who experience early life catch-up growth remain on average -0.5 SD shorter than AGA peers as adolescents, while adolescents with failure of early life catch-up growth are 1.7 standard deviations shorter than their AGA peers [101]. Head circumference is a proxy measure of brain size that corresponds well to frontal lobe volume [102]. Catch-up in head circumference occurs throughout the first 6-12 postnatal months [103], taking place during a period of rapid postnatal brain development [19, 104] and appears to be slower than catch-up of weight and height [99]. At three years of age term IUGR children have on average 0.9 cm smaller head circumferences than AGA peers [92].

Postnatal nutrition may be one factor limiting postnatal growth. Energy quotient (EQ, energy intake/kg body weight per day) in the first 10 days of life is higher (EQ above the median score of 90.5) in preterm SGA infants with catch-up growth, compared to those whose growth does not catch up (EQ < 90.5). Higher energy quotient in the first year of life, in turn, is highly predictive of developmental quotient and IQ in later life [103]. Unfortunately, the higher energy requirements associated with catch-up growth may mean that many preterm IUGR infants may be under-nourished relative to their requirements [105, 106], and this may also be the case in term-born IUGR infants. In the longer term, lower socioeconomic status is also associated with a greater incidence of catch-down growth (reduced postnatal growth rates compared to peers) and lower incidence of catch-up growth in preterm SGA [107]. Therefore, the postnatal environment can further exacerbate the consequences of poor prenatal growth.

1.6. Neurodevelopmental effects of IUGR and neonatal catch-up growth in humans

1.6.1. Effects of IUGR on brain structure

1.6.1.1. Fetal development and preterm infants prior to term age

IUGR has extensive effects on brain structure and organisation, including morphological and microstructural changes. From GW20 onwards, IUGR fetuses have smaller head circumference, smaller total intracranial, brain and left opercular volumes, relatively smaller corpus callosum volume, smaller biparietal diameters, and deeper insula and left cingulate fissures [96, 108-110] compared to normally-grown fetuses. Conversely, the cerebellum, brainstem and pons are larger in SGA than AGA fetuses at GW37, indicating that the volume changes do not just reflect a loss in total brain volume, but also altered brain structure [111]. Brain development *in utero*, particularly of grey matter, is delayed and discordant in SGA when compared to AGA fetuses of the same gestational age, with MRI indicating decreased cortical volume, thickness and surface area, but increased sulcation index in preterm IUGR compared to preterm AGA neonates at birth [112]. Between GW20 and GW40, the sylvian and parieto-orbital fissures are more developed in IUGR than control fetuses, suggesting accelerated cerebral maturation [109], but post-mortem examination shows a decreased trajectory of rate of gain in cell number in the cortical plate of the cerebrum [108]. In contrast, other measures of maturation, such as cerebral myelination, deeper cortical infolding in other regions, germinal cell division and glial cell migration do not differ between preterm IUGR and preterm non-IUGR fetuses at GW24-GW36 [113], indicating that altered brain development in the IUGR fetus is region-specific. At 37 weeks gestation, brain metabolism of N-acetylaspartate and choline containing compounds, such as glycerophosphocholine differs between SGA fetuses without placental restriction and IUGR fetuses, but there is still

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evidence of neuronal loss or injury in both compared to AGA fetuses [114]. It is worth noting that term equivalent age preterm AGA neonates also have abnormal neurodevelopment, compared to term-born AGA neonates, including reduced grey matter, particularly in deep cortical regions [115]. These effects of preterm birth do not recapitulate those of IUGR however, and preterm IUGR neonates have neurodevelopmental dysfunction that is additional to that associated with preterm birth.

1.6.1.2. Neonates

Altered *in utero* brain development results in distinctive differences between IUGR and non-IUGR neonates at term. Term-born IUGR neonates have ~2 cm smaller head circumference than term-born non-IUGR neonates [92]. Brain volume of preterm IUGR neonates measured by MRI at term equivalent age is reduced by 42 mL compared to AGA, which appears to be largely due to decreased grey matter volume [116, 117], and preterm SGA neonates similarly have decreased grey matter volume compared to preterm AGA [118]. Preterm-born IUGR neonates at term-equivalent age also have reduced grey matter volume in the left and right hippocampus, and grey matter volume in these regions is positively associated with both overall cortical grey matter volume and with birth weight [119].

1.6.1.3. Infants

Neurodevelopmental trajectories of IUGR infants remain discordant from those of AGA infants in early postnatal life. At one year of corrected age, absolute grey matter volume is reduced in preterm IUGR infants, compared to both term and preterm non-IUGR, in part as a consequence of the smaller head circumference and cortical volumes of these children, and their relative grey matter volumes are not decreased [117]. Closer examination shows that the

loss of grey matter within the preterm IUGR infant brain, compared to preterm AGA, is region-specific, including bilaterally within the temporal and insular lobes, superior gyrus of the parietal lobe, hippocampus and amygdala, and in the right perirolandic area and prefrontal lobe [117, 120]. In addition to grey matter loss, IUGR impairs development of white matter. Compared to term AGA, white matter volume in preterm IUGR babies is decreased in the corpus callosum, left hippocampus and cerebellum, and there are microstructural changes to white matter, possibly indicative of higher fibre density, in the forceps minor and anterior corona radialis [117, 120]. There are also increases in white matter in the left temporal region in preterm IUGR compared to preterm AGA, indicating that rather than purely causing loss of white matter, the abnormal neurodevelopment in IUGR infants also results in abnormal gains of white matter in some regions [120]. These changes in white matter microstructure are not observed in preterm non-IUGR infants [117], suggesting that white matter differences between preterm IUGR and term AGA reflect the effects of IUGR. There are other region-specific microstructural changes to white matter in year old term SGA infants, with MRI studies indicating both increases and decreases in white matter fibre numbers in different regions, compared to AGA [100]. Connectomic modelling using MRI acquisitions from this SGA group also indicated decreased regional network efficiency (defined as the average inverse of the shortest fibre bundle path length between one brain region and its neighbouring regions) compared to AGA, which in turn predicted poorer neurodevelopmental scores at two years of age [100].

1.6.1.4. Children and adolescents

Region-specific differences in brain structure continue throughout the life of the IUGR child. At a gross level, compared to non-IUGR, term-born IUGR children have smaller head circumferences, indicative of smaller brain volume, than non-IUGR infants at three years of age [92], and term-born IUGR adolescents continue to have a smaller brain volume at age 15 [121]. Compared to AGA, 18 - 22 year old SGA term-born young adults also have reduced fractional anisotropy (FA) in the right external capsule and right anterior limb of the internal capsule, and bilaterally in the uncinate fasciculus, inferior fronto-occipital fasciculus, inferior longitudinal fasciculus and a number of areas within the right hemisphere [122]. Decreased FA is generally indicative of reduced myelination, axon injury and other damage to white matter bundles [123-125], and these tracts connect the cerebral cortex with other regions of the brain, including the limbic system and spinal cord. In term-born AGA young adults there is a positive correlation between third trimester brain growth and FA, whereas this is not the case in term-born SGA young adults [122], suggesting that in SGA either the discordant growth in late pregnancy has highly variable effects on myelination, or that changes persist into adolescence. Maturation of white matter, including myelination, occurs largely postnatally in the human, with the prefrontal cortex being the final region to complete myelination at 20 years of age [126], and thus long after the initial period of *in utero* restriction. This suggests that changes in developmental and maturational trajectories are initiated *in utero* and persist into later life.

1.6.1.5. Cerebral lateralisation

One of the more obvious gross anatomical alterations present in both SGA and IUGR children and adults is altered cerebral lateralisation. Reductions in brain volume induced by IUGR are asymmetric at GW37, with the left but not right opercular volume reduced in IUGR compared to non-IUGR fetuses [96]. There is also more pronounced insular cortical asymmetry, with greater cortical thickness in the left than right hemisphere in IUGR than non-IUGR fetuses [127]. This is probably region-specific, because in another study preterm IUGR and non-IUGR babies had right-favouring asymmetry of hippocampal volume, at term-equivalent age, and this was not affected by IUGR status [119]. While cerebral asymmetry has not been directly compared in term-born IUGR and non-IUGR infants or children, preterm IUGR infants have hemisphere- and region-specific grey matter loss compared to term AGA at one year of corrected age, with reductions in grey matter in the right hemisphere in the perirolandic area and frontal lobe, and in the left hemisphere in the parietal and occipital lobe, putamen, caudate and pallidium [117]. While this comparison is between preterm IUGR and term-born non-IUGR infants, the differences do not appear to be a consequence of the preterm birth alone, as comparisons between preterm and term-born AGA in the same study revealed very few differences in grey matter volume [117]. There is also some evidence that cerebral asymmetry persists after IUGR. At 15 years of age, term-born SGA adolescents have reduced right but not left hemispheric surface area compared to term-born AGA individuals [121]. In general, it appears that the cerebral asymmetry in IUGR reflects a reduction of cortical volume in the right hemisphere, particularly of grey matter, but more study is required to fully characterise these changes in cerebral lateralisation.

1.6.1.6. – Summary

To summarise, it appears that the effects of IUGR on neurodevelopment throughout the lifespan are two-fold. *In utero* growth restriction decreases brain volume, particular of grey matter, and induces other microstructural changes at birth. Secondly, the IUGR brain undergoes an abnormal developmental and maturational trajectory postnatally, such that white matter, in particular, develops gross microstructural abnormalities that were not present at birth. It is, however, unclear to what extent the changes in brain development that develop postnatally following IUGR are due to changes initiated *in utero* or due to the postnatal environment, and therefore to what extent it may prove possible to intervene after birth in IUGR infants to improve their neurodevelopmental outcomes.

1.6.2. Effects of IUGR on cognitive function

1 6.2.1 – Neurobehaviour in neonates and infants

Poor neurodevelopmental outcomes in IUGR children result in functional neurobehavioural and cognitive deficiencies from birth. Preterm IUGR infants have lower, indicating less mature, Assessment of Preterm Infant Behaviour (APIB) and Neonatal Behavioural Assessment Scale (NBAS) scores, and higher incidence of abnormal NBAS scores than preterm AGA infants. Scores on these two scales reflect a neonate's autonomic, motor, state organisational, attention-interaction, habituation, social-interactive and self-regulation systems [112, 116, 119, 128]. The attention capacity of IUGR infants is also altered, although the degree to which this differs is dependent on the assessment scales used, as preterm IUGR infants have higher APIB scores but lower NBAS scores than preterm AGA neonates [112, 116, 128]. This suggests that the different scales may measure different aspects of the altered capacity of the IUGR infants. Additionally, IUGR infants with confirmed placental

insufficiency have poorer outcomes than those born SGA without evidence of placental restriction. Within preterm-born groups, IUGR but not SGA neonates have poorer NBAS than AGA neonates [128]. As 1-3 year old toddlers, both preterm- and term-born IUGR have poorer neurobehavioural scores than AGA infants, including lower Bayley Scale of Infant Development (BSID) scores, with specific deficiencies including abnormal muscle tone, motor development, hearing and vision, delayed speech and poorer cognition [92, 100, 129]. There is also a higher incidence of cerebral palsy and cognitive delay in IUGR toddlers than the AGA population, with both low birth weight and gestational age separately contributing to this risk [129]. Conversely, development of auditory recognition memory is accelerated in preterm-born IUGR toddlers compared to age-matched non-IUGR toddlers [100].

Functional neurodevelopmental outcomes are highly predicted by the brain morphology of the fetus and neonate. Corpus callosum size at GW37 is reduced in IUGR compared to AGA fetuses, and these size measures correlate positively with NBAS scores at 42-43 postnatal weeks of age [110]. APIB scores at term equivalent age are positively correlated with cortical development at birth, which is impaired in preterm IUGR neonates compared to preterm non-IUGR [112]. Cortical surface area, in particular, predicts motor organisational, attention and self-regulation scores in this group [112], with lower cerebral grey matter volume associated with higher attention-interaction scores in preterm IUGR than AGA infants [116]. This may be due to poor subsystem differentiation, as, for example, low scores on attention-interaction suggest that efforts towards focussed attention come at the cost of motor and autonomic regulation in these infants [130]. Additionally, the volume increases in the brainstem and cerebellum in GW37 SGA fetuses are associated with poorer neurobehavioural outcomes measured 3-6 weeks after delivery, most notably of motor skills [111]. In the longer term, in one year old preterm-born IUGR infants, the impact of IUGR on the neural circuitry of

recognition memory appears more strongly related to birth weight rather than to head size [100]. At this age, both fibre number and FA brain connectivity correlate positively with BSID scores [100]. Sizes of some specific brain regions also predict later cognitive outcomes. For example, mental development index in preterm IUGR toddlers at 24 months of corrected age correlates positively with total hippocampal volume at term equivalent age [119].

1.6.2.2 – Cognition in children

Deficits in neurodevelopment and cognition after IUGR continue throughout childhood. Term-born IUGR, SGA and LBW children between 7-10 years of age have 6-9 point lower average IQs and greater learning difficulties compared to AGA and non-IUGR children [93, 98, 131, 132]. This deficit is worse in preterm IUGR. At ages 5-8 years, preterm IUGR children have lower full scale and verbal IQ compared to both preterm and term non-IUGR children [133]. Language, memory and executive function are also poorer in 9-10 year old term-born IUGR children compared to non-IUGR children [93, 98, 134]. Nine year old term-born IUGR children have a deficit in global and intermodality short-term memory processing in the Visual-Aural Digit Span Test, compared to age matched non-IUGR children, with processing of auditory input particularly impaired [134]. Nine year old term-born IUGR children also have poorer aural-oral and aural-written memory scores, compared to non-IUGR children, and this appears to be a consequence of poorer executive function rather than hippocampal damage [135]. Visuomotor skills are also impaired in 6-10 year old term-born IUGR, SGA, and LBW children compared to AGA and non-IUGR children [93, 98, 131, 136, 137]. Six year old term-born IUGR children perform worse than non-IUGR children in radial arm maze tasks evaluating both spatial memory and executive function, as they are less likely to use an algorithmic approach to solve each forced-choice task [137]. Motor skills are also

impaired in term-born SGA seven year olds, who demonstrate poorer coordination and fine motor skills than AGA peers [136].

In addition to direct effects of restricted intrauterine growth on brain structures required for memory, learning and executive function, cognitive outcomes may be impaired in term-born SGA and IUGR children as a result of their lower attentional scores [93, 136] and hyperactivity [136]. The incidence and severity of attention deficit hyperactivity disorder (ADHD) symptoms are higher in both preterm- and term-born SGA compared to AGA children at 24 and 56 months of age [122, 138], and in 5-8 year old preterm IUGR children, but not preterm non-IUGR children, compared to term-born non-IUGR [133]. Preterm IUGR children also have higher rates of behavioural and conduct problems compared to preterm AGA [133]. Term-born SGA children have a higher incidence of mental illness such as anxiety disorders compared to term-born AGA [122]. In combination, these behavioural effects of IUGR are likely to impede learning and lead to academic difficulties. Consistent with this hypothesis, in studies of 5-8 year old children, including term, preterm IUGR, and preterm non-IUGR individuals, behavioural deviance and attentional deficit disorder scores correlated negatively with full scale IQ scores [133].

1.6.2.3. – Cognition in adolescents and young adults

Unlike neurological and morphological outcomes, which appear to worsen with time, cognitive and functional deficits induced by IUGR remain consistent throughout postnatal life. The incidence of learning difficulties and attentional problems is increased, and reading scores are lower, in 14 year old term-born SGA children compared to AGA, particularly those whose birth weight was below the third centile [139]. Effects of IUGR on IQ in adolescents and young adults differ between studies. No differences in IQ were reported

between term-born SGA and AGA children at 14 years of age [139], but IQ is 6.3 points lower in term-born SGA and 14 points lower in term-born IUGR young adults at 19-20 years of age, when compared with AGA [140]. Also at 19-20 years of age, a number of IQ subscales were lower in both term-born SGA and IUGR compared to AGA, including vocabulary, comprehension, working memory and perceptual organisation [140]. When considering IUGR specifically, executive function scores and performance and total IQ scores were lower, but verbal IQ scores were similar in IUGR, compared to non-IUGR in groups of 18-year-olds containing both term and preterm-born individuals [141]. In contrast, in another study of young adults, 18 year olds born at term with low birthweight had better long term memory than AGA individuals [142].

Morphological brain measures appear predictive of functional outcomes in adolescents and adults. There is a negative correlation between FA and total IQ in term-born SGA but not AGA young adults, which Eikenes and colleagues propose is representative of less efficient compensatory rewiring of the white matter circuits in the SGA brain [122]. At 28 years of age term-born young adults whose birthweights were in the lowest quartile also have greater corticospinal sensitivity to transcranial magnetic stimulation, when compared with those of the highest two birth weight quartiles [143]. These LBW young adults also have increased functional asymmetry of motor threshold, compared to those of higher birth weight, with higher resting motor threshold of the right hemisphere than left [143]. In combination, this suggests gross changes to the functionality of the motor system. The general pattern therefore suggests long-term deficits in cognitive function following LBW or IUGR, although the specific areas of impairment are not consistent and differences between SGA, LBW and IUGR require further clarification.

1.6.2.4. – Academic outcomes

The combined cognitive and neurodevelopmental deficits throughout the life of the SGA and IUGR child and adolescent ultimately contribute to poorer academic outcomes. Term-born IUGR children have poorer primary school achievement [93], including in mathematics, reading and verbal knowledge [98] and lower grades in secondary school at 16 years of age than non-IUGR peers [141]. By 19-20 years of age there is more than a six-fold greater likelihood that term-born SGA and IUGR individuals have received special education compared to AGA [140]. SGA and IUGR term-born young adults also have five-fold higher rates than AGA of unemployment or long term sick leave at 20 years of age [140]. At 20 years of age, term-born adults of the lowest quartile of birthweight have a lower education level compared to those of the highest quartile [143].

1.6.2.5. – Functional lateralisation

In addition to morphological asymmetry, IUGR also appears to alter functional lateralisation, although this has not yet been well characterised, and it is unclear to what extent the functional outcomes correlate with altered structural asymmetry. Some of the visuomotor impairments observed in IUGR and SGA infants appear to be lateralised. Low birth weight, term-born adolescents have reduced corticospinal tract sensitivity to transcranial magnetic stimulation in the right hemisphere, and thus require a greater stimulus to elicit a motor response in the left hand compared to those of normal birth weight [143]. This may be responsible for the poorer motor skills term-born SGA adolescents have in their non-dominant hand relative to AGA adolescents [144], which suggests that SGA individuals have increased right-side motor lateralisation at the cost of function in the left. Other forms of lateralisation appear to be affected differently by reduced birth weight, with increased right

hemisphere dominance, measured by blood flow under situations of stress, in term-born low birth weight children compared to normal birth weight children [145]. In contrast, term-born SGA adolescents have reduced right ear dominance and poorer capacity to attend to unilateral stimuli presented to one ear only [145], suggesting reduced lateralisation of auditory function. As of yet the specific effects of IUGR on functional lateralisation are unclear, because these outcomes have only been reported in low birth weight and SGA individuals. Although these studies have demonstrated that poor prenatal growth is associated with adverse outcomes, there may be additional consequences for lateralisation due to the impaired placentation and thus reduced nutrition and hypoxia experienced during the IUGR pregnancy.

1.6.3. Implications of brain sparing for neurodevelopment and cognition

While brain sparing increases head size relative to body weight, it does not restore brain function or neurodevelopment of IUGR children to that of non-IUGR children. In fact, redistribution of middle cerebral artery blood flow, an indicator of brain sparing, in SGA and IUGR fetuses predicts decreased cerebral myelination [113], lower neurobehavioural scores, higher incidence of neurodevelopmental impairment and other adverse outcomes [90, 92, 146]. The majority of the neurodevelopmental impairments associated with brain-sparing of head circumference are within the frontal brain, which is restricted in SGA fetuses and the volume of which correlates positively with birth weight [102]. Indeed, even in healthy, term-born, non-IUGR infants, increased blood flow to the frontal brain, measured by fractional moving blood volume, is associated with lower neurobehavioural scores [147]. At 40 weeks corrected gestational age (term-equivalent age), IUGR neonates with brain sparing have poorer habituation, attention, motor and social interactive scores than non-IUGR neonates, whereas IUGR neonates without brain sparing do not differ from non-IUGR on any of these

measures [128]. At two years of age, both preterm and term-born SGA infants with cerebral blood flow redistribution have suboptimal neurodevelopment quotients, including poorer problem solving, communication and personal-social neurodevelopmental scores, compared to both AGA and SGA infants without cerebral blood flow redistribution [95, 148]. Although there are studies indicating that head size relative to body weight at birth positively predicts neurodevelopmental performance and IQ at nine years of age [93], this may indicate the benefits of having a larger brain rather than brain sparing.

Roselló and colleagues have suggested that the incidence of brain sparing should be considered as a symptom of more severe IUGR, and as an indicator of likely occurrence of brain damage [88], rather than a mechanism that exists to rescue neurodevelopment, as was initially assumed. Consistent with this, there are higher instances of fetal distress, Caesarean section, and perinatal complications in those fetuses in which redistribution of blood from the middle and anterior cerebral arteries has occurred [146]. A much higher incidence of brain sparing occurs in groups that have the most severe perinatal outcomes, with one study observing 78% incidence of brain-sparing observed in the most severely growth restricted groups [91].

1.6.4. Neonatal catch-up growth and cognitive outcomes

Catch-up growth during the early postnatal period is highly predictive of better cognitive outcomes in later life. Height at one year of age positively predicts IQ in term-born IUGR children at 9-10 years of age [149], and the correlation between IQ at this age and current head circumference is positive and stronger than the correlation with head circumference at birth [149]. Differences in cognitive outcomes between IUGR and non-IUGR appear to be consequences of altered (restricted) patterns of growth, not just the final size achieved at birth, and both prenatal and postnatal growth appear to be important. When separately comparing term-born IUGR and non-IUGR SGA young adults to AGA individuals who had similar birth weight, the IUGR but not non-IUGR SGA had lower IQs than the AGA group [140]. This suggests there are direct consequences of prenatal restriction regardless of birth weight. Additionally, within this cohort, head circumference at birth and adult IQ were not correlated, providing further evidence that postnatal factors also influence outcomes [140]. IUGR and SGA preterm and term-born children that underwent catch-up growth have IQs that are 3 points higher at age two, and are ~8 points higher by ages 8-10, in comparison to peers with suboptimal or failure of catch-up growth [93, 149, 150]. In the long term, regardless of gestational age at birth, SGA young adults with catch-up growth have higher intellectual performance scores than those without catch-up growth [151] and better academic outcomes as measured by more years of attained schooling [152]. There is, however, also evidence of a quadratic relationship between rapid catch-up growth in weight in the first 16 weeks of life and IQ at seven years of age in term-born SGA children, suggesting that while catch-up growth is generally beneficial, too much or too little growth in early life may result in suboptimal intellectual outcomes [132].

While catch-up growth is highly predictive of cognitive outcomes, there is conflicting evidence as to whether it fully restores outcomes in SGA individuals to those of AGA. In one study, there were no differences in IQ between eight year old preterm-born SGA and AGA children following catch-up growth [150]. However, in another study, IQ and reading ability were lower in 7-9 year old term- and preterm-born SGA children with catch-up of head growth compared to AGA and to SGA born with normal head circumference [153]. Results of in-depth batteries of cognitive tests suggest that the benefits of catch-up growth in term-born IUGR children may be quite specific, improving visuomotor skills, but not frontal cortex-dependent functions such as executive function and language [98].

1.7. Programming the brain: common outcomes and gaps in knowledge from animal studies of IUGR

1.7.1. Abstract

IUGR in humans is associated with impaired pre- and postnatal neurodevelopment, and subsequent postnatal cognition, resulting in lower IQ, poorer memory, visuomotor and executive function skills, as well as behavioural and attentional problems. Experimental models of IUGR are needed to allow direct testing of causality and interventions, and have benefits in reducing both confounding by comorbidities such as prematurity, and variation due to environment and genetics. This review describes and discusses experimental models of IUGR in which neurodevelopmental and cognitive outcomes of IUGR have been reported. We consider the timing of neurodevelopment relative to birth and to the period of restriction, as well as the effects of each experimental perturbation on the fetal environment and development, before discussing neurodevelopmental and cognitive outcomes for progeny as fetuses, neonates and into adolescent and adult life. Experimental IUGR induces broadly similar outcomes to human IUGR, with altered brain morphology, in particular grey matter loss and discordant trajectory of white matter development, and poorer cognition and memory reported in various studies. Nevertheless, there remain gaps in knowledge of neurodevelopment in experimental models. We end the review with recommendations for the design of future studies to further investigate the mechanisms underlying adverse neurodevelopmental consequences of IUGR, and to evaluate interventions that may subsequently improve outcomes of IUGR in humans.

1.7.2. Introduction

Intrauterine growth restriction (IUGR) occurs in approximately 15% of births worldwide, and 7% of pregnancies in developed countries [154]. IUGR is characterised by a restrictive environment that prevents the fetus from meeting its genetic potential for growth [76], and often results in a neonate who is small relative to gestational age [SGA, born with a birth weight in the lowest 10th centile of the population, 155]. While IUGR can be induced by maternal undernutrition [156], in developed countries IUGR is predominantly associated with maternal, fetal and uterine factors [reviewed in 77], that lead to poor placental function. This includes reduced uterine artery, placental and umbilical blood-flows [77, 157], and decreased fetal oxygen and nutrient supply [78-80, 158]. Fetal nutrient demand increases with growth as gestation progresses, and late in gestation demand approaches placental capacity even in normal pregnancy. Accordingly, placental blood flow and efficiency increases in later pregnancy [3, 4], such that there is a positive relationship between placental and birth weight in humans and sheep [3, 7], and placental size and efficiency increase with advancing pregnancy [3]. These progressive placental adaptations appear less successful in the pregnancies with an IUGR fetus, which have lower blood flow relative to fetal size developing in later pregnancy [3]. Because the level of placental dysfunction in IUGR increases as pregnancy progresses [83] substrate deficiency in human IUGR pregnancies is greatest during the third trimester, which corresponds with maximal *in utero* rates of neurodevelopment [18], with lifelong structural and functional consequences.

SGA status is often used as a proxy for IUGR in human studies due to limited data on fetal growth trajectories, but will also capture individuals born with a low birth weight who have not undergone the pathological exposure to a restrictive fetal environment [159]. Fetuses, neonates, children and adolescents who were subjected to IUGR and/or born SGA have reduced head circumference and reduced total and regional brain volumes compared to controls [92, 96, 108, 109, 117, 120, 121]. This is largely due to grey matter loss, as well as discordant white matter development and

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microstructural changes, suggesting reduced myelination and axon injury [100, 108-110, 112, 116, 117, 120-122]. The impaired functional outcomes in IUGR and SGA infants, children and adults are highly correlated with these morphological outcomes [110, 112, 116, 119, 122, 143]. Compared to infants born at a size appropriate for their gestational age (AGA), IUGR and SGA infants have more immature neurobehavioural scores [92, 100, 112, 116, 128, 129] and, as children, have lower IQ and poorer language, working and short-term memory, executive function and visuomotor skills [93, 98, 131, 132, 134-137, 140, 141]. There are also higher incidences of cerebral palsy, attention deficit hyperactivity symptoms and behavioural problems in offspring of IUGR pregnancies compared to AGA [93, 122, 129, 133, 136, 138]. In addition, low birth weight (<2500 g) interacts with a genetic risk for depression; in combination these are associated with a higher incidence of depressive symptoms [160], although this has not been examined in IUGR or SGA offspring. Cognitive and behavioural consequences ultimately contribute to poorer academic outcomes in IUGR and SGA children than in those who were born AGA [93, 98, 140, 141].

In addition to the limitations of human studies, where IUGR may not be clearly differentiated from other causes of low birth weight, there are a number of confounding factors limiting the capacity to fully characterise the consequences of IUGR and their underlying mechanisms in humans. Firstly, IUGR is rarely a discreet condition and comorbidities are common. The incidence of preterm birth is 11-20% in the SGA population [159, 161], compared to overall rates of 6-10% worldwide [155, 159, 161], and the incidence of SGA is 25% in very preterm children [159], compared to rates of 15% overall [162]. Because IUGR and preterm birth are each independently associated with adverse morphological, cognitive and motor outcomes [115-117, 133, 163], it can be difficult to separate the consequences of each. Secondly, human studies are confounded by environmental factors that are correlated with prenatal growth, postnatal growth and neurodevelopment. For example, lower family socioeconomic status and poorer maternal education are each associated with increased risk of IUGR or SGA pregnancy [159, 164-166], poorer postnatal growth in AGA and SGA children

[107], and poorer cognitive and academic outcomes in healthy children [164, 167, 168]. Postnatal neurodevelopmental outcomes such as IQ correlate positively with incidence and rate of catch up growth of head circumference [93, 149-152], a proxy measure of brain size that corresponds well to frontal lobe volume [102]. Catch up growth of head circumference occurs during the first 6-12 postnatal months [103], during a period of rapid postnatal brain development [19, 104], but is frequently incomplete, such that IUGR children fail to catch up to non-IUGR individuals [92]. In addition, preterm IUGR and very low birth weight children are at increased risk of failure of catch-up growth of head circumference [103, 117, 121, 169]. There is therefore confounding due to the effects of both postnatal environment and gestational age on postnatal growth, which adds to the difficulty in defining effects of prenatal exposures on neurodevelopment in human cohorts.

Animal models are therefore necessary to control for, or minimise, these confounding factors, and also allow direct testing of causality and greater investigation of underlying mechanisms. To enable translation of the findings from these preclinical models to defining mechanisms that may apply in humans, and to evaluate and identify effective interventions to improve long-term outcomes, it is important to consider the timing of neurodevelopment relative to both birth and the gestational age at onset of the restricted intrauterine growth. This review compares the different animal models used to study effects of prenatal growth restriction on neurodevelopment, describes the neurodevelopmental and cognitive outcomes of these, and the gaps in knowledge and suggests future directions for research in this field.

1.7.3. Timing of neurodevelopment in animal models of experimental IUGR

Rats, guinea pigs, rabbits and sheep are the non-human species most commonly used to examine the effects of IUGR on neurodevelopmental outcomes. However, the timing of neurodevelopmental events and gestation lengths vary between these species, and from those in humans (Fig. 1-4). These inherent differences make comparisons between models difficult, and extrapolating findings from one species to another largely invalid. For example, rats are one of the most frequently utilised model species, but many neurodevelopmental events that occur during gestation in humans occur postnatally in this species (Fig. 1-4) [18]. Brain growth rate accelerates in the last trimester in humans, peaking around birth, but occurs comparatively later in the rat, peaking around postnatal days 7-8 [18]. Similarly, fetal neurogenesis and white matter development begin later in gestation in rats than humans [15]. Central myelination occurs entirely postnatally in the rat [18], but begins in the human brain-stem at 29 weeks gestation (Fig. 1-4) [20]. As in humans, central myelination commences in late gestation in rabbits and guinea pigs and is sensitive to hypoxic damage *in utero* (Fig. 1-4) [170-172]. However, myelination in peripheral as well as central and higher brain regions commences before birth in the sheep. Myelination of the majority of higher brain regions in humans commences postnatally, so sheep neurodevelopment is comparatively more advanced at birth than it is in humans (Fig. 1-5) [18, 173]. Neurodevelopment in pigs shares some similarities to human, including occurrence of prenatal neurogenesis and both peri- and postnatal myelination, although humans have more advanced development relative to percentage of gestation [reviewed in 174]. Some cognitive and neurodevelopmental consequences have been studied in pigs with spontaneous, naturally occurring growth restriction either due to large litter size or variable growth within a litter. These share similarities with outcomes reported in human IUGR, including brain sparing at birth [175], morphological changes including decreased grey matter [176], and altered cognition [176-178]. In depth discussion of this model is omitted from this paper however, as changes in the fetal environment has not yet been well characterised.

Fig. 1-4. Timing of neurodevelopment in humans and in species utilised in animal models of IUGR.

N = onset of neurogenesis, green panel = onset of myelination, hollow arrow indicates onset of puberty. Data on onset of neurogenesis and onset of myelination were taken directly from the literature for rats and sheep [15, 18, 173].Timing of neurogenesis and myelination of the guinea pig and rabbit was extrapolated using the most recent models predicting developmental timing across species from available information from mapped developmental events and based on data on white matter development after the apparent onset of myelination in these species [170, 171, 179-181]. Data on onset of puberty were taken from data using species-appropriate measures in human [182], rat [183], guinea pig [184, 185], rabbit [186, 187] and sheep [188, 189]. Diagram does not show maturation of myelination, which continues into adolescence in the majority of species for which data is available [e.g. rats and humans, 18].
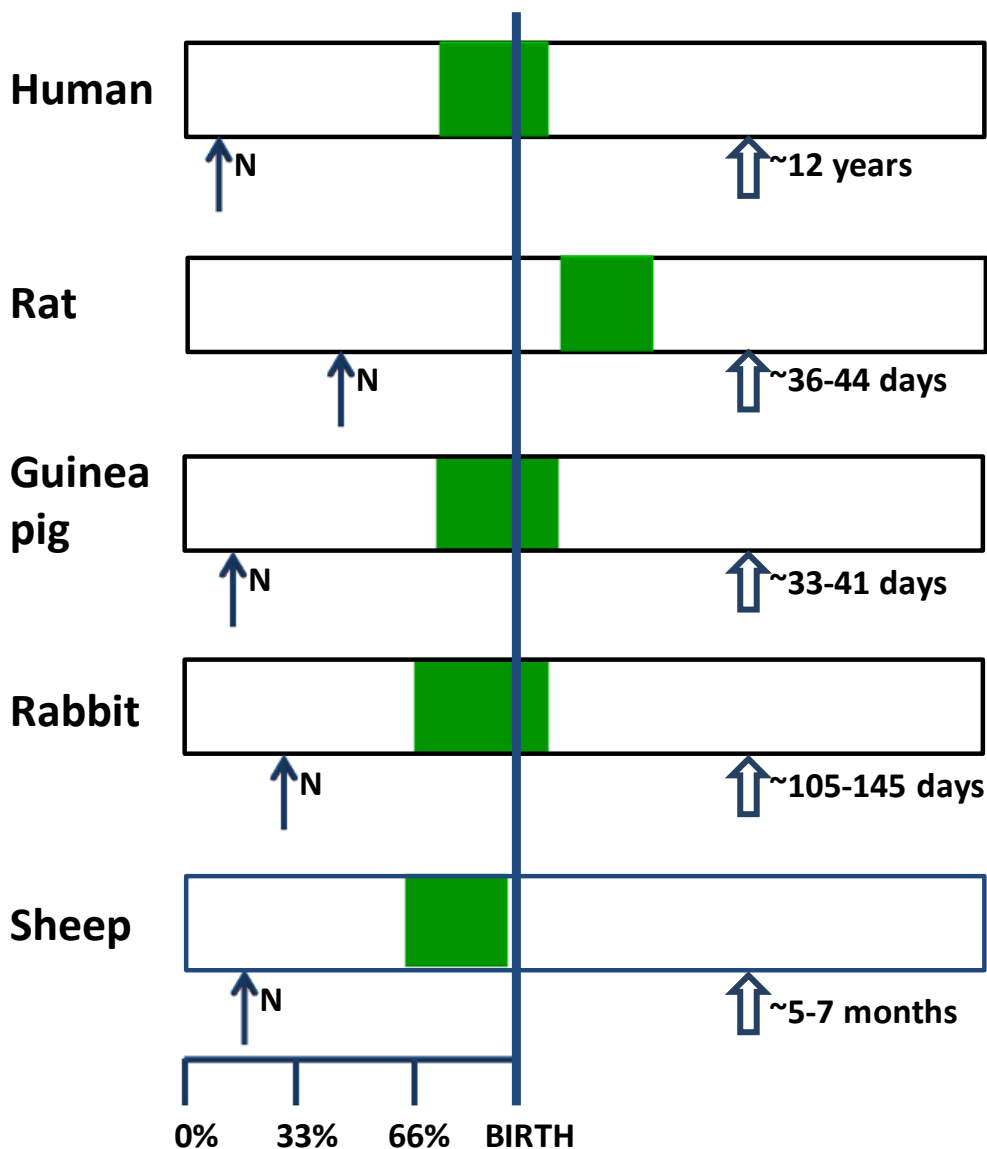
1.7.4. Methods and timing of experimental IUGR in animal models

A variety of paradigms of experimental IUGR have been utilised in studies of neurodevelopmental and cognitive outcomes. Experimental IUGR is generally induced by restricting fetal nutrient availability via global or nutrient-specific undernutrition of the mother, or by surgical or pharmaceutical induction of placental insufficiency to restrict placental capacity to transfer nutrients from mother to fetus (Fig. 1-5). Fetal and neonatal body and brain weights are reduced in the majority of these preclinical models, as is seen in human IUGR (Table 1-1, 1-2), although each model affects neurodevelopment, and in turn cognitive outcomes to varying degrees. While there are additional animal models of perturbed prenatal development in which neurodevelopment and/or cognitive outcomes have been investigated, for example those investigating effects of periconceptional and early gestational undernutrition in the sheep [190-192], these models do not restrict fetal growth in late gestation or reduce size at birth as occur in human IUGR and are therefore not discussed further in this review. Similarly, this review is limited to those models of IUGR in which neurodevelopmental and/or cognitive outcomes have been reported. This section describes key features of these models, including effects on fetal nutrient supply and metabolism, and development and timing relative to neurodevelopment. Specific neurodevelopmental and cognitive outcomes induced by IUGR in each model are described in following sections.

Fig. 1-5. Timing of placental restriction (PR) in human IUGR and animal models of IUGR.

UPL = uteroplacental vessel ligation, THROM = thromboxane A_2 analogue (STA_2) administration, UPE = uteroplacental vessel bed embolisation, CX = carunclectomy, N = onset of neurogenesis, hollow arrow = onset of puberty, green bar = period of majority of myelination, solid red bar = period of acute restriction, with multiple bars indicating different periods of restriction used in the same IUGR model, red gradient = chronic restriction with gradually increasing strength, purple box = period of catch up growth in species in which it has been reported (no data are available for rabbit or guinea pig following UPL). Periods of restriction depicted in this figure were chosen as most representative of the timing described in the literature: rat UPL [193, 194], guinea pig UPL [179, 195, 196], rabbit UPL [197-199], sheep UPE [200-202] and sheep CX [203-205]. Maternal global feed or protein restriction have been applied for multiple periods in rats, encompassing whole or part of gestation and may end at delivery or continue throughout lactation [206-215] – due to the variety of timing used in these studies they are not shown above.
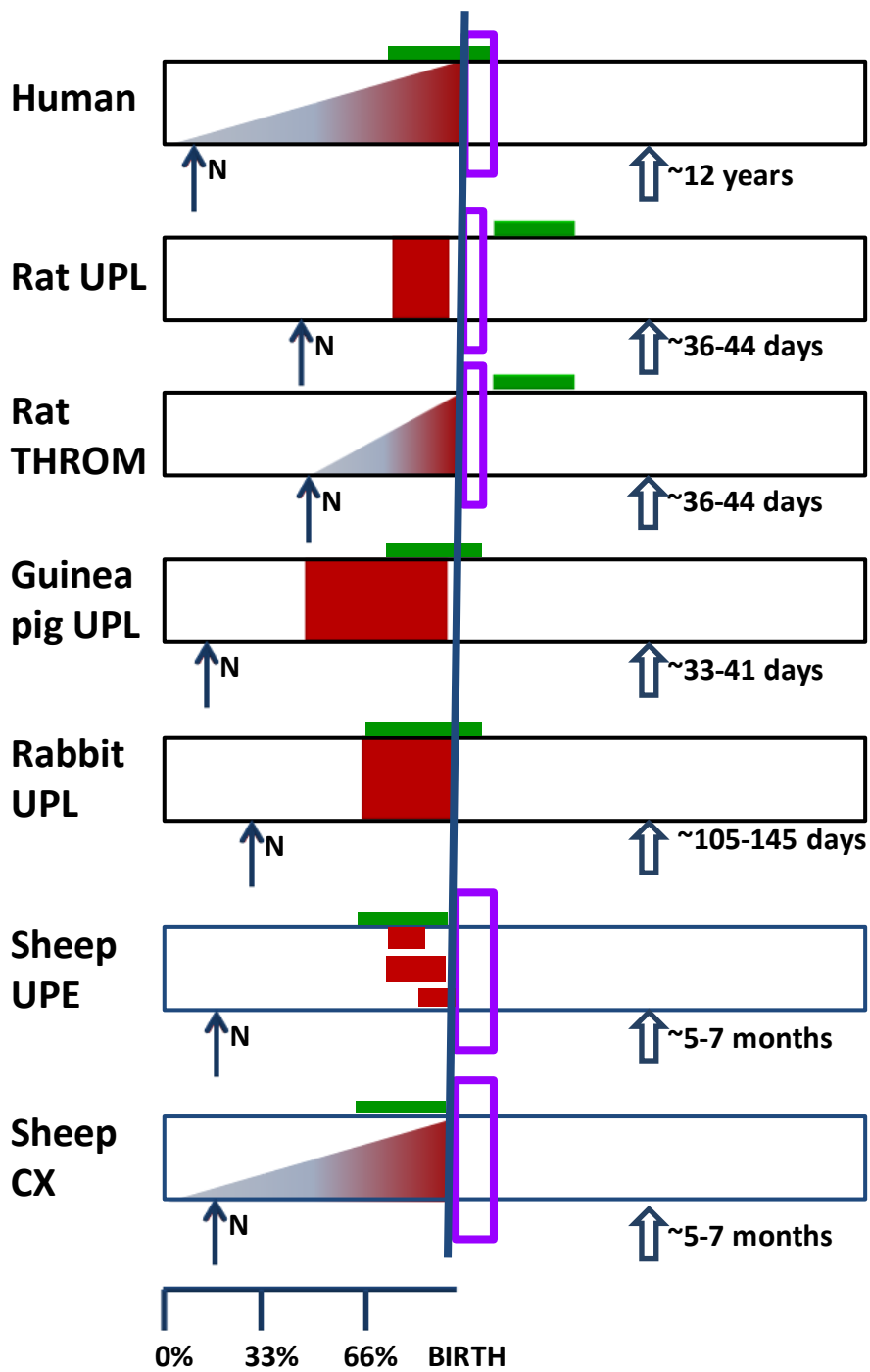
Fig. 1-5 – Timing of placental restriction (PR) in human IUGR and animal models of IUGR.

Table 1-1. Fetal growth outcomes in animal models of IUGR.

↓ decreased compared to healthy controls, ↑ increased compared to healthy controls, = unchanged/not different to controls, + present in this model. Days of pregnancy are designated by embryonic day, eg. E10.

	Rat maternal feed restriction	Rat maternal protein restriction	Sheep maternal feed restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation	Sheep uteroplacental embolisation	Sheep carunclectomy
Fetal weight	↓13% [216]	↓5-35% [213, 217, 218] ↑7-25% [219]	= [207, 209] ↓11% [209]	↓[220]	↓8-31% [193 , 221-223] = E19, ↓E21 [224]	↓22-63% [179, 196, 225-233]	↓20-36% [234-236]	↓20-42% [200, 237, 238]	↓15-43% [3, 204, 239, 240]
Placental weight		↓9.5-35% [217, 218] = [213] ↑↓ during pregnancy [219]	= [241] ↓size as pregnancy progresses [209]		↓20% [221]	↓21-40% [225, 226, 228-230, 232]	= [234, 235] ↓44% [242]	↓35% [200]	↓36-64% [3, 203, 204, 239, 240]
Fetal brain size		= [213] ↑12% [219]	↓E90, = E135 [209]	= E16, E18 [220] ↓E20 [220]	= E19, E22 [222, 224]	↓10-20% [227-231, 233] = [226, 232]	↓10-22% [234-236] = E25 [234]	= [201, 237, 238] ↓8.5% [200]	↓14-17% [3]
Brain sparing		+ [213] - [219]	= [207, 209]		+ [224]	+ [179, 226-229, 231-233]	+ [234, 235]	+ [200, 201, 237, 243]	+ [3]
Hypoxia			= d113-116 [207]			+ peripheral blood, severity varies in brain [226]	+ [234]	+ [200, 202, 237, 238, 243] + transient [201]	+ [3, 239, 244]
Fetal glucose		↑E14, = E21 [218]	↓[209], = [207]		↓E22 [222]	↓E49-51 [225]		↓[200, 237, 238]	↓[204, 244]
Fetal insulin		↑E14, = E21 [218]	= E90, ↓E135 [209]		= E22 [222]	↓E49-51 [225]			↓[204]
Fetal amino acids		↑↓[217]	= protein [209]						
Gestation length	= [206, 214]		= [241]					=/↓ [245] ↓3-16 [202, 243]	= [246-248] ↓2.2 days [249]

Table 1-2. Neonatal and long-term growth outcomes in animal models of IUGR.

↓ decreased compared to healthy controls, ↑ increased compared to healthy controls, = unchanged/not different to controls, + present in this model.

	Rat maternal feed restriction	Rat maternal protein restriction	Sheep maternal feed restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation	Sheep uteroplacental embolization	Sheep carunclectomy
NEONATE									
Birth weight	= [250] ↓4-23% [206, 214, 215, 251-254]	= [219, 255] ↓7-52% [256-263]	↓9.5-14% [210, 241] = [211]	↓[220, 264-266]	↓8-40% [193, 194, 222, 224, 260, 267-269]	↓36-42% [231, 270]	↓18-44% [197-199, 235, 242, 271]	↓42-48% [202, 243, 245]	↓17-28% [203, 246-249, 272-275]
Brain size	= [250] ↓cerebrum, 11% [206]	↓11-66% [260, 263, 276] = [219, 255]		↓[265]	= [222, 224] ↓33% [260] ↓forebrain [267]	↓14% [231] ↓forebrain [270]	↓10-34% [197, 199, 235, 242]		↓5% skull width [248, 272-274]
Brain sparing				+ [265]	- [224]	+ [231]	+ [197, 235] = [199]	+ [245]	+ [272]
Catch up growth	+ [212, 252-254] - [216, 251]	- [261, 276] + [256, 262]	= [241]	- [265] + [266]	- [193, 267] + [194, 222, 224, 269] =/- [268]			+ [245] - [243]	+ [246-248, 272-275, 277]
ADULT									
Adult body weight	= [206, 253] ↓8% [212, 251, 252]	= [255, 256, 258] ↓4-53% [256, 257, 260, 261]	= [210, 211]	= [266]	= [194, 224, 269] ↓14-33% [193, 260] = / ↓ [268]	↓15% [231]	= [198, 271]		= [246, 248] =/↓sex specific [277]
Adult brain size	= forebrain [206, 212] ↓4% [251]	= [255]				↓12% [231]	= [271]		= skull width [248]

1.7.4.1. Maternal undernutrition

Models of IUGR based on maternal undernutrition (UN) differ from human IUGR associated with poor placentation, in that restriction is largely of nutrients without substantial restriction of oxygen. There is also considerable variability in the length, degree and timing of nutritional restriction between studies [206-215], with some studies restricting throughout gestation or the entire length of pregnancy studied [206, 209, 213, 241], whilst others may only restrict during part of gestation [207, 210, 211, 215], or extend maternal nutrient restriction into lactation [212, 214]. The patterns of restriction in these models also differ from that in human IUGR due to placental insufficiency, which progressively worsens during pregnancy (Fig 1-5) [83]. Differing types of nutrient restriction have been utilised, particularly in rats, with some restricting dietary protein, while others impose global nutrient restriction [206, 213, 215, 259, 262, 276]. Variation between studies in the severity and nature of the nutrient restriction accounts in part for variable reductions in birth weight (Table 1-2). These range from 4 to 34% in progeny of globally nutrient-restricted rats [206, 214, 215, 251-254], whilst more severe restriction is seen in models of maternal protein restriction, with 7 to 52% reduction in birth weight in progeny [256, 257, 259-263]. The reported decrease in birth weight following the levels of maternal nutrient restriction used in neurodevelopmental studies in sheep and rabbits is milder ranging from 9.5% to 17.5% [210, 235, 241, 278]. Effects on fetal nutrient supply and metabolism also differ between the various models of IUGR (Table 1-1). For example, fetal blood glucose does not appear to be reduced by maternal protein restriction in rats [218, 279], but is reduced in other models of IUGR, such as utero-placental ligation in the guinea pig and in both utero-placental embolisation and carunclectomy-induced placental restriction in sheep [3, 200, 204, 225, 237, 238, 280].

One particular limitation of models of maternal nutrient restriction in rodents is that the restriction is imposed only during earlier stages of neurodevelopment than are affected by IUGR in humans. For example, if maternal undernutrition is imposed in rats only during gestation, this does not

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impact the period of myelination, which occurs postnatally in rats, but commences prior to birth in humans (Fig. 1-4) [20]. This can be addressed by continuing maternal undernutrition postnatally throughout lactation in the rat, but many studies do not do this.

1.7.4.2. Placental restriction induced during mid to late pregnancy

IUGR can be induced by restricted placental growth and/or function (PR). In small animals this is induced by restriction of uteroplacental blood flow during late pregnancy, which in the rat involves uterine artery ligation (ie. uteroplacental vessel ligation, UPL), usually at day 17 of the 21-22 day pregnancy [193, 194, 269, 281]. In the rabbit, the period of restriction similarly comprises a relatively short proportion of gestation, with 40-50% of uteroplacental vessels ligated at day 25 of the 31 day rabbit pregnancy, and pups surgically delivered five days later (Fig. 1-5) [197-199, 235]. Placental insufficiency is induced at an earlier stage of gestation in the guinea pig, with the uterine artery of one horn ligated at mid-gestation (at day 30-35 days of the 68 day pregnancy, Fig. 1-5) [179, 195, 196, 225]. IUGR can be induced during pregnancy in sheep by uteroplacental embolisation (UPE), where occlusion of the uteroplacental blood vessels is induced via repeated infusion of microspheres into the placental vascular bed, titrated to maintain a defined level of hypoxia [201, 237, 243, 280]. In the majority of studies, reduced placental blood flow is not maintained until term (Fig. 1-5), with the duration of embolisation ranging from 6-30 days, and generally commencing on day 110-120 of gestation [200-202, 237, 243, 245 , 280].

All of these experimental models reduce fetal and neonatal growth, placental growth and fetal substrate supply (Table 1-1, 1-2), and induce clear signs of neurodevelopmental disruption in progeny (Tables 1-3 and 1-4) that persists into adulthood in small animal models (Table 1-5). To date, there are no reports of outcomes in adulthood in large animal models, such as the UPE sheep. Compounding this, the varying timing of restriction induced by UPL or UPE, and species-specific

differences in temporal aspects of neurodevelopment, results in perturbations at different stages of neurodevelopment in each model (Fig. 1-5). For example, IUGR induced by UPL in late gestation in rats occurs at a neurodevelopmental stage similar to mid-gestation in the human [18]. In contrast, late pregnancy placental restriction in the UPL guinea pig and rabbit, and UPE sheep, affects neurodevelopmental at stages similar to those occurring during late gestation in the human, including neurogenesis and white matter development (Fig. 1-5) [170, 172, 173, 282].

One major drawback to all these models of IUGR induced in mid to late pregnancy is the need for surgical intervention during pregnancy, which may have additional consequences for fetal development. Even sham surgeries are associated with reduced fetal weight compared to controls in rats [283], due to mechanisms potentially including maternal stress. The UPL and UPE models are also predominantly models of late-pregnancy restriction, imposed acutely on previously unrestricted pregnancies. Pharmaceutical interventions may provide another, less acute avenue to introduce placental restriction, although this has only been examined in rats to date. Placental restriction induced by intraperitoneal infusion of synthetic thromboxane A_2 (STA_2) analogues in the rat constricts placental blood vessels, which reduces birth and brain weight (Table 1-2). This in turn alters neurodevelopment in the fetus and neonate (Table 1-3, 1-4), and impairs neuromotor, cognitive and behavioural development at least to adolescence (Table 1-6). Pumps to infuse STA_2 are implanted at day 13 of gestation, thus the period of placental restriction is longer than uterine artery ligation models in rats, and with shorter and less invasive surgery, which reduces maternal compromise [220, 265, 266, 284]. Further experimentation is needed to delineate the adult outcomes and underlying neurodevelopmental changes in this model of IUGR.

Table 1-3. Fetal neurodevelopmental outcomes in animal models of IUGR.

Gestational age is shown as embryonic day, eg E20 for day 20 of gestation. CA1, CA2, CA3, CA4 = cornu ammonis fields 1-4 respectively, DG = dentate gyrus, ↓ decreased compared to healthy controls, ↑ increased compared to healthy controls, = unchanged/not different to controls, + present in this model.

Outcomes	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Sheep uteroplacental embolisation
VOLUME				
Total	↓26.9% E20 [220]		↓9% [227]	↓9.5% [200]
Cerebrum	↓44.5% E20 [220]		↓13.5% [227], = [231]	= [200]
Hippocampus			↓26% [227]	
Cerebellum			= [231]	= [200]
Striatum			↓13% [227]	
Ventriculomegaly			+ [227]	
NEURONAL DENSITY				
Cortex		↓parietal cortex [224]	↓ [172, 285]	
Hippocampus			↓dentate gyrus [172]	= [238]
Cerebellum				↓ Purkinje neurons and molecular layer width [237]
HIPPOCAMPAL DEVELOPMENT				
Synaptogenesis			↓CA1, CA3, DG [196]	
Synaptic maturation			↓CA1, DG [196]	
Dendrite length			↓ apical and basal arbor, CA1, DG [228]	
Dendrite number			= apical, ↓basal intersections, CA1 [228]	
Dendritic branches			= basal, ↓ apical, CA1 [228]	
Dendritic spines			↑ CA1, DG [228]	
Region measurements			↓stratum oriens, mossy fibre layer [229]	= [237, 238]

Outcomes	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Sheep uteroplacental embolisation
WHITE MATTER				
Volume			↓ cerebrum, E60 [231] ↓cerebellum E60 [229, 231]	
Myelination			↓cerebrum, cerebellum, CA1 hippocampus, dorsal fornix, dorsal fimbria, corpus callosum, periventricular white matter, parasagittal white matter [179, 196, 230, 232] =/↓ spine, age dependent [179] = subcortical white matter, d65 [230] Delayed maturation of myelin [231]	↓cerebral cortex, striatum [237] Thinner sheaths, signs of degeneration [237]
Damage				+ lesions in cerebrum [237] + lesions, gliosis, axonal degeneration [200]
Oligodendrocytes			↑numbers in cerebellum [231]	
ASTROGLIOSIS				
Cerebrum			= E52 [179] ↑E60, E62 [179, 231] = E65 [230]	↑cortex [200, 237]
Striatum				↑ [237]
Cerebellum			= [179] ↑E60 [231]	
Hippocampus			= E65 [230]	

Table 1-4. Neonatal and pre-weaning neurodevelopmental outcomes in animal models of IUGR.

↑ increased compared to healthy controls, = unchanged/not different to controls, + present in this model. VMH = ventromedial hypothalamic nucleus, PVH = paraventricular hypothalamic nucleus, CC = corpus callosum, CA1, CA2, CA3, CA4 = cornu ammonis fields 1-4 respectively, DG = dentate gyrus. Age indicated in days from birth where appropriate, eg. d10 for day 10 postnatal age.

Outcomes	Rat maternal feed restriction	Rat maternal protein restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation	Sheep uteroplacental embolisation
VOLUME							
Brain	↓11% [206]	↓11% [276]	↓17.3% [220, 265]			↓10-18% [197, 242]	= [202]
Forebrain	↓10-15% [206, 212]		↓ [265]		↓13-16% [231, 270]	↓19% [242]	= [202]
Cortex			↓31% [220]			↓20% [267]	
Striatum						↓12% [242]	
Hippocampus	= [212]	= [259]		↓ CA1, males, d0 [286] = CA2, CA3, d0 [286]		↓22.5% [242]	
Cerebellum			↓ [265]		↓23% [231, 270]		= at birth [202] ↓22%, 8 weeks [202]
Hypothalamus		↓18% [259]					
Dentate gyrus				↓ females, d0 [286]			
Corpus callosum				↓ [269]			
NEURONAL COUNT							
Cortex	↓ [206]		↓ density, d0 = density, d7 [265]			= [199]	= density, 8 weeks [202]
VMH and PVH		↑ density [276]					

Outcomes	Rat maternal feed restriction	Rat maternal protein restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation	Sheep uteroplacental embolisation
Dentate gyrus	= [250]			↓females, d0 [286]			
Hippocampus	= CA1, CA3 [250] ↓CA2, CA4 [250]			↓ CA1, CA3, males d0 [286]	↓19% CA1 [270]		
Cerebellum					↓17% molecular layer, ↓22.5% granule layer [270]		= density, delayed migration, 8 weeks old [202]
Cell proliferation	↑↓hippocampus, hypothalamus, age and region specific [216]			=/↑ cingulate white matter, dependent on severity of restriction [268]			
WHITE MATTER							
Volume					↓cortex, cerebellum, hippocampal CA1 and stratum oriens [231, 270]		↓hippocampal stratums oriens width [202]
Structural damage and lesions				+ [193] ↑ axonal degeneration [269]			+ cerebrum, cerebellum [202]
Apoptosis				↑d0, d3 [193, 268, 287]			
MYELINATION							
Brain						↓ [197]	
Cerebrum					= [231]		= [202]
Corpus callosum				↓d7 [193, 268] ↓d14 [288]			

Outcomes	Rat maternal feed restriction	Rat maternal protein restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation	Sheep uteroplacental embolisation
Pre-oligodendrocytes				↓cingulum and CC d7 [193, 268]			
Oligodendrocytes				↓ CC d14 [268, 288]			
				↑↓ cingulum, p7, dependent on severity [268]			
				↑↓ CA1, sex specific [286]			
				= immature oligodendrocytes, CA3, DG, d0 [286]			
ASTROGLIOSIS							
Cerebrum							+ parietal, frontal and temporal lobes [202]
Hypothalamus		↓ [276]					
Hippocampus				↑CA3, males, d0 [286]			
Dentate gyrus				↑males, d0 [286]			
Corpus callosum				↑d21 [288]			
Cingulum				↑d7, d13, d14, d21, adults [193, 268, 269]			
Internal capsule				↑d7, d14 [193]			
External capsule				= [193]			

Table 1-5. Adolescent and adult neurodevelopmental outcomes in animal models of IUGR.

Gestational age is shown in days of gestation, eg d20 for day 20 of gestation. CA1, CA2, CA3, CA4 = cornu ammonis fields 1-4 respectively, DG = dentate gyrus, ↓ decreased compared to healthy controls, ↑ increased compared to healthy controls, = unchanged/no different to controls, + present in this model.

Outcomes	Rat maternal protein restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation
VOLUME					
Brain	↓ [251]			↓[231]	= [271]
Cerebrum	= [206, 212] ↓ [251]				
Midbrain	↓ [251]				
Hippocampus	= [212, 251]				
Cerebellum	= [206, 212] , ↓ [251]			↓[231]	
Corpus callosum			↓ [194]	↓width [231]	
NEURONAL DENSITY					
Cerebrum	= [206]				↓ insular, temporal and occipital cortex, indirect evidence [198]
Hippocampus	= [212]	↑ neuronal proliferation, adolescent females [266]	= [194, 281] ↑ degenerating neurons, CA3 [281]		↓ indirect evidence [198]
Dentate gyrus			= [101]		
Cerebellum	= [206]				↓ indirect evidence via MRI [198]
Fornix			↑ degenerating neurons [281]		
Entorhinal cortex			↓ [194, 281] ↑ degenerating neurons [281]		
Cingulate cortex			= [281]		
External capsule			↑ degenerating neurons [281]		

Outcomes	Rat maternal protein restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Guinea pig uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation
Prefrontal cortex			= [194]		↓ indirect evidence [198]
GABAergic interneurons			↑ prefrontal cortex [194]		
WHITE MATTER					
Axonal density					↓ left hemispheric anxiety and memory pathways [198]
Axonal degeneration			+ cingulate and somatosensory cortices, internal capsule, pontocerebellar tract [194]		
Microstructural reorganisation					+ [271]
MYELINATION					
Cerebrum				= [231]	↓ [198]
Corpus callosum			= [288], ↓d60 [40]		
Cingulum			↓d60 [193]		
Internal and external capsule			= d60 [193]		
ASTROGLIOSIS					
Hippocampus			↑CA1 [194, 281]		
Dentate gyrus			↑ [194, 281]		
Entorhinal cortex			↑ [194, 281]		
Cingulum			↑ [194, 281], = [94]		
Fornix			↑ [281]		
Motor cortex			= [194]		
Somatosensory cortex			↑ [194]		

Table 1-6. Neurobehavioural and cognitive outcomes in animal models of IUGR.

Postnatal age is shown days where appropriate, eg. d10 for 10 postnatal days of age. ↓ decreased compared to healthy controls, ↑ increased compared to healthy controls, = unchanged/not different to controls, + present in this model.

Outcome	Rat maternal feed restriction	Rat maternal protein restriction	Sheep maternal feed restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation	Sheep uteroplacental embolisation	Sheep carunclectomy
Neonatal neuro-behaviour	= reflexes [206] ↓righting reflex, d3-4 males, d3 females [214] ↓cliff avoidance, d7 females, d8, both sexes [214] ↓negative geotaxis, d7-8 males [214]	= reflexes d10-21 [289]		↓surface righting, d2-9 ↓ negative geotaxis d4-15 [265]		↓righting reflexes, locomotion, head turning and smell test scores as d1 neonates [197]		
Neuromotor		↓grip strength, adult males [289]		↓motor learning, males [265]	↓motor learning, adults [288]			
Spatial learning	= adult males [253]	= adults [256, 289]			= adult males [290]		↓initial simple maze tests (lambs) [243] = extended simple maze testing, obstacle course tasks, t-maze tasks (lambs) [243]	↓initial simple maze tests (male lambs and young adults) [291]
Reversal learning	↓male pups [206]	↓adult males, with ↑perseverative errors [256]	↓in maze tasks, adult males [211] = maze tasks, adult females [211]					↑lambs, young adults [291]

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Outcome	Rat maternal feed restriction	Rat maternal protein restriction	Sheep maternal feed restriction	Rat maternal thromboxane	Rat uteroplacental vessel ligation	Rabbit uteroplacental vessel ligation	Sheep uteroplacental embolisation	Sheep carunclectomy
Fear and avoidance learning	↑male pups [206] = adult males [253]			↓ [265]				
MEMORY								
Recognition					↓adults [194, 281, 292]	↓adults [198]		
Spatial	= adult males [253]	= adult males [256]		↓adolescent females [266]	↓adult males [194, 290]			= lambs and adults [291]
Short term		= adult males [256]			= adult males [290]			
BEHAVIOUR								
Behavioural anxiety	= male pups [206] =/↑adult males [251, 252]	↓adults [257, 258]	↑reactivity to physical restraint and surprise, adults [211]	↑adolescent females = adolescent males [266]		↑adults [198]		↑low birth weight female lambs [291]
Spontaneous ambulation	= adult males [252, 253]	↑females [289]	↑in isolation tasks, adults [211]		↑adults [194, 269, 281] ↓adult males [224]			
Hyperactivity		↑adult females [289]			↑adults [194, 269, 281]			
Exploratory behaviour		↑adult females [258]			↑adults [194, 269, 281]	↓adults [198, 271]		
Response to novelty			↓novelty seeking, adults [211]		= adults [281]			

1.7.4.3. Placental restriction throughout pregnancy

The carunclectomy model of placental restriction (CX) in sheep is induced by removal of the majority of uterine caruncles (placental attachment sites) prior to pregnancy, which reduces placental size, in spite of compensatory hypertrophic growth of remaining placentomes [203]. Reduced placental size in turn impairs placental blood flow, and the efficiency and delivery of nutrients to the fetus (Table 1-1). Neonates from CX pregnancies are smaller than controls at birth with reductions of 20-30% in birth weight [205, 249], and smaller decreases (5%) in skull width, indicative of brain sparing [205, 247, 272, 273]. The advantages of this model are that, similar to human IUGR, the fetuses are hypoxic, and restriction is chronic and increases throughout the course of pregnancy (Fig. 1-5) [3]. Moreover, no surgical intervention is required during pregnancy. Additionally, CX sheep offspring have similar postnatal endocrine and growth outcomes to the IUGR human, including insulin resistance [205, 274], increased visceral adiposity [247], and neonatal catch-up growth [246-248, 272-275].

1.7.5. Neurodevelopmental and cognitive consequences of experimental IUGR

1.7.5.1. Fetal neurodevelopment

Fetal neurodevelopment has been examined more frequently in the UPL guinea pig and UPE sheep models than rat models of IUGR, but not at all in the UPL rabbit or CX sheep. In both the UPL guinea pig and UPE sheep there are morphological signs of disrupted development, increased apoptosis and decreased expression of neurotropins, such as brain-derived neurotrophic factor (Table 1-2) [195, 200, 229, 230, 237]. In the late gestation guinea pig fetus, UPL decreases overall and neuronal volume of the whole brain, cerebrum and hippocampus (Table 1-3), consistent with the human IUGR fetus [96, 108-110, 112]. The impaired development of the hippocampus, myelination and white matter development in the UPL guinea pig have been investigated in detail, with both delays and decreases in myelination reported (Table 1-3). Region-specific changes in
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concentration and metabolism of neurotransmitters and catecholamines in the brain also occur in the UPL guinea pig. UPL elevates serotonin concentration in the frontal and temporal cortex, increases noradrenaline in the caudate nucleus, and alters dopamine and noradrenaline metabolism in a number of regions [226]. Similar patterns of volume loss and neurodevelopmental damage, including decreases in cortical myelination, and decreases in mitotic division and increased post-mitotic cell death in the cerebellum, but not hippocampus, have been reported for the UPE sheep (Tables 1-3 and 1-4). Specific attention has been paid to examining damage in the hippocampus, and to a lesser extent cerebellum in the UPE sheep. Similar damage is seen in both regions in UPE sheep, including white matter lesions, gliosis, loss of neurons, and decreased gross volume [200, 202, 270]. These models thus demonstrate causal effects of restricted placental function on fetal neurodevelopment by specifically manipulating this variable without genetic or environmental confounders associated with IUGR in human cohorts.

1.7.5.2. Neonatal neurodevelopment and cognitive outcomes

The majority of rat and rabbit studies have examined outcomes in neonates, whereas neonatal outcomes have not been examined in any great detail in the guinea pig, or at all in sheep models of IUGR. In all rat IUGR models, and in the UPL rabbit, neonatal brain volume is decreased overall and within specific brain regions (Table 1-4). In addition to loss of volume, neuron number is also further impacted by decreased neuronal density in a number of brain regions, at least in progeny of rat pregnancies subject to maternal undernutrition or UPL (Table 1-4).Studies in the STA_2 rat suggest this may be due to delayed neuronal migration [264], which may be due to the decreased expression of neural cell adhesion molecule and brain derived neurotrophic factor, which guide neuronal differentiation and migration, observed in these animals [293]. Studies in the UPL rat have continued into early postnatal life to examine the onset of myelination. In early postnatal life, structural damage, decreased myelin volume, and region specific changes to numbers of pre-

oligodendrocytes and oligodendrocytes, are evident in the UPL rat, indicating discordant brain development (Table 1-4). The UPL rat also has a loss of white matter volume in the corpus callosum at birth and during the first two weeks of postnatal life, as is the case in human IUGR neonates [117], whilst in the UPL rabbit there is decreased white matter volume in the hippocampus at birth (Table 1-4). While cognitive studies are not possible at this young age, neonatal neurobehaviour, including reflex development, is impaired in IUGR rats induced by either maternal global UN or STA$_2$ rat, and UPL rabbit models of IUGR (Table 1-6), consistent with observations in human IUGR neonates and toddlers [92, 100, 128, 129].

1.7.5.3. Adolescent and adult neurodevelopment and cognitive outcomes

Outcomes in the adolescent or adult have not been examined in the majority of experimental models of IUGR. Importantly, and consistent with persistent functional consequences of IUGR, SGA and low birth weight in humans [139-141, 143], existing studies do suggest long-term structural damage following experimental IUGR. These include damage which occurs during exposure to restriction and persist from fetal life, such as decreased neuronal density [198], which can be contributed by grey matter loss *in utero* resulting in decreased neuron numbers in later life. This also includes further changes that develop after birth, including decreased myelination [193, 198, 285]. Studies in adolescent and adult animals (Table 1-5) also provide evidence of causation for long-term effects of a restricted environment *in utero*, by providing a common postnatal environment including diet and environmental stimuli in which all progeny are assessed. The adult UPL rat and UPL rabbit both have decreased neuronal density and myelination in multiple brain regions (Table 1-5). Maternal global or protein feed restriction in rats induces limited changes in brain volume in the adult (Table 1-6), in contrast to the volume losses and decreased levels of myelination seen in adolescent and young adult humans affected by IUGR and SGA [121, 122]. It is not clear whether these comparatively limited effects of maternal undernutrition on brain structure are a consequence of

relatively mild restriction in this model, or are a characteristic of this species, since volumes of specific brain regions have not been reported for other experimental rat models of IUGR. There are also few gross structural consequences of IUGR in the adult CX sheep, in which grey and white matter areas remain unchanged in the prefrontal cortex (Chapter 4). The addition of structural studies in other experimental models of IUGR and detailed histological studies to assess more subtle changes will assist in comparisons of lasting neurodevelopmental consequences between these experimental models of IUGR and with human IUGR.

The majority of studies examining postnatal cognition have been conducted using rat models of IUGR. Maternal global or protein feed restriction in rats impairs reversal learning (a measure of executive function, in which rules or discriminations to solve a task are initially learned and then reversed), in pups and adult progeny, but in the majority of models there are no signs of spatial learning or memory impairments (Table 1-6). The opposite is true in the sheep (Table 1-6), in which initial learning but not memory is impaired during simple maze tasks in UPE lambs [sexes combined, 243], and during diamond maze tasks in male CX lambs and young adult sheep [291], but reversal learning is not impaired.

1.7.6. Gaps in knowledge and future directions

Taken in combination there are clear gaps in knowledge when comparing outcomes between animal models, and to human IUGR. Firstly, the different ages studied make it difficult to make comparisons between species, in part due to the differing neurodevelopmental trajectories (Fig. 1-4). Models and studies differ in the timing of exposure to restriction, whilst the variable timing at which outcomes are evaluated determine what outcomes it is possible to observe. For example, in the majority of rat studies, brains are studied at postnatal day 0 and 1. Thus examination of white matter development is impossible, as central myelination has not yet commenced at this age in the

rat [18]. Earlier timing of neurodevelopment in other species, such as the guinea pig (Table 1-3) and rabbit (Table 1-4), mean that these species are useful in determining effects of experimental IUGR on fetal and neonatal neurodevelopment and reflexes. Sheep undergo neurodevelopment even earlier and may prove particularly useful for fetal studies in experimental IUGR. The lamb has previously been used to investigate white matter injury following asphyxia and preterm birth [294-298], and effects of perinatal exposure to corticosteroids [299-302] due to the onset of myelination in late pregnancy. There is therefore a considerable body of literature in this species examining possible mechanisms by which IUGR may influence outcomes, such as via hypoxia. Comparable neurodevelopmental data in the human is not currently available. To date, studies of the IUGR human fetus and neonate have largely examined grey matter volume, whereas the greatest effects of IUGR on neurodevelopment in toddlers and adolescents are on white matter [100, 120, 122].

The techniques used to study neurodevelopment and cognition in each experimental species also differ, which further complicates comparisons between species. Animal models are the only means by which mechanisms of damage associated with IUGR can be examined at the tissue or molecular level, as human studies rely on rare donations of tissue from miscarried fetuses, and thus are obtained at varying stages of prenatal development, and often exposed to pathological conditions [108]. Assessment of neurodevelopmental outcomes in the rat and guinea pig frequently analyse microstructural, histological and gene expression outcomes [196, 206, 212, 220, 224, 228, 229, 231], but have not yet directly studied functional outcomes into adult life. In UPL rabbits, MRI and imaging techniques have been utilised [198, 271]; methods that are also used to assess brain morphology following IUGR in humans [100, 118, 119, 121]. Nevertheless, as is the case in humans, MRI studies do not permit for examination of causality. Studies that incorporate these imaging techniques concurrently with histological studies and measures of learning outcomes could prove a valuable way to relate structure (eg. myelination) with functional outcomes in future. It simply is not clear at present how the fetal and neonatal structural outcomes observed in rats,

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rabbits, sheep and guinea pigs translate to functional outcomes, nor what mechanisms underlie the structural and functional outcomes of IUGR.

Comparison of cognitive outcomes is also difficult between models, due to study at different ages and with varying tests. Neonatal neurobehavioural outcomes, such as development of reflexes, have been studied in the IUGR rat following maternal global or protein feed restriction or STA_2 administration [206, 214, 265, 289] and in the UPL rabbit [170, 171], but similar studies are not possible in guinea pigs and sheep, which are born more developed and with these reflexes already established [181]. Impairments of later memory and visuomotor skills have been observed in the majority of animal models of IUGR (Table 1-6), although some differences exist in outcomes between species and studies. Initial and reversal learning and memory are impaired in maze testing in progeny of maternal global feed restricted and UPL rats [194, 206, 256, 281, 290]. In contrast, although UPE and CX in sheep impair initial learning of maze routes in progeny [243, 291], reversal tasks are solved more quickly by CX than control progeny [291]. It is not clear whether this reflects differences in the type and timing of restriction, or behavioural differences between species. For example, in T and Y-maze tasks sheep rapidly acquire bias towards entering one arm preferentially and become averse to entering the other maze arm in reversal tasks [243, 303, 304]. In contrast, rats find novelty far more attractive, and are therefore more likely to explore maze arms they have not previously been able to access [305].

Understanding and comparison of cognitive outcomes of IUGR may also be limited by availability of validated tools for cognitive testing in many species, with few tools able to be utilised in both experimental and human IUGR. The majority of human studies report IQ, memory and other cognitive measures taken via written, oral or manual dexterity tests [93, 98, 131, 132, 134-137, 140, 141], which are obviously not possible in animal models. Perhaps more importantly, the vast majority of human motor and cognitive assessments were designed to detect relatively frank

disability, and may well miss more subtle but still physiologically-relevant neurodevelopmental impairments. No group differences in mean neurodevelopmental scores exist between preterm IUGR and preterm AGA infants at twelve months corrected age [169], although the incidence of abnormal scores is increased in IUGR compared to AGA infants [128, 129]. Limited capacities in infancy limit the ability to measure subtle changes in development and cognition, particularly prior to language development. Tools such as the Assessment of Preterm Infants Behaviour therefore assess measures such as motor tone, attention and self-regulation in neonates [130] rather than cognition. There is a sharp trajectory of cognitive development after age six into adolescence, during which humans develop more complex cognitive abilities, especially executive functions. This enables use of a wider battery of testing tools in children than infants, which detect lower scores in IUGR children for a number of IQ subscales from the age of six onwards [306]. Few human IUGR studies have examined neurodevelopmental or cognitive outcomes past childhood and into adulthood, however. Maze testing is a useful measure of learning and memory and has been utilised in IUGR rats and sheep [243, 253, 290, 291], but to date only one study has utilised this in human IUGR with toddlers completing a maze task directly comparable to those tests used in animal studies [137]. Object recognition tests have been utilised in UPL rats and rabbits, allowing discrimination between different kinds of memory, specifically recognition and spatial memory [194, 198, 281]. Although maze [291, 304, 307-309], and executive function tasks [303, 308] have been utilised in studies of sheep behaviour, not all of these tools have been yet applied to IUGR models. Use of a greater variety of tests in animal models of IUGR, to evaluate outcomes including executive function, dexterity, learning and non-spatial forms of memory, are necessary to enable better comparisons of functional deficits between human and experimental IUGR.

In all of these experimental models of IUGR, there is currently a lack of detailed longitudinal studies of cognitive changes throughout the lifespan in parallel with studies of structural neurodevelopment. Such studies are needed both to allow comparisons of outcomes with those of

human IUGR, and to evaluate long-term consequences of interventions. Such longitudinal studies in large animal models may be precluded by husbandry costs and the lifespan, and be more feasible in small animal models due to their rapid neurodevelopment. Although longitudinal assessment of brain structure and reflex development has been performed in the UPL rabbit using MRI acquisition [197, 271], concurrent functional assessments are not yet available. To date, there have been few longitudinal studies of cognitive outcomes in any species, and due to the cost of maintaining animal cohorts, the same animals are generally tested at multiple ages. Experimenters therefore also need to account for effects of prior learning during analysis of data, as species such as sheep are capable of remembering both visual cues [310] and strategies required to solve maze tasks [291] for periods ranging from a month to a year after initial learning.

Finally, it is vital that more studies examine cognition in intact post-pubertal adults of each sex. In the rat, maternal UN has sex-specific effects on cognition [211], and these may in part be due to interactions with sex steroids. Sex hormones, particularly testosterone, affect behavioural stress responses in sheep [311, 312], whereas in rats both oestrogen and testosterone appear to independently affect both stress response and spatial learning [313-316]. Therefore, studies utilising one sex or pre-pubertal animals are unlikely to produce data applicable to human adults. Additionally, stress induced by human contact and isolation during the course of testing may impact outcomes differently dependent on species. Sheep find proximity to observers aversive [317-320], and minimising stress is critical to avoid confounding during cognitive testing. Further complicating this issue, prenatal exposures also have sex-specific effects on stress responses. For example in adult sheep progeny of maternal globally-feed restricted pregnancies, UN males have a greater locomotion response than control males in response to sudden movement (reactivity test) [211]. Both UN and control females share this rapid locomotion response, but this persists for a shorter duration in UN than control females [211]. Low birth weight (in term-born children and thus likely to reflect restricted growth *in utero*) also has sex-specific effects on the cortisol response to stress in

pre-pubertal human children [321]. As adults, low birth weight women have greater systolic blood pressure during stress tasks than controls, and also greater heart rate during the luteal but not follicular phase of the menstrual cycle [322]. Responses to the same stress tasks do not differ between control and low birth weight men [322]. Stress affects cognitive outcomes including memory [323], and both stress response and effects of IUGR appear to be sex-specific and reactive to levels of sex steroids. It is therefore important to include gonadally-intact animals of both sexes and evaluate outcomes before and after puberty to fully characterise the effects of IUGR on cognition [211].

1.7.7. Conclusions and recommendations

Animal models of IUGR have enabled examination of causal links between IUGR and morphological and cognitive outcomes, and minimisation of environmental and genetic confounders and variation. There are merits and drawbacks to each currently utilised experimental model of IUGR. Nevertheless, in the majority of models, experimental IUGR produces progeny with broadly similar outcomes to human IUGR, including altered brain morphology, particularly grey matter loss and discordant trajectory of white matter development, and poorer cognition and memory. These preclinical studies have been limited, however, by lack of concurrent and detailed characterisation of mechanisms and functional outcomes, and a paucity of longitudinal studies including pre- and post-pubertal animals of both sexes.

In order to further investigate the mechanisms underlying adverse neurodevelopmental and functional consequences of IUGR, and to evaluate interventions that will subsequently improve outcomes of IUGR in humans, we recommend that preclinical studies need to incorporate the following design considerations:

1. The method of restriction should induce similar changes in the intrauterine environment to those seen in human IUGR, including decreased nutrient and oxygen availability.

2. The timing of growth restriction relative to neurodevelopment should be similar to that seen in human IUGR.

3. Neurodevelopmental and cognitive outcomes should resemble those reported following human IUGR, including incidence of brain sparing in more severe cases of restriction, reduction of brain volume at birth, particularly grey matter volume, delayed and discordant white matter development, and impaired learning, memory, visuomotor and executive function skills.

4. Species-appropriate cognitive tests that minimise confounding by factors including stress should be used.

5. Outcomes should be evaluated across the life course and in gonadally-intact animals of both sexes

1.8. Conclusions, thesis aims and hypotheses

IUGR results in long-term neurodevelopmental changes, starting during pregnancy but progressing throughout postnatal life. The mechanistic link between cognitive and morphological changes remains unclear, but appears linked to altered trajectories of postnatal development. Use of animal models remain the most practical way to untangle causality and minimise confounding, with placental restriction models in the sheep, particularly the carunclectomy model of PR, offering promise in this endeavour. Future studies that would be most helpful to filling the gaps in the literature would ideally be longitudinal studies in both sexes, designed to minimise environmental stress and control for prior learning.

The aims of studies described in this thesis were therefore:

1: To investigate cognition in male and female healthy sheep before and after puberty to more clearly define age and sex-differences in learning and behaviour.

Hypotheses:

- Forty week-old sheep re-tested on cognitive tasks previously learned at 18 weeks of age will recall these tasks, evidenced by better learning than sheep learning these tasks for the first time at 40 weeks of age.

2. To compare cognitive outcomes in male and female control sheep to those of PR sheep at the same ages, in order to determine whether PR, size at birth and neonatal growth influence cognition in a sex-specific manner, and how this differs with age.

Hypotheses:

- PR sheep will have impaired learning, memory and cognitive flexibility compared to CON sheep.

- Low birth weight and slow neonatal growth will be associated with poorer learning, memory and cognitive flexibility.

3. To investigate the effects of PR and pre- and postnatal growth on cerebral asymmetry and behavioural lateralisation in the same groups, and determine whether any morphological changes are correlated with behavioural lateralisation.

Hypotheses:

- PR, low birth weight and slow neonatal growth of the skull will each increase the strength of behavioural lateralisation at 18 and 40 weeks of age.

- Slow perinatal growth will increase structural lateralisation of the brain at 52 weeks of age to the side contralateral to behavioural lateralisation.

- Behavioural lateralisation will correlate with structural laterality of the prefrontal cortex and caudate nucleus.

Chapter 2 – General methods

2.1. Preamble

Chapter 2 describes the methods used to general the animal cohort, the behavioural tests utilised in chapters 3, 4 and 5, and the apparatus used for these. I also describe post-mortem collection of the brain and methods of morphological analysis of the brains used for morphological examination in Chapter 5.

2.1. Cohort generation

2.1.1. Ethics Statement

All procedures were jointly approved by the University of Adelaide Animal Ethics Committee (M-2009-145 and M-2011-055) and the SA Pathology Animal Ethics Committee (135a/09) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* [324].

2.1.2. Animals

Restriction of placental growth and function was induced by surgical removal of all but four visible endometrial placental attachment sites (caruncles) from each uterine horn of primiparous Merino x Border Leicester ewes [203, 325]. At least 10 weeks recovery occurred prior to time-mating to Merino rams. Control (CON) ewes were un-operated and were also included in the time-mating program.

Fig. 2-1 describes the experimental cohort and timeline. Groups of lambs were spontaneously born at term at five-week intervals between July 2010 and December 2012. Pregnant CON and PR ewes were housed indoors in individual pens in an animal holding room with a 12 h light:12 h dark cycle from day 110 of pregnancy until four weeks after birth. Ewes and lambs were then transferred to group housing within the same facility until lamb weaning at 13 weeks of age. Ewes were fed 1 kg Rumevite pellets/day (Ridley AgriProducts, Victoria, Australia), and had *ad libitum* access to lucerne chaff and water. Weaned lambs were housed in outside paddocks in groups of the same sex and similar ages. Lambs were group-fed each day with 0.5 $kg.d^{-1}.lamb^{-1}$ Rumevite pellets, with *ad libitum* access to oaten hay, seasonal pasture and water.

Individual measures of growth were taken every second day from birth to 16 days of age, then weekly until weaning, and then finally at 5 week intervals thereafter. Cognitive and behavioural testing occurred at 18 and 40 weeks of age. All lambs also underwent studies of behavioural and physiological stress response at 18 and 40 weeks of age (unpublished data), immune function between 20 and 28 weeks of age [249], and studies of metabolic function at 48-52 weeks of age [248], outcomes of which are not discussed in this thesis. Finally, sheep were humanely killed at 52 weeks of age and underwent post-mortem.

2.1.3. Experimental groups

Learning, memory, reversal learning and side preference were evaluated at 18 and 40 weeks of age. Additionally, sheep underwent an isolation test, evaluating social stress (results not reported in this thesis). All animals underwent the same sequence of behavioural testing at both ages, as outlined in Table 2-1, except in cases of illness.

Delays in construction of the behavioural testing apparatus led to the generation of two sub-groups: naïve sheep that only underwent behavioural testing at 40 weeks of age (40N) and sheep that were tested at 18 weeks of age (18N) then re-tested at 40 weeks of age (40E). Learning differences between these groups in CON sheep are described in Chapter 3. As there were clear effects of prior learning observed in controls, the 40N group were excluded from subsequent analyses comparing cognitive outcomes in PR and CON sheep in Chapters 4 and 5. The 40N group was, however, included in analysis of brain morphology at 52 weeks of age.

In terms of sheep tested at each age, among the controls one male sheep was ill and unable to finish cognitive testing at 40 weeks and so is only included in 18N cognitive data, but in both datasets for lateralisation data. One CON 40N female failed task R2 and so was not included in analysis of cognitive data in this task (Chapter 3). All other CON and PR sheep tested at 18 weeks completed all tasks at both 18 and 40 weeks.

Sample sizes decreased between behavioural testing at 40 weeks of age and post-mortem at 52 weeks of age due to death of some sheep during this period (Fig. 2-1). Of this group, only photographs of appropriate quality had brain morphology analysed at 40 weeks of age (Fig 2-1).

2.2. Behavioural testing

2.2.1. Habituation to experimenters

All lambs in this cohort were well habituated to human contact. Lambs were handled frequently from birth in order to take growth measures, and tests of immune function occurred between 20-28 weeks of age, in both instances providing close contact with experimenters involved in the behavioural testing. Daily feeding in small groups during pen and then paddock housing also provided frequent human contact. Finally, habituation to both experimenters and the maze apparatus occurred as part of the cognitive testing battery at 18 and 40 weeks of age. The obstacle avoidance task and maze side preference tasks (Days 1 and 2 of the battery, Table 2-1) served to habituate sheep to handling, the brief isolation necessary within each trial of testing, and to the maze used for cognitive testing (Fig. 2-1) prior to commencement of the learning, memory and reversal tasks. Sheep were not

habituated to prolonged isolation out of sight of flockmates however, which was the premise for the isolation tests (unpublished data).

Fig. 2-1. Experimental cohort and timeline.

M = male, F = female, N = naïve sheep undergoing testing for the first time at this age, E = 40 week sheep that underwent behavioural testing at 18 weeks that were re-tested at 40 weeks. Results from immune testing, DEXA and metabolic tests are not reported in this thesis, but have been published in co-authored papers (Appendix 5 and 6 respectively).

Ewe treatments	55 unoperated	**Merino x Border Leicester Ewes**	33 carunclectomy

Progeny born

Control progeny
Males: 4 singleton,
23 multiple
Females: 5 singleton,
23 multiple

PR progeny
Males: 7 singleton,
7 multiple
Females: 14 singleton,
6 multiple

Behavioural testing (18 weeks)

18N CON
M: 1 singleton,
22 multiple
F: 1 singleton,
17 multiple

18N PR
M: 5 singleton,
5 multiple
F: 10 singleton,
3 multiple

Immune testing (20-28 weeks)

Behavioural testing (40 weeks)

40N CON
M: 3 singleton,
1 multiple
F: 3 singleton,
7 multiple

40E CON
M: 1 singleton,
22 multiple
F: 1 singleton,
16 multiple

40N PR
M: 2 singleton,
2 multiple
F: 4 singleton,
3 multiple

40E PR
M: 5 singleton,
5 multiple
F: 10 singleton,
3 multiple

DEXA (45 weeks)

Metabolic testing (48-52 weeks)

Post mortem, brain photographed (52 weeks)

CON
M: 4 singleton,
21 multiple
F: 5 singleton,
22 multiple

PR
M: 7 singleton,
7 multiple
F: 13 singleton,
5 multiple

Brain photographs analysed (52 weeks)

CON
M: 4 singleton,
19 multiple
F: 5 singleton,
20 multiple

PR
M: 7 singleton,
7 multiple
F: 13 singleton,
5 multiple

2.2.2. Apparatus

All behavioural tests utilised the same purpose-built apparatuses (Fig. 2-2), which were covered by a 3 m high shade-cloth pergola, to remove the confounding effects of shadow or light glare on side-preference that were observed during preliminary trials. The floor of the maze and obstacle avoidance laneways were lined with sawdust that was raked between each test, to ensure visual symmetry. Sheep not being tested were kept in a group holding pen that was positioned directly adjacent to the reward pen. In all tests, the experimenter stood directly behind the starting pen, such that sheep ran away from the experimenter and towards flockmates in the reward pen.

A diamond maze was used for learning, memory and reversal learning (Chapters 3, 4) and one of the two lateralisation tasks (Chapter 5). The diamond-shaped maze was constructed of portable metal fencing panels covered in black shade-cloth. At the start of the maze was a small holding pen, in which the sheep undergoing testing waited for the commencement of the trial. The sheep then entered the maze through this pen, and navigated towards the reward pen. Both maze arms were constructed to be identical mirror images, and therefore the major spatial cue was position of each maze arm relative to the starting arm containing the entry gate. Sheep could see and hear flock mates through the open or closed gates, but the exit gates were not visible from the Y-intersection. The sheep therefore had to commit to picking a lane at the intersection, walk 1.5 m along that lane-way from the intersection, and turn a corner before the exit gates came into sight.
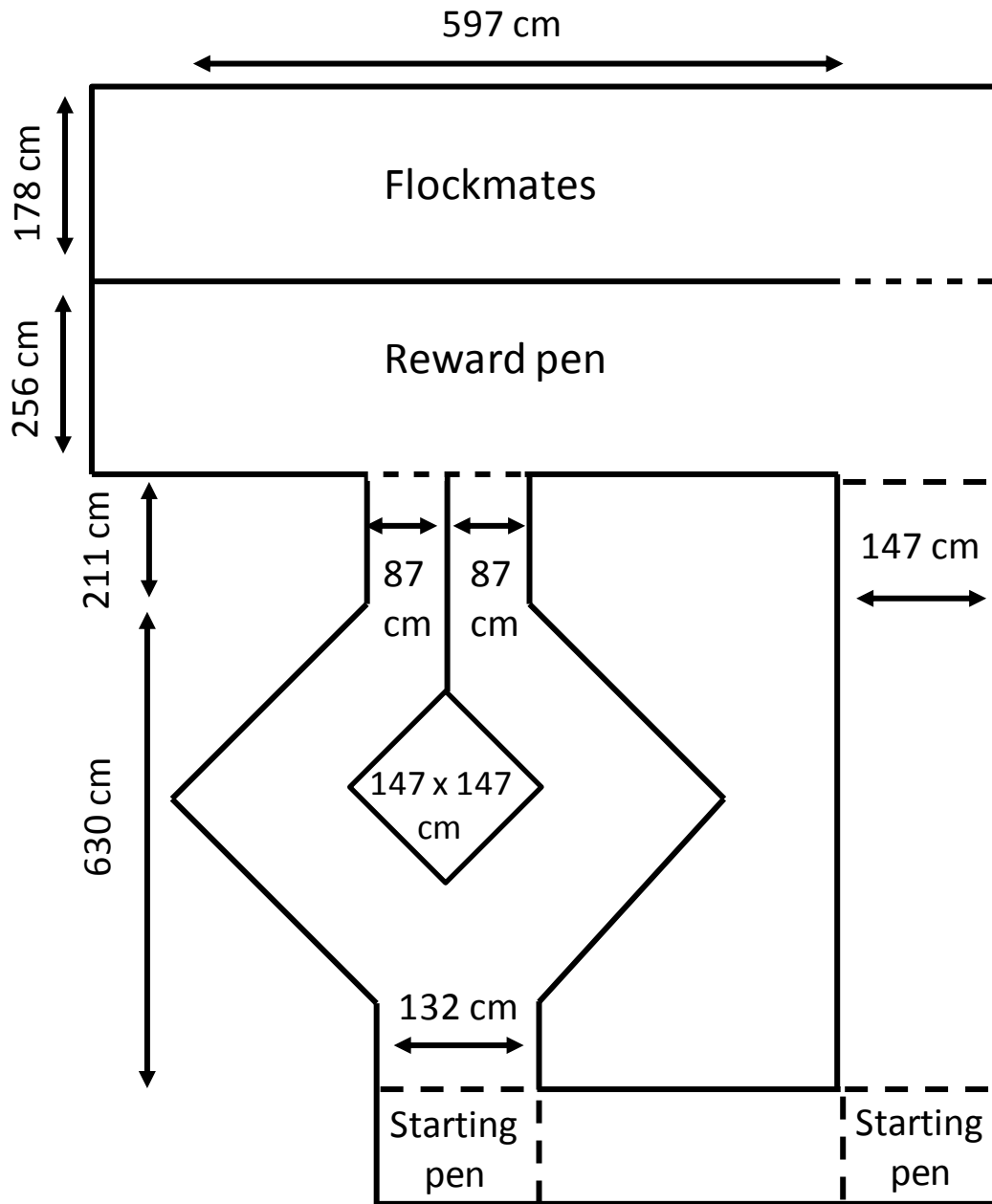
Fig. 2-2. Behavioural testing apparatus.

Solid lines indicate walls. Dashed lines indicate gates, which were used to close off appropriate regions of the apparatus for the test being conducted. The obstacle avoidance task took place using the long laneway on the right of the apparatus, which later served as the route to enter the diamond maze on the left during cognitive testing.

The obstacle avoidance lateralisation task (Chapter 5) took place in a 147 x 841 cm laneway built from portable fencing panels lined with opaque black shade-cloth. At one end of the lane was a holding pen, where the sheep undergoing testing waited for the commencement of the trial, and the other was the reward pen. An obstacle (an upturned opaque orange plastic crate, 610 x 407 x 275 mm) was placed in the centre of the laneway, approximately 1/3 of the distance from the start pen, and was removed in between trials.

2.2.3. Behavioural test battery

2.2.3.1. Obstacle avoidance task

The obstacle avoidance task used the methods of Versace et al. [326]. The task consisted of 10 trials in which the sheep ran from the holding pen to the reward pen, navigating around the obstacle (an upturned opaque orange plastic crate, 610 x 407 x 275 mm) in the centre of the laneway, approximately 1/3 of the distance from the start pen. The sheep then remained in the reward pen for 30 seconds before being returned to the starting pen for the next trial. Side preference was determined based on the number of trials out of the ten in which the sheep passed to the left or right side of the obstacle.

2.2.3.2. Maze side preference task

This task was based on the methods of Hernandez et al. [192]. Sheep were guided into the diamond maze, which had both exited gates open, and were permitted to exit the maze through whichever arm they preferred. Five trials took place, with side preference recorded as the arm the sheep chose to exit the maze through during that trial. This task also acted as

habituation for the maze cognitive tasks, as it habituated to the handling protocol and the maze apparatus.

2.2.3.3. Cognitive tests

The maze cognitive tasks were modified from those described by Erhard et al. [211] and Hernandez et al. [192], and immediately followed the maze exit preference task. The sequence of testing is summarised in Table 2-1. Having already determined side preference in the maze during preceding tasks, guided trials followed. During these, each sheep was trained to turn around at dead ends by being guided to a closed gate, turned around, and then guided to the open gate. The closed arm chosen to start the guided runs sequence was randomised, to prevent any introduction of side bias. For subsequent tests, the recorder was positioned in a set position behind the maze entrance, with a clear view of the entire maze; and the handler moved the sheep being tested to the starting pen at the beginning of each trial, and remained out of sight during the entire trial.

The guided runs were followed by a learning task, in which the sheep were required to exit the maze only through their preferred side (Task L). The sheep had six trials to learn to exit the maze only through this arm. Criterion for success on this task, and in subsequent memory and reversal tasks was three consecutive exits from the maze in three minutes or less during the allocated number of trials for the task. The reward for solving the maze was the capacity to exit into the reward pen, allowing for close proximity with flock-mates, and access to a food reward (lucerne chaff) during the ≥10 second rest period between runs. The only penalty for not solving the maze was the inability to leave the maze during that trial, consistent with previous studies [192, 211]. Once the failed trial was complete (after 3 minutes without

exiting), sheep were steered out of the correct exit to the reward pen, and remained there for a ≥ 10 second rest period as above.

Table 2-1. Sequence of behavioural and cognitive tests.

All sheep participated in each day of testing sequentially, although the majority of sheep completed tasks M2 and R2 successfully on Day 4 and so did not undergo testing on days 5 and 6.

	Experiment	**Apparatus**
Day 1	Obstacle avoidance	Obstacle avoidance laneway
Day 2	Maze side preference Guided runs Initial learning (Task L)	Diamond maze
Day 3	Memory task 1 (Task M1) Reversal task 1 (Task R1)	Diamond maze
Day 4	If Day 3 tasks failed, repeat If Day 3 tasks passed then: Memory task 2 (Task M2) Reversal task 2 (Task R2)	Diamond maze
Day 5	If tasks on Day 3 or 4 failed, repeat. If Day 4 passed, no testing	Diamond maze
Day 6	If tasks on Day 3 or 4 failed, repeat. If Day 4 or 5 passed, no testing	Diamond maze
Rest days	0-2 rest days before isolation testing. Heart rate electrodes fastened to skin.	NA
Day 7	Isolation testing	Isolation chamber

On the following day of testing, the sheep first performed a memory task (Task M1) which involved repetition of task L from the previous day. This was followed by a reversal task, requiring completion of the maze with the open gate switched to the non-preferred side (Task R1). On day 3, the sheep performed a memory task (Task M2); repeating task R1 with the gate on the non-preferred side. The open gate was then switched back to the preferred side for the final reversal task (Task R2). Each task consisted of ten trials, and successfully completing the day's tasks resulted in graduation to the next day of testing in the sequence. Failure to complete either task on Days 2 or 3 resulted in the sheep repeating that day's tests on following days until successful on both tasks. Sheep had a maximum of five days to finish the entire three day sequence.

Measures recorded during each test included total trials and time taken to complete each trial, numbers of vocalizations in each trial, and number of arm entries and average time per trial in criterion trials. Sheep were also classified according to the method they used to exit the maze during the majority of criterion trials, these being either a direct exit method (utilised a direct route to the open gate) or indirect method (initially entered the closed maze arm before turning around and exiting via the open gate).

2.2.3.4. Isolation tests

Results for this test is not included in this thesis, however it was a part of the cognitive and behavioural battery at both ages tested, and thus is described briefly as follows. Emotional reactivity was examined via an isolation test, as per [211]. Each sheep was tested individually in a 4 x 4 metre pen with 2 metre high opaque black walls, with the test filmed using two digital cameras (Sony HDR-PJ10E, Sony, Japan) mounted above the walls at opposite sides of

the pen. The test sequence consisted of three phases: an initial *isolation* phase for three minutes, in which the stressor was social isolation. The *novelty* phase followed, in a novel object (a closed red umbrella) was inserted into the pen through a hole in the gate. This phase lasted until the sheep approached within 20 cm of the umbrella, at which point umbrella was rapidly open and the *suddenness* phase commenced. During this phase, the umbrella was held in place for a further three minutes while behaviour was recorded. If the sheep failed to approach the umbrella within three minutes the test ended, and the sheep was returned to the holding pen. Salivary cortisol samples were taken immediately prior and after testing, and all sheep wore heart rate monitors during tests.

Number of rest days between the conclusion of maze testing and the commencement of isolation testing (Table 2-1) were variable between individuals, and dependent on weather, as this test could not take place on days with excessive wind or rain. All groups of sheep were tested during in the same period of the day (10-2 pm) to minimise effects of circadian rhythm on cortisol.

2.2.4. Brain morphological analyses

2.2.4.1. Post-mortems and tissue acquisition

Post-mortems took place at 52 weeks of age, following four weeks of metabolic experiments (data not reported in this thesis). Sheep were weighed, had their biparietal skull width measured, and then were humanely killed by an intravenous overdose of thiopentone (Troy Laboratories, New South Wales, Australia). Brains were rapidly dissected out, weighed, and sectioned coronally at the rostral end of the sylvian sulcus, bisecting the prefrontal cortex,

lateral ventricles, rostral caudate nucleus and corpus callosum. The slice of brain immediately rostral to this section was placed, unfixed, caudal face upwards against a 5 mm reference grid and digitally photographed using a Sony HDR-PJ10E camera.

2.2.4.2. Image analysis

Photos were analysed using Axiovision 4.8 (Carl Zeiss, Jena, Germany), with blinding to treatment and sex. Photos of insufficient quality were excluded for analysis (Fig. 2-1), and so structural brain analysis occurred completed in 48 control (23 male and 25 female) and 32 PR sheep (14 male and 18 female). Boundaries of regions of interest (total, grey and white matter, the left and right caudate nuclei, and the corpus callosum) were manually outlined, with measurements taken within each hemisphere and overall. From these measures, ratios of left to right hemisphere total area, grey matter and white matter areas and grey:white matter area ratio for each hemisphere were calculated.

Chapter 3 – Do I turn left or right? Effects of sex, age, experience and exit route on maze test performance in sheep

3.1. Preamble

Chapter 3 describes behavioural outcomes in healthy sheep that were utilised as control sheep in chapters 4 and 5. This chapter describes outcomes differences between sexes and ages, and the effects of prior learning, which led to the subsequent decision to exclude naïve learners at 40 weeks of age in later chapters. I acted as data recorder and observer in all behavioural tests in this chapter, analysed the data, wrote the manuscript and wrote all subsequent redrafts and edits of the manuscript. This chapter has been published in Physiology and Behavior (Appendix 2), with myself as first author, and text and figures are presented unaltered as per University of Adelaide guidelines.

Hunter, D. S**.**, Hazel, S.J., Kind, K.L., Liu, H., Marini, D., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2015). "Do I turn left or right? Effects of sex, age, experience and exit route on maze test performance in sheep." Physiology and Behavior 139: 244–253.

3.2. Statement of Authorship

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Name of Principal Author (Candidate)	Damien Hunter		
Contribution to the Paper	Performed experiments, performed analyses on all data, interpreted data, wrote manuscript and created all figures		
Overall percentage (%)	50.00%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	6/10/15

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

 i. the candidate's stated contribution to the publication is accurate (as detailed above);

 ii. permission is granted for the candidate in include the publication in the thesis; and

 iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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3.3. Abstract

Brain development and function are susceptible to perturbation by environmental factors. Sheep are increasingly being used as a neurodevelopmental model due to timing similarities with humans, but effects of age, experience and sex on cognition are not well characterised in this species. We therefore studied memory and reversal learning in sheep using a modified Y-maze at two ages: naive 18 week olds (18N: 23 male, 17 female), experienced 40 week old sheep that had previously been tested at 18 weeks (40E: 22 male, 17 female), and naive 40 week olds (40N: 4 male, 10 female). Younger naive animals (18N) required more trials to solve the first reversal task (R1) than 40E ($P = 0.01$).Experience also improved outcomes, with 40N sheep requiring more time to solve tasks L ($P = 0.03$) and R1 ($P = 0.002$) than 40E. Increasing age (40N cf. 18N) decreased bleat frequency in tasks R1, M2 and R2 (each $P < 0.05$). In females, 40N sheep took more trials overall than 18N ($P = 0.008$). In 40N females, outcomes also differed by exit method in task R1, with those that exited via an indirect route taking less time to pass tasks R1 ($P = 0.009$) and R2 ($P = 0.001$) than those that used a direct route. Age plus experience improved learning outcomes, demonstrating knowledge retention for 22 weeks in this species, whilst age alone affected mostly behavioural responses. These results provide comparison data, and can be utilised to improve experimental design, for studies of neurodevelopment in the sheep.

3.4. Introduction

Brain development and function are susceptible to perturbation by various exposures and environmental factors in early life. For example, in humans preterm birth [327, 328], prenatal undernutrition [329] and intrauterine growth restriction [328, 330] are associated with a lower intelligence quotient (IQ) and poorer learning, memory and executive function in children and adolescents. Techniques such as magnetic resonance imaging [331] and transcranial magnetic stimulation [143] allow for study of the morphological and some functional determinants of these cognitive capacities. Use of non-human species extends this to enable studies of the molecular basis and causal pathways that underlie associations between brain development and postnatal function, and early testing of interventions. Further, non-human species enable minimisation of confounding factors affecting neurodevelopment and postnatal function and loss due to drop out and the more rapid follow-up of long term outcomes than is possible in humans.

The sheep is an appropriate species in which to test early life environmental effects on brain development and function, in part due to similar timing of key neurodevelopmental events with humans. In humans and sheep, neurogenesis commences in the first third of gestation [15, 173], and myelination by the last third of gestation [126, 173, 296], although in humans myelination commences after birth in some of the higher brain structures including cerebrum [126]. In contrast, in the rat, neurogenesis does not commence until the last third of gestation (~57% of term), and myelination occurs postnatally [15, 18, 19]. Sheep are also an intelligent species, capable of fine object, brightness, face and plant species discrimination, can extrapolate visual information to recognise individuals from different viewpoints or earlier ages [332], and can be trained to make use of these skills [310, 332-336]. Sheep can learn to navigate mazes [192, 211, 243, 307, 308], and are the only large animal model aside from

primates in which complex executive function has been demonstrated, with cognitive flexibility demonstrated using tests of reversal learning and intra- and extra-dimensional set-shifting [192, 211, 303, 308]. Together these characteristics make them a suitable species in which to examine effects of early life factors on neurodevelopment and subsequent behaviour.

To date there have been few studies examining cognition even in healthy sheep as they mature, limiting their usefulness for developmental studies. Memory and reversal performance in maze tasks improved with age in pre-pubertal lambs [308]. Changes in side-preference and reversal learning have also been examined in 4- and 18-month old control and periconceptionally undernourished sheep [192], however the effects of prior learning were not controlled for, and therefore effects of age and experience could not be separated. Because learning may also differ between sexes, and this effect may also differ with ageing due to effects of sex steroids on reactions to stressful situations [311, 312] and effects of stress hormones on learning [323, 337, 338], it is also important to characterise learning development in both sexes.

We therefore tested learning, memory and cognitive flexibility in 18- and 40 week old sheep, ages corresponding to pre-puberty and young adulthood [188, 339], in a cohort that were habituated to frequent human contact and handling. We also recorded numbers of entries into each arm of the maze, allowing us to assess patterns of entries prior to successful exit of the maze. Three comparisons were performed to differentiate effects of age and experience and their interactions with sex. Firstly, we compared performance in the same sheep tested twice, at 18 and 40 weeks of age, where differences reflect age and experience. Second, we compared performance in sheep tested at 18 weeks and in those tested only at 40 weeks,

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where both groups were naive to the task, to assess effects of age alone. Third, we compared performance of 40 week old sheep between those that had been tested previously (at 18 weeks of age) and those who were tested only at 40 weeks of age, to examine the effects of experience alone.

3.5. Methods

3.5.1. Ethics Statement

All procedures were jointly approved by the University of Adelaide Animal Ethics Committee (M-2009-145 and M-2011-055) and the SA Pathology Animal Ethics Committee (135a/09) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* [324].

3.5.2. Animals

Merino x Border Leicester ewes were mated with Merino rams in a timed mating program [246], and delivered spontaneously at term between July 2010 and November 2012. Animal management was as described previously [249]. Briefly, ewes were housed in individual pens in an animal holding room with a 12 h light:12 h dark cycle from day 110 of pregnancy until four weeks after birth, then group housed in pens until the lambs were weaned at 13 weeks of age. Ewes were fed 1 kg Rumevite pellets/day (Ridley AgriProducts, Victoria, Australia), and had *ad libitum* access to lucerne chaff and water. Weaned lambs were housed in outside paddocks in groups of the same sex and similar ages. Lambs were group-fed each day with 0.5 kg.d^{-1}.lamb^{-1} Rumevite pellets, with *ad libitum* access to oaten hay, seasonal pasture and water.

Lambs were handled frequently from birth, having direct contact with the experimenters during individual measures of growth every second day from birth to 16 days of age, weekly to weaning, and then at 5 week intervals. Studies of immune function between 20 and 28 weeks of age [249] also required frequent handling of small groups of sheep in yards adjacent to paddocks. Daily feeding in small groups during pen and then paddock housing also provided frequent human contact and ensured lambs were habituated to the presence of humans. Learning was tested in these sheep either at both 18 and 40 weeks, or at only 40 weeks of age, resulting in three groups – naive 18 week old (18N: 23 male, 17 female), experienced 40 week old (40E: 22 male, 17 female) and naive 40 week old (40N: 4 male, 10 female). The 40N group was generated due to delays in maze construction at the start of the project, which meant that the maze was not available for testing of the first three lambing groups when they reached 18 weeks of age, and so animal numbers in this group were limited. One male sheep died between 18 and 40 weeks of age and is only included in 18N data. All other sheep tested at 18 weeks completed all tasks at both 18 and 40 weeks. Of the sheep tested only at 40 weeks (40N), one 40N female failed Task R2, and results from this animal were excluded only for this task.

3.5.3. Learning Evaluation

A diamond-shaped maze was constructed of opaque panels under a 3 m high pergola covered by shade-cloth, to remove the confounding effects of shadow or light glare on side-preference that were observed during preliminary trials (data not shown). Both maze arms were constructed to be identical mirror images, and therefore the major spatial cue was position of each maze arm relative to the starting arm containing the entry gate. Exit gates were not visible from the Y-intersection, and the sheep had to commit to picking a lane, walk 1.5 m

along that lane-way from the intersection, and turn a corner before these came into sight. The gates were non-opaque, such that sheep could see and hear flock mates through the gate, be it open or closed.

We adapted the assessments described previously by Erhard et al. [211] and Hernandez et al. [192]. Briefly, the test protocol consisted of 3-5 days of testing. The first day consisted of an initial habituation task, in which sheep were habituated to the handling protocol and the maze apparatus, and trained to exit the maze through the open gates. The initial side chosen for the guided runs was randomized to prevent any introduction of side bias. Following this, a sequence of memory and reversal tasks took place on subsequent days (Table 3-1). Successfully completing the day's tasks resulted in graduation to the next day's testing in the sequence. Failure to complete either task on Days 2 or 3 resulted in the sheep repeating that day's tests on following days until successful on both tasks. Sheep had a maximum of five days to finish the entire three day sequence, with a maximum of six trials to learn Task L on day 1, and a maximum of ten trials to solve each task on sequence days 2 and 3. If sheep failed to solve a task, it repeated the entire day's sequence of tasks on the subsequent day, until the sheep either passed both tasks for that day or had finished five consecutive days of testing. The criterion to complete each task consisted of three consecutive exits from the maze in three minutes or less, within the allocated number of trials to learn this task (Table 3-1). These final three consecutive successful trials of each task (i.e. successfully exiting the maze in < 3 minutes per trial) were defined as the criterion trials in subsequent analyses. The reward for solving the maze was the capacity to exit into the reward pen, allowing for close proximity with flock-mates, and access to a lucerne chaff food reward during the ≥ 10 second rest period between runs. The only penalty for not solving the maze was the inability to leave the maze during that trial, consistent with previous studies [192, 211]. Once the failed trial

was complete (after 3 minutes without exiting), sheep were steered out of the correct exit to the reward pen, and remained there for a ≥10 second rest period as above.

Measures recorded during the maze tasks included total trials and time taken to complete each trial, numbers of vocalizations in each trial, and number of arm entries and average time per trial in criterion trials. Sheep were also classified according to the method they used to exit the maze (complete the task) within the three minutes available. Those sheep that exited the maze via a direct route to the open gate on the majority of criterion trials were classified as using a direct exit method (Fig. 3-1). Sheep which initially entered the closed maze arm before reversing direction and exiting via the open gate in the majority of their criterion trials were classified as using an indirect exit method (Fig. 3-1).

3.5.4 Statistical analysis

Effects of sex and task on continuously distributed outcomes were initially analyzed within each group for data from all tasks using mixed model analysis, including repeated measures for multiple measures on each individual sheep, with post-hoc Bonferroni comparisons used to compare differences between each task. Effects of sex plus group (18N vs. 40E, in which differences reflect both age and experience), age (18N vs. 40N) or experience (40N vs. 40E) on continuously distributed outcomes were then analysed for data within each task separately using mixed effects models including repeated observations on each individual sheep for the comparison of 18N and 40E groups. These data were log-transformed prior to analysis to reduce skew. Variables that were counts of events (ie. total trials per task) were analyzed using a Poisson distribution with log link. Where effects of group, age, or experience differed

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Table 3-1. Learning tasks and testing schedule.

Day	Task	Description	Maximum number of trials	Maze exits open
1	*Training*	Habituation task in which both gates were open. Sheep could exit maze out of either, side most frequently used to exit was used in later tasks as their preferred side.	5	Both
	Guided runs	Habituation task in which sheep were guided down one of the maze arms to a closed gate and trained to turn around at this dead end and exit through the other.	2	Left and right sequentially. Initial direction randomized
	Learning task (Task L)	Training sheep to exit maze via preferred side.	6	Preferred side
2+	*Memory Task 1 (Task M1)*	Learning consolidation, in which task L was repeated.	10	Preferred side
	Reversal task 1 (Task R1)	Reversal 1 training, in which the open gates were swapped and the lamb had to unlearn the previous route out of the maze and learn to exit through the other gate	10	Non-preferred side
3+	*Memory Task 2 (Task M2)*	Reversal 1 learning consolidation – repetition of R1	10	Non-preferred side
	Reversal task 2 (Task R2)	Re-reversal, with open gate swapped once more to preferred side.	10	Preferred side
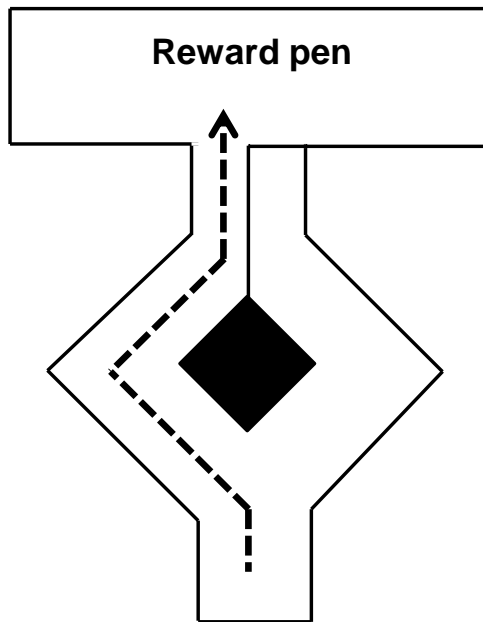
Direct exit method   Indirect exit method
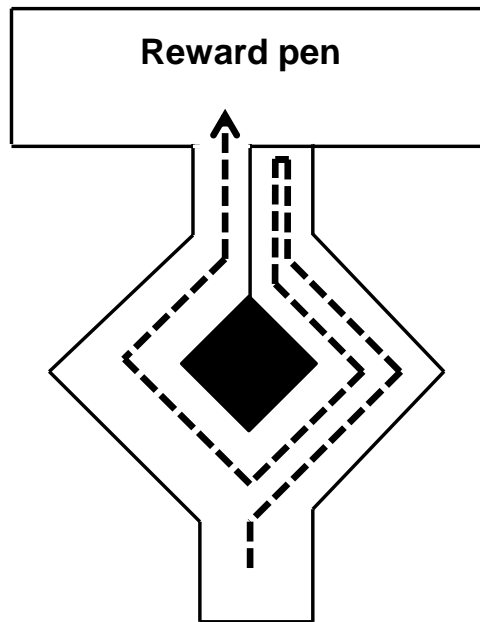
Fig. 3-1. Exit method.

Sheep were classified as using a direct or indirect exit method for each task, according to the route they used to exit the maze in the majority of their criterion runs for task R1.

between sexes (e.g. age*sex interactions), subgroup analysis was carried out. Chi-squared

tests of association were used to examine proportions of animals within each group and sex

with each exit method, and to determine whether exit method in Tasks L (learning task) and

R1 (reversal task) predicted exit method utilised in later tasks. Effects of exit method in Task

R1 on outcomes in task R1 and the subsequent tasks, M2 and R2, were analysed by mixed

effects models for effects of sex and exit method within each group (18N, 40N, and 40E). As

there were no male 40N sheep that used a direct route exit method, and only 4 male sheep in

the 40N group in total, effects of exit method in the 40N group were examined in females

only. All analyses were carried out using SPSS 20.0 (IBM, Armonk, USA). Data are

presented as mean ± SEM unless otherwise stated, and statistical significance was accepted at

$P < 0.05$.

3.6. Results

3.6.1. Between task differences

Data for outcomes in each task is shown in Figs. 3-2 – 3-6. The number of trials required to

complete tasks differed between tasks in 18N and 40E (each $P < 0.001$) but not 40N

($P > 0.2$), whilst the total time required to complete the task differed between tasks in all

groups of sheep (18N, 40E and 40N; $P < 0.01$ for all). In 18N sheep, task R1 required more

trials and longer total time to complete than all other tasks ($P \leq 0.005$ for all). Also in 18N

sheep, task R2 required more trials than task L ($P = 0.02$) or task M2 ($P = 0.007$), and took

longer to complete than task M1 ($P = 0.01$). These naive 18-week old sheep also bleated

more in each trial for task R1 than tasks L, M1 or M2 (each $P \leq 0.005$) but not task R2

($P > 0.1$), and female 18N sheep bleated more than male 18N sheep overall ($P = 0.005$). In

40N sheep, the number of trials required to complete tasks did not differ between tasks

(P > 0.2), but the total time required to solve task R1 was longer than for task M2 (P = 0.01) with a similar trend for task M1 (P = 0.09). Also in 40N sheep, task differences in bleat frequency differed between sexes (task*sex interaction P = 0.04), but did not differ between tasks in either sex when analysed separately (males P > 0.4, females P = 0.08). In 40E sheep, task R1 required more trials and longer total time to compete than task L (P = 0.02 and P = 0.002 respectively) and required longer total time than task M1 (P = 0.003). Also in 40E sheep, task R2 required more trials and longer total time than task L (P = 0.01 and P = 0.01 respectively) and required longer total time than task M1 (P = 0.008). These experienced 40-week old sheep bleated more in task R1 than task M1 (P = 0.03) but bleats per trial did not differ between other tasks.

3.6.2. Outcomes in learning task (Task L)

3.6.2.1. Effects of age and experience (18N vs 40E)

For task L, numbers of trials, total time required to solve the task and average time per criterion trial did not differ between groups (each P > 0.4) or sexes (each P > 0.5) for task L (Fig. 3-2). Bleat frequency in task L (Fig. 3-2) was greater in 18N than 40E (P = 0.02) and did not differ between sexes (P > 0.4). Effects of group on the number of arm entries per trial (Fig. 3-2) differed between sexes (group*sex interaction P = 0.04), such that 18N males made less arm entries per trial than 40E males (P = 0.001), whilst arm entries per trial did not differ between 18N and 40E females (P > 0.9).
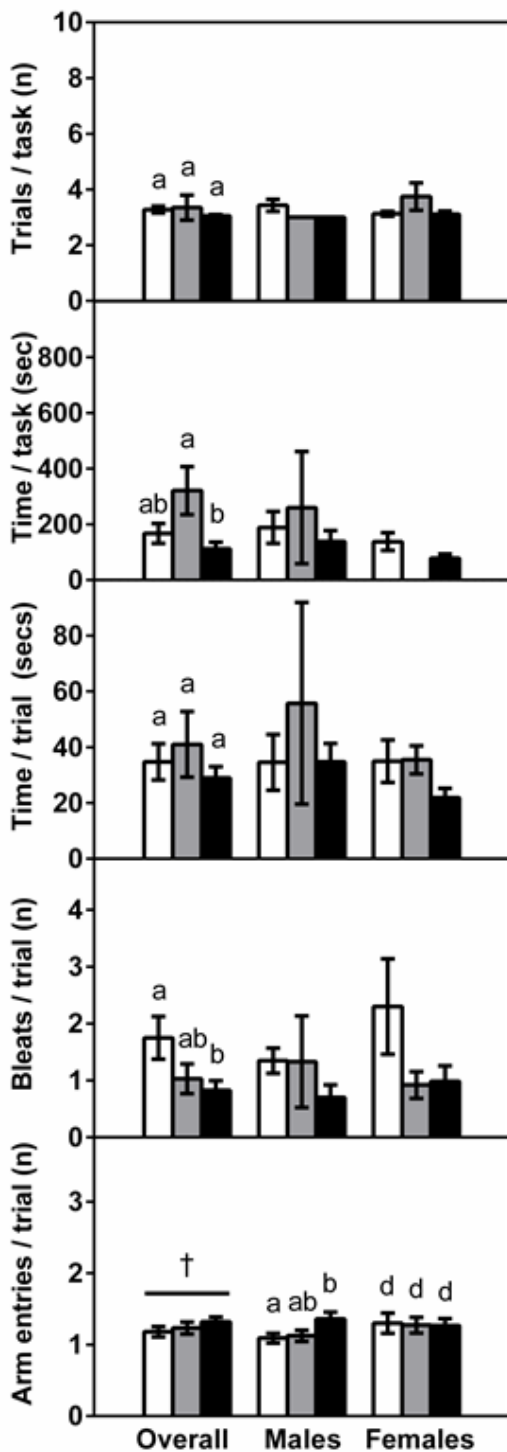
Fig. 3-2. Performance and behaviour in task L in naive 18 week-old sheep (18N, white bars), naive 40week-old sheep (40N, grey bars) and experienced 40week-old sheep (40E, black bars).

Comparisons between groups (18N vs. 40E), ages (18N vs. 40N) and experience (40N vs. 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group ($P < 0.05$, †), sex*experience ($P < 0.05$, &).
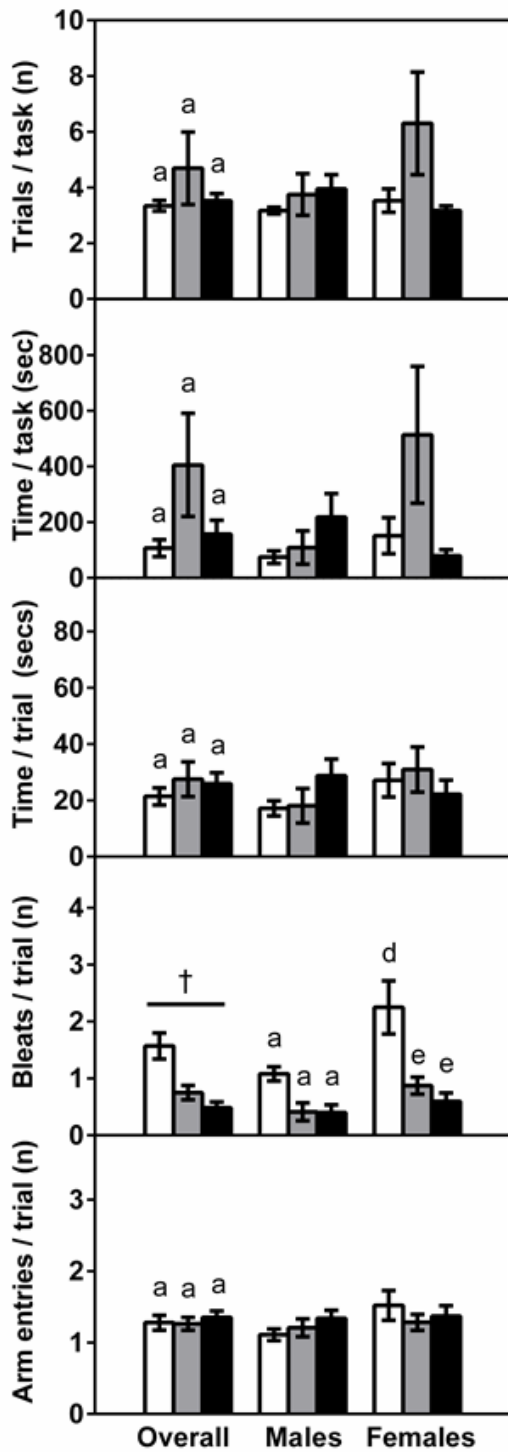
Fig. 3-3. Performance and behaviour in task M1 in naive 18week-old sheep (18N, white bars), naive 40week-old sheep (40N, grey bars) and experienced 40week-old sheep (40E, black bars).

Comparisons between groups (18N vs. 40E), ages (18N vs. 40N) and experience (40N vs. 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group ($P < 0.05$, †), sex*experience ($P < 0.05$, &).

3.6.2.2. Effects of age in naive learners (18N vs 40N)

Age and sex did not affect any outcomes in task L (number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial and arm entries per trial) in comparisons of 18N and 40N sheep (each $P > 0.1$, Fig.3-2).

3.6.2.3. Effects of experience in 40-week old sheep (40N vs 40E)

Greater experience reduced the total time required to solve task L (40N > 40E, $P = 0.03$) but experience did not affect number of trials, average time per criterion trial, bleat number per trial or arm entries per trial (each $P > 0.1$, Fig. 3-2). Outcomes in task L did not differ between sexes in comparisons of 40N and 40E sheep (each $P > 0.1$).

3.6.3. Outcomes in first memory task (Task M1)

3.6.3.1. Effects of age and experience (18N vs 40E)

Group and sex did not affect (each $P > 0.1$) number of trials, total time required to solve the task, average time per criterion trial, or arm entries per trial in task M1 in comparisons of 18N and 40E sheep (Fig. 3-3). Effects of group on bleat frequency in task M1 differed between sexes (group*sex interaction $P = 0.008$). Bleat frequency did not differ between 18N and 40E males ($P > 0.4$), whilst 18N females bleated more than 40E females ($P = 0.001$, Fig. 3-3).

3.6.3.2. Effects of age in naive learners (18N vs 40N)

Age and sex did not affect (each P > 0.1) number of trials, total time required to solve the task, average time per criterion trial, or arm entries per trial in task M1 in comparisons of 18N and 40N sheep (Fig. 3-3). Naive 18-week old sheep bleated more often in task M1 than naive 40-week old sheep (P = 0.006, Fig. 3-3) and females bleated more than males in comparisons of 18N and 40N sheep (P = 0.04).

3.6.3.3. Effects of experience in 40-week old sheep (40N vs 40E)

Experience and sex did not affect number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial or arm entries per trial for task M1 in comparisons of 40N and 40E sheep (each P > 0.1, Fig. 3-3).

3.6.4. Outcomes in first reversal task (Task R1)

3.6.4.1. Effects of age and experience (18N vs 40E)

More trials and greater total time were required for 18N than 40E sheep to solve task R1 (P = 0.007 and P < 0.001 respectively), and 18N sheep bleated more frequently than 40E sheep in this task (P < 0.001), but average time per criterion trial and arm entries per trial in task R1 did not differ between 18N and 40E sheep (each P > 0.1, Fig. 3-4). Outcomes in task R1 did not differ between sexes in comparisons of 18N and 40E sheep (each P > 0.09).

3.6.4.2. Effects of age in naive learners (18N vs 40N)

Age did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial in comparisons of 18N and 40N sheep (each $P > 0.5$, Fig. 2-4). Younger naive sheep (18N) bleated more frequently than 40N sheep in task R1 ($P < 0.001$, Fig. 2-4). Female sheep tended to require more trials ($P = 0.07$) and more total time ($P = 0.08$) to solve task R1 than males ($P = 0.07$ and $P = 0.08$ respectively), and bleated more often than males ($P = 0.003$), whilst average time per criterion trial and arm entries per trial did not differ between sexes (each $P > 0.1$).

3.6.4.3. Effects of experience in 40-week old sheep (40N vs 40E)

In 40 week old sheep, effects of experience on the number of trials required to solve task R1 differed between sexes (experience*sex interaction $P = 0.05$), with 40N and 40E males requiring similar number of trials to complete this task ($P > 0.8$), and 40N females requiring more trials to complete task R1 than 40E females ($P = 0.04$, Fig. 3-4). Bleat number per trial also differed between 40N and 40E sheep in a sex-dependent manner (experience*sex interaction $P = 0.009$), with 40N males bleating less than 40E males ($P = 0.05$) and no difference in bleat frequency between 40N and 40E females (Fig. 3-4). Total time required to complete task R1 was greater in 40N than 40E ($P = 0.002$, Fig. 3-4) and did not differ between sexes ($P > 0.3$). Experience and sex did not affect average time per criterion trial and numbers of arm entries per trial in task R1 in comparisons between 40N and 40E sheep (each $P > 0.1$, Fig. 3-4).

3.6.5. Outcomes in second memory task (Task M2)

3.6.5.1. Effects of age and experience (18N vs 40E)

Compared to 40E sheep, 18N sheep required fewer trials and less total time to solve task M2 ($P = 0.001$ and $P = 0.05$ respectively), and bleated more ($P = 0.004$, Fig. 3-5). Average time per criterion trial and numbers of arm entries per trial in task M2 did not differ between 18N and 40E sheep (each $P > 0.2$). Sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial in comparisons of 18N and 40E sheep (each $P > 0.4$), and female sheep bleated more per trial than males ($P = 0.005$).

3.6.5.2. Effects of age in naive learners (18N vs 40N)

Age and sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial in comparisons of 18N and 40N sheep (each $P > 0.09$, Fig. 3-5). Younger naive sheep (18N) bleated more frequently than 40N sheep ($P = 0.002$, Fig. 3-5) and females bleated more than males in task M2 ($P = 0.02$).

3.6.5.3. Effects of experience in 40-week old sheep (40N vs 40E)

Experience and sex did not affect any outcomes in task M2 (number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial and arm entries per trial) in comparisons of 40N and 40E sheep (each $P > 0.1$, Fig. 3-5).
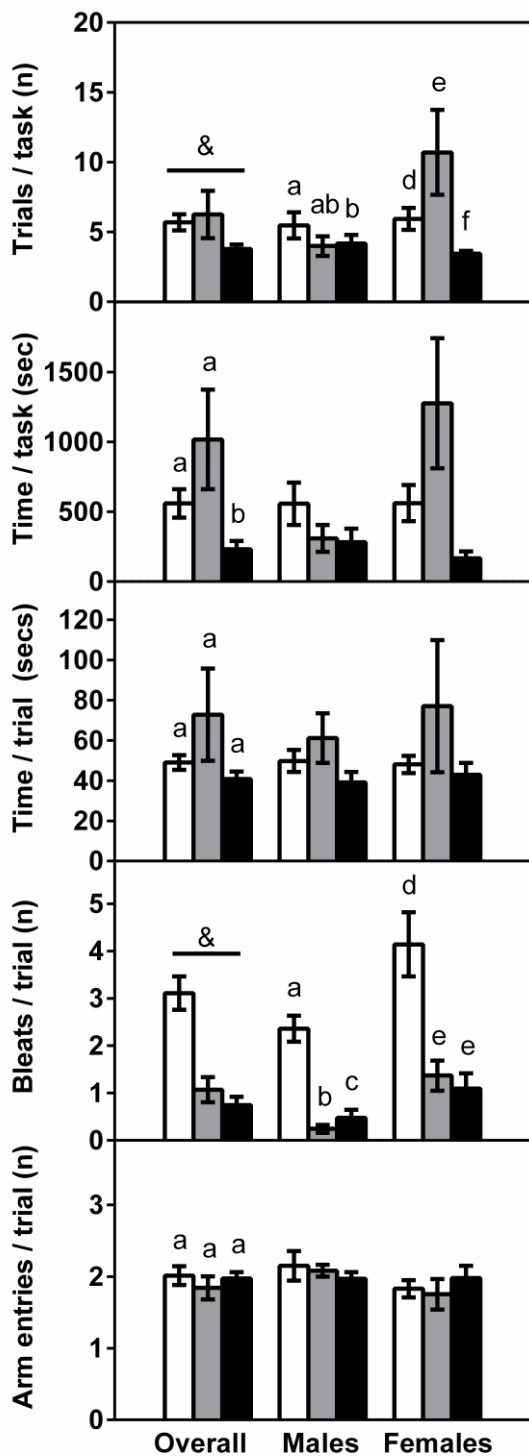
Fig. 3-4. Performance and behaviour in task R1 in naive 18 week-old sheep (18N, white bars), naive 40 week-old sheep (40N, grey bars) and experienced 40 week-old sheep (40E, black bars).

Comparisons between groups (18N vs. 40E), ages (18N vs. 40N) and experience (40N vs. 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group ($P < 0.05$, †), sex*experience ($P < 0.05$, &).
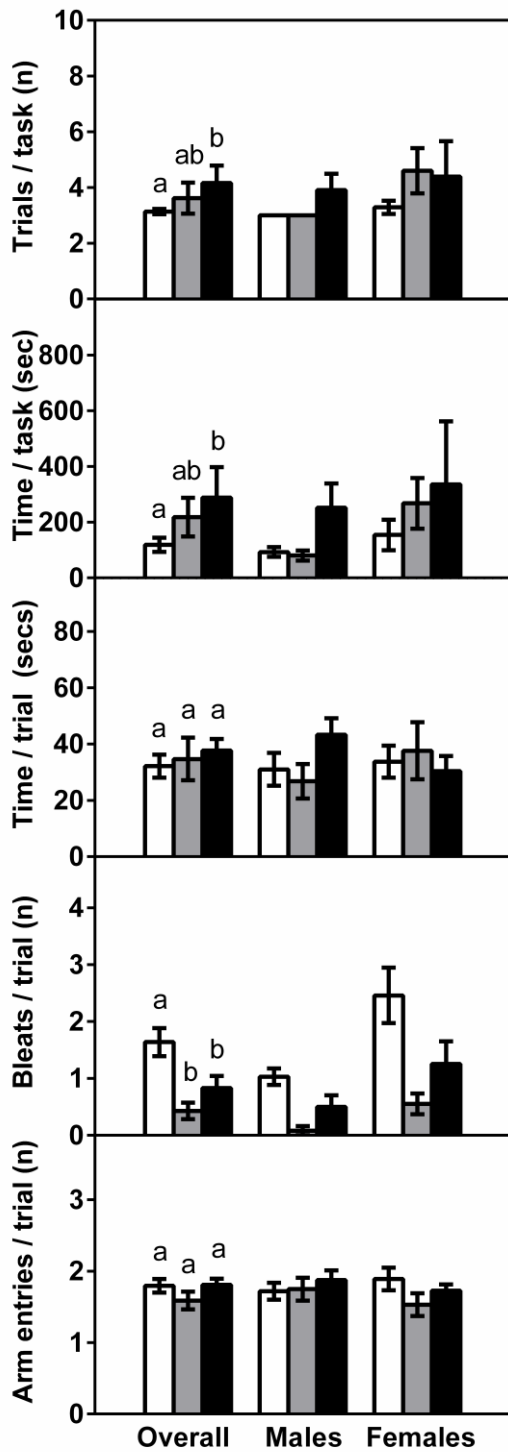
Fig. 3-5. Performance and behaviour in task M2 in naive 18 week-old sheep (18N, white bars), naive 40 week-old sheep (40N, grey bars) and experienced 40 week-old sheep (40E, black bars).

Comparisons between groups (18N vs. 40E), ages (18N vs. 40N) and experience (40N vs. 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group ($P < 0.05$, †), sex*experience ($P < 0.05$, &).
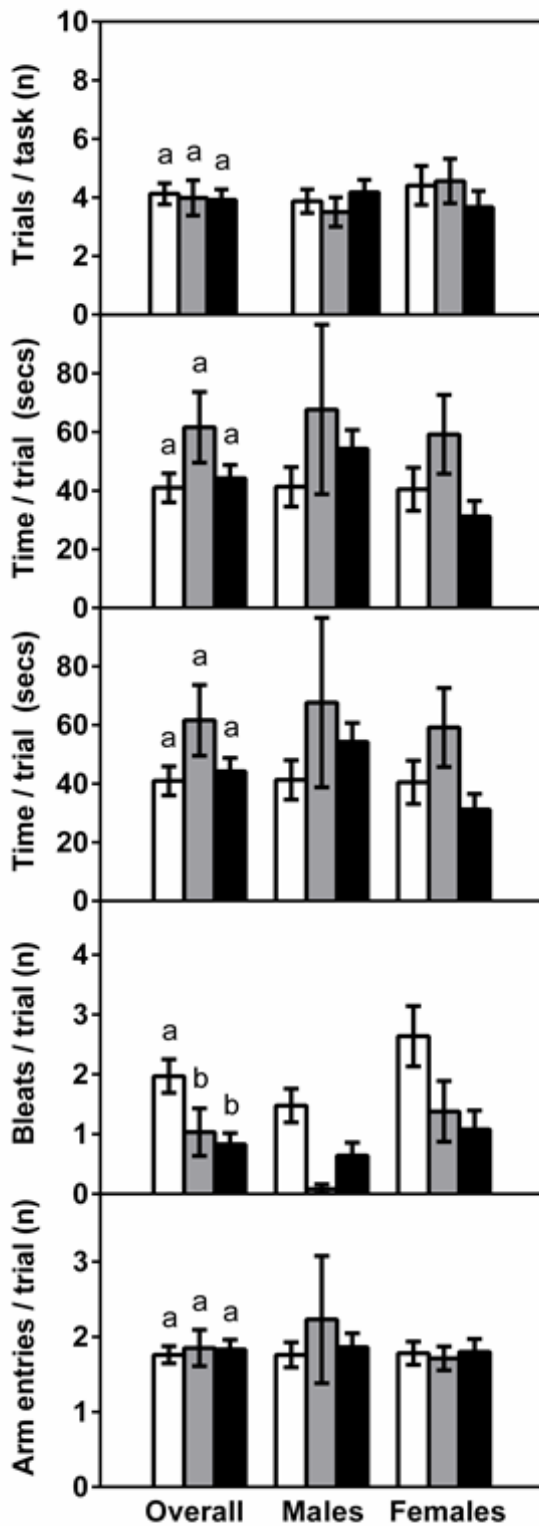
Fig. 3-6. Performance and behaviour in task R2 in naive 18 week-old sheep (18N, white bars), naive 40 week-old sheep (40N, grey bars) and experienced 40 week-old sheep (40E, black bars).

Comparisons between groups (18N vs. 40E), ages (18N vs. 40N) and experience (40N vs. 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group ($P < 0.05$, †), sex*experience ($P < 0.05$, &).

3.3.6. Outcomes in second reversal task (Task R2)

3.3.6.1. Effects of age and experience (18N vs 40E)

Group and sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial for task R2 in comparisons of 18N and 40E sheep (each $P > 0.1$, Fig. 3-6). Younger naive sheep (18N) bleated more frequently than older experienced sheep (40E, $P = 0.007$, Fig. 3-6) and females bleated more than males in task R2 ($P = 0.03$).

3.3.6.2. Effects of age in naive learners (18N vs 40N)

Age and sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial for task R2 in comparisons of 18N and 40N sheep (each $P > 0.1$, Fig. 3-6). Younger naive sheep (18N) bleated more frequently than older naive sheep (40N, $P = 0.03$, Fig. 3-6) and bleat frequency did not differ between sexes for task R2 ($P > 0.1$).

3.3.6.3. Effects of experience in 40-week old sheep (40N vs 40E)

Experience and sex did not affect any outcomes in task R2 (number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial and arm entries per trial) in comparisons of 40N and 40E sheep (each $P > 0.1$, Fig. 3-6).

3.3.7. Exit method

The exit method used for the criterion trials (final three successful trials) of each task did not differ between groups, age, experience or sex. In task R1, 25 sheep (27%) sheep that exited directly via the open arm (direct exit method) to complete the task, and 67 (73%) sheep entered the closed arm first before exiting via the open arm (indirect exit method) in order to solve the task. Exit method in Task L predicted exit method in Task M1 only (χ^2 (2) = 30.0, P < 0.001). Exit method in Task R1 predicted exit method in Tasks M2 (χ^2 (1) = 16.8, P < 0.001) and R2 (χ^2 (1) = 8.78, P = 0.03). Exit method in Task R1 was therefore used as the factor for subsequent analyses of effects of exit method on maze performance.

3.3.8. Effects of exit method

3.3.8.1. 18N

Differences between sheep using direct and indirect exit methods did not vary with sex within any group. In naive 18 week-old sheep, sheep that took a direct exit route in criterion trials of task R1 required similar numbers of trials and total time to complete tasks R1 and M2 (each P > 0.1), but required more trials to complete task R2 (P = 0.02) with a similar trend for total time to complete task R2 (P = 0.07) compared to 18N sheep that exited indirectly in task R1 (Fig. 3-7). Also in naive 18-week old sheep, those that took a direct exit method in task R1 had faster average times per criterion trial in tasks R1 (P < 0.001) and M2 (P = 0.03) but not task R2 (P > 0.2), and bleated less often in task R1 (P = 0.012) but not later tasks, compared to sheep that exited indirectly in task R1 (Fig. 3-7).

3.3.8.2. 40N

Effects of exit method were only examined in 40N females owing to the small sample size of 40N males. In female naive 40 week-old sheep, those that took a direct exit route in criterion trials of task R1 required more trials and time to complete task R1 than those who exited indirectly (P = 0.001 and P = 0.009 respectively, Fig. 3-7). Similar effects and trends were seen for higher total trial number (P = 0.08) and total time (P = 0.02) in task R2 for sheep that used a direct exit method in task R1, whilst performance in task M2 did not differ between these groups (Fig. 3-7). Average time per criterion trial and bleat frequency in tasks R1, M2 and R2 did not differ between exit methods for female naive 40 week-old sheep (Fig. 3-7).

3.3.8.3. 40E

In experienced 40 week-old sheep, total time and number of trials required to solve tasks R1, M2 and R2 did not differ between those that took direct cf. indirect exit routes in criterion trials of task R1 (each P > 0.1, Fig. 3-7). Sheep in the 40E group that used a direct exit method in task R1 took less time per criterion trial (P = 0.005) and bleated less (P = 0.001) in task R1, but not in subsequent tasks (each P > 0.3), than those that used an indirect exit method (Fig. 3-7).
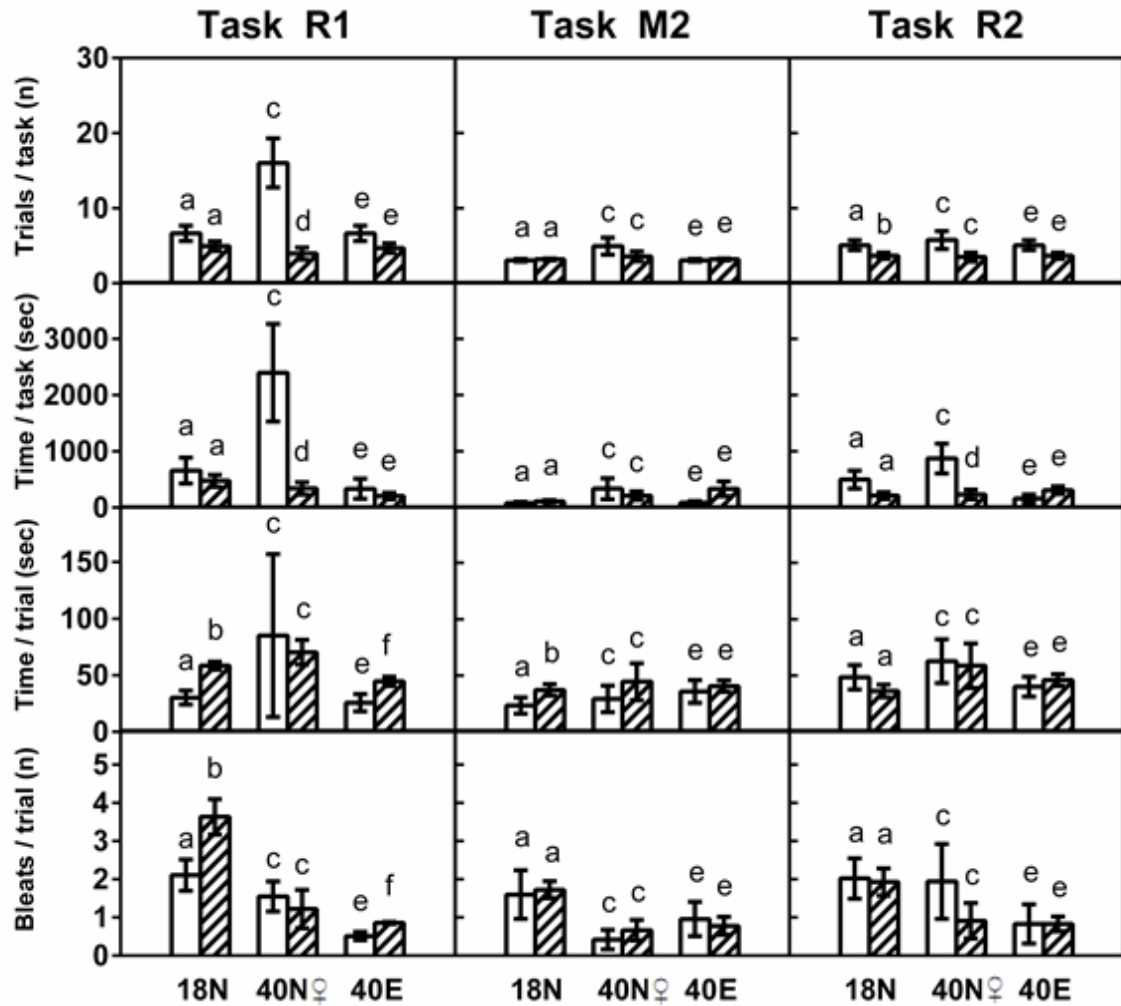
Fig. 3-7. Performance and behaviour in task R1, task M2 and task R2 in sheep that used a direct (plain bars) or indirect (striped bars) exit method in task R1.

Outcomes were compared within each group (18N, 40N and 40E) between sheep using a direct and indirect exit method in criterion trials of task R1, and are shown as the mean ± SEM for each exit method group, for males and females combined in 18N and 40E sheep, and in females only for 40N sheep. Bars with the same letter within each group do not differ.

3.4. Discussion

There are two novel findings from this study. Firstly, we have shown that young sheep are capable of retaining knowledge of complex tasks when re-tested 22 weeks after initially learning these tasks, as experienced 40 week olds (40E) completed the majority of tasks more quickly than naive 40 week old sheep (40N) not previously exposed to the tasks. This suggests sheep may be useful for examining long term memory, and indicates the necessity of controlling for effects of prior learning in this species. Secondly, whilst the combination of age and experience improved learning outcomes, with the same sheep performing better as experienced 40 week-olds than in their first exposure to tests as 18 week-olds, age alone affected mostly behavioural responses. Naive sheep bleated more at 18 weeks of age than at 40 weeks of age but these groups differed in learning outcomes only in females, and only in the first reversal task.

Poorer performance in the reversal versus the learning and memory tasks in the present study was unsurprising as reversal learning is a comparatively demanding task compared to simple maze navigation. Reversal learning in Y-maze tasks requires initially learning a rule (e.g. which arm to enter to reach the open gate in Task L and M1 of the present study) and then reversing the use of this rule (e.g. entering the previously unrewarded maze arm in Task R1 and R2 in the present study). In rats reversal learning requires greater use of working memory and more complex attentional processes than simple spatial learning [reviewed in 340]. While reversal tasks are rapidly acquired by sheep in T-maze experiments, a higher proportion of sheep fail reversal tasks in early trials compared to more readily learned spatial learning tasks, further illustrating the greater difficulty and cognitive demand of this type of task [303, 308]. As such, reversal learning measures one aspect of executive function. In the rat, reversal learning activates the orbitofrontal cortex function in areas distinct from those

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associated with complex executive functions such as intra- and extra-dimensional set-shifting [341], and this task may thus provide a measure of function of this area in sheep.

Reversal learning in the present study in sheep does, however, involve differences in behaviours and stimuli than those experienced in rodents during maze learning. In rodent and primate studies, poor performance in reversal trials has been interpreted as unnecessary perseverance at an action that is no longer rewarded [340, 342], particularly as rats have a tendency to explore arms not visited in previous trials [305]. In contrast, sheep are reluctant to enter lanes that were not rewarded during training in maze tests [303], and the majority of sheep in the present study made very few arm entries per trial, generally waiting in the closed arm within sight of flock-mates during failed trials. Furthermore, sheep find social isolation stressful [320], and reversal learning in the present study required sheep to move away from visible flock-mates if they initially entered the incorrect arm of the maze, therefore involving two types of aversive stimuli. Stress probably magnifies group differences in inherent learning capacity during reversal tasks, since stress decreases the likelihood of approaching aversive stimuli in sheep [343], and 18N and 40E sheep in the present study vocalised more often in the first reversal task than in learning and memory tasks, indicating they may have found this task stressful. Assessing reversal learning in sheep therefore requires assessing their ability to successfully exit the maze to reach the reward, rather than proportions of time in correct and incorrect arms of the maze, as reported in many rodent studies where the reward is within the maze itself [e.g. 344, 345].

Learning performance also differed with age and experience in the present study. Older experienced sheep (40E) learnt the first reversal task (R1) more quickly and required fewer trials than young naive sheep (18N). This suggests the 40E sheep were recalling executive

function skills in Task R1 that they learnt at 18 weeks of age. We doubt faster learning speed in 40E sheep compared to their performance at 18 weeks is solely an effect of habituation to human handling, as sheep were handled regularly from birth, although habituation to the maze test itself may have reduced stress and improved learning. While it has been established previously that sheep can identify and recall the faces of individuals for over two years [310] retention of more complex tasks over long periods has not previously been demonstrated. Our results suggest executive function skills learned at 18 weeks are remembered for at least five months after the initial learning in sheep.

Age alone did not affect learning performance in comparisons between naive 40 week-old (40N) and 18 week-old sheep (18N), but bleat frequency was higher in the younger group in all tasks except the initial learning task. Johnson and colleagues reported that 14 week-old lambs learned more quickly than 9 week-olds regardless of sex [308]. Since the lambs in that study were all pre-pubertal in age, these age-related improvements may have been a consequence of pre-adulthood brain maturation resulting in gain of function in this younger group [308]. Both ages in the present study were older than the animals tested by Johnson, and our findings suggest learning does not differ between these older ages in sheep.

The sex-specific effects of age and experience on learning in sheep in the present study, with fewer trials required to solve the first reversal task in 18N females than 40N females, and in 40E than 18N or 40N females, but not males, may be related to effects of sex steroids, particularly after puberty. Merino ewes enter puberty between 23-43 weeks of age, at an average age of 31 weeks [339], and therefore 18N and 40N females were exposed to different hormonal environments during their initial learning. Oestrus, but not dioestrus, female rats have impaired performance in Morris Water Maze hidden platform tasks compared to males,

and this seems to be a consequence of estrogens interfering with task acquisition rather than recall [313]. This may also explain why in females, 40E sheep that had already learned this reversal task performed better than 40N sheep that were learning the task for the first time. Age and sex differences may also be mediated by emotional reactivity, which is reduced by testosterone in sheep [311, 312]. Lower emotional reactivity due to testosterone in rams would decrease their stress responses and protect their capacity to overcome aversive stimuli [343] and hence will improve reversal performance within this maze design. Our observation of similar bleat frequencies in females in both 40-week old groups, suggests that differences in emotional reactivity do not explain sex-specific effects of experience in learning outcomes in the present study, however. These conclusions are limited by small numbers in the naive 40 week-old group, particularly the males, however, and effects of sex and its interactions with prior learning and age need to be confirmed in subsequent larger studies. Generation of the 40N group was opportunistic with a relatively small sample size, due to the timing of maze construction after part of the flock had passed 18 weeks of age. In addition, testing at different phases of the oestrus cycle may provide clearer information about the probable effects of sex steroids on learning in sheep, as previous studies of hormonal status have examined changes in emotional reactivity [311, 312] but not learning.

In addition to effects of group, experience, age and sex on maze performance, we also observed differences according to the exit method sheep used to leave the maze during the criterion trials. Because we did not observe sex or age differences in numbers of arm entries per trial in any memory or reversal tasks throughout the protocol in these sheep, we suggest arm entries are not an indicator of general activity in sheep, unlike the rat [305]. Surprisingly, sheep that learned to turn around at dead ends in the first reversal task (indirect exit method) were quicker to learn reversal tasks (less total time and trials required in task R1 in 40-week-

old naive females and fewer trials required in task R2 in naive 18 week-old sheep) than those learnt to directly enter the open arm of the maze. Consistent with a longer path length to exit in the indirect group, time per criterion trial was greater in 18N and 40E indirect exit method sheep than in those who used a direct exit method for the first reversal task. Behaviour also differed between direct and indirect learners in the first reversal task, when sheep using an indirect exit method bleated more often than those who exited directly, in the 18N and 40E groups. Interestingly, this difference in bleat frequency was not observed in 40N sheep in task R1 or task R2 or in 18N sheep in task R2, where indirect learners completed the task faster than direct learners. This suggests that different exit methods might reflect temperament differences such as lower flocking instinct and hence a greater willingness to leave sight of flock mates at the closed gate, and/or cognitive differences such as superior executive function and hence better reversal learning, and that temperament differences might mask differences in cognitive outcomes under conditions of stress.

In summary, effects of age and experience on learning performance in maze tasks vary between sexes in healthy sheep. Our data emphasises the importance of studying both sexes, and with gonadally-intact animals, if the intention is to draw comparisons to the human condition, particularly after puberty, and to control for prior learning and handling in studies of behavioural outcomes in the sheep. These results provide comparison data for studies of neurodevelopment in the sheep, as well as longitudinal information that will allow for improved experimental design.

3.5. Acknowledgements

Chapter 4 - Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behaviour in sheep in an age- and sex-specific manner.

4.1. Preamble

Chapter 4 describes behavioural outcomes in PR and control sheep, and compares differences between treatments within each sex at 18 and 40 weeks of age. I was involved in animal husbandry from birth, recording birth weight and postnatal growth measures. I conducted all behavioural tests in this chapter, as data recorder and observer, analysed the data, wrote the manuscript and contributed significantly to all redrafts and edits to the manuscript.

This chapter has been published in Physiology and Behavior, with myself as first author, and text and figures are presented unaltered (Appendix 3):

Hunter, D. S**.**, Hazel, S.J., Kind, K.L., Liu, H., Marini, D. Giles, L.C., De Blasio, M. J., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2015). "Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behaviour in sheep in an age- and sex-specific manner." Physiology and Behavior 152(Pt A): 1-10.

This was the third paper describing this PR cohort, with papers describing immunological [249] and metabolic [248] outcomes already published, with myself as a co-author.

4.2. Statement of Authorship

Title of Paper	Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behaviour in sheep in an age- and sex-specific manner.
Publication Status	Published
Publication Details	Hunter, D. S., et al. (2015). "Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behaviour in sheep in an age- and sex-specific manner." Physiology and Behavior **152**(Pt A): 1-10.

Principal Author

Name of Principal Author (Candidate)	Damien Hunter		
Contribution to the Paper	Performed experiments, performed analyses on all data, interpreted data, wrote manuscript and created all figures		
Overall percentage (%)	50.00%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	6/10/15

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Susan J. Hazel		
Contribution to the Paper	Assisted with experiments, interpreted data, edited manuscript		
Signature		Date	6/10/15

Name of Co-Author	Karen L. Kind		
Contribution to the Paper	Assisted with experiments, interpreted data, edited manuscript		
Signature		Date	28/9/15

Name of Co-Author	Hong Liu		
Contribution to the Paper	Helped set up sheep cohort, assisted with experiments, helped edit manuscript		
Signature		Date	2/10/2015

Name of Co-Author	Danila Marini		
Contribution to the Paper	Assisted with experiments, helped edit manuscript		
Signature		Date	29/9/15

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Name of Co-Author	Lynne C. Giles		
Contribution to the Paper	Design and assistance with statistical analysis, helped edit manuscript		
Signature		Date	29/9/15

Name of Co-Author	Miles J. De Blasio		
Contribution to the Paper	Helped establish sheep cohort and edit manuscript		
Signature		Date	29/9/15

Contribution to the Paper	Assisted with data interpretation, helped edit manuscript		
Signature		Date	1/10/2015

Name of Co-Author	Julie A. Owens	
Contribution to the Paper	Supervised development of cohort and research plan, helped edit manuscript	
Signature		Date	29 9 15

Name of Co-Author	Kathy Gatford		
Contribution to the Paper	Supervised development of cohort and research plan, assisted with experiments, helped interpret data, helped write manuscript, acted as corresponding author		
Signature		Date	01 October 2015

4.3. Abstract

Intrauterine growth restriction and slow neonatal growth in humans are each associated with poorer learning, memory and cognitive flexibility in childhood and adulthood. The relative contributions of pre- and post-natal growth to cognitive outcomes are unclear, however. We therefore compared performance in learning, memory and reversal tasks using a modified Y-maze at 18 and 40 weeks of age in offspring of placentally-restricted (PR: 10 M, 13 F) and control (23 M, 17 F) ovine pregnancies. We also investigated relationships between size at birth, neonatal growth rates and cognitive outcomes. PR had limited effects on cognitive outcomes, with PR males requiring more trials to solve the initial learning task than controls ($P = 0.04$) but faster completion of reversal tasks in both sexes at 18 weeks of age. In males, neonatal growth rate correlated inversely with numbers of trials and total time required to solve memory tasks at 40 weeks of age. In females, bleat frequency in the first reversal task at 18 weeks of age correlated positively with birth weight ($r = 0.734$, $P < 0.05$) and neonatal growth rate ($r = 0.563$, $P < 0.05$). We conclude that PR induces limited effects on cognitive outcomes in sheep, with some evidence of impaired learning in males, but little effect on memory or cognitive flexibility in either sex. Rapid neonatal growth predicted improved memory task performance in males, suggesting that strategies to optimise neonatal growth may have long-term cognitive benefits but that these may be sex-specific.

4.4. Introduction

Intrauterine growth-restriction (IUGR) is associated with impaired neurodevelopment, with life-long consequences for cognitive function [140]. Small size at birth corrected for gestational age (SGA, size at birth below the 10th centile for gestational age) is often used as a surrogate marker of IUGR in humans when repeated measures of fetal growth are not available. Children born small for gestational age have, on average, IQs 6-11 points lower than their peers, poorer language skills, impaired spatial learning and memory, and higher incidences of behavioural and attentional problems [98, 103, 134, 346]. These deficits have functional consequences, as SGA is also associated with poorer academic outcomes in children [347] and adults [143, 348].

The effects of IUGR on neurodevelopmental outcomes may be ameliorated by catch-up growth in early life, suggesting an important role for post-natal growth. Catch-up growth following IUGR is common across species, including humans, where it occurs mostly during the first two months after birth [349, 350]. Catch-up growth is associated with better visuomotor and problem solving skills, intelligence quotients, IQ and academic performance in SGA children, starting from 18 months and continuing into adulthood, compared to those with failure of catch up growth [98, 103, 151, 153]. SGA children do not always catch up in head circumference compared to peers born at an appropriate weight for their gestational age (AGA) [103, 149, 351, 352], even if they are among the 86% of SGA children that catch up in height and weight [99, 101]. Head circumference is an important surrogate marker for neurodevelopment, because it is strongly correlated with IQ, language, visuomotor and neurodevelopmental scores in SGA children [149, 153], a relationship that strengthens with age [149].

Disentangling the influences of fetal and postnatal growth on neurodevelopmental outcomes is complicated by the common comorbidity between IUGR and preterm birth (birth before 37 completed weeks of gestation) in humans, both of which separately impair neurodevelopment and learning outcomes [98, 353], with compounding effects in combination [150, 354]. Human studies can also be confounded by shared prenatal and postnatal environments, and complicated by variation due to genetics and environmental factors. For example, lower socioeconomic status is associated with increased risk of SGA, a reduction in postnatal catch-up growth [107, 355, 356], and poorer cognition and executive function in both healthy [168], and SGA children [98, 103, 347, 354]. Therefore, an animal model of fetal growth restriction, with IUGR offspring born at term, is required to further investigate the influence of fetal and neonatal growth on neurodevelopmental outcomes.

Sheep have a similar ontogeny of neurodevelopment to humans with neurogenesis, oligodendrocyte development and myelination commencing prenatally in both species [173, 296]. Importantly, sheep demonstrate higher cognitive processing, including executive functions and problem solving [303, 308], and learning, memory and cognitive flexibility can be tested in this species using maze tasks [192, 211, 243, 304, 308]. Impaired placentation, which reduces the supply of nutrients and oxygen reaching the fetus, is a major cause of IUGR in developed countries [76]. Restriction of placental growth (PR) in sheep, by surgical removal of placental attachment sites prior to pregnancy, reduces nutrient and oxygen supply and is associated with similar fetal outcomes as occurs in human IUGR, including endocrine adaptations [3, 204, 239, 244]. PR results in delivery of full-term lambs with reductions in average birth weight of 20-31% [205, 249]. PR lambs also undergo neonatal catch-up growth, with incomplete catch-up of skull width [273, 277], consistent with growth patterns in IUGR infants [246, 249, 273, 277]. This model allows effects of IUGR to be tested independent of confounders such as preterm birth and environmental differences, since all individuals share a common postnatal environment. We therefore tested the hypothesis that in adolescent and

adult sheep, PR, low birth weight and slow neonatal growth each impair learning, memory and cognitive flexibility.

4.5. Methods

All procedures were jointly approved by the University of Adelaide Animal Ethics Committee (M-2009-145 and M-2011-055) and the SA Pathology Animal Ethics Committee (135a/09) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* [324].

4.5.1. Animals

Generation and management of this cohort has been described previously [249]. Briefly, placental growth and function of primiparous Merino x Border Leicester ewes was restricted by surgical removal of all but four visible endometrial placental attachment sites (caruncles) from each uterine horn [203, 325] at least 10 weeks prior to timed mating to Merino rams. Control ewes were un-operated and were also included in the timed mating program. Pregnant control (CON) and PR ewes were housed indoors from day 110 of gestation until their spontaneously-born lambs were weaned at 13 weeks of age. Groups of lambs were born at five-week intervals between July 2010 and December 2012. Ewes were fed 1 kg Rumevite pellets daily (Ridley AgriProducts, Melbourne, Australia), with *ad libitum* access to lucerne chaff and water. Gestational ages in days (GA), birth weight (BW) and litter sizes were recorded. After weaning, progeny were housed in outside paddocks in same sex groups of similar ages and fed 0.5 kg Rumevite pellets/sheep daily, with *ad libitum* access to oaten hay, pasture and water. Progeny were handled frequently from birth, with measures of weight recorded every second day from birth to 16 days of age to calculate fractional growth rate for weight (FGR)[272], followed by weekly weighing until weaning. All animals were fed daily

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by an animal technician, providing frequent human contact and ensuring lambs were habituated to humans.

4.5.2. Learning evaluation

Maze tests were performed at 18 and 40 weeks of age as described previously for control animals [304] using a protocol modified from Erhard et al.[211] and Hernandez et al. [192]. Here we report outcomes from animals tested at 18 weeks of age and retested at 40 weeks of age; consisting of 40 control progeny (1 male and 1 female from singleton births, 22 male and 16 female from multiple births) and 23 PR progeny (5 male and 10 female from singleton births, 5 male and 3 female from multiple births).

Briefly, the test protocol consisted of 3-5 days of testing [304]. The first day commenced with a habituation task, in which sheep had five trials to exit the maze through either of the open gates at their own leisure, allowing them to habituate to the human handling (guidance from handler to the start position at the start of each trial and presence of recorder behind start position), the maze itself and maze protocols (travelling from the start position when start gates were opened through the diamond maze to an open gate). The gate most frequently exited in this task was recorded as their preferred side. Time was not recorded for this task, however the majority of sheep exited the maze in under 30 seconds for each trial. During all behavioural testing two experimenters were involved in the protocol, which consisted of a series of five tasks, with progression to subsequent tasks requiring successful completion of earlier tasks (Supplementary Fig. 4-1). On day 1, sheep first completed one guided run on each side of the maze (handler guiding sheep first to the closed gate and then back around diamond maze to the open gate). For subsequent tests, the recorder was positioned in a set position behind the maze entrance, with a clear view of the entire maze; and the handler moved the sheep being tested to the starting pen at the beginning of each trial, and remained out of sight during the entire trial. The guided runs were followed by a learning task, in which

the sheep were required to exit the maze only through their preferred side (Task L). On day 2, sheep first performed a memory task (Task M1) which involved repetition of task L from the previous day. This was followed by a reversal task, requiring completion of the maze with the open gate switched to the non-preferred side (Task R1). On day 3, the sheep performed a memory task (Task M2); repeating task R1 with the gate on the non-preferred side, and then the open gate was switched back to the preferred side for the final reversal task (Task R2).

The criterion that had to be met to complete each task was three consecutive correct exits from the maze within either 6 trials (Task L) or 10 trials (Tasks M1, R1, M2 and R2), with each trial completed within three minutes, similar to previously published definitions [192, 304]. In order to complete the task and exit the maze in three consecutive trials within three minutes, sheep need to overcome one or both of two aversions, particularly for reversal tasks, which are the most challenging in the series and require the most trials before success (Fig. 4-1). For a sheep to succeed in the task by entering the maze arm with the open gate on the reversal task, also referred to as direct exit method [304], the sheep needs to overcome its side preference. In the initial side-preference task, 78% of 18 week old and 74% of 40 week old sheep entered their preferred side in >80% of trials, consistent with strong side preference in this species, particularly in lambs [326]. Alternatively, sheep can exit via an indirect route [304], by reversing when they come to the closed gate at the end of the incorrect arm - this requires the sheep to overcome flocking instinct and move away from sight of flockmates. The reward for solving the maze was access to the reward pen for 10 s, allowing access to flock-mates in the neighbouring pen and a food reward. The only penalty for not solving the maze was the inability to leave the maze during that trial. Sheep that failed a trial (>3 minutes in maze), were then steered through the correct exit to the reward pen, where they stayed for 10 s before the next run. Successfully completing the tasks for each day resulted in graduation to the next day of testing in the sequence, whereas failure to complete tasks M1, R1, M2 or R2 resulted in the sequence being repeated, with a maximum of five days permitted to

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complete the sequence (Supplementary Fig. 4-1). Cognitive measures included total time and number of trials taken to solve each task and average time per criterion trial (i.e. the final three successful trials of each task). Behavioural measures included number of bleats [as a measure of stress, eg. 357] and maze arm entries per trial, indicating the number of times the sheep entered each maze arm.

4.5.3. Statistical analysis

Effects of treatment (control or PR), sex and litter size (singleton or multiple birth), and interactions between these variables on gestational age, size at birth and neonatal growth were analysed using generalized linear mixed models, including the mother as a random factor. Effects of treatment, sex, litter size and age on maze task outcomes were analysed for data within each task separately using generalised linear mixed models, including the mother as a random factor, with only main effects for litter size, and recognizing the multiple measures on each individual sheep, with post-hoc Bonferroni comparisons used to compare differences between each treatment, sex or age. Continuously distributed variables (i.e. time and growth measures) were log-transformed prior to analysis to reduce skew and were analysed assuming a normal distribution and identity link, while variables that were counts of events (i.e. total trials per task) were analysed using a Poisson distribution with log link. Subgroup analyses were run when interactions were significant. Correlations between BW, GA and FGR were tested by multiple linear regression for continuously distributed variables, and Poisson regression for count variables. Ewe identity did not influence these correlations with continuously distributed variables and was therefore excluded from correlation analysis. Effect of treatment on litter size was analysed by χ^2-test. All analyses were carried out using SPSS 20.0 (IBM, Armonk, USA). Data are presented as mean ± SEM unless otherwise stated and statistical significance was accepted at $P < 0.05$.

Fig. 4-1. Proportion of control (white circles) and placentally restricted (PR, grey circles) sheep completing each task within a given number of trials at 18 weeks of age (A) and 40 weeks of age (B).

Task completion required three maze exits in $\leq$ 3 min. Trial 3 is the first possible trial in which each task can be completed, and the X axis for each task starts at trial number 3 with the five sequential tasks shown from left to right being the learning task (Task L), first memory task (Task M1), first reversal task (Task R1), second memory task (Task M2), and second reversal task (Task R2). Sheep were allowed to attempt each task up to 10 times on each day; vertical dashed lines indicate the transition between consecutive days of testing for some tasks where not all sheep completed the task on the first day of testing. The horizontal dotted line indicates 100% completion of a task.

A

Task completed (% of sheep)

Task L (number of trials)

Task M 1 (number of trials)

Task R 1 (number of trials)

Task M 2 (number of trials)

Task R 2 (number of trials)

B

Task completed (% of sheep)

Task L (number of trials)

Task M 1 (number of trials)

Task R 1 (number of trials)

Task M 2 (number of trials)

Task R 2 (number of trials)
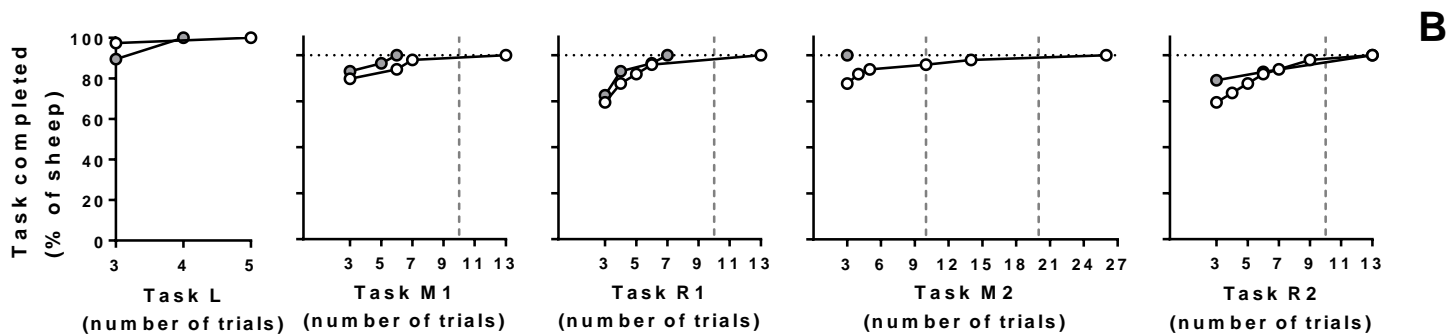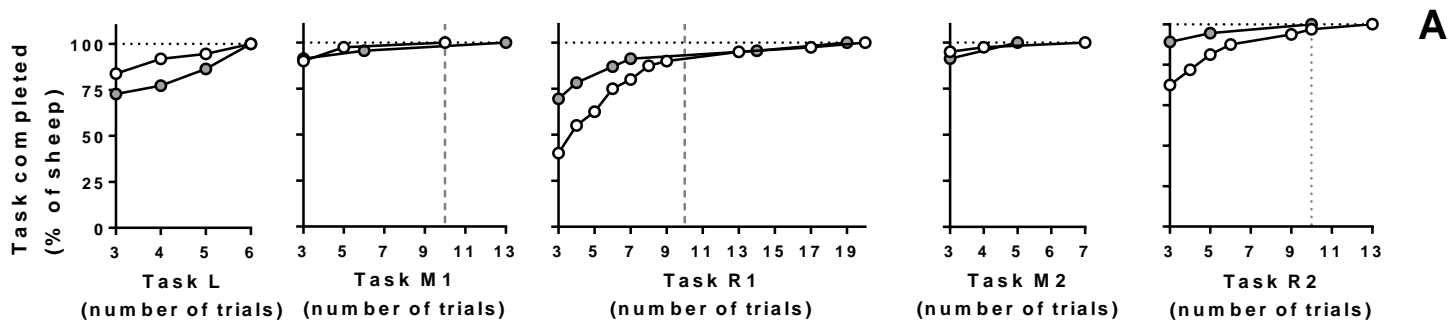
4.6. Results

4.6.1. Effects of PR on size at birth and neonatal growth

Overall, PR did not alter BW, FGR or GA, which overlapped between treatments (Fig. 4-2). PR did not alter skull width at birth ($P > 0.7$), but males had a larger skull width than female ($P = 0.019$), and singletons had a larger skull width than twins ($P = 0.027$). There were no effects of treatment, sex or litter size on skull length at birth (all $P > 0.1$). The greater proportion of twins in CON than PR adult offspring ($P < 0.001$) may have contributed to the lack of differences in size at birth and neonatal growth rates, however, PR did not alter BW (CON: 5.5 kg ± 0.5, PR: 4.3 kg ± 0.2, $P = 0.08$), skull width (CON: 6.4 ± 0.2 cm, PR: 6.4 ± 0.1 cm, $P > 0.9$), skull length (CON: 12.9 ± 0.4 cm, PR: 12.9 ± 0.2 cm, $P > 0.9$), FGR (CON: 7.0 ± 1.3 %/d, PR: 8.3, ± 0.6 %/d, $P > 0.3$, or GA (CON: 146.5 ± 0.8, PR: 145.7 ± 0.4d, $P > 0.3$) in singletons alone. Similarly PR did not alter these measures in twins. BW correlated positively with GA ($r = 0.440$, $P < 0.001$), and FGR correlated inversely with BW ($r = -0.515$, $P < 0.001$) but not with GA.

At 35 days of age, the period after catch-up growth has occurred in this model [273], weight, skull width and skull length did not differ between PR and CON sheep (all $P > 0.1$). Males had narrower ($P = 0.002$), but not longer ($P > 0.1$), skulls than females at 35 days old, and twins were smaller than singletons in weight ($P = 0.008$), but not skull measures (each $P > 0.05$).
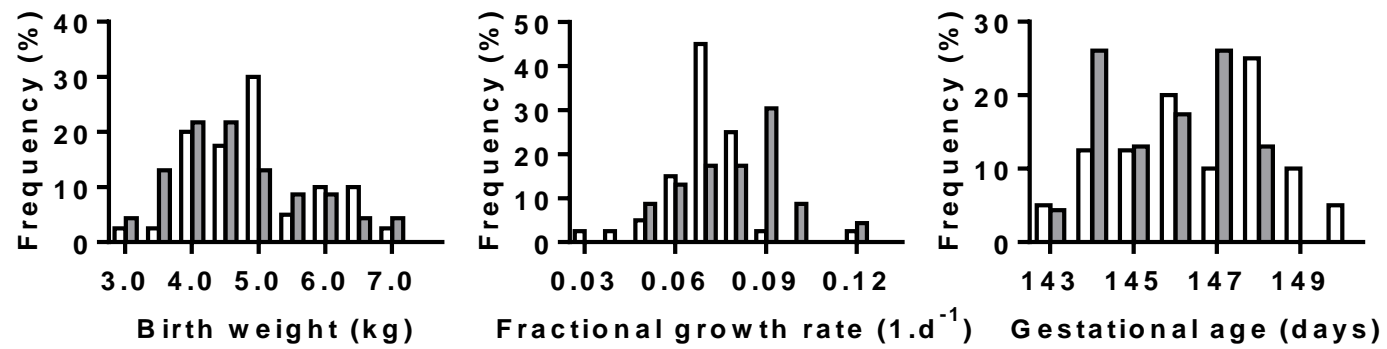
Fig. 4-2. Distribution of birth weight, fractional growth rate and gestational age in control (CON, n = 40, white bars) and placentally restricted (PR, n = 16, grey bars) sheep.

A: Birth weight (kg), B: Fractional growth rate for weight (1 day^{-1}), C: gestational age (days).

4.6.2. Effects of PR on cognitive and behavioural outcomes

4.6.2.1 Learning task (task L)

Effects of treatment and age on the number of trials required to solve task L differed between sexes (interactions: treatment*sex, P = 0.023, age*sex P = 0.02, Fig. 4-3) and did not differ between singleton- and multiple-birth sheep (P > 0.6). PR males required more trials than CON males (P = 0.04) and 18 week-old males required more trials than 40 week-old males (P = 0.001) to complete task L. In females, treatment did not affect the number of trials required to complete task L, and similar to the pattern in males, 18 week-old females required more trials than 40 week-old females in the two treatment groups combined (P = 0.04). The total time required to solve task L did not differ between males and females (P = 0.07) or between treatments, litter sizes or ages (each P > 0.1, Fig. 4-3). The average time in criterion trials did not differ between treatments, litter sizes, sexes or ages (each P > 0.1, Fig. 4-3). Younger sheep (18 week olds) bleated more (P = 0.004) and made fewer arm entries per trial (P = 0.002) than older sheep (40 week olds), and these outcomes did not differ between treatments, litter sizes or sexes (each P > 0.2, Fig. 4-3).

4.6.2.2. First memory task (task M1)

The number of trials required to solve task M1 differed between treatments and ages in a sex-specific manner (interaction: treatment*sex*age P = 0.03) and did not differ between litter size groups (P > 0.5, data not shown). In males, there was an interaction between treatment and age (P = 0.04), but treatment did not affect the number of trials required to solve task M1 in either 18 week-old males (P > 0.1) or 40 week-old males (P > 0.7). The number of trials required to solve task M1 did not differ between ages in either CON or PR males (each P > 0.1). In females, the number of trials required to solve task M1 did not differ with age or

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treatment. The total time required to solve task M1 and average time in criterion trials differed between treatments and ages in a sex-specific manner (interaction for total time: treatment*sex*age P = 0.03; interaction for time per criterion trial: treatment*sex*age P = 0.03), and did not differ between litter sizes (each P > 0.5, data not shown). Despite the overall interaction, when each sex was analysed separately effects of treatment and age on these outcomes were not different in either sex (all P > 0.6). Bleat frequency also differed between treatments and ages in a sex-specific manner (interaction: treatment*sex*age P = 0.005), and did not differ between litter sizes (P > 0.1, data not shown). In males, bleat frequency did not differ between treatments (P > 0.8) and was greater at 18 weeks of age than at 40 weeks of age (P = 0.02). In females, effect of treatment changed with age (interaction: treatment*age P = 0.008). Bleat frequency was not different in CON and PR females within either age group (each P > 0.3). Bleat frequency decreased from 18 to 40 weeks of age in CON females (P < 0.001), but not in PR females (P > 0.6). Arm entries per trial in task M1 did not differ between treatments, litter sizes, sexes or ages (each P > 0.2).

4.6.2.3. First reversal task (task R1)

The number of trials required to solve task R1 did not differ between treatments, sexes or litter sizes (all P > 0.2), and was greater at 18 than 40 weeks of age (P = 0.003, Fig. 4-4). The total time required to solve task R1 differed between treatments and sexes in an age-specific manner (interactions: treatment*age P = 0.009; sex*age P = 0.003, Fig. 4-4), and did not differ between singleton-born and multiple-born sheep overall (P = 0.07). In males, effects of treatment differed between ages (interaction: treatment*age P = 0.009), such that in 18 week-old males, control sheep required more time to solve task R1 than PR sheep (P = 0.02), but in 40 week-old males, treatment did not affect this outcome (P > 0.9). In females, both treatment and age affected the total time required to solve task R1. Overall, control females required

more time to solve task R1 than PR females (P = 0.03), and 18 week-old females required more time to solve task R1 than 40 week-old females (P < 0.001). Time per criterion trial in task R1 differed between treatments in an age- and sex-specific manner (interaction: treatment*sex*age P = 0.01, Fig. 4-4). In males, effects of treatment on average time in criterion trials changed with age (interaction: treatment*age P = 0.009). At 18 weeks of age, control males were slower in criterion trials than PR males (P = 0.02), and at 40 weeks of age, control and PR males completed criterion trials in similar times (P > 0.5, Fig. 4-4). In females, time in criterion trials was unaffected by treatment (P > 0.1), age (P = 0.05), or litter size (P = 0.09, data not shown). Bleats and arm entries per trial in task R1 did not differ between treatments, litter sizes or sex (each P > 0.1, Fig. 4-4). Bleat frequency was greater at 18 than 40 weeks of age (P < 0.001), but there was no age difference in arm entries per trial (P > 0.1).

4.6.2.4. Second memory task (task M2)

The number of trials required to solve task M2 did not differ between sexes or litter sizes (each P > 0.7), and effects of treatment varied with age (interaction: treatment*age, P = 0.04). At 18 weeks, the number of trials required to solve task M2 did not differ between treatments, sexes or litter size groups (each P > 0.9). At 40 weeks of age, the number of trials required to solve task M2 did not differ between treatments (P = 0.06), nor between sexes or litter size groups (each P > 0.2). CON sheep required more trials to solve task M2 at 40 than 18 weeks of age (P = 0.01), but the number of trials to solve task M2 did not change with age in PR sheep (P = 0.08). The total time required to solve task M2 did not differ between treatments or litter size groups (each P > 0.2), and effects of age differed between sexes (interaction: age*sex P = 0.02). In males, 40 week-olds took more time to solve task M2 than 18 week-olds overall (P = 0.04), and in females, this outcome did not change with age.

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Average time in criterion trials similarly did not differ between treatments or litter size groups (each P > 0.6), and effects of age differed between sexes (interaction: age*sex P = 0.04). In males, 40 week-olds had longer average time in criterion trials than 18 week-olds (P = 0.02), but time in criterion trials did not change with age in females (P = 0.08). Bleat frequency did not differ between treatments and litter size groups (each P > 0.7), was greater in females than males (P = 0.008) and greater at 18 than at 40 weeks of age (P < 0.001). Arm entries per trial did not differ between treatments and litter size groups (each P > 0.2), and differed between ages in a sex-specific manner (interaction: age*sex P = 0.03). Numbers of arm entries did not change with age in males (P > 0.1) or females (P = 0.06).

4.6.2.5. Second reversal task (task R2)

The number of trials required to complete task R2 and arm entries per trial in task R2 did not differ between treatments, sexes, litter size groups or ages (all P > 0.1). The total time required to complete task R2 was greater in CON than PR sheep (P < 0.05) and did not differ between sexes, ages or litter size groups. Average time per criterion trial did not differ between treatments, litter size groups or ages (all P > 0.1), and was greater in males than females (P < 0.05). Bleat frequency was greater at 18 than 40 weeks of age overall (P = 0.003) and did not differ between treatments, sexes and litter size groups (all P > 0.1).
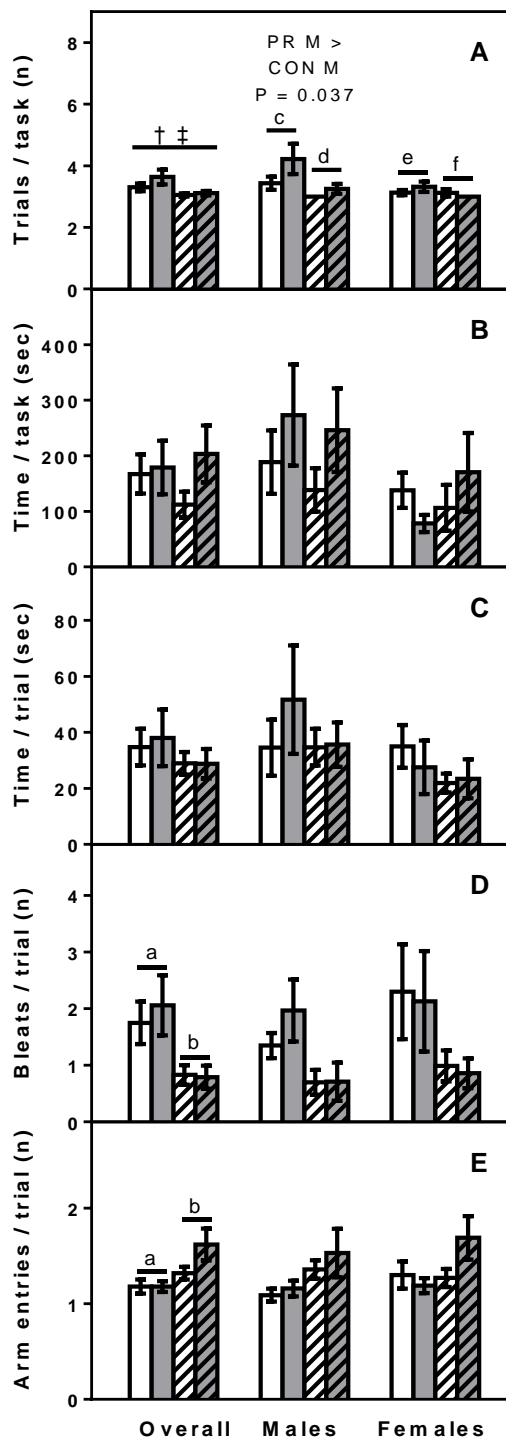
Fig. 4-3. Performance (A, B, C) and behaviour (D, E) in learning task (Task L) in control (white bars) and placentally-restricted (grey bars) sheep at 18 (unhashed bars) and 40 (hashed bars) weeks of age.

Performance measures consisted of the total number of trials (A) and total time (B) required to complete task L, and average time per trial in the three (consecutive successful exits) criterion trials for task L (C). Behavioural measures consisted of average bleat frequency per trial (D) and average number of arm entries made in each trial during task L. Comparisons between treatments and ages are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences are shown separately for males and females. Treatment effects are shown in text above the overall data or sex-specific data as appropriate. Different letters above bars indicate groups that differ overall (a, b), within males only (c, d) or within females only (e, f). Interactions are indicated as follows: sex*treatment ($P < 0.05$, †), sex*age ($P < 0.05$, ‡), and sex*treatment*age ($P < 0.05$, Φ).

Fig. 4-4. Performance (A, B, C) and behaviour (D, E) in first reversal task (Task R1) in control (white bars) and placentally-restricted (grey bars) sheep at 18 (unhashed bars) and 40 (hashed bars) weeks of age.

Performance measures consisted of the total number of trials (A) and total time (B) required to complete task L, and average time per trial in the three (consecutive successful exits) criterion trials for task L (C). Behavioural measures consisted of average bleat frequency per trial (D) and average number of arm entries made in each trial during task L. Comparisons between treatments and ages are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences are shown separately for males and females. Treatment effects are shown in text above the overall data or sex-specific data as appropriate. Different letters above bars indicate groups that differ overall (a, b), within males only (c, d) or within females only (e, f). Interactions are indicated as follows: sex*treatment ($P < 0.05$, †), sex*age ($P < 0.05$, ‡), and sex*treatment*age ($P < 0.05$, Φ).

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4.6.3. Relationships of cognitive outcomes with birth weight, neonatal growth rate and gestational age

Associations of cognitive outcomes with BW, neonatal FGR and GA in multiple linear regression analyses changed with age and differed between sexes. At 18 weeks, BW, FGR and GA rarely predicted cognitive outcomes (total trials, total time, time/criterion trial; Table 4-1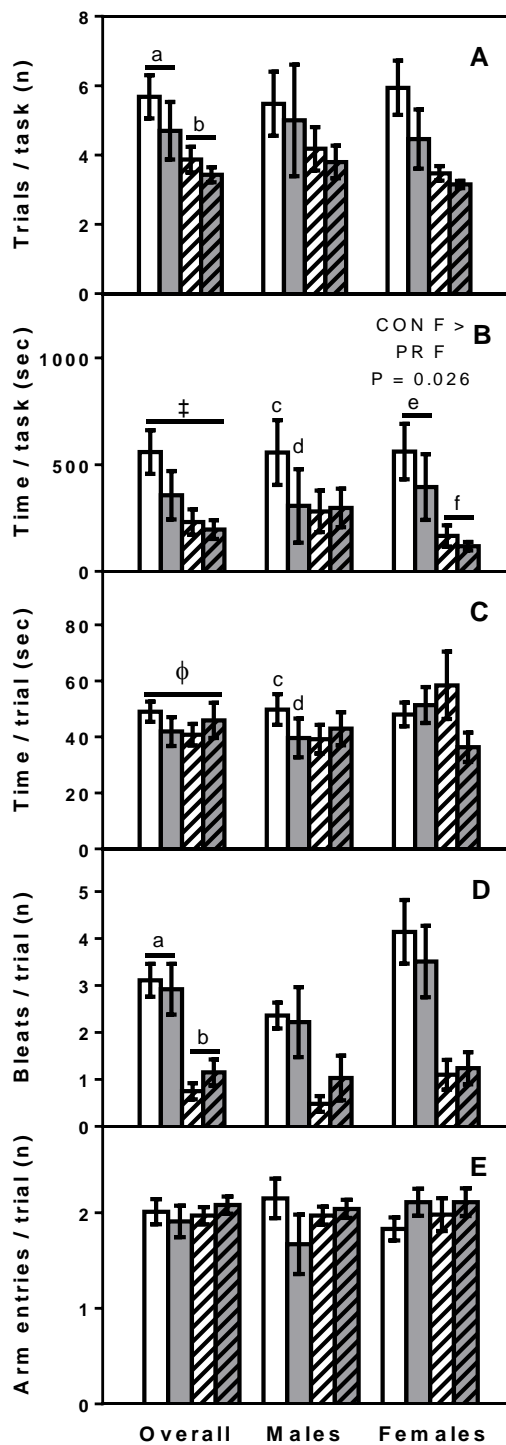). In females, time per criterion trial in task R1 correlated inversely with GA and positively with BW (Table 4-1). At 40 weeks, associations between cognitive outcomes, BW, FGR and GA differed between sexes (Table 4-2). In females, the total number of trials to solve Task R2 correlated positively with BW, whereas time per criterion trial in the same task correlated inversely with BW (Table 4-2). In contrast, female performance in the learning, memory and the first reversal task was not associated with BW, FGR and GA. In 40 week old males, time per criterion trial in Task L correlated positively with FGR, and the number of trials, and total time required to solve the first memory task (M1) correlated inversely with FGR (Table 4-2). Outcomes in memory or reversal tasks did not correlate with BW or GA in these older males, however.

4.6.4. Relationships of behaviour during maze tests with birth weight, neonatal growth and gestational age

Correlations between behaviour, BW, neonatal FGR and GA in multiple linear regression analyses changed with age and differed between sexes. In 18 week old males (Table 4-1), bleat frequency did not correlate with BW, FGR or GA. In 18 week old females, bleat frequency during task M1 correlated positively with GA ($P < 0.05$), and bleat frequency during task R1 correlated positively with BW and FGR (Table 4-1).

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Table 4-1. Associations of maze test outcomes at 18 weeks of age with gestational age, birth weight and neonatal growth.

Associations of count data (total trials) with each factor are presented as standardized beta, and associations of continuous data (total time, time / criterion trial, bleats/trial and arm entries/trial) with each factor are presented as partial R. Model r was obtained from models for continuous data but was not generated in models of count data. Significance of associations between outcomes and each factor are indicated by symbols: # $P < 0.1$, * $P < 0.05$, ** $P < 0.01$

Measure	Males				Females			
	Model r	BW	FGR	GA	Model r	BW	FGR	GA
Task L								
Total trials		-0.004	0.082	-0.086,		0.019	0.051	-0.014
Total time	0.292	0.042	0.271	-0.058	0.249	-0.025	0.119	0.213
Time / criterion trial	0.308	-0.020	0.255	0.043	0.362	0.036	0.190	0.323
Bleats / trial	0.391	0.087	0.221	-0.340	0.232	0.089	0.199	0.124
Arm entries / trial	0.508*	-0.101	0.378*	0.217	0.463	-0.241	-0.024	0.448*
Task M1								
Total trials		-0.033	0.157	-0.143		-0.237	-0.142	0.068
Total time	0.380	0.062	0.312	-0.225	0.312	-0.280	-0.194	0.212
Time / criterion trial	0.408	-0.023	0.332	-0.134	0.317	-0.123	-0.090	0.303
Bleats / trial	0.306	0.133	0.200	-0.278	0.499#	0.246	0.245	0.395*
Arm entries / trial	0.570*	-0.325#	0.324#	0.036	0.503#	-0.121	-0.100	0.484*
Task R1								
Total trials		0.293	0.202	-0.197		-0.107	-0.130	0.007
Total time	0.204	0.183	0.112	-0.181	0.148	-0.048	-0.126	-0.077
Time / criterion trial	0.314	-0.310	-0.203	0.185	0.551*	0.447*	0.080	-0.399*
Bleats / trial	0.111	-0.053	-0.111	0.045	0.751**	0.734*	0.563*	0.011
Arm entries / trial	0.548*	-0.517*	-0.156	0.439*	0.205	0.156	0.019	-0.122
Task M2								
Total trials		0.211	0.167	-0.149		-0.103	-0.080	-0.010
Total time	0.180	-0.139	-0.001	0.005	0.220	-0.210	-0.109	0.024
Time / criterion trial	0.239	-0.212	-0.172	0.196	0.249	-0.119	0.082	0.118
Bleats / trial	0.232	0.180	0.140	0.007	0.387	0.336#	0.194	0.093
Arm entries / trial	0.404	0.344#	-0.377*	-0.252	0.374	0.358#	0.328#	-0.017
Task R2								
Total trials		0.017	0.064	0.123		-0.064	-0.107	-0.072
Total time	0.223	0.063	0.153	-0.189	0.129	-0.053	-0.099	-0.085
Time / criterion trial	0.327	0.217	0.305	-0.227	0.108	-0.062	-0.079	0.070
Bleats / trial	0.085	0.075	0.039	-0.011	0.326	0.310	0.188	0.007
Arm entries / trial	0.226	-0.019	0.173	0.068	0.236	0.046	-0.089	0.105

Table 4-2. Associations of maze test outcomes at 40 weeks of age with gestational age, birth weight and neonatal growth.

Associations of count data (total trials) with each factor are presented as standardized beta, and associations of continuous data (total time, time / criterion trial, bleats/trial and arm entries/trial) with each factor are presented as partial *R*. Model *r* was obtained from models for continuous data but was not generated in models of count data. Significance of associations between outcomes and each factor are indicated by symbols: # P < 0.1, * P < 0.05, ** P < 0.01.

Measure	Males				Females			
	Model *r*	BW	FGR	GA	Model *r*	BW	FGR	GA
Task L								
Total trials		0.037	0.031	-0.025		-0.024	-0.029	-0.007
Total time	0.206	-0.150	0.039	0.127	0.317	-0.191	-0.012	-0.108
Time / criterion trial	0.492#	0.357#	0.428*	-0.058	0.252	-0.152	-0.110	-0.163
Bleats / trial	0.443	-0.372*	-0.400*	0.307#	0.288	0.088	0.093	0.247
Arm entries / trial	0.273	-0.002	0.188	0.130	0.292	-0.131	-0.098	-0.222
Task M1								
Total trials		-0.238#	-0.233*	0.197#		0.050	0.011	-0.072
Total time	0.444	-0.326#	-0.409*	0.329#	-0.096	-0.013	0.004	-0.080
Time / criterion trial	0.180	0.077	-0.013	0.087	0.146	-0.129	-0.041	0.073
Bleats / trial	0.230	-0.080	0.088	0.169	0.286	0.206	0.244	0.130
Arm entries / trial	0.289	0.112	0.277	0.080	0.228	-0.227	-0.149	0.044
Task R1								
Total trials		-0.114	-0.182	0.198		-0.008	-0.021	0.061
Total time	0.373	-0.181	-0.306	0.305	0.143	-0.087	-0.004	0.119
Time / criterion trial	0.311	0.233	0.298	-0.155	0.361	0.123	0.252	0.280
Bleats / trial	0.148	-0.079	0.020	0.127	0.439	0.365#	0.323#	0.184
Arm entries / trial	0.288	0.051	0.118	0.128	0.232	0.064	0.193	-0.045
Task M2								
Total trials		-0.091	-0.199#	0.177		-0.238	-0.206	0.020
Total time	0.351	-0.038	-0.281	0.202	0.206	-0.197	-0.180	0.03
Time / criterion trial	0.260	-0.071	-0.252	0.052	0.203	-0.162	-0.048	0.146
Bleats / trial	0.117	-0.018	0.060	0.075	0.330	0.075	0.123	0.299
Arm entries / trial	0.421	-0.202	-0.279	-0.157	0.359	-0.310	-0.142	-0.054
Task R2								
Total trials		-0.014	-0.024	0.055		0.498*	0.416#	0.108
Total time	0.137	-0.067	0.002	0.128	0.409	0.324#	0.337#	0.173
Time / criterion trial	0.069	-0.045	-0.048	-0.008	0.439	-0.395*	-0.184	0.291
Bleats / trial	0.199	-0.141	-0.154	0.001	0.358	0.219	0.095	0.192
Arm entries / trial	0.337	-0.161	0.066	-0.138	0.422	-0.358#	-0.233	0.322#

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In 18 week old males, numbers of arm entries in task R1 correlated inversely with BW and positively with GA (Table 4-1). In these young males, associations between numbers of arm entries and FGR differed between tasks, such that number of arm entries in task L correlated positively with FGR, whereas arm entries in task M2 correlated inversely with FGR. In 18 week old females, numbers of arm entries did not correlate with BW and FGR, whilst numbers of arm entries in tasks L and M1 correlated positively with GA (Table 4-1). Few associations were observed between behaviour during maze tests at 40 weeks and BW, FGR and GA. In 40 week old males, bleat frequency in task L, but not other tasks, correlated inversely with BW and FGR. In 40 week old females, bleat frequencies in maze tasks did not correlate with BW, FGR or GA (Table 4-2). Numbers of arm entries were not correlated with BW, FGR or GA in either sex or in any task in these older animals.

4.7. Discussion

In the present study, PR had limited effects on cognitive outcomes, whilst effects of size at birth and neonatal growth on cognitive and behavioural outcomes were sex- and age-specific. Because the present study was conducted in term-born animals raised in a common postnatal environment, the results of the present study are independent of confounders common in human studies. PR impaired initial learning performance, but did not impair measures of memory or reversal learning, and in fact we saw evidence of improved performance in reversal learning tasks in PR compared to control sheep. In 40 week-old males only, early postnatal growth rate positively predicted performance in the memory task conducted the day after the initial learning task, suggesting that early postnatal growth benefits learning retention in adult male sheep. In females, size at birth and early postnatal growth correlated positively and much more strongly with behavioural outcomes than cognitive outcomes in these tasks. The reversal tasks are the most challenging and stressful in the maze test series

[303, 304], and required more trials to complete than learning of memory tasks (Fig. 3-1). We hypothesise that altered emotional reactivity, including sex-specific changes to stress responses, might contribute to adverse effects of IUGR seen in humans undertaking more complex learning tasks requiring higher-order executive function than used here in sheep [98, 141, 153].

The effects of PR were sex specific and limited to impaired initial learning performance, but we did not see impaired memory or reversal learning in PR sheep. In males, PR sheep required more trials than CON to solve task L, the initial learning task in the maze series. Impaired initial learning after PR is consistent with results of human studies, where LBW (<2500 g) and SGA children (lowest 10th percentile of population birth weight) had poorer visuomotor skills compared to AGA [358], including poorer maze learning, evidenced by a greater proportion of incorrect arm entries in a radial maze and poorer spatial orientation, based on Kaufman-ABC results [137]. The fact that SGA children also have a greater incidence of learning deficits compared to AGA [135] suggests they also have learning difficulties in areas additional to the spatial learning we examined in task L of the present study. Our observation of learning deficits in males only in the PR sheep contrasts with results of human studies, where SGA is associated with learning impairments in both sexes, although there is some evidence from those studies in which sex-specific outcomes have been reported that SGA affects different cognitive outcomes more severely in different sexes [98, 139], whilst other studies report similar effects of prenatal growth on cognitive outcomes [134, 347]. Our cohort of surviving adult progeny included more twins in control than PR groups, probably reflecting a greater adverse effect of PR on survival in twins than singletons [273] as well as decreased twin conceptions. This led to considerable overlap in size at birth

between the groups, since twinning also restricts nutrient delivery to ovine fetuses [359], and may also have reduced differences between the groups.

Somewhat surprisingly, we saw some evidence of better performance in reversal tasks in PR than CON. In both R1 and R2, PR took less total time per task than CON; seen in task R1 overall at 18 weeks and also at 40 weeks but only in females; and overall (across ages and treatments) in task R2. We have reported previously that the reversal tasks, particularly task R1, are the most challenging for sheep within the series of maze tests performed in the present study [304]. It was therefore surprising that PR decreased the time required to solve this reversal learning task, because in humans SGA children and adults have lower test performance on measures of executive function than AGA individuals [98, 141]. SGA children also show greater perseverative errors than AGA controls in the Wisconsin Card Counting test, a measure of problem solving and executive function [153]. Perseverative errors are characteristic of failure of reversal learning, particularly following damage to the prefrontal cortex and hippocampus [360] and these outcomes in SGA children suggest that their reversal learning is also likely to be similarly impaired, although this aspect of performance was not reported separately in that study [153]. The lower total time in PR than CON sheep in the reversal task did not reflect fewer trials to solve the task. Greater general speed of PR sheep also does not appear to explain the faster overall completion of the reversal tasks, because average trial time for criterion trials was greater in PR than CON only in 18-week old males, and not in 18-week old females or in 40-week-old sheep of either sex. PR and CON animals also did not differ in bleat frequency, a measure of behavioural stress response [361], in either reversal task in the present study. This suggests that differences in perceived stress also do not explain the better performance of PR than CON sheep in reversal learning tasks. We hypothesise that the faster completion of reversal learning tasks in PR than

CON sheep actually reflects weaker initial learning during the first learning task at 18 weeks of age, reducing proactive interference during learning of the reversed route in the subsequent reversal task.

Measures of early postnatal growth positively predicted performance in memory tasks, conducted the day after initial learning tasks, suggesting that faster early postnatal growth benefits learning retention in sheep, albeit in a sex-specific manner. Slow neonatal growth predicted poorer cognitive outcomes in memory tasks (total time and trials required) at 40 weeks of age in males, with a similar trend for effects of low birth weight. Birth weight and neonatal growth did not predict memory task performance in females. Our data suggests that neonatal growth as well as prenatal growth affects adult memory, in males but not females. Impaired memory may therefore be one mechanism explaining the adverse effects of poor neonatal growth on IQ and intellectual performance, consistent with the observation that SGA children that do not undergo catch-up growth have lower IQ and intellectual performance at 2-4 [103] or 8 years of age [150] compared to SGA with catch-up growth or AGA, and these effects persisted until adulthood [103]. Although working memory at 7-9 years of age does not differ between SGA children who did or did not catch-up in head circumference within the first 9 months of life [153], our data suggest that learning retention to the next day (long-term memory), may be positively affected by neonatal growth, at least in terms of body weight. In the present study, skull width, an indirect measure of brain size, did not differ between CON and PR sheep at birth or at 35 days of age, although in a previous larger cohort of singleton animals skull width was lower in PR than control lambs and had not caught up at 35 days of age [273]. Geva and co-authors [98] have suggested that poorer memory in IUGR compared to AGA children might be explained by their lower grey matter volume [362], including in areas important for memory, such as the hippocampus, as

observed in preterm humans and in animal models [119, 270]. In neonatal IUGR guinea pigs, loss of hippocampal grey matter is characterised by decreased axonal and dendritic sprouting as well as neuronal and glial cell loss [270]. Because neurogenesis is completed before birth in sheep and humans [173, 363, 364], improvements in cognitive function associated with neonatal growth in these species might therefore be via postnatal synaptogenesis or glial cell division. Myelination has commenced or is complete in the majority of regions in the ovine brain prior to birth [173], and abundance of myelin basic protein in the cerebral cortex is decreased ~70% in IUGR compared to control sheep fetuses [237]. There is some evidence that white matter can recover during neonatal life following prenatal insults in the sheep, as seen after prenatal and maternal viral infection with Border disease, where axonal myelination of progeny, while not normalised, was improved at six months postnatal age compared to birth [365]. Whether accelerated neonatal growth improves white matter remodelling and this underlies the beneficial relationships observed between neonatal growth and memory in the present study remains to be investigated. It is not clear why we only observed relationships between neonatal growth and memory task performance in males, as in SGA children effects of catch-up growth on IQ and intellectual performance were apparent in both sexes [103, 150].

In contrast to the positive relationships between size at birth, neonatal growth and memory task performance in males, low birth weight and slow neonatal growth weakly predicted better outcomes in task R2 in females. Reversal task outcomes were not correlated with size at birth or neonatal growth in males. These negative relationships of birth weight and neonatal growth with reversal learning in females were seen only in task R2, where animals reverse to exit the maze on their preferred side, and not in task R1, where animals need to exit on the non-preferred side. We therefore hypothesise that these negative correlations may

reflect stronger lateralisation in female sheep of low birth weight and slow neonatal growth. Consistent with this, SGA individuals have stronger visuomotor lateralisation than AGA as adolescents, whilst decreasing birth weight centile correlated with stronger cortical lateralisation in young adults [143, 144]. To date, effects of neonatal growth on lateralisation have not been directly explored in human cohorts.

Pre- and postnatal growth in terms of weight correlated more strongly with behavioural than cognitive outcomes, and these relationships were sex-, age- and task-specific. Low birth weight and slow neonatal growth predicted lower behavioural stress, measured as bleat frequency in the first reversal task in females at both ages and not in males. While bleats are an indirect behavioural measure of stress response, bleating is observed as a behavioural response to exposure to frightening situations or exposure aversive stimuli [318, 366] and has been used in arena tests as a behavioural indicator of greater emotional reactivity to stress [361]. These proxy measures are important because sheep find close contact with humans aversive and seek to maintain a minimum distance from handlers [318], and therefore behavioural measures of stress response are necessary to remove the confounding effect of stress associated with the handling required to take blood or saliva samples to measure cortisol response. Reversal learning, particularly the first reversal task, is the hardest task in the test sequence used in the present study [304], and therefore the most likely to expose effects of pre- and post-natal growth on stress responses. Conversely, these indicators of restricted pre- and neonatal growth correlated with greater bleat frequency in the initial learning task in males, and only as adults, and not in females. There is also evidence that prenatal growth alters postnatal stress axis function in a sex- and age-specific manner in humans. Low birth weight is associated with reprogramming of the stress axis, including increased circulating cortisol in cord blood, increased morning peak (unstressed) cortisol

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levels in girls, larger stress-induced increases in cortisol in boys and greater and more sustained increases in cortisol following ACTH-stimulation in aged men [321, 367, 368]. In humans, both high and low levels of cortisol impair recall of memorised traits [323]. In the present study, greater behavioural stress responses in adult males of low birth weight and with slow neonatal growth may have impaired learning during task L and may therefore have contributed to their poorer maze performance in task M1 the following day. Reprogramming of the stress axis may particularly inhibit learning in more complex executive function tasks (e.g. set-shifting), which are more sensitive to disruption by acute stress than reversal learning [369].

The strong negative correlation between birth weight and arm entries in the first reversal task in 18 week-old males provides further evidence that restricted prenatal growth affects behaviour. Arm entries in this maze task in sheep are unlikely to reflect general activity, as sheep make very few arm entries within each individual trial [304]. More frequent arm entries in low birth weight adolescent males than in those of higher birth weight may therefore indicate changes to exploratory drive or flocking instinct, since reversal from one arm to the other requires sheep to move away from flock mates. Unlike bleat frequency, neonatal growth was not correlated with arm entries for this task and was in fact positively correlated with arm entries for task L and M1 in 18 week-old males, suggesting that pre- and post-natal growth do not have consistent effects on this behavioural outcome. Consistent with adverse effects of restricted prenatal growth on behaviour, low birth weight and SGA children have higher incidences of behavioural disruption, ADHD and conduct disorders than AGA children [138, 346], particularly in girls [139]. It appears likely, therefore, that while memory may be directly impaired by poor pre- and postnatal growth, behavioural disruption
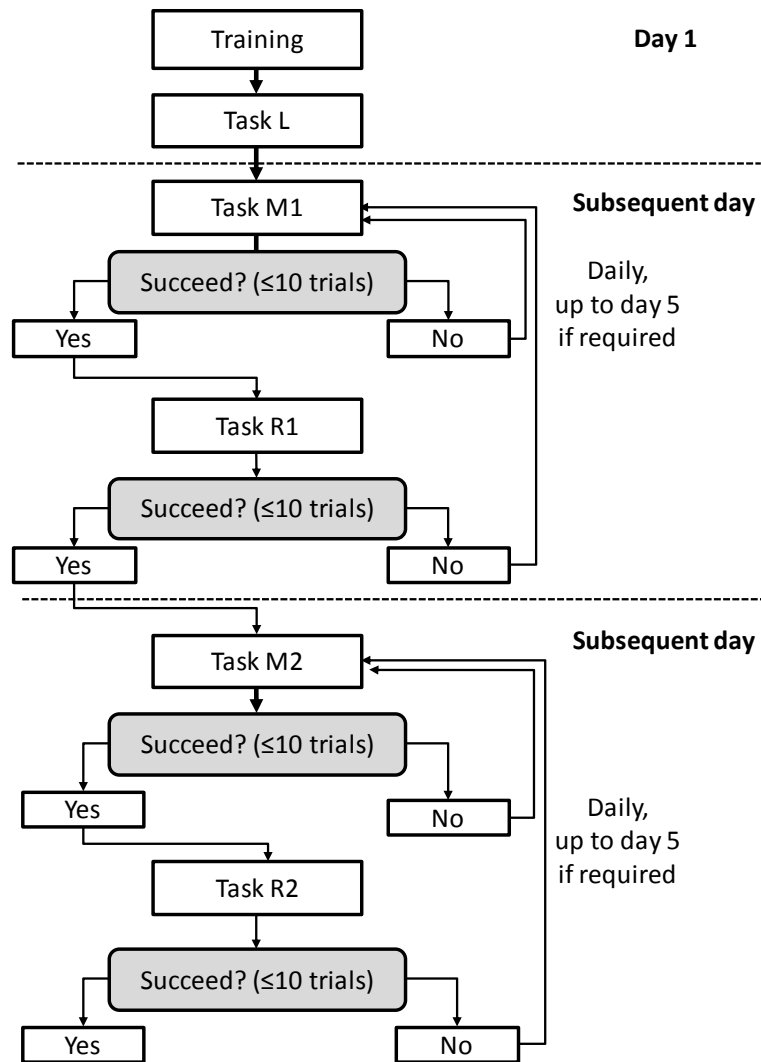
– including that linked to poor attention and altered stress responses – may also contribute to learning problems after IUGR.

In conclusion, surgical restriction of placental growth had limited effects on cognition in adolescent and adult sheep. PR impaired cognitive outcomes in a learning task but not in memory or reversal tasks, in a cohort of sheep born at term and raised in a common postnatal environment, and despite PR not reducing birth weight in this cohort. Neonatal growth correlated positively with memory task performance in adult males only, suggesting that accelerated neonatal growth may benefit cognitive function, even after completion of neurogenesis. This is consistent with the observation that neurodevelopmental outcomes from childhood to adulthood are better in SGA individuals with catch-up growth compared to SGA without catch-up [103, 150]. Low birth weight and slow neonatal growth were associated with lower behavioural stress in females during reversal tasks, measured as bleat frequency, but conversely with increased behavioural stress in males during the initial learning task in the present study. IUGR in humans alters function of the stress axis and increases incidence of attention problems and behavioural disruption [136, 138, 139]. Given the evidence for impaired memory recall with either low or elevated circulatory cortisol levels [323], we hypothesise that adverse effects of impaired prenatal and neonatal growth on complex learning are at least in part due to altered stress axis function, and suggest that additional studies of stress responses are warranted in ovine models of IUGR.

4.8. Acknowledgements

4.9. Supplementary Figure

Supplementary Fig. 4-1. Maze test protocol showing task sequence.

Progression from task M1 to R1 (same day of testing), R1 to M2 (subsequent day of testing) or M2 to R2 (same day of testing) required successful completion of preceding tasks within that days' testing, by exiting the maze via the open maze arm in ≤ 3 min in ≤ 10 trials. Failure on a task resulted in that day's sequence of events being repeated the following day, with a maximum of five consecutive days of testing possible for each sheep.

Chapter 5 - Effects of induced placental and fetal growth restriction, size at birth and early neonatal growth on behavioural and brain structural lateralization in sheep.

5.1. Preamble

Chapter 5 describes differences in structural and functional lateralisation PR and control sheep, and compares differences between treatments within each sex at 18 and 40 weeks of age. I was involved in animal husbandry from birth, recording birth weight and postnatal morphological growth measures. I acted as data recorder and observer for all behavioural tests in this chapter, was involved in all brain collection, processed and analysed all data, wrote the manuscript and contributed significantly to all subsequent redrafts and edits to the manuscript.

This chapter has been published in Laterality, with myself as first author, and text and figures are presented unaltered (Appendix 4):

Hunter, D. S**.**, Hazel, S.J., Kind, K.L., Liu, H., Marini, D. Giles, L.C., De Blasio, M. J., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2016). "Effects of induced placental and fetal growth restriction, size at birth and early neonatal growth on behavioral and brain structural lateralization in sheep." <u>Laterality</u> Oct 19: 1-30. [Epub ahead of print].

5.2. Statement of Authorship

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Name of Principal Author (Candidate)	Damien Hunter		
Contribution to the Paper	Performed experiments, dissected the brain during the majority of post-mortems, performed analyses on all data, interpreted data, wrote manuscript and created all figures		
Overall percentage (%)	50.00%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	1/9/16

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	14/9/16

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Contribution to the Paper	Supervised development of cohort and research plan, helped edit manuscript		
Signature		Date	13/9/16

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Name of Co-Author	Kathryn L. Gatford
Contribution to the Paper	Supervised development of cohort and research plan, assisted with experiments, helped interpret data, helped edit and write manuscript

		Date	12/9/16
Signature			

5.3. Abstract

Poor perinatal growth in humans results in asymmetrical grey matter loss in fetuses and infants and increased functional and behavioural asymmetry, but specific contributions of pre- and postnatal growth are unclear. We therefore compared strength and direction of lateralisation in obstacle avoidance and maze exit preference tasks in offspring of placentally-restricted (PR: 10 M, 13 F) and control (CON: 23 M, 17 F) sheep pregnancies at 18 and 40 weeks of age, and examined gross brain structure of the prefrontal cortex at 52 weeks of age (PR: 14M, 18F; CON: 23M, 25F). PR did not affect lateralisation direction, but 40 week old PR females had greater lateralisation strength than CON ($P = 0.02$). Behavioural lateralisation measures were not correlated with perinatal growth. PR did not alter brain morphology. In males, cross-sectional areas of the prefrontal cortex and left hemisphere correlated positively with skull width at birth, and white matter area correlated positively with neonatal growth rate of the skull (all $P < 0.05$). These studies reinforce the need to include progeny of both sexes in future studies of neurodevelopmental programming, and suggest that restricting in utero growth has relatively mild effects on gross brain structural or behavioural lateralisation in sheep.

5.4. Introduction

Intrauterine growth restriction (IUGR) is a prenatal insult with long-term consequences for brain development and function. These include reduced total and grey matter volume [116, 117, 121], white matter macro- and micro-structural changes [100, 117, 120], lower IQ, impaired memory and learning deficits [93, 98, 131]. Evidence from human and animal studies indicates that impaired prenatal growth does not affect all brain regions or hemispheres equally, however [1, 120, 197, 202, 229]. Although the proportions of left and right-handed individuals appear unchanged in IUGR humans compared to control populations [370], there is evidence that IUGR individuals are more strongly functionally lateralized, possibly due to disrupted neurodevelopment *in utero*. This is consistent with findings of studies in non-mammalian species where manipulation of the fetal environment, such as via controlling light exposure, has profound effects on development of ordinary functional asymmetries. Light exposure to the right eye of the embryonic domestic chicken and pigeon, which directly faces the shell and membranes, during sensitive periods of development is necessary for the development of asymmetry in visual systems [reviewed in 371]. Preventing this light exposure in turn disrupts development of reliant lateralised behaviours that develop post-hatching [371].

Region-specific structural asymmetries in volume and cortical thickness exist in the brains of healthy adult humans [34]. Human IUGR fetuses have more pronounced right-favouring asymmetry of insular volume and insular fissure depth, reduced left opercular volume, and more pronounced leftwards asymmetry of posterior insula cortical thickness in magnetic resonance imaging (MRI) analyses compared to non-IUGR fetuses [96, 127]. Grey matter volume specifically is reduced in the right insula and perirolandic area, and left parietal and

temporal lobes in preterm IUGR compared to preterm non-IUGR human infants at one year of corrected age [117]. There are also region and hemisphere-specific connectome differences between IUGR and control infants, which predict neurobehavioural scores at two years of age [100].

To date, human studies examining the effects of IUGR on structural asymmetry have predominantly focused on fetuses and neonates [96, 100, 117, 127]. Studies of adolescents born small for gestational age (SGA) reveal very little of the structural asymmetry observed in fetuses and neonates, noting only reduced cortical surface area of the right hemisphere, suggesting structural asymmetry at least partially recovers during postnatal neurodevelopment [121]. Nevertheless, compared to young adults born at a weight appropriate for gestational age (AGA) , SGA humans have reduced white matter volume, particularly in the right hemisphere, which appears to be a consequence of later life growth rather than poorer brain growth in late gestation [122]. Postnatal growth may similarly affect functional lateralisation, as neonatal and childhood catch-up growth positively predict cognitive outcomes [149, 153, 372]. The effects of catch-up growth on morphological and functional lateralisation have not yet been reported, however.

Altered structural lateralisation is likely to be associated with altered functional lateralisation, which includes behaviour and cognition that predominantly or exclusively utilizes one hemisphere over the other for a task, as well as visuospatial or perceptual behaviour. Spatial recognition dependent on geocentric cues is lateralised to the right hemisphere in several species including chicken, mice and humans [66, 373-375] . Some lateralisation may be species-specific, however, as side preference in spatial tasks varies between species. In T-maze or Y-maze tasks, the majority of reindeer show a left side preference [376], whereas

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rats and four species of minnow show a right side preference [344, 377]. Some species do not seem to have a dominant population side bias, with similar proportions of beef cattle having left and right side preferences in Y-maze tasks [378, 379]. In horses, side preference in obstacle avoidance tasks differs between sexes, with males generally possessing a left side bias and females a greater right side bias [378]. Side preference in sheep also varies according to age, sex and litter size [192, 326], but there is generally a majority right side preference in maze and obstacle avoidance tasks [309, 326]. The caudate region of the brain appears important for lateralisation of side preference. In rodents, lesions in the caudate nucleus ipsilateral to side preference increase strength of behavioural lateralisation in the T-maze, whereas lesions to the contralateral side reverse side preference [67]. Side preference in turn affects spatial learning, with rats solving maze and reversal tasks faster when the reward is placed ipsilateral to side preference, whereas performance is poorer for the contralateral arm [344].

Results of a limited body of human studies support the hypothesis that IUGR alters motor and perceptual lateralisation. SGA adolescents have poorer sensorimotor skills in their non-dominant hand compared to adolescents born at AGA [144]. In term-born healthy young adults, lateralisation of motor-cortical excitability shifted rightwards with decreasing birth weight, such that low birth weight individuals required proportionally greater transcranial magnetic stimulation intensity to elicit a motor cortex response to the left hand [143]. In other tasks the reverse was found, such that SGA adolescents had decreased right ear dominance, and a poorer capacity to modify attentional preference to attend to auditory stimuli presented in one ear only [145], suggesting that alterations to behavioural lateralisation are task-specific. Stress may also exacerbate the effects of prenatal growth on functional asymmetry. Indirect measures of cerebral blood-flow in term-born children at 8-9 years of age were not

correlated with birth weight under basal conditions, but low birth weight predicted a higher

right:left ratio of blood flow during the Trier Social Stress Test [380].

In human studies, it is difficult to remove effects of confounders such as socioeconomic

status (SES) and preterm birth which each affect birth weight, brain development and

cognition [98, 107, 159, 168, 353]. Due to limited human data available from term-born

IUGR individuals, data from preterm IUGR and SGA individuals is often used. Use of data

from SGA populations defined on the basis of low birth weight rather than pathology may,

however, dilute effects of IUGR, due to the presence of small but non growth-restricted

individuals. Preterm birth is an additional confounder, as it consistently reduces lateralisation

towards the left hemisphere, which may in turn contribute to developmental delays in motor

function, speech and language [112, 381]. Variation in postnatal environment is an additional

problem for human cohorts, since synaptogenesis, synaptic pruning, and myelination

continue postnatally in humans [18, 19, 126, 382], and low SES is associated with higher

incidence of IUGR and SGA, and poorer childhood growth [107, 164-166, 383]. Animal

studies are therefore needed to remove environmental confounders, but as yet, effects of

experimental IUGR on lateralisation have not been reported.

Poor placental development and/or function is a common cause of human IUGR [76].

Restricted placental growth and function (PR) can be induced experimentally in sheep by

surgical removal of the majority of placental attachment sites prior to mating [203, 325]. PR

in sheep produces similar outcomes to human IUGR, including fetal endocrine adaptations [3,

204, 239, 244] and hypoxia [239]. Postnatally, birth weight is reduced by 20-31% [205, 249],

and, similar to SGA human infants, PR lambs undergo neonatal catch-up growth, with

incomplete catch-up of skull width [103, 136, 273, 277, 351]. Few studies have examined

lateralisation in sheep, and these studies have examined behavioural lateralisation only in progeny of normal pregnancies [309, 326], or in those subjected to maternal undernutrition during pregnancy or peri-conception [192, 211]. This study is therefore the first to examine cerebral and behavioural lateralisation in the PR sheep.

We hypothesized that PR, low birth weight and slow neonatal growth of the skull, a proxy measure of brain size in humans [102], would each increase the strength of behavioural lateralisation. We further hypothesized that these measures of slow perinatal growth would increase structural lateralisation to the side of the brain contralateral to behavioural lateralisation. Finally, we tested the hypothesis that behavioural lateralisation would be correlated with structural laterality of the prefrontal cortex and caudate nucleus, because these regions are of particular importance in spatial learning, obstacle navigation and memory [384, 385]. We therefore measured the direction and strength of behavioural lateralisation using two side preference tasks in a cohort of control and PR sheep as adolescents at 18 weeks of age, and again as young adults at 40 weeks of age. We also measured size and structural lateralisation of the prefrontal cortex and caudate of the brains of the same cohort of animals as young adults at 52 weeks of age.

5.5. Methods

All procedures were jointly approved by the University of Adelaide Animal Ethics Committee (M-2009-145 and M-2011-055) and the SA Pathology Animal Ethics Committee (135a/09) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* [324].

5.5.1. Animals

Generation and management of the sheep cohort has been described previously [249, 291]. Briefly, all but four visible endometrial placental attachment sites (caruncles) from each uterine horn were surgically removed from primiparous non-pregnant Merino x Border Leicester ewes (PR) [203, 246, 325]. Un-operated control (CON) and PR ewes were time-mated to Merino rams, after at least 10 weeks of post-surgical recovery for PR ewes. Pregnant ewes were housed indoors from d 110 of gestation until their lambs were weaned at 13 weeks of age. Ewes were fed 1 kg Rumevite pellets daily (Ridley AgriProducts, Melbourne, Australia), with *ad libitum* access to lucerne chaff and water. Gestational age, lamb weight and litter sizes were recorded at birth. Groups of CON and PR lambs were born spontaneously at term, at 5 week intervals between July 2010 and December 2012. Males were intact, and the oestrous cycle of females was not manipulated. Lambs were weighed every second day from birth to 16 d and fractional growth rate (FGR_{weight}) for weight calculated by linear regression [272]. Lambs were weighed at least weekly until weaning. Biparietal skull width was measured at birth and at d16, and fractional growth rate calculated as daily change in skull width divided by skull width at birth ($FGR_{skull\ width}$) [272]. After weaning, progeny were housed in outside paddocks in same sex groups of similar ages and fed 0.5 kg Rumevite pellets per sheep daily, with *ad libitum* access to oaten hay, seasonal

pasture and water. Progeny were handled frequently for growth measures from birth, and fed daily by an animal technician uninvolved in behavioural testing in later life, providing frequent human contact and ensuring lambs were habituated to the presence of humans.

5.5.2. Behavioural tests

Behavioural tests of lateralisation were performed twice on a subset of the cohort, at 18 and 40 weeks of age (Fig. 5-1), corresponding to pre- and post-puberty respectively [188, 339]. These behavioural tests comprised an obstacle avoidance task and a diamond maze exit side preference task. At both ages, lateralisation tasks were performed by observers blinded to treatments before subsequent learning, memory and cognitive tests in the maze, as described previously [291, 304]. All available progeny were tested at both ages; 40 CON progeny (1 male and 1 female from singleton births, 22 male and 16 female from multiple births) and 23 PR progeny (5 male and 10 female from singleton births, 5 male and 3 females from multiple births). Each sheep underwent the same number of trials for each lateralisation test at each age. The same experimenter (D.H.) was involved in all behavioural tests, including data recording, and stood directly behind the start gates of each pen, in sight of the animal being tested (Fig. 5-2A, 5-2B). Other experimenters assisted with animal handling (primarily D.M, K.L.G and H.L.), all of whom the sheep were habituated to from routine husbandry and other experimental procedures [249]. These experimenters were not visible to sheep during the tests.

Fig. 5-1. Experimental cohort and timeline.

We have reported previously that prior experience in cognitive tests at 18 weeks altered behaviour at 40 weeks of age in this cohort [291]. Behavioural lateralisation data was therefore included in analysis for the present paper only for those sheep that were tested at both ages.
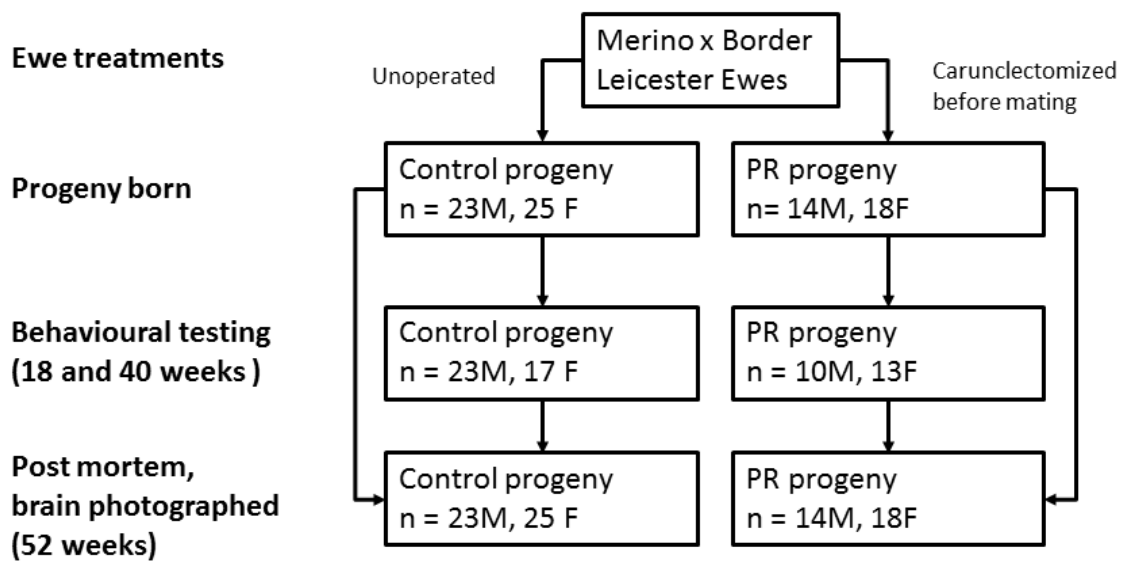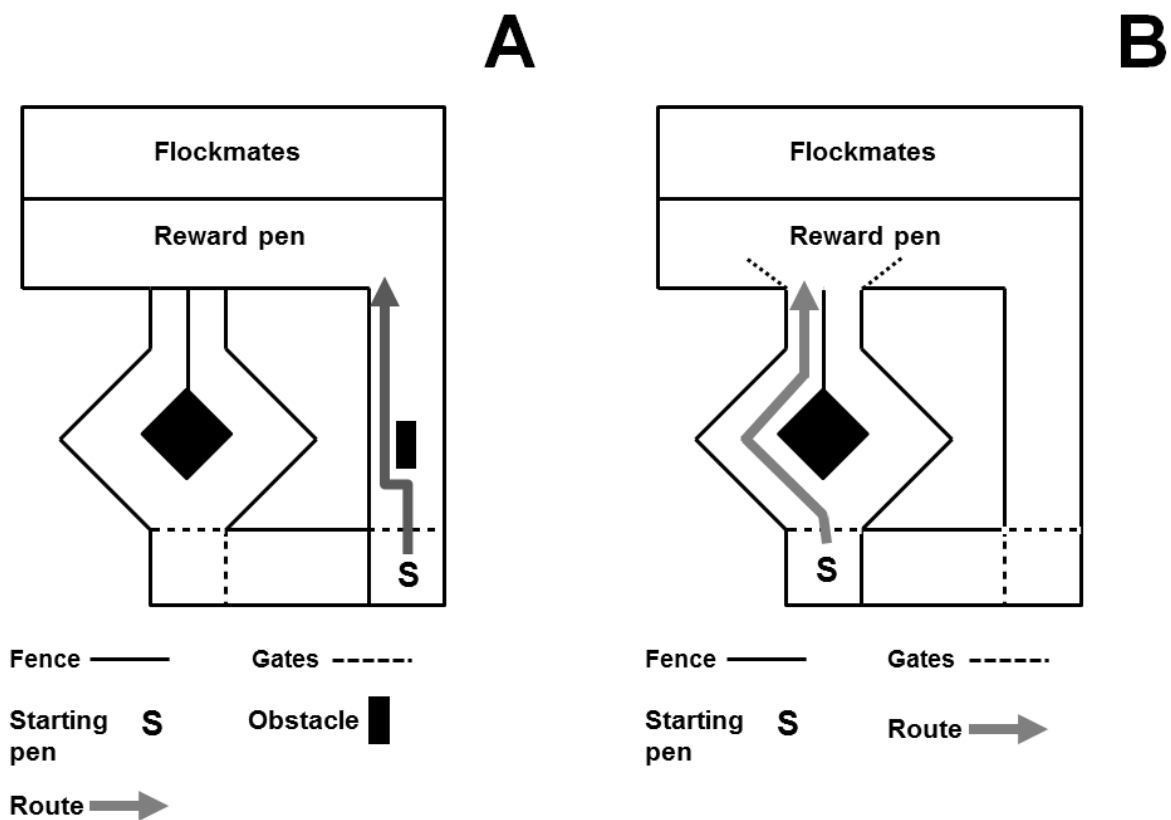
5.5.2.1. Obstacle avoidance task

This lateralisation test was performed as previously described by Versace et al. [326], with flockmates located directly ahead of the test subject, and took place in a 147 x 841 cm laneway lined with opaque black shade-cloth (Fig. 5-2A). The laneway was constructed under a shade-cloth covered pergola to remove confounding effects of shadow and sunlight, and the sawdust floor surface was raked before each test such that the lane was visually symmetrical. At one end of the lane was a holding pen, where the sheep being tested waited for the commencement of the trial, and at the other end was the reward pen, which was adjacent to flockmates and contained a food reward. An obstacle (an upturned opaque orange plastic crate, 610 x 407 x 275 mm) was placed in the center of the laneway, approximately 1/3 of the distance from the start pen. The task consisted of 10 trials in which the sheep ran from the holding pen to the reward pen, navigating around the obstacle. The recorder stood directly behind the starting pen and opened the gates, such that the sheep travelled away from the recorder during each trial. The sheep then remained in the reward pen for 30 seconds before being returned to the holding pen for the next trial. Sheep were classified on side preference (direction of lateralisation) based on the number of trials out of the ten in which they passed to the left side of the object (left lateralised: 8-10 trials; neutral: 3-7 trials; right lateralised: 0-2 trials). A lateralisation direction quotient was calculated as ((number of trials on which sheep passed to the left of the object)/total trials)*100), consistent with published methods [326, 386]. This produced a score indicating how strong their left side preference was, e.g. a score of 90% indicates the sheep passed around the left side of the obstacle on 9 out of 10 trials. Sheep were also classified for strength of lateralisation (regardless of direction), based on the proportion of runs on their preferred side, such that a higher number indicates greater strength of lateralisation as follows (0: equal numbers of runs on each side,

1: 6 of 10 runs on preferred side, 2: 7 of 10 runs on preferred side, 3: 8 of 10 runs on preferred side, 4: 9 of 10 runs on preferred side, and 5: 10 of 10 runs on preferred side).

Fig. 5-2. Schematic of behavioural testing apparatus.

A: Route used for obstacle avoidance tasks. The sheep being tested was walked from the group pen holding flockmates into the starting pen. During the trial the obstacle was in a fixed position in the lane and the gates were opened, such that the sheep ran past the obstacle towards the reward pen. B: Prior to the maze task the sheep being tested was walked down the empty obstacle avoidance lane to the maze starting pen. During the trial the gates to the maze were opened and the sheep through the maze towards flockmates, although these were not visible until the sheep had moved past the intersection and entered a maze arm. In both tasks, the experimenter stood behind the starting pen to operate gates, such that the sheep ran directly away from the experimenter and towards flockmates.

5.5.2.2. Diamond maze exit side preference task

Side preference in T-maze, Y-maze and diamond maze tasks are generally used to examine spatial lateralisation (eg [70, 192, 387]). This task was performed 1-3 d after the obstacle avoidance task, using a diamond maze with both exit gates open (Fig. 4-2B), such that sheep could exit via their preferred route [291, 304]. Neither exit gates nor flockmates were visible from the starting gate or intersection, and therefore could not influence choice of direction. A total of five trials were completed at each age, consistent with previous studies using the diamond maze [192, 291, 304]. For each trial, sheep were guided to the starting pen and then allowed to exit through a maze arm of their choosing, with the exit side recorded. Sheep were classified for side preference (direction of lateralisation) based on the number of trials in which they exited through the left-hand gate of the maze (left side preference: 4-5 trials; neutral/no side preference: 2-3 trials; right side preference: 0-1 trials). A lateralisation direction quotient was calculated as described for the obstacle avoidance task above. Sheep were also classified for strength of lateralisation, based on the proportions of runs on the preferred side, where a higher number indicated greater strength of lateralisation (1: 3 of 5 runs on preferred side, 2: 4 of 5 runs on preferred side, 3: 5 of 5 runs on preferred side).

5.5.3. Brain structure

Brain structure was assessed in a larger group of animals, which included animals that completed behavioural tests of lateralisation only at 40 weeks of age for logistical reasons (construction of testing apparatus), in addition to those that completed behavioural tests at both ages (Fig. 5-1). Structural brain analyses were completed in 48 control (4 male and 5 female singletons, 19 male and 20 female multiples) and 32 PR sheep (7 male and 13 female singletons, 7 male and 5 female multiples). At 52 weeks of age, sheep were weighed,

biparietal skull width measured, and sheep were humanely killed by an intravenous overdose of thiopentone (Troy Laboratories, New South Wales, Australia). Brains were dissected, weighed and sectioned coronally at the rostral end of the sylvian sulcus, bisecting the prefrontal cortex, lateral ventricles, rostral caudate nucleus and corpus callosum. The slice immediately rostral to the section was placed caudal face upwards against a 5 mm reference grid and digitally photographed, unfixed, using a Sony HDR-PJ10E camera. Photos were analyzed using Axiovision 4.8 (Carl Zeiss, Jena, Germany). The boundaries of regions of interest (total, grey and white matter, and the left and right caudate nuclei) were outlined manually and measured overall and within each hemisphere (Fig. 5-3A, 5-3B, 5-3C). The area of the corpus callosum (single area per brain, Fig. 5-3C), an area within white matter which is reduced in human IUGR fetuses [110] was also measured. From these measures, ratios of left to right hemisphere total area, grey matter and white matter areas and grey:white matter area ratio for each hemisphere were calculated. All analyses were conducted by a single investigator who was blinded to treatment groups.

5.3.4. Statistical analysis

Effects of treatment (control or PR), sex and litter size (singleton or multiple birth), and interactions between these variables on size and gestational age at birth, neonatal growth, adult size and brain structure were analyzed using generalized linear mixed models, including the mother as a random factor. Sheep from twins and triplet litters were grouped together as

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Fig. 5-3. Coronal slice of ovine brain collected at 52 weeks of age.
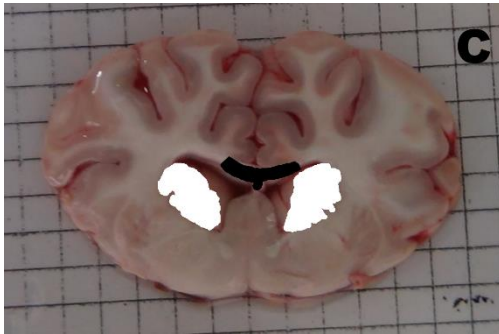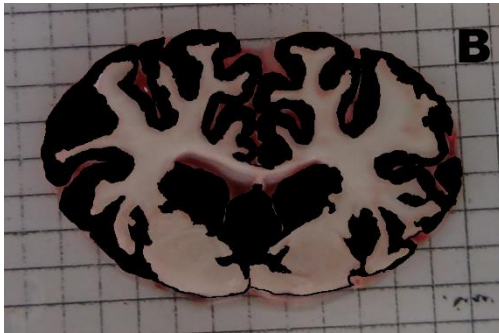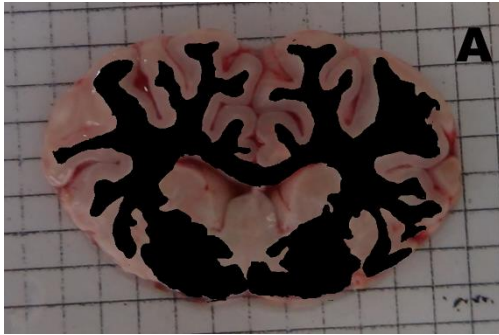
Coronal slices were laid caudal face upwards on a 5 mm grid to be photographed. Areas of interest were traced on each digital image: A - White matter (black fill), B – grey matter (black fill), C – corpus callosum (black fill), caudate nucleus (white fill).

multiple-birth progeny due to limited numbers of triplets within the overall cohort, and because fetal and placental size are similarly restricted in twin and triplet ovine fetuses [388]. Interactions between litter size and other variables were not examined due to the limited number of singleton controls and surviving twin PR lambs. Direction and strength of lateralisation were compared between treatments separately within each task, sex and age using Fisher's Exact Tests. Consistency of side preference between the two behavioural lateralisation tasks within each age and consistency of side preference between ages within the same task were also compared between treatments using Fisher's Exact Tests. The effects of birth weight and neonatal fractional growth rate for weight on lateralisation quotient and strength of lateralisation for each task and age, and on post-mortem brain measures at 52 weeks of age within each sex were analyzed by multiple linear regression. Gestational age did not correlate significantly with any outcomes and therefore was excluded from final analyses. Similarly, the effects of birth skull width, neonatal fractional growth rate for skull width and gestational age on lateralisation quotient and strength of lateralisation for each task and age, and on post-mortem brain measures at 52 weeks of age within each sex were analyzed by multiple linear regression. Relationships of strength and direction lateralisation scores for each task and age with brain measures at 52 weeks of age, were examined within each sex by linear regression. Relationships of strength and direction lateralisation scores for each task and age with the areas of the caudate nucleus and measures of brain asymmetry at 52 weeks (ratios of left:right hemisphere total, grey matter, white matter and caudate nucleus areas) were examined within each sex by linear regression. All analyses were carried out using SPSS 21.0 (IBM, Armonk, USA). Data are presented as mean ± SEM. Statistical significance was accepted at $P < 0.05$.

5.6. Results

5.6.1. Size at birth and neonatal growth

Neonatal outcomes have been reported previously for the subset of sheep in which behavioural lateralisation was measured [291]. Gestational age at birth ranged from 143-150 d in CON sheep, and 143-148 d in PR. Gestational age, birth weight (CON: 5.1 ± 0.2 kg, PR: 4.7 ± 0.2 kg) and neonatal FGR_{weight} (CON: 7.1 ± 0.4 %/d, PR: 7.9 ± 0.3 %/d) did not differ between PR (n = 23) and CON (n = 40) lambs in this subset, between sexes, or singletons or multiples, as reported previously [291]. Skull width at birth did not differ between treatments ($F_{(1, 57)}$ = 0.122, P > 0.7), but males had wider skulls than females (male: 6.4 ± 0.1 cm, female: 6.2 ± 0.1 cm; $F_{(1, 57)}$ = 5.86, P = 0.02), and singletons had wider skulls than multiples (singletons: 6.4 ± 0.1 cm, multiples: 6.2 ± 0.1 cm, $F_{(1, 57)}$ = 5.18, P = 0.03). $FGR_{skull\ width}$ did not differ between treatments, sexes or litter sizes (all P > 0.6).

In the larger cohort, in which brain structure was assessed, PR reduced gestational age at birth (CON: 146.9 ± 0.3 d, n = 48, PR: 145.5 ± 0.3 days, n = 32, $F_{(1, 84)}$ = 7.70, P = 0.007) and birth weight (CON: 5.4 ± 0.2 kg, PR: 4.6 ± 0.2 kg, $F_{(1, 84)}$ = 9.83, P = 0.002), and increased neonatal FGR_{weight} (CON: 6.8 ± 0.2 %/d, PR: 7.8 ± 0. 3 %/d, $F_{(1, 84)}$ = 7.66, P = 0.007). Litter size did not affect gestational age or FGR_{weight}, while singleton lambs were heavier at birth than multiples overall (singletons: 5.4 ± 0.2 kg, multiples: 4.7 ± 0.2 kg, $F_{(1. 84)}$ = 7.00, P = 0.01). Gestational age at birth, birth weight and fractional growth rate did not differ between male and female lambs. Treatment effects on skull width at birth differed between sexes (treatment*sex interaction, P = 0.03). There was no difference in skull width between CON and PR males (CON: 6.4 ± 0.6 cm, PR: 6.4 ± 0.7 cm, $F_{(1, 37)}$ = 0.094, P = 0.7), whereas CON females had wider skulls than PR females (CON female: 6.4 ± 0.1 cm,

PR female: 6.2 ± 0.1 cm, $F (1, 45) = 9.15$, $P = 0.004$). PR males also had wider skulls than

PR females ($F (1, 30) = 4.51$, $P = 0.04$), whereas there were no sex differences in skull width

in CON sheep ($F (1, 52) = 0.036$, $p > 0.8$). Singletons had wider skulls than multiples

(singletons: 6.5 ± 0.1 cm, multiples: 6.2 ± 0.4 cm, $F (1, 83) = 25.12$, $P < 0.001$). There were

no effects of treatment, sex or litter size on $FGR_{skull\ width}$ (all $P > 0.4$). At 52 weeks of age,

body weight and skull width did not differ between PR and CON sheep or between singletons

and multiples (each $P > 0.2$). Females weighed less (males: 55.4 ± 1.5 kg, females: 43.3 ± 1.4

kg, $F (1, 81) = 33.64$, $P < 0.001$) and had smaller skull widths than males (males: 15.6 ± 0.2

cm, females: 14.4 ± 0.2 cm, $F (1, 77) = 15.44$, $P < 0.001$) as adults.

5.6.2. Direction and strength of behavioural lateralisation

In both ages and sexes, there was a mixture of left- and right- lateralised animals for both

tasks, with very few non-lateralised animals, and no consistent predominance of right- or left-

lateralisation (Table 5-1). Within each sex, PR did not alter the direction of lateralisation at

either age in the obstacle avoidance task (males and 40 week-old females, each $P > 0.2$, 18

week-old females $P = 0.08$, Table 5-1) or maze exit preference task (each $P > 0.1$, Table 5-1).

Within each treatment, the direction of lateralisation did not differ between sexes for either

task or age (each $P > 0.1$).

At 18 weeks of age, PR did not alter the strength of lateralisation of sheep within each sex

and task (each $P > 0.1$, Table 5-2). At 40 weeks of age, PR did not affect strength of

lateralisation in either task in males (each $P > 0.1$, Table 5-2). In contrast, PR increased the

strength of lateralisation in females at 40 weeks of age for the obstacle avoidance task ($P =$

0.04, Table 5-2), with a similar trend to increased strength of lateralisation in the maze exit

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Table 5-1. Direction of lateralisation in CON and PR male and female sheep performing obstacle avoidance and maze exit preference tasks at 18 and 40 weeks of age.

Direction of lateralisation within each task was classified according to the number of trials in which the sheep passed to the left side of the object. Within the obstacle avoidance task, left lateralised: 8-10 trials; neutral: 3-7 trials; right lateralised: 0-2 trials. Within the maze exit side preference task, left lateralised: 4-5 trials; neutral: 2-3 trials; right lateralised: 0-1 trials. Effects of treatment within each sex and age were analyzed by Fisher's Exact Test.

Task and age	Lateralisation direction	Male			Female		
		CON	PR	Significance for treatment effect	CON	PR	Significance for treatment effect
Obstacle avoidance task, 18 weeks	Left	7 (30.4%)	4 (40.0%)		8 (47.0%)	2 (15.5%)	
	Neutral	1 (4.3%)	0 (0.0%)	0.793	1 (6.0%)	0 (0%)	0.085
	Right	15 (65.2%)	6 (60.0%)		8 (47.0%)	11 (84.5%)	
Obstacle avoidance task, 40 weeks	Left	9 (41.0%)	5 (50%)		7 (41.2%)	2 (15.4%)	
	Neutral	1 (4.5%)	0 (0.0%)	0.999	0 (0.0%)	0 (0.0%)	0.229
	Right	12 (54.5%)	5 (50%)		10 (58.8%)	11 (84.6%)	
Maze exit preference task, 18 weeks	Left	10 (43.5%)	2 (20%)		8 (47%)	4 (30.5%)	
	Neutral	0 (0%)	0 (0%)	0.259	0 (0%)	0 (0%)	0.465
	Right	13 (56.5%)	8 (80%)		9 (53%)	9 (69%)	
Maze exit preference task, 40 weeks	Left	11 (50.0%)	7 (70.0%)		13 (76.5%)	7 (53.8%)	
	Neutral	0 (0%)	0 (0%)	0.446	0 (0%)	0 (0%)	0.255
	Right	11 (50.0%)	3 (30.0%)		4 (23.5%)	6 (46.2%)	

preference task (P = 0.06, Table 5-2). At 40 weeks of age, female PR sheep were more strongly lateralised than PR males in both obstacle avoidance (P = 0.02) and maze exit preference tasks (P = 0.04), whereas there were no differences in lateralisation strength between sexes in CON sheep at this age, or between sexes in 18 week-old sheep within either treatment (each P > 0.3).

5.6.3. Consistency of behavioural lateralisation between tasks and ages

In CON and PR males and CON females, side preference in the obstacle avoidance task did not correspond with side preference in the maze exit preference task at either age (each P > 0.4). In PR females, side preference in the obstacle avoidance task tended to correspond with side preference in the maze exit preference task at 18 weeks (P = 0.08), although not at 40 weeks of age (P > 0.4).

Within CON sheep, side preference within the obstacle avoidance task was consistent between ages (P < 0.05), but was not consistent between ages for the maze exit preference task (P > 0.9). Within PR males there was no consistency in side preference between ages in either task (each P > 0.5). PR females, however, had consistent side preference in the obstacle avoidance task at 18 and 40 weeks of age (P = 0.01), with a similar tendency to prefer the same side in the maze exit preference task at both ages (P = 0.07).

Table 5-2. Strength of lateralisation in CON and PR male and female sheep performing obstacle avoidance and maze exit preference tasks at 18 and 40 weeks of age.

Strength of lateralisation was classified based on the difference between numbers of runs on the preferred side compared to numbers on the non-preferred side, where a higher number indicates greater strength of lateralisation. Within the obstacle avoidance task, 0: equal numbers of runs on each side, 1: 6 of 10 runs on preferred side, 2: 7 of 10 runs on preferred side, 3: 8 of 10 runs on preferred side, 4: 9 of 10 runs on preferred side, and 5: 10 of 10 runs on preferred side. Within the maze exit route task, 1: 3 of 5 runs on preferred side, 2: 4 of 5 runs on preferred side, 3: 5 of 5 runs on preferred side. Effects of treatment within each sex and age were analyzed by Fisher's Exact Test.

Task and age	Strength	Male			Female		
		CON	PR	Significance for treatment effect	CON	PR	Significance for treatment effect
Obstacle avoidance task, 18 weeks	0	1 (4.4%)	0 (0%)	0.449	1 (5.9%)	0 (0%)	0.302
	1	0 (0%)	1 (10%)		0 (0%)	2 (15.4%)	
	2	1 (4.4%)	0 (0%)		0 (0%)	2 (15.4%)	
	3	2 (8.7%)	0 (0%)		2 (11.8%)	0 (0%)	
	4	3 (13.0%)	0 (0%)		1 (5.9%)	1 (7.7%)	
	5	16 (69.6%)	9 (90%)		13 (76.5%)	8 (61.5%)	
Obstacle avoidance task, 40 weeks	0	1 (4.6%)	0 (0%)	0.175	0(0%)	0 (0%)	0.041
	1	5 (22.7%)	3 (30%)		4 (23.5%)	0 (0%)	
	2	0 (0%)	2 (20%)		1 (5.9%)	1 (7.7%)	
	3	2 (9.1%)	2 (20%)		2 (11.8%)	0 (0%)	
	4	4 (18.2%)	0 (0%)		0 (0%)	3 (23.1%)	
	5	10 (45.5%)	3 (30%)		10 (58.8%)	9 (69.2%)	
Diamond maze exit route task, 18 weeks	1	8 (34.8%)	2 (20%)	0.227	3 (17.6%)	1 (7.7%)	0.601
	2	9 (39.1)	2 (20%)		7 (41.2%)	4 (30.8%)	
	3	6 (23.1%)	6 (60%)		7 (41.2%)	8 (61.5%)	
Diamond maze exit route task, 40 weeks	1	5 (22.7%)	4 (40%)	0.258	6 (35.3%)	1 (7.7%)	0.062
	2	5 (22.7%)	0 (0%)		1 (5.9%)	5 (38.5%)	
	3	12 (54.5%)	6 (60%)		10 (58.8%)	7 (53.8%)	

5.6.4. Relationships between behavioural lateralisation, size at birth and neonatal growth

The direction and strength of behavioural lateralisation in each task did not correlate with birth weight or neonatal FGR_{weight} in either sex (data not shown). Lateralisation direction quotient in the obstacle avoidance task in female sheep at 18 weeks of age correlated positively with gestational age at birth, such that sheep that had a longer gestation were more left-side oriented (partial $r = 0.383$, $P = 0.04$).

There were few significant correlations between direction or strength of behavioural lateralisation in either task and either neonatal skull width or $FGR_{skull\ width}$. In 18 week old female sheep, lateralisation direction quotient in the maze exit preference task correlated negatively with neonatal skull width, such that those sheep with a larger neonatal skull width were more right lateralised (partial $r = -0.392$, $P = 0.04$). There were no other correlations with lateralisation strength or direction in 18 or 40 week old female sheep. In males, direction of lateralisation was positively correlated with gestational age at birth (partial $r = 0.383$, $P < 0.05$), such that males with longer gestational ages were more likely to be left lateralised. There were no other predictors of lateralisation strength or direction at any age or in any task.

5.6.5. Brain structural lateralisation

Brain weight in young adult sheep did not differ between treatments or sexes, nor between singletons and multiples (each $P > 0.1$, data not shown). Similarly, cross-sectional areas of the brain slice and of grey and white brain matter, both overall and in either hemisphere, did not differ between treatments or sexes (overall mean ± SEM: total slice area = 15.0 ± 2.2 cm^2, total grey matter = 9.2 ± 1.7 cm^2, total white matter = 5.7 ± 1.5 cm^2, each $P > 0.1$). Brain

areas and white matter areas overall and within left and right hemispheres did not differ between singletons and multiples (each P > 0.1). Grey matter area overall (P > 0.1) and within the right hemisphere (P > 0.5) were similar in singletons and multiples, whilst grey matter area in the left hemisphere was greater in singletons than multiples (singletons: 4.8 ± 0.1 cm^2, multiples: 4.5 ± 0.1 cm^2, F (1, 61) = 7.97, P = 0.006).

Prefrontal cortex brain slice, white matter and grey matter areas were consistently larger in the right hemisphere than the left hemisphere (Left:right ratio of total slice = 0.93 ± 0.01, white matter = 0.99 ± 0.14, grey matter = 0.90 ± 0.11, in which ratio > 1 indicates left-favouring asymmetry). The left:right hemisphere ratios of brain slice area, grey matter and white matter areas did not differ between treatments or sexes (each P > 0.1). The left:right ratio brain area was greater in singletons than multiples (singletons: 0.96 ± 0.01, multiples: 0.92 ± 0.01, F (1, 72) = 4.97, P = 0.03), as was the ratio of left:right grey matter area (singletons: 0.97 ± 0.02, multiples: 0.87 ± 0.02, F (1, 61) = 12.49, P = 0.001), whilst the ratio of left:right white matter area did not differ between litter size groups (P > 0.1). Grey:white matter area ratios overall and within each hemisphere (overall = 1.63 ± 0.51, left = 1.61 ± 0.59, right = 1.74 ± 0.52, in which ratios > 1 indicate more grey than white matter) did not differ between treatments (each P > 0.2) or sexes (each P > 0.2). Grey:white matter area ratios were greater in singletons than multiples for the total brain (singletons: 1.83 ± 0.10, multiples: 1.49 ± 0.09, F (1, 63) = 5.82, P = 0.019) and in the left hemisphere (singletons: 1.91 ± 0.12, multiples: 1.40 ± 0.10, F (1, 61) = 10.64, P = 0.002), but did not differ between litter sizes for the right hemisphere (P > 0.1). The area of the corpus callosum did not differ between treatments, sexes or litter size (0.2 ± 0.1 cm^2, all P > 0.05). The areas of the left and right caudate also did not differ between treatments or litter sizes (left = 0.4 ± 0.2 cm^2, right = 0.5 ± 0.2 cm^2, all P > 0.5), however, female sheep tended to have both a larger left (F (1, 69)

= 3.95, P = 0.05) and right (F (1, 68) = 2.84, P = 0.1) caudate than male sheep. The ratio of left:right caudate area did not differ between sex, treatment or litter size (each P > 0.1).

5.6.6. Relationships between brain structure, skull size at birth, and neonatal growth of the skull

Neonatal skull measures were stronger predictors of adult brain measures in males than females (Table 5-3). In males, total slice area, left hemisphere area, left hemisphere white matter area, and left:right area ratios for the entire slice and for grey matter only, each correlated positively with skull width at birth (all P < 0.05). Also in males, left and right hemisphere area, total white matter area, and white matter areas of both hemispheres correlated positively with $FGR_{skull\ width}$ (Table 5-3). Total grey:white matter area ratio correlated negatively with $FGR_{skull\ width}$ (Table 5-3). There were no significant correlations between caudate areas and neonatal skull size measures in females. In males, right caudate nucleus area correlated positively with fractional growth rate of the skull, but not with skull size at birth. This relationship was not significant for left caudate area (Table 5-3). There was no relationship between skull width at birth or $FGR_{skull\ width}$ with left:right ratio of the caudate nucleus in either sex.

Table 5-3. Correlations of brain measures in coronal slices of the prefrontal cortex at 52 weeks of age with birth skull width, neonatal FGR$_{skull\ width}$ and gestational age.

Data was analysed by multiple linear regression, and are presented as model R^2, followed by partial R for correlations with birth skull width (Skwid), fractional growth rate of skull width (FGR$_{skull\ width}$) and gestational age (GA). Significance of associations between outcomes and each factor are indicated by symbols: † P < 0.1, * P < 0.05, ** P < 0.01.

Measure	Males			Females		
	Model R^2	Skwid	FGR$_{skull\ width}$	Model R^2	Skwid	FGR$_{skull\ width}$
Slice area (cm^2)						
Total	0.230*	0.476*	0.155	0.089	0.263	0.251
Left hemisphere	0.283*	0.470*	0.394*	0.086	0.292	0.131
Right hemisphere	0.273*	0.278	0.522*	0.110	0.331†	0.178
Left : right ratio	0.164	0.399*	-0.002	0.052	-0.090	0.022
Grey matter area (cm^2)						
Total area	0.240*	0.321	-0.259	0.031	0.159	0.021
Left hemisphere	0.106	0.310	0.163	0.072	0.125	-0.146
Right hemisphere	0.121	-0.035	0.307	0.156	0.225	-0.184
Left : right ratio	0.203†	0.427*	-0.092	0.032	-0.136	0.031
White matter area (cm^2)						
Total area	0.321*	0.375†	0.559*	0.088	0.208†	0.286
Left hemisphere	0.320*	0.389*	0.555*	0.065	0.247	0.172
Right hemisphere	0.283*	0.331†	0.527*	0.069	0.190	0.251
Left : right ratio	0.010	0.100	0.041	0.291	0.069	-0.228
Corpus callosum	0.144	0.179	0.379†	0.054	0.118	0.231
Grey:white matter ratio						
Total area	0.324**	-0.144	-0.560**	0.016	0.094	-0.021
Left hemisphere	0.157	-0.161	-0.389†	0.001	0.022	0.013
Right hemisphere	0.153	-0.302	-0.363†	0.032	0.120	-0.051
Caudate area (cm^2)						
Left caudate	0.133	-0.060	0.310	0.078	0.271	0.068
Right caudate	0.254*	0.088	0.489*	0.068	0.007	0.011
Left : right ratio	0.154	-0.133	-0.128	0.262	0.159	0.261

5.6.7. Relationships between brain structural lateralisation and functional lateralisation

Few significant relationships were observed between functional lateralisation in the behavioural tasks and brain structure, and these were age- and sex-specific (Table 5-4). In 18 week old males, area of the right caudate nucleus correlated positively with lateralisation strength but not direction in the obstacle avoidance task ($r = 0.418$, $P < 0.05$, Table 5-4). This relationship was not apparent at 40 weeks of age, and no other brain measures correlated significantly with direction or strength of lateralisation in either the obstacle avoidance or maze task.

In female sheep, at 18 weeks, direction but not strength of lateralisation in the obstacle avoidance task correlated negatively with left:right hemisphere ratio of total brain area ($r = -0.401$, $P < 0.05$, Table 5-4). At 40 weeks left:right asymmetry of total brain area no longer predicted direction of side preference, but correlated positively with strength of lateralisation in these female sheep ($r = 0.417$, $P < 0.05$). At 40 weeks of age only, area of both the left ($r = 0.491$, $P < 0.05$) and right ($r = 0.424$, $P < 0.05$) caudate correlated positively with direction of side preference, but not strength in female sheep. Also only in 40 week females, area of the right caudate nucleus correlated negatively with strength of lateralisation ($r = -0.406$, $P < 0.05$). Ratio of left:right caudate area did not predict strength or direction of lateralisation in female sheep at either age.

Table 5-4. Correlations of directions of behavioural lateralisation in obstacle avoidance and maze tasks with measures of cerebral asymmetry in coronal slices of the prefrontal cortex at 52 weeks of age.

Data was analyzed by linear regression separately for each brain measure, and is presented as R for correlations. Significance of associations between outcomes and each factor are indicated by symbols: † $P < 0.1$, * $P < 0.05$

Measure	Cerebral asymmetry (left:right hemisphere area ratio)			Caudate area (mm2)		Caudate asymmetry ratio
	Total	**Grey matter**	**White matter**	**Left**	**Right**	**Left:right hemisphere**
Obstacle avoidance task, 18 weeks						
Male						
Direction	-0.177	-0.334†	0.120	0.167	0.072	0.125
Strength	0.171	0.142	0.092	0.321†	0.418*	-0.073
Female						
Direction	-0.401*	-0.196	-0.169	0.275	0.222	0.059
Strength	0.062	-0.112	0.134	0.113	-0.012	0.232
Obstacle avoidance task, 40 weeks						
Male						
Direction	-0.214	-0.358†	0.069	-0.218	-0.286	0.059
Strength	0.324†	0.089	0.358†	0.029	0.227	-0.262
Female						
Direction	-0.303	-0.125	-0.156	0.491*	0.424*	0.154
Strength	0.417*	0.182	0.243	0.330	0.147	0.356†
Maze task, 18 weeks						
Male						
Direction	0.003	0.034	-0.028	-0.340†	-0.182	-0.252
Strength	0.032	0.230	0.303	0.213	0.234	0.049
Female						
Direction	-0.010	-0.380†	-0.378†	-0.192	-0.156	-0.097
Strength	0.006	-0.395†	-0.460*	0.132	0.054	0.121
Maze task, 40 weeks						
Male						
Direction	-0.208	-0.084	-0.360†	-0.189	-0.226	0.078
Strength	0.036	-0.037	0.046	-0.139	-0.173	0.073
Female						
Direction	-0.187	0.218	-0.468*	-0.115	-0.124	-0.051
Strength	0.235	0.257	0.096	-0.329	-0.406*	0.008

5.7. Discussion

Consistent with our first hypothesis, PR increased the strength of lateralisation, however, this was observed in young adult female sheep only, not in males or during adolescence. The lack of consistent effects of PR on behavioural lateralisation may be in part because there were no differences in birth weight between groups within the subset of animals used for behavioural testing. Lower survival of PR lambs, particularly in twins [273], is likely to have contributed to lack of birth weight difference in these survivors. Birth weight (reflecting prenatal growth) and neonatal growth rates of weight and skull width were poor predictors of either strength or direction of behavioural lateralisation in adolescence and adulthood. Consistent with the limited effects of PR on behavioural lateralisation, PR also did not affect structural lateralisation of either the prefrontal cortex or caudate nucleus in young adult sheep, although in this larger group of animals birth weight was reduced by PR. Skull size at birth and neonatal fractional growth rate of the skull were each predictors of total brain slice and white matter cross-sectional areas in adulthood in adult males but not females. Overall, our data show that placental restriction and poor prenatal growth and neonatal growth have few effects on the particular behavioural lateralities studied, or on gross structural lateralisation of the prefrontal cortex region of the brain in the sheep, and those that do occur are sex-specific.

The patterns of behavioural lateralisation we observed in the present study show some differences to those previously reported in sheep using the maze exit preference task [192], or obstacle avoidance task [326].We found that while most individual sheep had a strongly preferred side for a given task and age, similar numbers of individuals at each age were right- or left-lateralised. Our findings differ from previous reports that most suckling lambs and adolescent sheep preferred the right side in obstacle avoidance tasks [326], and of sex- and litter-size specific side preferences in maze exit preference tasks [192]. The lack of

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population-level side bias in the present study is however consistent with findings in beef cattle in which there was also no population level side bias in obstacle avoidance tasks, although the majority of individuals had lateralised side preference [379]. We did not observe any differences in side preference between control and PR sheep, which may be due to the lack of significant difference in birth weight between the control and PR sheep in the subset which underwent behavioural testing. Additionally, the differences in birth weight between groups in the total cohort indicates that only moderate restriction (>15%) existed in the PR sheep in the present cohort. This appears largely a consequence of high rates of twinning in the present study, which restricts fetal nutrient supply and growth [359], coupled with lower survival of PR twins than singletons [273]. The consequently greater proportion of twins in control than PR sheep included in behavioural studies would therefore reduce differences in the level of in utero restriction between groups, and increase the sample size that would be required to observe more subtle effects. Future studies with larger sample sizes or utilizing only singleton progeny are necessary to confirm findings observed in the present study.

Whilst sheep showed clear and strong side preference within each task, there was no overall consistency in side preference between tasks, such that an individual could be strongly lateralised to different directions in the obstacle avoidance and maze tasks. In addition, while individuals were lateralised there was no population level lateralisation. Previous ovine studies comparing preferred directions in obstacle avoidance, foreleg preference, and jaw movements during rumination, also report that side preference varies between tasks [326]. Side preference similarly varies between tasks in dogs, when completing food retrieval and tape removal tasks [389], whilst forelimb preference in rats differs between tasks and with side preference in spatial tasks [70]. Individual differences in direction or strength of side preference between two tasks utilized in the present study do not appear to be due to the

effects of relative position of flockmates in the obstacle avoidance task. Were this the case, a greater frequency of left side preference would be expected in the obstacle avoidance task as sheep sought to remain closer to flockmates. The lack of right side bias also suggests that perceptual lateralisation, at least of faces/flockmates, is unlikely to be a major component in this task. This was suggested as a possible contributor to the right-side bias noted by Versace and colleagues [326], since processing of faces is lateralised to the right hemisphere in sheep [390], and their facial perception is therefore biased towards the left eye. The greater distance of the obstacle from flockmates in our study compared to those of Versace [326] may also reduce the contribution of facial perception bias to lateralisation in this task. Furthermore in our second test of behavioural lateralisation, the maze task, position of flockmates relative to exits could not influence choices of arms used to exit, as the diamond design meant that sheep being tested were unable to see flockmates until after choice of a maze arm (Figure 2). This suggests differences in behavioural lateralisation between tasks are due to different cognitive demands of each task.

We also found that, with the exception of PR males, sheep were consistent in side preference between ages within the obstacle avoidance task. This is in agreement with previous reports that side preference within the same motor or spatial task is consistent with age in dogs and sheep respectively [192, 389]. It is unclear why side preference differed with age in the maze exit task for control sheep and PR males, given that a previous study utilizing the same task observed that more than 75% of control sheep were consistent in their side preferences when studied at 4 and 18 months of age [192]. It is also unclear why 40 week old PR females had greater strength but not altered direction of side preference in the present study. Previous studies have found adult sheep to be less strongly behaviourally lateralised than lambs, but acknowledged that this may have been a consequence of the younger lambs running to be

reunited with their mother, whereas older sheep were running towards flockmates [326]. Accordingly, greater motivation of younger lambs to be reunited with their mother was cited as a potential reason for their stronger lateralisation, owing to differential role of each hemisphere dependent on emotional valence [326], and strength of right side lateralisation is increased in newly hatched chicks running towards unfamiliar conspecifics compared to familiar [391]. That was not the case in the present study, in which we tested older sheep, always running towards flockmates.

PR did not affect gross structural lateralisation of the prefrontal cortex in 52 week old sheep in the present study. In addition to the already flagged issues resulting in limited growth restriction in these animals, this may also be a consequence of studying adult animals, as the prenatal consequences of PR are likely to lessen with time due to postnatal environmental influences on brain development. Studies of the fetus, neonate and growing animals are necessary to ascertain the impact on PR on neurodevelopment earlier in life. We did observed limited effects of gestational age in the present study, and thus excluded it from our final models. This therefore suggests that while our PR sheep were born a day earlier than control sheep, this was not sufficient to affect neurodevelopmental outcomes, although studies in fetuses and neonates would be needed to confirm this, as such differences may also no longer be evident in adults. Future studies would also benefit from MRI examination, to look at microstructure and connectivity within each hemisphere, or quantitative assessment of neurochemicals such as dopamine within the caudate nuclei. Study of motor lateralisation also is a logical functional outcome to next examine in the PR sheep, as reduced cerebellar volume and developmental delays have been observed in the ovine uteroplacental embolisation model of IUGR [202]. In addition, cerebellar connectivity to and inhibition of

the motor cortex is highly lateralised [63, 64], and may explain impaired or more highly lateralised motor functions in SGA and low birth weight young adults [143, 370].

The present study revealed that skull size at birth, a proxy measure of prenatal brain growth, correlated weakly but significantly with leftwards favouring asymmetry in male sheep only. As the sheep in the present cohort had larger right hemispheres than left, this translates to lambs with poorer prenatal growth having increased right side structural lateralisation as adults. This did not appear to be a consequence of grey matter loss, which is observed in humans following IUGR [120], which suggests that a restricted prenatal environment favours growth of the right hemisphere. As adults, although grey matter area was not decreased, greater right favouring asymmetry was observed in low birth weight male sheep. It is questionable as to whether this translates to functional outcomes, however, as correlations of total and grey to white matter asymmetry with lateralisation strength and direction failed to reach statistical significance. Very few of the measures of cerebral lateralisation we examined in the present study correlated with functional outcomes. Left to right caudate area ratio did not correlate with strength or direction of side preference in either task, while the relationships between gross caudate area and these same outcomes were limited in the present study. This seems counter to previous evidence indicating clear relationships between intact caudate nucleus function and side preference in T-maze spatial tasks in rats [67, 392], which resemble our diamond maze task. Whether this is indicative of different brain regions utilized in the tasks used in the present study, or a species difference is unclear.

We observed a weak positive relationship with skull size at birth and left hemispheric white matter area in male sheep, suggesting this hemisphere to be more reactive to the prenatal environment than the right. It is unclear whether this relationship is likely to reflect outcomes

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in the human, however, since myelination occurs during late gestation in the sheep [173] but not in the human, in which the majority of myelination occurs postnatally [20, 126]. Gestational age did not affect any structural outcomes in the present study, likely because none of the sheep were born preterm, and therefore brain development was unlikely to be significantly impacted. Surprisingly, we observed that postnatal growth correlated weakly but positively with white matter area in male sheep, despite the prenatal myelination in this species. This suggests capacity for postnatal recovery from white matter loss, with neither hemisphere being favoured preferentially by postnatal growth. Unlike the cerebral cortex, right caudate nucleus area did not seem affected by prenatal growth, but rather by postnatal skull growth in male sheep. This further emphasizes the importance of postnatal environment on outcomes, although the present study suggests functional consequences of this to be transient, with poor outcomes of IUGR in adolescence recovering by adulthood. Whether this indicates postnatal recovery continuing in later life or compensatory mechanisms is unclear.

Females appeared to have completely different relationships between structural and functional spatial lateralisation than males, which is consistent with sex differences in spatial lateralisation in other species, such as chickens and horses [373, 378]. As neither structural nor functional lateralisation were affected by pre- or postnatal environment in our female sheep, this suggests that females may have a greater resilience towards perinatal insult. Previous studies in female sheep have observed greater resting and post-stressor levels in adrenocorticotrophic hormone secretion in ewes with strong motor lateralisation compared to those that were weakly lateralised [393]. Higher resting cortisol levels and elevated stress responses are observed in low birth weight compared to normal birth weight girls and adult women [321, 322]. Whether stress hormones contributed to the stronger lateralisation observed in female PR sheep is therefore worth further examination. Unlike male sheep,

caudate area at 40 weeks predicted functional outcomes in females for either strength or direction of side preference in each task, and suggests sex specific mechanisms by which each sex solved these tasks. Quantification of dopamine within these structures, as per rodent studies (e.g. [392]) may further elucidate these findings, as gross area of the caudate provides only a limited explanation for these relationships. Similarly, use of MRI to examine the connectome of each hemisphere within each sex, and comparing this data to spatial learning outcomes may identify underlying mechanisms. As each sex does appear to utilize different brain regions for these tasks, this suggests that brain regions not examined in the present study are also likely to be differently altered by IUGR in each sex. This emphasizes the need to study each sex separately in human studies.

In conclusion, PR and measures of perinatal growth had limited effects on direction of the behavioural lateralities studied in sheep, but increased strength and consistency of these in females. The relationship of prenatal and postnatal growth with adult brain structures and functional outcomes is highly sex-specific, and suggests that inherent differences exist between determinants of side preference in male and female sheep, each with different sensitivities to perinatal environment. Additional investigation of brain morphology is required to determine whether PR and perinatal growth in sheep induce similar hemisphere-specific microstructural changes as have been reported in human IUGR, such as altered fibre organization in the left anterior limb of the internal capsule, left external capsule, and right frontal course of the uncinate fasciculus [117], and/or changes in volume and three dimensional structure of the more rostral regions of the prefrontal cortex, in which asymmetric loss of grey matter has been observed by MRI [117]. This is the first report of effects of an experimental model of placental restriction on spatial lateralisation. Strengths of the present study include the longitudinal observations, inclusion of both sexes, and analysis

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of morphological as well as behavioural outcomes. Our data also provides evidence of sex-specific effects on lateralisation strength and reinforces the need to include progeny of both sexes in future studies of neurodevelopmental programming.

Chapter 6 - General discussion

6.1 Introduction

IUGR is a condition associated with adverse neurodevelopmental outcomes, including learning disabilities and lower IQ [93, 129, 141]. Examination of causality is confounded in humans due to frequent comorbidity of SGA and IUGR with preterm birth [159, 161], and greater risks of IUGR and low birth weight in socially disadvantaged groups [164-166], who are also at increased risks of poor cognitive and academic outcomes [164, 167, 168]. Animal models are therefore necessary to better characterise outcomes in the absence of confounding and to enable control of the postnatal environment, and to trial interventions. The carunclectomy model of PR in sheep is an established model of intrauterine growth restriction [203, 204, 277, 394]; however, the effects on neurodevelopmental and cognitive outcomes have not previously been examined. While sheep are increasingly used to study effects of hypoxia on white matter damage [297, 298], and effects of IUGR on fetal brain development [202, 238] and postnatal cognition [243], the long-term effects of IUGR on brain and cognitive outcomes have not yet been examined. Chapter 1 presented the background for this research, and described human neurodevelopmental outcomes, issues of confounding in the human, and evaluation of animal models of IUGR, including the placentally restricted (PR) sheep.

The studies described in this thesis therefore initially sought to examine the effects of age and sex on cognition in healthy pre-pubertal and young adult sheep, in order to provide necessary information for subsequent studies in the sheep (Chapter 3). Following this, effects of PR on neurodevelopmental outcomes (learning, memory, executive function and behaviour) were investigated (Chapter 4). Studies described in Chapter 4 also investigated correlations

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between size at birth, neonatal growth and cognitive outcomes. Finally, effects of PR on behavioural and cerebral lateralisation and correlations of these with size at birth and neonatal growth were examined in the same cohort (Chapter 5). This was the first study to examine lateralisation in an animal model of placental restriction. This final chapter summarises my findings in relation to the broader body of literature, and suggests future directions for study.

6.2 Effects of sex, age and prior learning on cognition in control sheep

Sheep are increasing in use as a research model of effects of prenatal perturbation, but comparatively little is known about cognition in sheep, compared to other animal models such as the rat and guinea pig. Most previous studies of sheep behaviour have investigated behavioural outcomes for welfare or production purposes [395]. In the past decade there have been studies investigating maze learning [303, 307, 308] and behavioural lateralisation [309] in sheep. There had not previously been attempts to describe systematically the longitudinal changes with age, sex differences in animals before and after puberty, however, or the effects of prior learning on subsequent learning, and these were the overall aims of studies described in Chapter 3.

A key finding of the cognitive studies in control sheep was that sheep recalled a reversal task taught at 18 weeks when re-tested at 40 weeks of age [304]. Additionally, sheep learned better when first asked to solve the reversal learning tasks in the maze at 18 weeks (18N) than at 40 weeks of age (40N). Naive 40 week olds (40N) performed more poorly than experienced 40 week old sheep (40E) on the first reversal task, and also on the initial learning task, whereas 18N and 40E did not differ in time or trials taken to solve this task [304]. While learning in similar tasks has been previously examined at 4 and 18 months of age [192],

direct comparisons were not made between the age groups, nor did that study compare sheep being re-tested and those that were naive to the task at 18 months. While sheep are capable of remembering facial cues two years after training [310], my results have shown for the first time that sheep possess a similar capacity for long-term recall of reversal tasks, albeit over a shorter time-frame of 22 weeks. This is important to consider in design of neurodevelopmental studies in sheep, as studies looking at postnatal recovery or maturation will need to take into account the effects of prior learning when re-testing sheep, or else use independent groups for each age to avoid this effect. This demonstration of learning recall in sheep also indicates that examination of long-term memory is possible in this species, which will allow assessment of whether specific conditions result in poorer learning retention.

Additionally, the present study examined behavioural measures during the maze testing. I observed that older sheep bleated less than younger sheep, and additionally that sheep made very few arm entries within each trial. This differs from behavioural responses reported in rats, which display more exploratory behaviour during testing [305]. I hypothesise that the low numbers of arm entries per test in the studies reported in Chapter 3 are in part a consequence of a lack of desire to move away from flockmates visible through the closed maze arms, so that during failed trials sheep generally waited in the closed arm instead of seeking to explore the other arm. Tests on sheep therefore need to be designed with their species-specific behaviour in mind, as while this species is capable of complex learning, poorly designed tests may prevent its measurement. I also recorded maze exit route, and thus was able to examine means by which sheep solved reversal tasks. I discovered that in 40N females, sheep with an indirect exit route (turned around at dead ends) learned more quickly than sheep with a direct exit route (only exited the maze via going straight into the open arm). This appears to be because sheep that did not gain the capacity to self-correct (reverse at

closed gate), were also reluctant to enter the open maze arm, and thus continually failed trials until they overcame this bias and spontaneously entered the open arm, which they then repeated in subsequent trials to pass the test. Exit method may therefore be a useful measure of learning strategy and temperament differences, such as strength of flocking instinct, although further investigation is needed to examine the degree to which each contribute to this behaviour. The behavioural measures taken in the maze experiments provided additional information which previous studies utilising sheep generally neglected, in which success in maze tasks is often only described in terms of trials taken to solve the task [192, 243, 308, 318]. Measures of behavioural stress and exploratory behaviour assist in understanding how individual sheep learn, as well as the consequences of stress on learning.

6.3 Effects of PR, size at birth and neonatal growth on cognitive outcomes and lateralisation

My fourth and fifth chapters investigated effects of PR, size at birth and neonatal growth on cognitive outcomes and lateralisation. In the studies reported in these chapters, I found that PR had only minor and sex-specific effects on cognition and cerebral lateralisation. In addition, outcomes affected by PR differed from those that correlated with size at birth and neonatal growth. PR males learned more poorly than CON males in the initial spatial learning task, but there were no differences between CON and PR on memory tasks. Additionally, PR did better on reversal tasks, which may reflect as weaker initial learning and decreased proactive interference. The relationships between pre- and post-natal growth and cognitive outcomes were similarly sex-specific, and differed from the consequences of PR. Notably, slow neonatal growth rate in males was associated with poorer memory, whereas in females small size at birth and slow neonatal growth were associated with increased bleats, an

indicator of increased emotional reactivity. This emphasises the importance of studying gonadally-intact members of both sexes, as the present study suggests that the consequences of placental restriction are both sex- and task-specific. The additional measures of behaviour taken during testing also helped clarify that the poorer learning in PR male sheep in the initial spatial learning task was a function of poorer cognition rather than behavioural disturbances, as CON and PR sheep did not differ in terms of bleat frequency or number of arm entries per trial. Incidence of attentional problems, including ADHD symptoms [136, 138], emotional problems [160] and stress response [321] are increased in children that are LBW, SGA or IUGR, which likely contribute to their poorer learning. In the present study, I did not find evidence that altered behaviour disrupted learning in the tasks evaluated, suggesting that behavioural changes were minimal in PR sheep, or not severe enough to interfere with learning in simple maze tasks. In the absence of the behavioural measures that were collected (bleats, arm entries and speed per trial), our cognitive results could not have been interpreted, which suggests that similar behavioural measures should be included during cognitive testing in future sheep studies.

The fact that there was a relationship between neonatal growth and memory in males at 40 but not 18 weeks of age in a pooled sample of CON and PR sheep suggests that early life growth may program continued neurodevelopment throughout the life of the sheep. This is surprising to observe in sheep, which are born with myelination having commenced and largely completed in the majority of brain regions [173], whereas the majority of myelination in the human occurs postnatally [20, 126]. Further studies are needed to determine whether the relationship between neonatal growth and memory in adult males is a consequence of altered postnatal neurodevelopment in later life, or if this is a consequence of altered sex hormone abundance, as sheep in the present cohort were studied at ages pre- and post-pubertally.

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PR females had stronger and more consistent behavioural lateralisation than CON females, whereas lateralisation in males did not differ between treatments. Birth weight and neonatal weight gain were poor predictors of both brain measures and behavioural lateralisation, but skull width at birth and neonatal skull growth were predictive of limited brain measures, the majority of which were evident in males only. In males, skull width at birth correlated positively with slice area overall and in the left hemisphere, whereas neonatal growth rate of skull width correlated positively with overall area of the both hemispheres. Also in males, neonatal growth rate of the skull correlated positively with white matter area overall and in both hemispheres, negatively with grey to white matter ratio in the total brain slice, and positively with area of the right caudate. In females, skull width at birth and neonatal growth of the skull did not significantly correlate with any brain measures at 52 weeks of age. There were few relationships between skull measures and behavioural lateralisation in either sex. In males, strength but not direction of lateralisation in the obstacle avoidance task at 18 weeks correlated positively with right caudate area only, whereas in female sheep, at 40 weeks old only area of both caudates correlated positively with direction of lateralisation in the same task. This finding is difficult to reconcile with studies in the rat, in which lesions in the caudate contralateral to side preference resulted in a change of side preference [67]. In female sheep only, a greater left than right hemisphere area correlated with greater right side bias in the obstacle avoidance task at 18 weeks, whereas in the same task at 40 weeks this relationship didn't exist, with greater left side cerebral asymmetry instead predicting greater strength of lateralisation, regardless of side preference. In females only, greater white matter area in the left hemisphere than right predicted weaker strength of lateralisation in the maze task at 18 weeks of age, and stronger right side bias in the maze task at 40 weeks of age. In combination, this suggests that the determinants of structural and behavioural lateralisation

are sex-specific. While structures in the prefrontal cortex of the male were affected by poor prenatal growth or postnatal growth, these structures did not appear to be involved in the behavioural lateralisation tasks examined in Chapter 5, whereas in the female these structures appeared to be utilised, but were generally unaffected by poor early life growth.

6.4 Strengths and limitations

The main strengths and limitations of the studies described in this thesis were largely due to the nature of the cohort. Strengths of the cohort were consistent maternal age and genotype, and that all sheep were raised in the same environment. Ewes were housed together, lambing took place in the same facility, and all ewes were fed the same diet during pregnancy and lactation. Pregnancies were unproblematic, with all lambs born at term. Post-weaning, all lambs were raised communally in same-sex groups of similar ages and nutrition was consistent throughout the study. Accordingly, the environmental factors that confound human studies were generally absent or controlled.

Nevertheless, the modest difference in birth weight between PR and CON, significant only in the larger cohort included in brain measures, is less substantial compared to previous studies using the carunclectomy paradigm, which had 20-28% in which birth weight reductions [272, 275, 396]. This may in part reflect confounding by the high rate of twinning in the CON group, and low survival rate of PR twins, meaning the majority of PR progeny studied as adolescents and adult were singletons. This is consistent with the previously reported interaction between PR and litter size for survival, such that the adverse effect of PR on survival is far greater in twins than singletons [273]. Moreover, the lower incidence of PR twins compared to CON may also be a consequence of the carunclectomy surgery removing placental attachment sites [203] and hence reduction in implantation of multiple fetuses.

Unfortunately this means that the majority of our CON lambs have been subjected to a level of *in utero* growth restriction due to twin gestation [359], which likely also reduced differences in *in utero* growth and environment between the CON and PR sheep. While twinning does not induce as large a reduction in birth weight as PR, with birth weight in PR being 20% lower than CON, multiples are still 14% lighter than singletons [249]. Our CON and PR groups therefore have a greater overlap in birth weight than cohorts comprising of singletons alone [248]. Assessment of placental size via ultrasound or circulating markers such as placental lactogen might also be useful inclusions in future studies to assess the degree of restriction. Placental lactogen is produced by the placenta and an indicator of placental function, and concentrations in maternal circulation are markedly lower in late pregnancy in carunclectomised compared to control ewes, with levels correlating positively with placental weight [204].

Future studies would benefit from studying singleton sheep only, or by use of a breed of sheep in which twinning is less common. The Merino x Border Leicester first cross ewes utilised in this project, were chosen based on availability of sufficient numbers of the same age and background, including similar genotype (all ewes were sourced from a single stud), and hence minimisation of variation due to these factors. First-cross ewes also have excellent mothering abilities, and high milk production, which is important for greater survival of comparatively frail PR lambs, but are a breed with a frequent incidence of multiple births [397]. Choice of breeds for future studies needs to take these advantages and disadvantages into consideration.

While there were modest differences in birth weight in the full cohort (Chapter 5), birth weights were similar in CON and PR subset of sheep used in the behavioural studies (Chapter

4, 5). This may have in part been due to the smaller sample size limiting power to detect differences between these two groups, in addition to effects of twinning and survival discussed above. Furthermore, some comparisons were impossible due to the small sample size of some groups. The naive 40-week old (40N) group included in the study of CON sheep described in Chapter 3 were generated opportunistically. Behavioural testing commenced after the first few replicates of lambs (born at ~5-week intervals over a 3 year period) had passed 18 weeks of age, and numbers of animals in the 40N group were therefore limited to 4 males and 10 females [304]. This limited power to examine effects of exit strategy on learning within this sub-group in this first chapter. In the studies of PR sheep described in Chapters 4 and 5, litter size was uneven between groups amongst the survivors in whom behavioural testing was conducted at 18 and 40 weeks of age. As described in Chapter 3, prior learning improved maze performance at 40 weeks of age in CON sheep. Due to limited sample size and for logistical reasons, however, the CON and PR sheep analysed in Chapter 4 included only animals tested at 18 weeks and then sheep re-tested at 40 weeks, and we were unable to assess learning in naive PR 40 week old sheep. It is therefore possible that effects of prior learning may have masked learning impairments that would be evident in naive 40 week old PR sheep.

In Chapter 4, bleats were used as a measure of behavioural stress, in part due to prior convention and reported use of this measure in sheep behavioural studies [361, 366]. In the absence of physiological stress measures, however, it is difficult to assess the full degree of stress during testing in this cohort. This limitation would be difficult to rectify in future studies because handling to take a saliva or blood sample for cortisol analysis would itself result in elevated stress during testing. Although repeated handling during routine husbandry decreases stress responses including behavioural measures of fearfulness, stress responses to

more aversive events such as shearing are not prevented by habituation [398-400], and sampling could therefore potentially confound behavioural and learning assessments. In addition, this would increase the study duration and time between trials, and reduce the capacity to compare results to prior studies in which samples were not collected such as those by Hernandez and colleagues [192].

Finally, in Chapter 5, I was able to evaluate only a limited, two-dimensional region of the brain, and therefore analysis of cerebral structural asymmetry was conducted in only a comparatively small area of the brain. It is difficult to generalise my results to lateralisation of each hemisphere as a result, and therefore comments about the lack of difference between PR and CON can only be confined to the prefrontal cortex and caudate nuclei within the slices studied. There are other brain regions involved in spatial learning, such as the right hippocampus, parietal and temporal lobe [59], in which asymmetry may also influence behavioural outcomes, and these would be of benefit to examine in future studies. There may additionally be differences present in the relative utilisation of each hemisphere in spatial learning and navigation. In the human this has been examined utilising electroencephalograms to examine brain activity during navigation of a virtual maze, and functional MRI has been used to similarly examine relative hemisphere contribution movement control [47-49]. Such studies may be impossible to do in the sheep, however, for practical reasons. Similarly, while there are likely to be changes to other forms of functional lateralisation in the PR sheep, maze-based physical tasks remain the easiest to conduct. The lateralisation task utilised in Chapter 5 made use of a task previously used in non-PR sheep [326], and indeed it remains the only validated protocol of spatial lateralisation in sheep. In contrast, the Y-maze studies of Kendrick and colleagues measured perceptual lateralisation of faces not spatial lateralisation [401]. The diamond maze task that I used in studies described

in Chapter 5, however, specifically measures side preference and my results should therefore not be interpreted as conclusive evidence of the effects (or lack thereof) of PR on spatial lateralisation more generally.

6.5 Future directions

The first and most obvious future direction would be to repeat this study in a cohort comprising singletons only, in order to assess effects of PR without the natural restriction in CON animals and confounding by group differences in litter size. Additionally, three areas stand out as logical directions for future investigation. The first is the further examination of effects of PR on sheep cognition during more complex tasks, the second is further investigation on effects of stress on cognition following PR, and the third is to examine further behavioural and cerebral lateralisation using different measures.

The methods used in studies reported in Chapters 3 and 4 examined relatively simple spatial learning and memory tasks, with reversal learning investigated as a simple measure of executive function. Complex executive function, including intra- and extra-dimensional set-shifting has been demonstrated in healthy sheep [303, 308], and this may provide an avenue to investigate more complex learning outcomes in the PR sheep. These studies would be particularly interesting given the intriguing observation that PR sheep learned reversal tasks more quickly than CON sheep. Studies of executive function are likely to be more appropriate in older sheep than younger. Although lambs as young as 14 weeks old are capable of learning executive function tasks [401], we observed in Chapters 2 and 3 that younger sheep at 18 weeks of age were more emotionally reactive than older sheep at 40 weeks of age (eg. bleated more in the majority of tasks). Since stress can impair learning and memory [323, 402], subtle measures of learning such as executive function may be less able

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to detect treatment effects in pre-pubertal animals than in adults.

Additionally, having observed altered behavioural stress responses during reversal learning tasks in PR sheep, it would be worthwhile further investigating stress response and its effects on learning. The positive relationship between bleats and birth weight observed in female sheep in the reversal task in Chapter 4 suggests a dampened stress response to this task, which agrees with our findings in control sheep found both more difficult and more stressful than the other learning and memory tasks (Chapter 3, [304]). The positive relationship between bleats and birth-weight in female sheep differs from observations in humans, where stress responses in boys and circadian peak cortisol in girls were each inversely correlated with birthweight [321]. Physiological measures, such as cortisol response, are needed to confirm that bleating is a stress-related behaviour in this task, however. This may in turn impact learning, as studies in humans suggest there is a bell-curve relationship between cortisol and memory [323], such that excessively low or high levels of cortisol are equally likely to impair learning. Accordingly, the decreased stress response in low birth weight female sheep may reflect a shift of this bell-curve, which may in turn influence learning on a number of tasks.

Finally, the outcomes reported in Chapter 5 suggest that further examination of behavioural and cerebral lateralisation should be conducted, using different measures. The differences in behavioural lateralisation reported in Chapter 5 were generally task-specific, and suggest that PR alters specific aspects rather than inducing global changes of behavioural lateralisation. The maze and locomotion tasks I conducted assessed side preference in simple spatial decision-making tasks, however, there are other lateralised aspects of spatial learning, including working memory and navigation skills, as well as perceptual lateralisation. Studies

of executive function in sheep generally involve training sheep to navigate using visual cues [303], and studies in which these cues have been faces indicate that sheep generally show a left hemifield bias when a facial cue is utilised [390]. There is the potential to examine relative salience of cues presented in the left or right maze arm using the diamond maze in the present study, and the effects of such cues on working memory. Studies in rabbit models of IUGR have also examined object memory, using a task that assessed both memory of the object's appearance and the object's location [198], and a similar approach could be used to examine spatial lateralisation in sheep. Additionally, a radial arm maze task could be utilised to determine whether PR sheep have impaired memory of spatial cues or the internal map. Outcomes in such tasks may not necessarily correspond with the findings of the present study, which examined side preference alone, as each task utilises different regions of the brain, which in turn may be differently affected by IUGR.

6.6 Conclusions

The studies described in this thesis utilised a sheep model of IUGR to examine effects on learning, behaviour and brain morphology, and observed a number of outcomes that are useful both to further studies of sheep cognition and relevant to mechanisms underlying effects of human IUGR on neurodevelopment and function. Consistent with my first hypothesis, sheep at 40 weeks of age recalled tasks learned at 18 weeks. The enduring memory of tasks across 22 weeks and novel observation of different exit routes in healthy sheep confirm that sheep are an excellent model for complex behavioural studies of behaviour, and complement the neurodevelopmental studies already taking place in this species.

I observed only limited effects of PR on spatial learning and behavioural lateralisation, which were extremely sex-specific, and differed to those which were a consequence of low birth weight. Contrary to my hypotheses, there were no differences in brain morphology in prefrontal cortex brain slices, and low birth weight was a far less important predictor of outcomes in the sheep compared to skull width and growth in early life. Additionally, all morphological and behavioural measures were sex-specific, and effects of PR, birth weight and fractional growth rate differed with age, with some relationships and group differences only manifesting at a later age. Not only would these complex relationships be difficult to have learned by studying IUGR children due to environmental confounding, but these long-term consequences in this species are likely to have been overlooked, as the majority of studies have studied consequences of IUGR in infants and children,.

What this thesis ultimately presents is the observation that there are three separate factors influencing cognitive and neurodevelopmental outcomes: PR status, poor prenatal growth and poor postnatal growth, all of which acted in combination to impair outcomes in the adolescent and adult sheep. This implies that SGA and IUGR groups in humans should not be treated as interchangeable. Additionally, while my data confirms that rapid postnatal growth proves beneficial for cognition, it also emphasises the limits to which postnatal treatment may benefit outcomes following IUGR. This also indicates that prenatal intervention is a necessity in order to normalise neurodevelopment in IUGR children.

Chapter 7 - Appendices

This section contains the first pages of all published articles produced during the course of my PhD candidature, including those that make up section 1.6 of Chapter 1, and Chapters 3, 4 and 5 of this thesis.

Authored papers

Appendix 1

Hunter, D. S., Hazel, S.J., Kind, K.L., Liu, H., Marini, D., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2014). "Do I turn left or right? Effects of sex, age, experience and exit route on maze test performance in sheep." <u>Physiology and Behavior</u> 139: 244–253.

Do I turn left or right? Effects of sex, age, experience and exit route on maze test performance in sheep

CrossMark

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HIGHLIGHTS

- We studied the effects of age and experience on maze learning in sheep.
- Sheep remember maze tasks taught at 18 weeks of age for at least 22 weeks.
- Age plus experience improve learning, age alone affects mostly behavior.

ARTICLE INFO

ABSTRACT

Brain development and function are susceptible to perturbation by environmental factors. Sheep are increasingly being used as a neurodevelopmental model due to timing similarities with humans, but effects of age, experience and sex on cognition are not well characterised in this species. We therefore studied memory and reversal learning in sheep using a modified Y-maze at two ages: naive 18 weeks old (18N: 23 male, 17 female), experienced 40 week old sheep that had previously been tested at 18 weeks (40E: 22 male, 17 female), and naive 40 weeks old (40N: 4 male, 10 female). Younger naive animals (18N) required more trials and time to solve the first reversal task (task R1) than 40E (P = 0.007 and P < 0.001 respectively). Experience also improved outcomes, with 40N sheep requiring more time to solve tasks L (P = 0.034) and R1 (P = 0.002) than 40E. Increasing age (40N cf. 18N) decreased bleat frequency in tasks R1, M2 and R2 (each P < 0.05). In 40N females, outcomes also differed by exit method in task R1, with those that exited via an indirect route taking less time to pass tasks R1 (P = 0.009) and R2 (P = 0.015) than those that used a direct route. Age plus experience improved learning outcomes, demonstrating knowledge retention for 22 weeks in this species, whilst age alone affected mostly behavioral responses. These results provide comparison data, and can be utilised to improve experimental design, for studies of neurodevelopment in the sheep.

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1. Introduction

Brain development and function are susceptible to perturbation by various exposures and environmental factors in early life. For example, in humans preterm birth [1,2], prenatal undernutrition [3] and intra-uterine growth restriction [2,4] are associated with a lower intelligence quotient (IQ) and poorer learning, memory and executive function in children and adolescents. Techniques such as magnetic resonance imaging [5] and transcranial magnetic stimulation [6] allow for the study of the morphological and some functional determinants of these cognitive capacities. The use of non-human species extends this to enable studies of the molecular basis and causal pathways that underlie associations between brain development and postnatal function, and early testing of interventions. Further, non-human species enable minimization of confounding factors affecting neurodevelopment and postnatal function and loss due to drop out and permit more rapid follow-up of long term outcomes than is possible in humans.

The sheep is an appropriate species in which to test early life environmental effects on brain development and function, in part due to similar timing of key neurodevelopmental events as humans. In humans and sheep, neurogenesis commences in the first third of gestation [7,8], and myelination by the last third of gestation [7,9,10], although in humans myelination commences after birth in some of the higher

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Appendix 2

Hunter, D. S., Hazel, S.J., Kind, K.L., Liu, H., Marini, D. Giles, L.C., De Blasio, M. J., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2015). "Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behaviour in sheep in an age- and sex-specific manner." <u>Physiology and Behavior</u> 152(Pt A): 1-10.

Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behaviour in sheep in an age- and sex-specific manner☆,☆☆

CrossMark

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H I G H L I G H T S

- We studied cognitive effects of restricted placental and fetal growth (PR) in sheep.
- PR males at 18 and 40 weeks of age had impaired learning compared to controls.
- PR sheep performed better on reversal tasks than controls at 18 weeks of age.
- Rapid neonatal growth predicted better memory in males at 40 weeks of age.

A R T I C L E   I N F O

A B S T R A C T

Intrauterine growth restriction and slow neonatal growth in humans are each associated with poorer learning, memory and cognitive flexibility in childhood and adulthood. The relative contributions of pre- and post-natal growth to cognitive outcomes are unclear, however. We therefore compared performance in learning, memory and reversal tasks using a modified Y-maze at 18 and 40 weeks of age in offspring of placentally-restricted (PR: 10 M, 13 F) and control (23 M, 17 F) ovine pregnancies. We also investigated relationships between size at birth, neonatal growth rates and cognitive outcomes. PR had limited effects on cognitive outcomes, with PR males requiring more trials to solve the initial learning task than controls (P = 0.037) but faster completion of reversal tasks in both sexes at 18 weeks of age. In males, neonatal growth rate correlated inversely with numbers of trials and total time required to solve memory tasks at 40 weeks of age. In females, bleat frequency in the first reversal task at 18 weeks of age correlated positively with birth weight (r = 0.734, P < 0.05) and neonatal growth rate (r = 0.563, P < 0.05). We conclude that PR induces limited effects on cognitive outcomes in sheep, with some evidence of impaired learning in males, but little effect on memory or cognitive flexibility in either sex. Rapid neonatal growth predicted improved memory task performance in males, suggesting that strategies to optimize neonatal growth may have long-term cognitive benefits but that these may be sex-specific.

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1. Introduction

Intrauterine growth-restriction (IUGR) is associated with impaired neurodevelopment, with life-long consequences for cognitive function [1]. Small size at birth corrected for gestational age (SGA, size at birth below the 10th centile for gestational age) is often used as a surrogate marker of IUGR in humans when repeated measures of fetal growth are not available. Children born small for gestational age have, on average, IQ 6–11 points lower than their peers, poorer language skills, impaired spatial learning and memory, and higher incidences of behavioural and attentional problems [2–5]. These deficits have functional

Appendix 3

Hunter, D. S., Hazel, S.J., Kind, K.L., Owens, J.A., Pitcher, J.B., Gatford, K.L. (2015). "Programming the brain: Common outcomes and gaps in knowledge from animal studies of IUGR." Physiology & Behavior 164, Part A: 233-248.

Programming the brain: Common outcomes and gaps in knowledge from animal studies of IUGR

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HIGHLIGHTS

- IUGR has long-term effects on brain structure and function.
- We review animal models of IUGR used to study neurodevelopment and cognition.
- Timing of neurodevelopment and restriction impacts interpretation and translation.
- We end with a set of recommendations for design of future studies.

ARTICLE INFO

ABSTRACT

IUGR in humans is associated with impaired pre- and postnatal neurodevelopment, and subsequent postnatal cognition, resulting in lower IQ, poorer memory, visuomotor and executive function skills, as well as behavioural and attentional problems. Experimental models of IUGR are needed to allow direct testing of causality and interventions, and have benefits in reducing both confounding by comorbidities such as prematurity, and variation due to environment and genetics. This review describes and discusses experimental models of IUGR in which neurodevelopmental and cognitive outcomes of IUGR have been reported. We consider the timing of neurodevelopment relative to birth and to the period of restriction, as well as the effects of each experimental perturbation on the fetal environment and development, before discussing neurodevelopmental and cognitive outcomes for progeny as fetuses, neonates and into adolescent and adult life. Experimental IUGR induces broadly similar outcomes to human IUGR, with altered brain morphology, in particular grey matter loss and discordant trajectory of white matter development, and poorer cognition and memory reported in various studies. Nevertheless, there remain gaps in knowledge of neurodevelopment in experimental models. We end the review with recommendations for the design of future studies to further investigate the mechanisms underlying adverse neurodevelopmental consequences of IUGR, and to evaluate interventions that may subsequently improve outcomes of IUGR in humans.

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Contents

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  E-mail address: kathy.gatford@adelaide.edu.au (K.L. Gatford).

Appendix 4

Hunter, D. S., et al. (2016). "Effects of induced placental and fetal growth restriction, size at birth and early neonatal growth on behavioral and brain structural lateralization in sheep." Laterality Oct 19: 1-30. [Epub ahead of print].

Co-authored papers

Appendix 5

Wooldridge, A. L., Bischof, R.J., Meeusen, E.N., Liu, H., Heinemann, G.K., **Hunter, D.S**., Giles, L.C., Kind, K.L., Owens, J.A., Clifton, V.L., Gatford, K.L. (2014). "Placental restriction of fetal growth reduces cutaneous responses to antigen after sensitization in sheep." American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 306(7): R441-446.

Appendix 6

Liu, H., Schultz, C.G., De Blasio, M.J., Peura, A.M., Heinemann, G.K., Harryanto, H., **Hunter, D.S.**, Wooldridge, A.L., Kind, K.L., Giles, L.C., Simmons, R.A., Owens, J.A., Gatford, K.L. (2015). "Effect of placental restriction and neonatal exendin-4 treatment on postnatal growth, adult body composition, and in vivo glucose metabolism in the sheep." American Journal of Physiology - Endocrinology and Metabolism 309(6): E589-E600.

NOTE:

This publication is included on page 230 in the print copy
of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.1152/ajpendo.00487.2014

Conference presentations

Appendix 7

Maternal dietary methyl supplementation normalises brain structure in the placentally-restricted sheep

Damien Hunter, Hong Liu, Kathryn L Gatford, Julie A Owens, Karen L Kind, Julia Pitcher, Susan Hazel

Background: Intrauterine growth restriction (IUGR) retards neurodevelopment [1], and is linked to learning and short-term memory impairment in children [2]. IUGR children have reduced cortical volume and thickness [3], and a stronger right lateralisation at the expense of left-sided function [4]. Epigenetic mechanisms may underlie some effects of IUGR on later development. IUGR in rodents decreases expression of DNA methyltransferase 1 in fetal brain, and causes global and CpG island DNA hypomethylation in cerebrum at birth [5]. We therefore investigated effects of restriction of placental and fetal growth (PR) in sheep on brain structure, and tested whether supplementing the maternal diet with methyl donors, in order to increase supply to the fetus, would normalise brain structure of PR offspring.

Hypotheses: 1. PR in sheep will reduce brain grey matter area postnatally, and 2. maternal dietary methyl supplementation of PR sheep during late pregnancy will ameliorate this.

Method: PR was induced in Border Leicester x Merino ewes by removal of the majority of placental implantation sites from the endometrium ≥10 weeks before mating. From d 120 of pregnancy to term, a subset of PR ewes (MethylPR) were fed dietary methyl supplements (2 g.d^{-1} rumen-protected methionine, 300 mg.d^{-1} folic acid, 1.2 g.d^{-1} sulphur, 0.7 mg.d^{-1} cobalt). Offspring were humanely killed at 52 weeks, brains dissected, sectioned coronally and digitally photographed. Brain morphology in a cross-section at the sylvian gyrus, predominantly cerebrum, was assessed using Axiovision LE (Carl Zeiss MicroImaging GmbH, Germany) and effects of sex and treatment analysed by two-way ANOVA.

Results: Total and grey matter areas of brain and left hemisphere, and the ratios of grey:white matter areas overall and in the left hemisphere (Figure) were lower in PR than CON sheep (all P<0.03). Maternal methyl supplementation restored these outcomes in PR progeny (MethylPR cf. CON, all P>0.4). White matter areas were not affected by treatment. Brain areas and proportions did not differ between sexes.
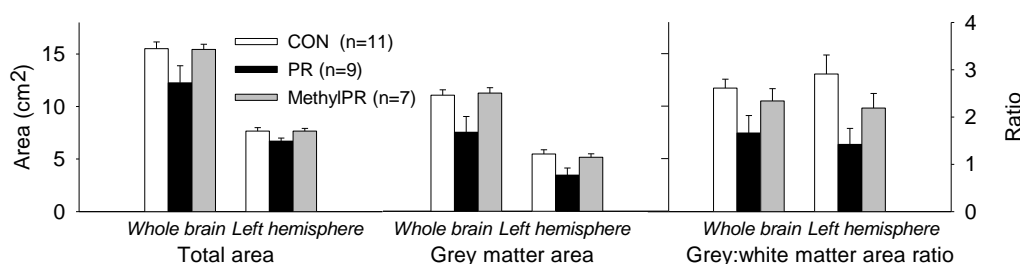
Conclusions: Loss of grey matter suggests loss of neuronal numbers after PR, particularly in the left hemisphere. Prenatal maternal dietary methyl supplementation ameliorates this, and may improve brain function, particularly for right-side preferenced tasks.

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Appendix 8

Placental restriction of fetal growth induces sex-specific changes in learning in maze tasks in adolescent and young adult sheep

Damien Hunter, Kathryn L Gatford, Karen L Kind, Hong Liu, Miles De Blasio, Julie A Owens, Julia Pitcher, Susan Hazel

Background: Intrauterine growth restriction (IUGR) retards neurodevelopment, particularly of the hippocampus [1]. IUGR children have impaired hippocampal-associated cognition, including learning and short term memory [2]. In humans, both deficient and excess cortisol levels impair learning [3]. IUGR has sex-specific effects on the stress axis in children. Low birth weight boys secrete more cortisol when stressed than those of normal birth weight [4], which may also contribute to cognitive impairment. However, examining these mechanisms, and the interactions between the two, is difficult in humans due to postnatal confounding factors such as socioeconomic status. We therefore investigated effects of restriction of placental and fetal growth (PR) in sheep on cognition utilizing a maze protocol.

Hypotheses: 1. Restriction of placental and fetal growth (PR) will impair learning speed, cognitive flexibility and short term memory in adolescent and young adult sheep. 2. PR will impair these outcomes to a greater extent in males than in females.

Method: PR was induced in Border Leicester x Merino ewes by removal of the majority of endometrial placental implantation sites ≥10 weeks before mating. Maze testing was performed at 18 and 40 weeks of age in control (CON, n = 16M, 14F) and PR progeny (n = 6M, 7F). Learning, memory, cognitive flexibility, and bleat frequency (a behavioural stress measure), were examined by sequential training, learning and reversal tasks. Effects of treatment, sex, age and task were analysed by repeated measures ANOVA.

Results: The effects of PR on learning measures and behavioural stress differed by sex. PR males took more trials to solve each task than CON males, whereas PR females took fewer trials than CON females (sex*treatment, p=0.018). When adjusted for animal speed (time for each task relative to time taken on the initial training task), 18 wk CON females took less time than PR females to learn reversal and retention tasks, related to learning extinction and memory respectively, whereas PR males took longer than CON males (sex*treatment, p<0.0001). At 18 weeks, PR males bleated more frequently than CON males, and PR females less than CON females (sex*treatment, p=0.045). Inclusion of bleat frequency as a covariate in analysis of maze performance removed the effects of PR on relative time to learn each task, but the effects of PR on the number of trials remained.

Conclusions: Consistent with our hypothesis, effects of PR on learning speed and cognitive flexibility differed with sex, with males but not females being impaired. Differences in apparent stress appear to account for some but not all of these sex-specific effects of PR. The greater relative time the PR males took during reversal tasks suggests poorer cognitive flexibility, whereas the more rapid learning extinction in females may indicate a cognitive advantage due to decreased stress following PR.

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Abstract for Fetal and Neonatal Workshop 2014

Low birth-weight and poor postnatal growth correlate with poorer memory and cognitive flexibility in male IUGR sheep in maze tasks

Damien S. Hunter, Susan J. Hazel, Karen L. Kind, Hong Liu, Danila Marini, Lynne Giles, Julie A. Owens, Julia B. Pitcher, Kathryn L. Gatford

Background: Catch-up growth in the first six months of life ameliorates the adverse effect of intrauterine growth restriction (IUGR) on learning, memory and cognitive flexibility in humans[1]. Assessing the relative contributions of prenatal and postnatal growth is difficult in humans due to a range of confounding factors that can affect neurodevelopment. We therefore examined the relationship of birth weight and neonatal growth with measures of learning, memory and cognitive function in adolescence, in control sheep and in sheep whose growth was restricted before birth.

Hypothesis: Birth weight and neonatal growth rate will correlate negatively with number of trials and time required to solve maze learning tasks in adolescent sheep.

Methods: Low birth weight was induced by surgical reduction of placental implantation sites (PR) and by natural twinning. Birth weight (BW), and fractional growth rate (FGR) during the first 16 days of life (during rapid neonatal catch-up growth in this species) were measured in control (23M, 17F) and PR (6M, 10F) sheep. Trials and time per task were recorded for initial learning (L), memory (M1, M2) and reversal (R1, R2) maze tasks at 18 and 40 weeks of age[2,3]. Relationships between BW and FGR and outcomes were analysed using multiple linear regression modelling (time per task) or Poisson regression modelling (numbers of trials per task), with significance for effects of BW or FGR accepted at $p<0.05$.

Results: When both sexes were examined together, BW and FGR did not affect learning at 18 weeks. Also in both sexes, time to solve Task M1 at 40 weeks correlated negatively with FGR (std β = -0.29, p=0.026) and tended to correlate negatively with BW (std β = -0.24, p=0.074). Other correlations at 40 weeks were sex specific: number of trials to solve Task M1 correlated negatively with FGR (std β = -0.23, p=0.025), and also tended to with BW (std β = -0.24, p=0.09) in males, but not females (both p>0.8). Number of trials to solve Task M2 tended to correlate negatively with FGR in males (std β = -0.20, p=0.098) but not females (p>0.3).

Conclusions: As with humans, slow neonatal growth rate in sheep correlated with impaired learning performance on a memory task. This was most evident in males, and after puberty. Effects of variation in BW were limited, with some indication of negative effects of low BW in males, but not females. Interventions to promote neonatal growth may be beneficial for cognitive outcomes, particularly for males; the sheep may provide a useful model for such investigations.

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