Effects of high-dose caffeine on the cerebrum of the immature ovine brain

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BSc (Hons)

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Notice 1

Notice 2

“It always seems impossible until it’s done”

-Nelson Mandela

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Summary

Preterm birth is a worldwide clinical problem and despite advances in obstetric care, infants born preterm have higher rates of morbidity including cerebral palsy and neurodisability. Infants born very preterm (<34 weeks) are at an increased risk of developing apnea of prematurity (AOP) a condition of irregular/intermittent breathing which can contribute to unfavourable neurodevelopmental outcomes. Caffeine administration is one of the few treatments available for these infants, with proven efficacy. The current treatment involves a loading dose of 20mg/kg caffeine (citrate) followed by a daily maintenance dose of 5-10mg/kg and this dose of caffeine is associated with improved neurodevelopmental oucomes. Infants that do not respond to the current clinical dosing of caffeine may require higher doses. However, the effects of higher doses of caffeine on the developing brain have not been subject to rigorous neuropathological evaluation in a suitable animal model. Thus there is an urgent need to evaluate the safety of a high-dose caffeine treatment on the development of the immature brain. The global aim of this thesis has therefore been to investigate the effects of high-dose caffeine administration on the development of the immature brain. To do this, I have used an ovine model, where caffeine was administered to fetal sheep at a stage of brain development similar to that of a very preterm infant.

In Chapter 3, the fetal physiological response to high-dose caffeine was assessed to determine if caffeine has a direct effect on the brain or an indirect effect through alterations in fetal physiology. As caffeine was administered *in utero*, assessment of maternal physiological response was necessary in order to determine if any changes in fetal physiology was a result of altered maternal status. The data presented in Chapter 3 demonstrated that the dosing regimen of caffeine base used

(25 mg/kg loading; 20 mg/kg maintenance) produced high fetal plasma caffeine levels, which were comparable to plasma caffeine concentrations measured in preterm infants administered higher than standard doses of caffeine. Exposure to high-dose caffeine did not adversely affect fetal blood chemistry over the 15-day administration period, nor did it affect fetal growth. High-dose caffeine did lead to transient alterations in fetal cardiovascular physiology in the first 3 days of caffeine exposure. As we did not measure cardiovascular variables after this period it is unknown whether these transient alterations continued. We conclude that any observed neuropathological effects of caffeine are likely to be a direct, rather than an indirect action on the brain.

Chapter 4 assessed the impact of high-dose caffeine on the developing white matter, a region of high vulnerability in the preterm infant. The results from this chapter suggested that daily administration of high-dose caffeine did not cause any structural alterations to the developing white matter, or alterations to associated glial cells, in the very immature ovine brain. We conclude that high-dose caffeine does not overtly injure the developing ovine white matter.

The potential effect of caffeine on the grey matter was assessed in Chapter 5. Here high-dose caffeine was associated with increased Ctip2-positive subcerebral projection neurons, GFAP-positive astrocytes and Olig2-positive oligodendrocytes in the cortical grey matter; there was no effect on somatostatin-positive GABAergic interneurons. It is possible that high-dose caffeine may increase proliferation of these cells, however this requires further investigation. The findings from this chapter suggest that high-dose caffeine may have adverse consequences for the ovine cortical grey matter. Since subcerebral projection neurons are excitatory glutamatergic neurons in the cortex, an increase in this cell type with no alterations in inhibitory GABAergic neurons may lead to cerebral dysconnectivity and thus cognitive and/or motor deficits. Thus further investigation is required to determine if these changes persist in the long-term.

In Chapter 6, the long-term effects of high-dose caffeine were investigated. The data from this chapter showed that although caffeine exposure did not compromise growth trajectory at 2 months after birth, it did decrease kidney weight by 14%. As nephrogenesis coincided with the period of caffeine administered, we conclude that early caffeine exposure may alter long-term kidney growth by increasing the functional demands of the kidney and subsequently disrupting developing nephrons. Thus the long-term renal effects of high-dose caffeine need to be assessed further. Prenatal high-dose caffeine exposure did not adversely affect white matter structure, detected using diffusion MRI at 2 months of postnatal age. Grey matter structure was not assessed in the present

study, however, given the increase in neuronal and glial cell density described in Chapter 5, this region requires further investigation.

In conclusion, the studies presented in this thesis increase our understanding of the effects of high-dose caffeine on neurodevelopment and provide substantial evidence that high-dose caffeine can affect fetal development. This thesis also provides a model that may help us better understand the effects of high-dose caffeine on the developing brain. As caffeine has been, and will remain, the treatment of choice for AOP, and infants who do not response to standard caffeine doses will continue to receive higher doses, we need to further investigate the neuropathological outcomes associated with caffeine use. The results from this thesis add to our understanding of the safety of high-dose caffeine. Studies must now focus on determining the maximal dose of caffeine that is not only effective for AOP, but also safe for developing and highly vulnerable preterm infants.

Declaration

I hereby declare, that no part of this thesis has been submitted for the award of any other degree or diploma at any university or equivalent institution. To the best of my knowledge, this thesis contains no material previously published or written by any other person, except where due reference is made in the text. All experiments reported in this thesis complied with the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes and had approval from the Monash University Animal Welfare Committee.

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List of Publications

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Conference abstracts:

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Atik A., Tolcos M., Cheong J., Harding R. and De Matteo R. (2011) Consequences of high dose caffeine treatment on fetal physiology and the immature brain. *Department of Anatomy & Developmental Biology Departmental Seminar, Monash University, Victoria, Australia*.

Atik A., Tolcos M., Cheong J., Harding R. and De Matteo R. (2011) Consequences of high dose caffeine treatment on the development of the immature brain. *Students of Brain Research Student Symposium, Melbourne Brain Centre, Parkville, Victoria, Australia*.

Atik A., Tolcos M., Cheong J., Harding R. and De Matteo R. (2011) Does high dose chronic caffeine treatment affect physiological status and brain development of the immature fetus? *Australian Society for Medical Research (ASMR) Student Symposium, Melbourne, Victoria, Australia*.

Atik A., Tolcos M., Cheong J., Harding R. and De Matteo R. (2011) Caffeine for apnoea of prematurity: does high dose caffeine treatment affect the development of the immature brain. *Department of Anatomy and Developmental Biology Postgraduate Student Symposium, Monash University, Victoria, Australia*.

Atik A., Tolcos M., Cheong J., Harding R. and De Matteo R. (2011) Does high dose chronic caffeine treatment affect physiological status and brain development of the immature fetus. *The Ritchie Centre 2011 Colloquium, Monash University, Victoria, Australia*

Atik A., Tolcos M., Cheong J., Harding R. and De Matteo R. (2011) Does high dose chronic caffeine treatment affect physiological status and brain development of the immature fetus? *Fetal and Neonatal Physiological Society (FNPS) Meeting, Palm Cove, Queensland, Australia*.

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Atik A., De Matteo R., Cheong J., Johnston L., Wright D., Harding R., Rees S. and Tolcos M. (2012) Long-term consequences of chronic caffeine exposure: effects on growth and the developing brain. *Fetal and Neonatal Physiological Society Workshop, Newcastle, New South Wales, Australia*.

Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2012) Caffeine for apnoea of prematurity: effects of chronic caffeine exposure on white matter in the very immature brain. *Federation of Asia and Oceania Perinatal Societies (FAOPS) & Perinatal Society of Australia and New Zealand (PSANZ) Congress, Sydney, New South Wales, Australia*.

Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2012) Does high dose caffeine administration affect the developing brain? *Department of Anatomy & Developmental Biology Postgraduate Student Symposium, Monash University, Victoria, Australia*.

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Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2012) Does chronic caffeine exposure affect white matter in the immature brain? *The Ritchie Centre 2012 Colloquium, Monash Institute of Medical Research, Victoria, Australia*.

Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2012) Caffeine for apnoea of prematurity: impact of chronic caffeine exposure on white matter in the very immature brain. *Pediatric Academic Societies (PAS) Meeting, Boston, Massachusetts, USA*.

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Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2013) Impact of high dose caffeine administration on the developing cerebral cortex. *Perinatal Society of Australia and New Zealand (PSANZ) Congress, Adelaide, South Australia*.

Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2013) High-dose caffeine for the treatment of apnoea of prematurity: is it safe for the developing brain. *Pediatric Academic Societies (PAS) Meeting, Washington, D.C, USA*.

Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2013) Caffeine for apnoea of prematurity: impact of high dose caffeine administration on the developing brain. *Monash Health Research Week, Monash Medical Centre, Victoria, Australia*.

Atik A., De Matteo R., Cheong J., Harding R., Rees S. and Tolcos M. (2013) Caffeine for apnoea of prematurity: does high dose caffeine treatment affect the development of the immature brain. *The Ritchie Centre 2013 Colloquium, Melbourne Cricket Grounds, Victoria, Australia*.

List of Abbreviations

~	Approximately
*****	Asterisk
°C	Degrees Celsius
=	Equals
-	Minus; negative
x	Multiply
%	Percent
/	Per; divide by
+	Plus
±	Plus or minus
®	Registered trademark
Σ	Sum of (sigma)
AMP	Adenosine monophosphate
ANOVA	Analysis of variance

AOP	Apnea of prematurity
AR	Adenosine receptor
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
BP	Blood pressure
BPD	Bronchopulmonary dysplasia
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CAP	Caffeine for apnea of prematurity
cAMP	Cyclic adenosine monophosphate
CC	Corpus callosum
cm	Centimetre(s)
cm^3	Centimetre(s) cubed
CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase
CN	Caudate nucleus
CNS	Central nervous system
CO_2	Carbon dioxide
CP	Cortical plate
CPAP	Continuous positive airway pressure
CRL	Crown-to-rump length
CREB	Ca^{2+}/cAMP response element binding protein
CSF	Corticospinal fluid
CST	Corticospinal tract

Ctip2	Chicken ovalbumin upstream promoter transcription factor-interacting protein 2
Cx	Cortex
DAB	3,3'-diaminobenzidine solution
DCD	Developmental coordination disorder
DG	Days gestation
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer xylene
EMG	Electromyography
Fezf2	Forebrain embryonic zinc finder-like 2
FLL	Forelimb length
Fr	Frontal lobe
FRC	Functional residual capacity
g	Gram(s)
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
Glu	Glucose
GM	Grey matter
Hi	Hippocampus
h	Hour(s)
H&E	Haematoxylin and eosin
HR	Heart rate
Iba-1	Ionized calcium binding adaptor molecule

I.D.	Inside diameter
IR	Immunoreactive
i.v.	Intravenous
IVH	Intraventricular haemorrhage
IZ	Intermediate zone
kg	kilogram(s)
L	Litre(s)
Lac	Lactate
LV	Lateral ventricle
M	Molar
MAG	Myelin associated glycoprotein
MAP	Mean arterial pressure
MB	Midbrain
MBP	Myelin basic protein
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
µm	Micrometre(s)
µM	Micromolar
mM	Millimolar
mm	Millimetre(s)
mm^2	Millimetre(s) squared
mmHg	Millimetre(s) of mercury

MOG	Myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MRI	Magnetic resonance imaging
MZ	Marginal zone
NaCL	Sodium chloride
NEC	Necrotizing enterocolitis
NeuN	Neuronal nuclei
NGF	Nerve growth factor
nIPC	Neurogenic intermediate progenitor cell
NMDA	N-methyl-D-aspartate
NT	Neurotrophin
O1	Oligodendrocyte marker 1
O4	Oligodendrocyte marker 4
Oc	Occipital lobe
O.C.T	Optimal cutting temperature
OD	Optical density
O.D.	Outside diameter
oIPC	Oligodendrocyte intermediate progenitor cell
OL	Oligodendrocyte
Olig2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte progenitor cell
p	p value; level of significance
P	Postnatal day(s)

$PaCO_2$	Arterial partial pressure of carbon dioxide
PaO_2	Arterial partial pressure of oxygen
PB	Phosphate buffer
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDGF	platelet-derived growth factor
PFA	Paraformaldehyde
pH	Power of hydrogen ions
PI	Ponderal index
PLP	Proteolipid protein
PNA	Postnatal age
PNS	Peripheral nervous system
Pu	Putamen
PV	Periventricular
SaO_2	Arterial oxygen saturation
Satb2	special AT-rich sequence-binding protein 2
SC	Subcortical
SEM	Standard error of the mean
SMI-312	Pan-axonal neurofilament marker
SST	Somatostatin
St	Striatum
SVZ	Subventricular zone
Tbr1	T-box brain gene 1

TG	Thoracic girth
Th	Thalamus
tHB	Total haemoglobin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VZ	Ventricular zone
w/v	Weight per volume
WM	White matter
ZFP	Zinc finger protein 191

1 Literature review

1.1 Overview

Very preterm infants are born during a critical period of susceptibility to both lung and brain injury during the first few days after birth; these injuries can have life-long effects. Thus it is important to understand how to manage these preterm infants with safe and effective therapies, in order improve the outcome of these infants. The studies included in this thesis were aimed at defining the effects of caffeine, a drug that is commonly used for the treatment of apnea of prematurity (AOP), on the immature, developing brain; a secondary aim was to assess the physiological effects of caffeine administration. A reproducible model using the ovine fetus was established so that the neuropathological outcome and physiological responses at the time of caffeine administration could be assessed. Developmental alterations or injury to the cerebral white matter (WM) and grey matter (GM) are of particular interest to these studies as they are regions that are highly vulnerable in preterm infants. Investigation of the effects that treatments such as caffeine have on the developing brain at the neurostructural level is essential in order to determine the impact of such treatments on postnatal outcome.

In this chapter I shall discuss the key events involved in the development of the human central nervous system (CNS), with particular reference to development of the cerebral hemispheres. I will then discuss the causes and consequences preterm birth, with particular emphasis on AOP.

Treatments for AOP will be introduced followed by a review of the literature relating to the use of caffeine as a treatment for AOP, and the mechanisms believed to be involved in mediating the effects of caffeine. This chapter will also review literature on the effects of caffeine on the developing CNS. I shall then highlight the relevance of the use of ovine models, the chosen species for studies in this thesis, and compare brain development in the sheep to that of the human. This chapter will conclude by presenting the project rationale and aims that were investigated in the studies described in this thesis.

1.2 Human brain development

In humans and most other mammalian species the major events in brain development include formation of the neural tube (neurulation), neuronal proliferation (neurogenesis), neuronal migration and differentiation, synaptogenesis, cell death and synaptic rearrangement, gliogenesis and myelination. However, brain development is a continuous process, and there is overlap between these stages of development (Figure 1.1).

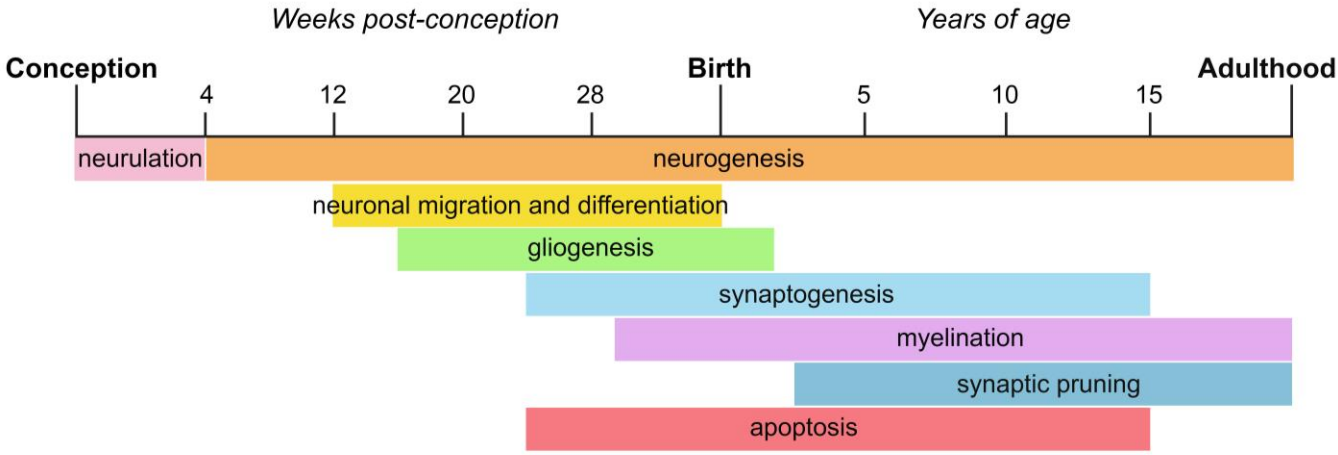


Figure 1.1 Timeline of key neurodevelopmental processes in humans

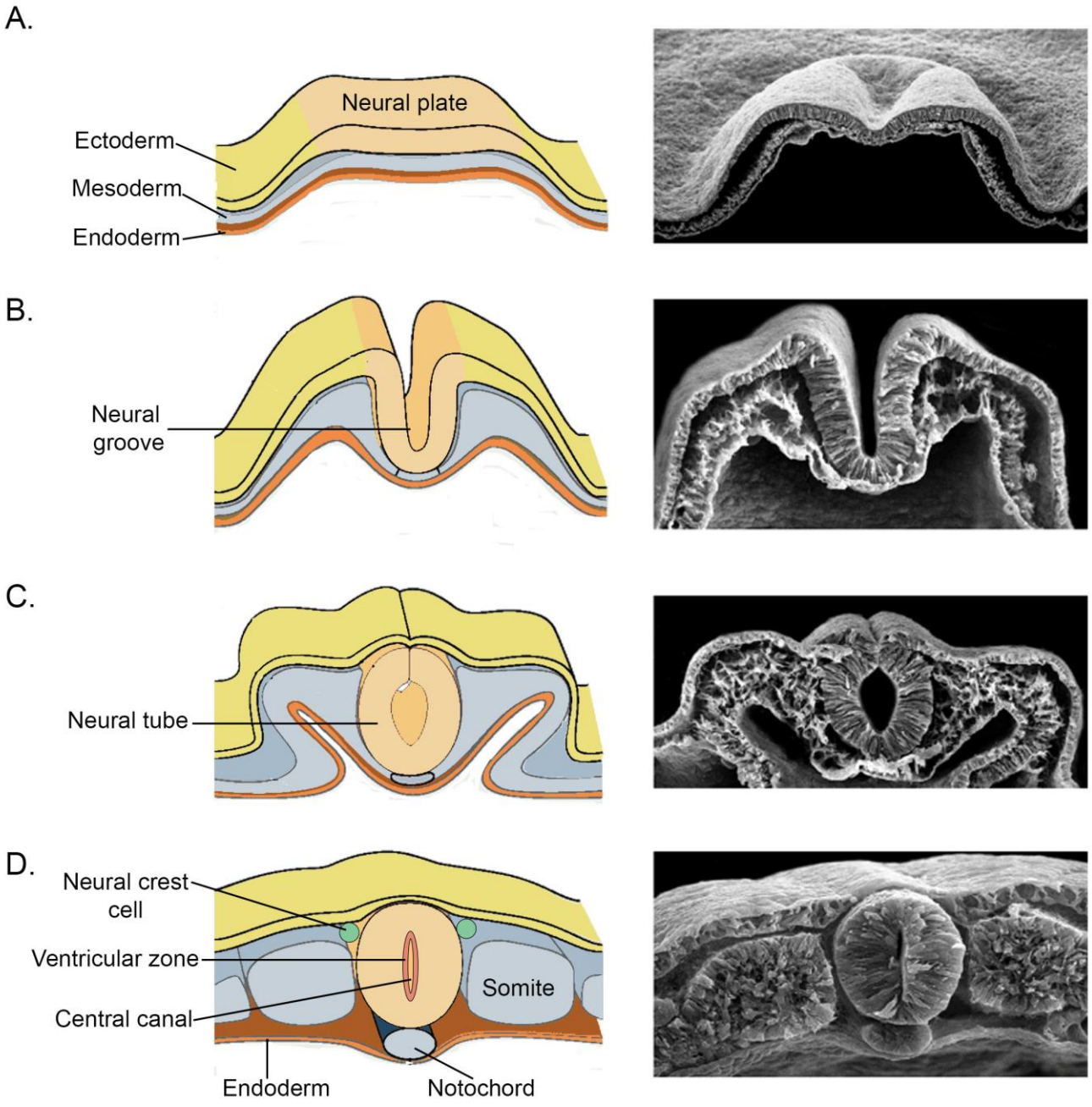
A diagrammatic representation of the key neurodevelopmental processes in humans and the time at which they occur. From gestation and up to adulthood (not to scale), there is a large overlap between each stage of neurodevelopment. The developmental timing may vary between different brain regions. Adapted from (Semple et al., 2013; Ronan et al., 2013).

1.2.1 Neurulation

Neurulation, occurring between 18 to 28 days of gestation, leads to the formation of the brain and spinal cord, marking the primary stage of CNS development (Figure 1.1) (Sanes et al., 2000; DeSesso et al., 1999; Rice and Barone, 2000). The process begins following gastrulation, which sees the establishment of a 3-layer embryo; these layers are the outer or ectodermal layer, which gives rise to the nervous system and skin, the middle or mesodermal layer, which develops to form the majority of the internal body components (connective tissue, muscles and bone) and the inner or endodermal layer which gives rise to the gastrointestinal system (Pomeroy and Kim, 2000; Sanes et al., 2000; Rice and Barone, 2000). At the commencement of neurulation, the proteins follistatin and noggin, released from the mesoderm, initiate the formation of a plate of tissue within the overlying ectoderm known as the neural plate (Smith and Schoenwolf, 1997; Sanes et al., 2000). As development continues, the chordal mesoderm influences the lateral margins of the neural plate to fold back over the plate; these folding margins close dorsally, in both the rostral and caudal direction, to form the neural tube which separates from the underlying ectoderm (Figure 1.2) (Smith and Schoenwolf, 1997; Sanes et al., 2000; Rice and Barone, 2000). Once closed, the neural tube gives rise to the CNS. The neuroepithelial cells within the wall of the tube (ventricular zone) subsequently give rise to the majority of neurons and glia (Kriegstein and Alvarez-Buylla, 2009). The lumen of the tube becomes the future ventricular system eventually containing cerebrospinal fluid (CSF), produced by the choroid plexus. With the help of sonic hedgehog (Smith, 1994), HOX genes (Pomeroy and Kim, 2000) and growth factors (Hatten, 1999) the anterior portion of the neural tube forms the brain and the posterior portion forms the spinal cord (Rice and Barone, 2000). Meanwhile, the neural crest cells, formed during the closure of the neural plate, bud off from the dorso-lateral aspect of the tube and migrate throughout the body and give rise to cells of the peripheral nervous system including dorsal root ganglia, sensory ganglia of cranial nerves, autonomic ganglia, Schwann cells and cells of the pia and arachnoid (Sanes et al., 2000; Rice and Barone, 2000). Following neurulation, specific areas of the CNS begins to form following a sequence of developmental processes including, neurogenesis, proliferation, migration, differentiation, synaptogenesis, apoptosis, synaptic rearrangement and myelination.

Figure 1.2 Stages of neural plate folding leading to the formation of the neural tube

Gastrulation leads to the formation of a three-layer embryo (A). At the commencement of neurulation the chordal mesoderm influences the lateral margins of the neural plate to fold back over the plate (B). The folding margins close dorsally in a rostral-caudal direction and separate from the ectoderm to form the neural tube (C). Following neural tube closure, the ventricular zone is established, from which cells of the CNS will be generated (D). The central canal becomes the future ventricular system containing CSF. The neural crest cells then bud off from the neural tube to form cells of the peripheral nervous system (D, neural crest cells are not visible on the micrograph). The plates on the left represent a diagrammatical representation of neural tube formation while the scanning electron micrographs on the right are taken from chick embryos. Adapted from (Kandel et al., 2000).


A.
Neural plate
Ectoderm
Mesoderm
Endoderm
B.
Neural groove
C.
Neural tube
D.
Neural crest cell
Ventricular zone
Central canal
Somite
Endoderm
Notochord

1.2.2 Neurogenesis

The closure of the neural tube initiates the next stage of CNS development, referred to as neurogenesis, beginning from 28 days of gestation (Rice and Barone, 2000) and continuing years postnatally and well into adulthood, in specific brain regions (Figure 1.1) (Herschkowitz et al., 1997; Gould et al., 1999; Guidi et al., 2005). Neurogenesis, involves a period of rapid mitotic cell proliferation of the single layer of neuroepithelial cells, producing approximately 250,000 cells per minute during the early stages of development (Cowan, 1979). This extensive proliferation leads to the formation of three vesicles by the fifth embryonic week; the prosencephalon, which becomes the forebrain, the mesencephalon, which becomes the midbrain and the rhombencephalon, which forms the hindbrain (Sanes et al., 2000; Rice and Barone, 2000). In the following weeks mitotic cell proliferation leads to the formation of two more vesicles derived from the prosencephalon, namely the telencephalon which forms the cerebral hemispheres and the diencephalon, which forms the thalamus and hypothalamus (Sanes et al., 2000; Rice and Barone, 2000). Furthermore, the rhombencephalon becomes the metencephalon (forming cerebellum and pons) and the myelencephalon (forming the medulla) (Sanes et al., 2000; Rice and Barone, 2000). These basic structures of the CNS form by 6-8 weeks of human gestation, marking the end of embryogenesis (Rice and Barone, 2000).

Neurogenesis then begins in the ventricular zone and later in the subventricular zone via symmetrical and asymmetrical cell division (Figure 1.3) (Noctor et al., 2004; Haubensak et al., 2004). Additionally, radial glia, generated from neuroepithelial cells via direct transformation, are the cells responsible for the generation of neurons, however some neuroepithelial cells are also likely to generate neurons early in development (Gotz, 2003). Firstly, asymmetrical division of radial glia gives rise to one radial glial cell and one postmitotic neuron, or one radial glial cell and one neurogenic intermediate progenitor cell (nIPC) that resides in the SVZ (Haubensak et al., 2004; McConnell, 1995; Pontious et al., 2008). Recent studies now suggest that nIPCs may be classified into two subpopulations, which include apical intermediate progenitor cells and basal intermediate progenitor cells with both types having distinct molecular profiles (Pilz et al., 2013; Reillo and Borrell, 2012; Shitamukai et al., 2011; Wang et al., 2011). Apical intermediate progenitor cells reside in the ventricular zone (VZ) and have short radial attachments to the apical (ventricular) surface, in contrast, basal intermediate progenitor cells delaminate from the VZ and migrate into the

SVZ (Kowalczyk et al., 2009; Hevner and Haydar, 2012). The second mode of neurogenesis is via symmetrical (indirect) division of nIPCs which gives rise to two postmitotic neurons, which like radial glial cells, are a major neurogenic cell population (McConnell, 1995; Haubensak et al., 2004; Arnold et al., 2008; Sessa et al., 2010; Sessa et al., 2008). Additionally, nIPCs can divide symmetrically with two rounds of division, resulting in two nIPCs (Haubensak et al., 2004; McConnell, 1995). These nIPCs can then divide symmetrically to produce neurons (Haubensak et al., 2004; McConnell, 1995).

Although the molecular mechanisms that trigger processes such as the transition of radial glial cells to nIPCs, progenitor cell division and determination of a progenitor cell's phenotype (i.e. neuron or glial cell) are still unclear; several transcriptional regulators such as insulinoma-associated protein 1 (INSM1), T-box brain protein 2 (TBR2) and TMF-regulated nuclear protein (TRNP1) appear to be involved (Sun and Hevner, 2014). As proliferation continues, proportionately more neurons and fewer neural stem cells are produced (Volpe, 2008; Kriegstein and Alvarez-Buylla, 2009). However, subpopulations of radial glia retain apical contact and continue to function as neural stem cells in the neonate (Gotz, 2003). As the cells withdraw from the mitotic cycle and cease proliferative activity, the neurons migrate along the radial glia fibres together in a column away from the ventricular zone, towards the intermediate zone to form the neuronal columns of the cerebral cortex (see Section 1.2.3) (Hansen et al., 2010; Noctor et al., 2004). The neural stem cells however, continue to proliferate as stem cells or undergo cell death (Kriegstein and Alvarez-Buylla, 2009; Pomeroy and Kim, 2000). During development, in most CNS regions the largest and deepest neurons are formed first, followed by the more superficial, smaller neurons and finally glia with the exception of radial glia, which are formed during the early stages of neurogenesis (Sanes et al., 2000). The majority of neurogenesis within the cerebral hemispheres occurs during the first half of gestation, however evidence now suggests that neurogenesis continues in the neurogenic zones in the forebrain (SVZ, subventricular zone; SGZ, subgranular zone of the DG, dentate gyrus; olfactory bulb) and cerebellum (EGL, external granular layer) during development (Gould et al., 1999; Guidi et al., 2005; Eriksson et al., 1998).

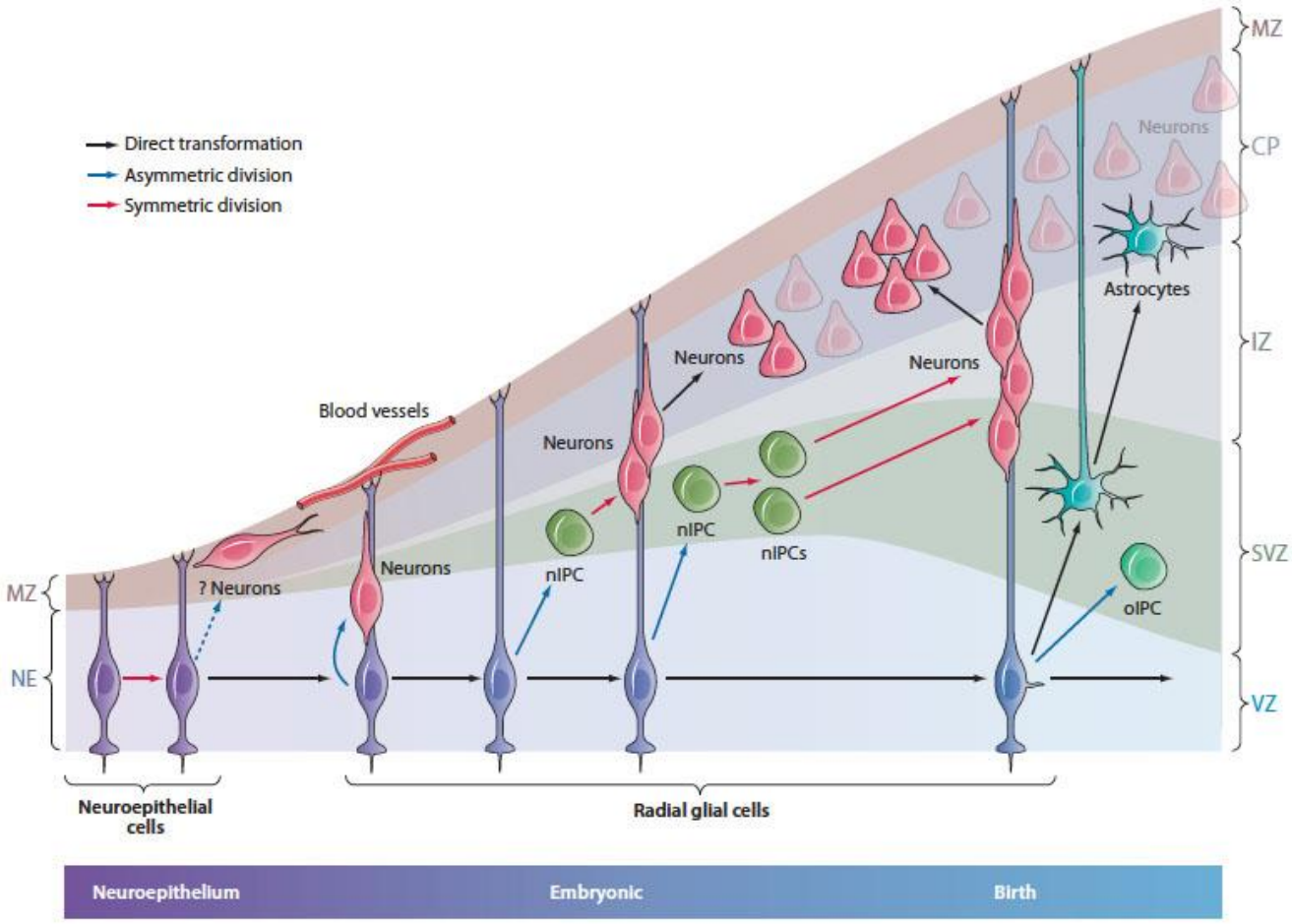


Figure 1.3 Modes of neurogenesis during cortical development

A diagrammatic representation of the three modes of neurogenesis. Radial glia generated from neuroepithelial cells generate neurons via direct transformation *(i)* asymmetric division of radial glia results in one radial glial cell and one neurogenic intermediate progenitor cell or one radial glial cell and one postmitotic neuron; *(ii)* symmetric division of neurogenic intermediate progenitor cells results in two neurons; or *(iii)* neurogenic intermediate progenitor cells divide symmetrically with two rounds of division, resulting in two neurogenic progenitor cells, with further amplification resulting in the generation of neurons. MZ, marginal zone CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone; nIPC, neurogenic intermediate progenitor cell; oIPC, oligodendrocyte intermediate progenitor cell. Taken from (Kriegstein and Alvarez-Buylla, 2009).

1.2.3 Neuronal migration and differentiation

Neuronal migration, occurring between 12 to 20 weeks gestational age in the cerebral cortex (Figure 1.1), marks the time whereby millions of neurons move from the ventricular zone towards their final destination, where they reside for life (O'Rourke et al., 1992). The development of the cortex occurs in an inside-out fashion; the deep cortical layers are formed first followed by the superficial cortical layers (Sanes et al., 2000; Nguyen and Hippenmeyer, 2013). Neuronal migration occurs in sequential steps leading to the formation of the six cortical layers (Ayala et al., 2007; Marin et al., 2010). The first wave of migration occurs via somal translocation (Nadarajah et al., 2001), where the earliest postmitotic neurons migrate out of the proliferative ventricular zone and form the preplate (Allendoerfer and Shatz, 1994; Nadarajah et al., 2001; Price et al., 1997). Following this a wave of postmitotic neurons moves towards the pial surface and splits the preplate into the marginal zone and the subplate (Nadarajah et al., 2001). Here the earliest born Cajal-Retzius cells form the marginal zone, while the subplate neurons form the subplate (D'Arcangelo et al., 1995). The cortical plate then begins to develop between these two layers, eventually giving rise to the multilayered neocortex (Nadarajah et al., 2001).

The second wave of migration leads to the formation of most of the cerebral cortex. During this time, neural progenitor cells in the proliferative ventricular zone, produce a complex variety of neuronal subtypes, with the help of transcription factors such as special AT-rich sequence-binding protein 2 (Satb2), T-box brain gene 1 (Tbr1), forebrain embryonic zinc finger-like 2 (Fezf2) and chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (Ctip2), among many others (McKenna et al., 2011). Although the precise relationship between individual progenitor cell populations and the resulting projection neuron subtype is not known, it is becoming increasingly evident that transcription factors, such as Satb2, Tbr1, Fezf2 and Ctip2 work together to control the identity of neural progenitor cells, towards a callosal, subcerebral or corticothalamic fate (McKenna et al., 2011; Molyneaux et al., 2007). Additionally, these transcription factors have layer and neuronal subtype specificity, enabling them to be used as layer markers (Figure 1.4) (Molyneaux et al., 2007). Specifically, Satb2 is expressed in callosal projection neurons, throughout the cortical layers with a prominent expression in cortical layers II-IV (Alcamo et al., 2008). Tbr1 is highly expressed in, and regulates the development of, corticothalamic neurons within the preplate and cortical layer VI (Hevner et al., 2001). Fezf2 is expressed in the ventricular zone prior to and during the generation of layers V and VI and later expressed in post-mitotic neurons of these layers

(Molyneaux et al., 2007). Additionally it has been shown that during development, Fezf2 is capable of instructing the birth of new neuronal subtypes from neural progenitors of the developing cortex and striatum (Leyva-Diaz and Lopez-Bendito, 2013; Chen et al., 2008). Once a neuronal subtype is generated, migration of neurons occurs in a radial fashion, with the assistance of a scaffold formed by radial glia (Nadarajah et al., 2001). Here neurons move past the subplate to reach their final destination in the cortical plate, where they settle at appropriate positions to build up the six cortical layers (Figure 1.4) (Price et al., 1997; Nadarajah et al., 2001). Once neurons reach their desired location the glycoprotein, reelin, secreted by the Cajal-Retzius cells in the marginal zone signals to the immature neurons to detach from the radial glial cells and settle in a specific cortical layer (Beffert et al., 2004; D'Arcangelo et al., 1999). The reelin signalling pathway acts at precise stages in the course of cortical projection neuron migration (Tissir and Goffinet, 2003). It has been suggested that reelin may act as a repellent cue (Ogawa et al., 1995; Schiffmann et al., 1997), a stop signal (Frotscher, 1997; Sheppard and Pearlman, 1997), to stimulate detachment of migrating cortical neurons from radial glial processes (Dulabon et al., 2000; Sanada et al., 2004) or may be involved in the regulation of radial glial-dependent somal translocation (Franco et al., 2011; Jossin and Cooper, 2011; Sekine et al., 2012), however the precise function of reelin in cortical neuronal migration is still unclear.

Once a neuron has reached its destination, they extend axonal and dendritic processes to target regions. Furthermore, the target region of a neuron is dependent upon the class of the cortical neuron. There are two broad classes of cortical neurons: interneurons, which are responsible for local connections and projection neurons, which extend axons to distant intracortical, subcortical and subcerebral targets (Molyneaux et al., 2007). Additionally, cellular interactions and guidance molecules regulate both axonal and dendritic outgrowth. Molecules such as laminin and fibronectin located in the extracellular matrix provide a substrate for axonal growth (Tonge et al., 2012), while neurotrophin (NT) 3, nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) influence dendritic growth (McAllister et al., 1995). Furthermore substrates that serve as chemoattractants (netrins) and those that serve as chemorepulsives (semaphorin/Plexin-B2) aid in both axonal and dendritic guidance (Jan and Jan, 2003). It is believed that both axons and dendrites are guided by the integrated and summed influence of both the attractant and repulsive cues (Ming et al., 2001; Keynes and Cook, 1995). Moreover, most neuronal processes consist of growth cones, which contain a flattened lamellapodia with several thin, finger-like filopodia located on its leading

edge (Sanes et al., 2000). These growth cones also guide neuronal processes toward its synaptic target for the commencement of synaptogenesis.

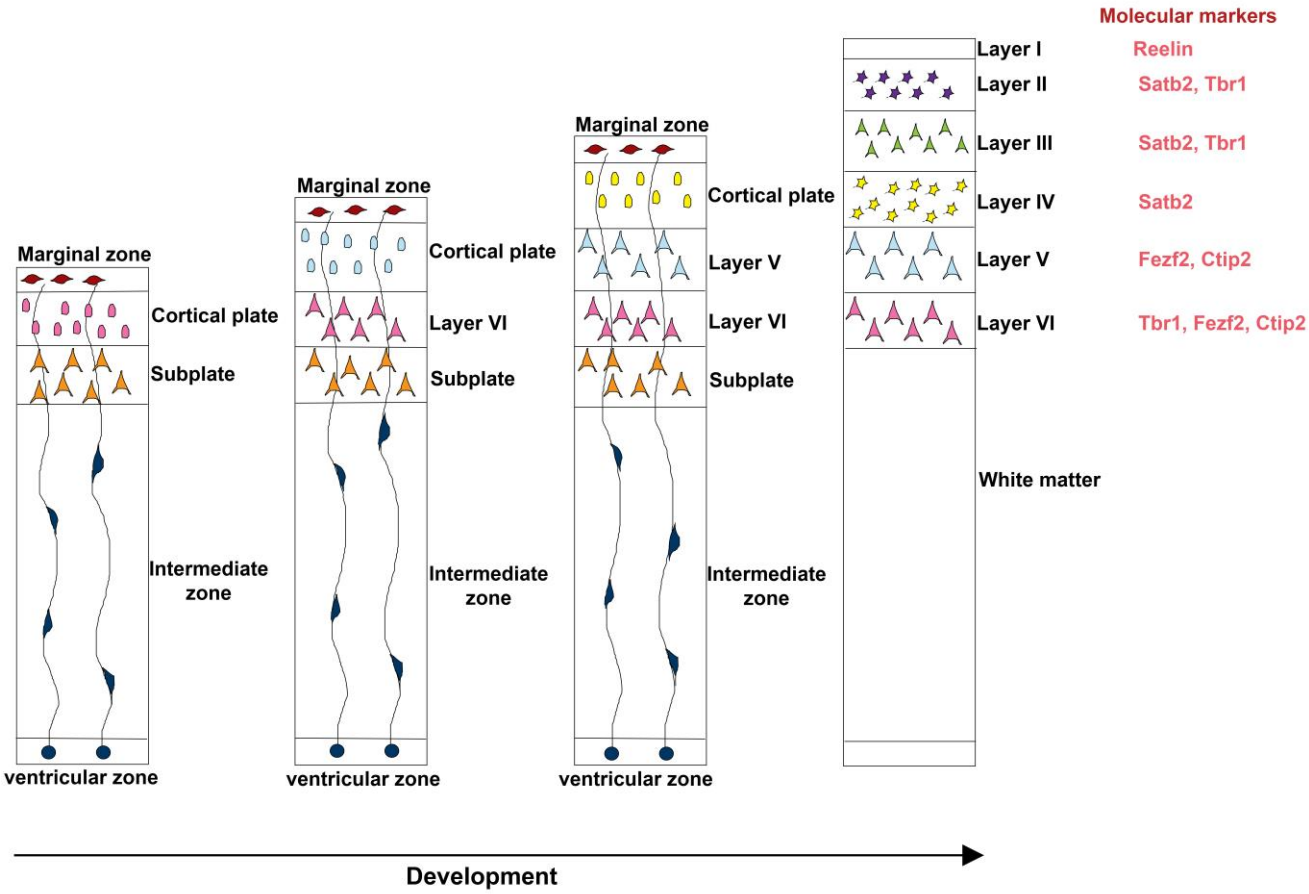


Figure 1.4 Developmental sequence of the formation of the six cortical layers

A diagrammatic representation of the developmental sequence that leads to the formation of the six cortical layers. Cortical layer formation occurs in an inside-out fashion, where the larger deeper cortical layers are formed first followed by smaller more superficial layers. The earliest neurons form the cortical plate by splitting the preplate into the marginal zone and the subplate. The next wave of neurons (future layer VI neurons), migrate from the ventricular zone via radial glia fibres and settle in the cortical plate, above the previously generated neurons. This sequence of neuronal migration is repeated until all cortical layers have been established. Adapted from (Bear and Connors, 2007; Nguyen and Hippenmeyer, 2013)

1.2.4 Synaptogenesis

Once an axon or dendrite has reached its appropriate target, synaptogenesis commences. During synaptogenesis, the CNS undergoes both biological and morphological changes which enables synapses to form between neurons (Semple et al., 2013). Synapse formation differs among regions in the brain, but generally begins before the completion of neurogenesis at ~20 weeks gestational age, and continues well into the second postnatal year (Figure 1.1) (Pomeroy and Kim, 2000; Herschkowitz et al., 1997; Lenroot and Giedd, 2006). However the number of dendritic spines and the sites of synaptic contact reach a peak at 34 to 36 weeks of gestation, followed by a rapid decline after birth (Volpe, 2008; Rice and Barone, 2000; Semple et al., 2013). This is because at birth there is an overproduction of neuronal arborisation and synaptic contacts in order to increase synaptic density, followed by an elimination or pruning phase of refinement after birth (see Section 1.2.5), due to inappropriate connections of some synapses and subsequent lack of synaptic activity (Semple et al., 2013). This is a fundamental step in development as it is hypothesized that this activity-dependent pruning of excess synapses contributes to synaptic plasticity and is a mechanism by which cortical circuitry is refined for the processing of adult cognition (Semple et al., 2013).

Synaptic formation is a complex interaction between the presynaptic component (growth cone) and the postsynaptic component (target tissue). The specificity of this synaptic connection is dependent upon molecular cues on the surface of neurons and neural processes which link axons to certain neurons via a specific chemical affinity known as chemoaffinity (Pomeroy and Kim, 2000). The synapse is then responsible for ensuring the survival of the neuron by supplying it with trophic support from the target tissue. This trophic support, which involves neurotrophic factors such as NGF, BDNF and the NT-3 and NT-4/5, is limited, making synaptogenesis a highly competitive process, where neurons that fail to make adequate connections and hence gain trophic support, undergo programmed cell death (Davies, 1996; Volpe, 2008; Sanes et al., 2000; Rakic and Zecevic, 2000).

1.2.5 Cell death and synaptic rearrangement

Following neurogenesis, at approximately 20 weeks of gestation, typically about fifty percent of neurons undergo programmed cell death before final maturation (Sanes et al., 2000). The subsequent loss of neurons serves two major functions during development; these include,

quantitative adjustments of interconnecting populations of neurons and elimination of projections that are aberrant or otherwise incorrect (Volpe, 2008; Sanes et al., 2000).

The organization of the CNS is further refined by a period of synaptic rearrangement, which is essential for normal neural function (Pomeroy and Kim, 2000; Semple et al., 2013). Over the duration of a few weeks, this synaptic rearrangement leads to a reduction in the number of synaptic inputs per cell, in turn eliminating weaker connections and strengthening the remaining connections through competition for neurotrophic factors, a process similar to those described for programmed cell death (Pomeroy and Kim, 2000). In humans, this process reaches a peak during the first few months of postnatal life and continues until the adult number of synapses is reached, which does not occur until adolescence (Huttenlocher, 1979; Semple et al., 2013). Activation of receptors, such as the N-methyl-D-aspartate (NMDA) type of glutamate receptor is believed to be important for the process of synapse elimination during development (Sanes et al., 2000).

1.2.6 Gliogenesis

In the CNS, glial cells consist of macroglia (astroglia and oligodendroglia), microglia and specialized glia (radial glia, ependymal cells). Macroglia and specialized glia are generally considered to develop from neuroepithelial progenitor cells in the ventricular zone (Roessmann and Gambetti, 1986a) and although the origin of microglia remains debatable, microglia are generally thought to develop from myeloid cells of the hematopoietic system (Cuadros and Navascues, 1998). Following neurogenesis, glial cell proliferation and differentiation (gliogenesis) begins, although there is some overlap between these two processes. The development of radial glia and microglia occurs in parallel with neurogenesis, oligodendrogenesis and astrocytogenesis however, develop well after the beginning of neurogenesis (Rice and Barone, 2000). Thus gliogenesis here is discussed in reference to macroglia and the development of microglia is discussed later in Section 1.2.6.2. Gliogenesis continues through the second half of gestation as well as after birth and the number of identifiable glial cells per unit area continues to increase in every part of the CNS (Roessmann and Gambetti, 1986a).

Gliogenesis is of major importance in the developing brain. Glial cells outnumber neurons in the CNS by approximately 1.25 to 1 and are almost the exclusive cell type in the WM (Volpe, 2008). Additionally, glial cells maintain their ability to regenerate (McCarthy and Leblond, 1988) and migrate (Walz and Bekar, 2001) throughout life, enabling them to respond quickly and effectively to developmental and pathophysiological changes within the CNS. The role of astrocytes and microglia during development are discussed in more detail below and the role of oligodendrocytes, critical to myelination, is discussed in more detail in Section 1.2.6.3.

1.2.6.1 Astrocytes

Astrocytes are responsible for numerous complex and essential functions within the healthy CNS. There is now evidence to suggest that loss of normal astrocyte function plays a crucial role in disease processes (Sofroniew and Vinters, 2010). Astrocytes play an important role in neural development, synapse function, energy and metabolism, regulation of blood flow, blood brain barrier formation as well as fluid, ion, pH and transmitter homeostasis. The two main subtypes of astrocytes, defined based on their morphology and anatomical locations are protoplasmic and fibrous astrocytes (Sofroniew and Vinters, 2010). Protoplasmic astrocytes are widespread throughout the GM and morphologically contain several stem branches that give rise to finely branching processes in a uniform spherical distribution (Sofroniew and Vinters, 2010). On the other hand fibrous astrocytes are found throughout the WM and morphologically consist of many fibre-like processes (Sofroniew and Vinters, 2010). The two types of astrocytes are similar in that they both make extensive contacts with blood vessels and they both form gap junctions between distal processes of neighbouring astrocytes (Sofroniew and Vinters, 2010). However, while the processes of protoplasmic astrocytes envelop synapses, the processes of fibrous astrocytes contact nodes of Ranvier along axons (Sofroniew and Vinters, 2010).

During development, astrocytes exert a number of essential functions in both the WM and GM. Astrocytes form molecular boundaries and thus play an important role in guiding the migration of developing axons (Powell and Geller, 1999). They are also essential for the function and development of synapses, as they release molecular signals such as thrombospondin, a protein known to increase synapse number (Ullian et al., 2004; Ullian et al., 2001). Additionally, astrocytes also influence developmental synaptic pruning as they release signals that induce the expression of

the protein C1q in synapses, which marks synapses for elimination by microglia (Stevens et al., 2007).

1.2.6.2 Microglia

Microglia are the resident immune cells of the CNS and play a major role in both brain development and responses to brain injury. These cells represent approximately 5-10% of the adult brain cell population, with a large population located within the WM (Czeh et al., 2011). Although the origin of microglia has long been debated, until recently the most accepted view has been that they originate principally, if not entirely, from bone marrow-derived monocytes (Monier et al., 2007; Simard and Rivest, 2004; Tomita et al., 2006). However, recent evidence now suggests that microglia originate in the yolk sac (Ginhoux et al., 2010). Microglia enter the brain in two waves during embryonic development and early postnatal life (Czeh et al., 2011). During embryonic development, microglia first enter the brain through the meninges, choroid plexus and ventricle and later in development, through blood vessel walls (Czeh et al., 2011; Verney et al., 2010; Monier et al., 2006). Studies of the developing human cerebrum have found that microglial cells during the second and third trimesters are primarily in an active state (i.e. amoeboid morphology), migrating progressively from ventricular-subventricular zones to the cerebral WM between 20-35 weeks and later to the cerebral cortex by radial and tangential migration via WM tracts and the cerebral vasculature (Billiards et al., 2006; Czeh et al., 2011; Rezaie and Male, 1999). Once amoeboid microglia have reached their destination, they transform into resting microglia, morphologically characterized by a small cell body with fine, long and ramified processes (Czeh et al., 2011). These resting microglia are responsible for screening the CNS for various changes in the environment in response to a threat to the functional and structural integrity of the CNS; they can then be rapidly activated and transformed back to their amoeboid (active) form (Czeh et al., 2011; Billiards et al., 2006). However, whether activated microglia have a beneficial or detrimental function is largely dependent on factors such as the kind of stress and damage signals, time or duration of impact, the microenvironment, interaction with other cell types and also the age or stage of brain development (Czeh et al., 2011). Some of the beneficial effects of activation include tissue repair or remodelling after injury and angiogenesis, while the detrimental effects include neuronal damage and reactive gliosis (Czeh et al., 2011). During brain development, microglial cells play key roles in vascularization (Rezaie and Male, 2002), apoptosis (Rakic and Zecevic, 2000), axonal development, including promotion of axonal growth and axonal guidance (Haynes et al., 2005),

synaptic pruning (Stevens et al., 2007; Paolicelli et al., 2011) and even myelination (Hamilton and Rome, 1994). Microglia also have a role in neurogenesis; they communicate extensively with neurons (Czeh et al., 2011) and secrete factors that induce cell proliferation including BDNF (Gomes et al., 2013; Dougherty et al., 2000) and insulin-like growth factor (O'Donnell et al., 2002).

1.2.6.3 Oligodendrocytes and myelination

Oligodendrocytes are derived from neuroepithelial cells that line the cerebral ventricles and spinal cord and are found throughout the mature CNS, particularly in the WM (Richardson et al., 2006; Emery, 2010a). These neuroepithelial cells give rise to oligodendrocyte progenitor cells (OPCs) which first appear in the ganglionic eminence around nine weeks of gestation in humans but continue to be produced after birth and into adulthood (Richardson et al., 2006). In the forebrain, OPCs appear in progressive waves originating from the medial ganglionic eminence, the lateral ganglionic eminence and the cerebral cortex, with the majority originating from the lateral ganglionic eminence and cerebral cortex (Richardson et al., 2006). The reasons underlying the loss of "competition" by the medial ganglionic eminence are unknown, but may involve the inability of these oligodendrocytes to compete effectively for the limited sources of growth factors (Richardson et al., 2006).

During brain development, oligodendrocyte maturation proceeds via a series of well-defined steps referred to as the oligodendrocyte lineage (Figure 1.5). OPCs are first generated from radial glia, the multipotent progenitor stem cells in the neuroepithelium of the brain and spinal cord (Richardson et al., 2006). OPCs then divide and migrate along the radial glial cells through the parenchyma to reach their final destination (Richardson et al., 2006). OPCs terminally differentiate into pre-oligodendrocytes, which are the dominant cell type between 18 and 28 weeks of gestation and account for 90% of the total oligodendroglial population (Richardson et al., 2006; Semple et al., 2013; Back and Volpe, 1997). Pre-oligodendrocytes further differentiate into pre-myelinating (or immature) oligodendrocytes, before finally taking on their mature myelinating form (Richardson et al., 2006). Oligodendrocytes at pre-oligodendrocyte and pre-myelinating oligodendrocyte stage of the lineage are most prevalent by 28 to 40 weeks of gestation (Semple et al., 2013).

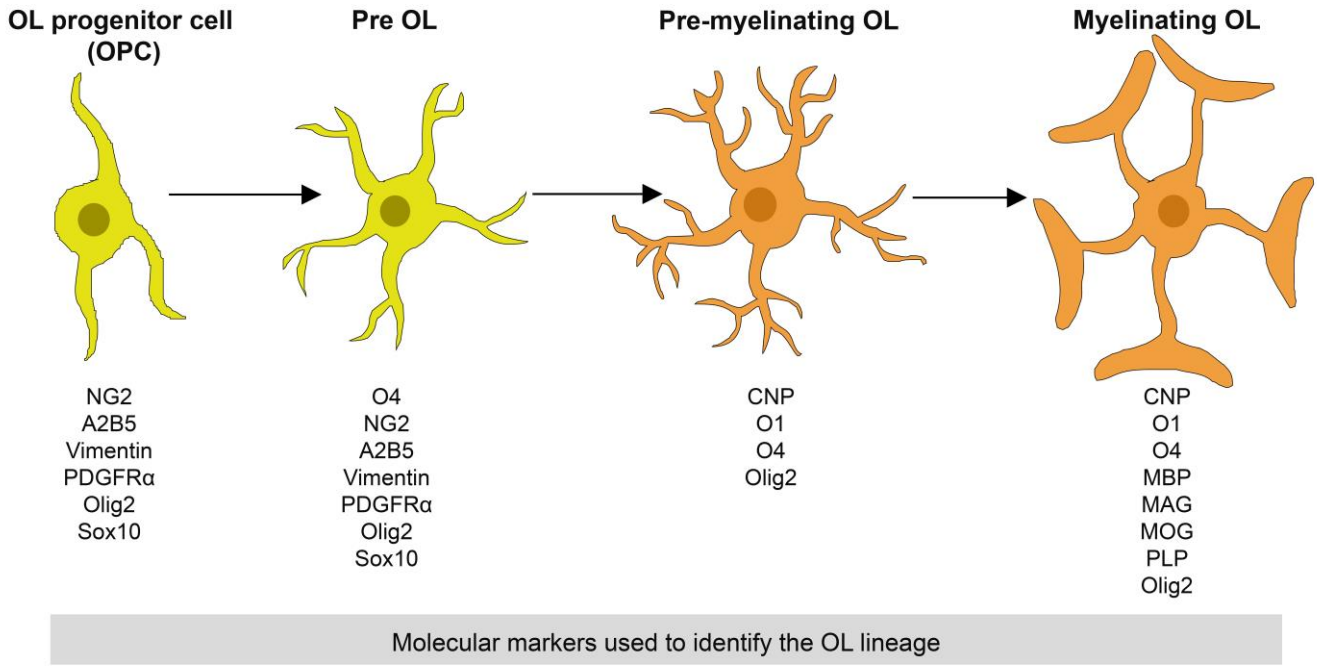


Figure 1.5 Oligodendrocyte lineage maturation

A diagrammatic representation of the maturation of the oligodendrocyte (OL) lineage. The principle stages from the OL progenitor cell to mature myelinating OL and the corresponding morphology and developmental profile are shown. Cells at each stage of the OL lineage can be identified by immunoreactivity using specific antigenic markers. Adapted from (Back and Volpe, 1997).

Progress through the oligodendrocyte lineage is regulated by a variety of growth factors, hormones, cytokines, surface receptors, secreted ligands, transcription factors and signalling pathways (Back and Volpe, 1997; Emery, 2010a). These include, but are not limited to, basic fibroblast growth factor, NT-3, PDGF, insulin-like growth factors, NGF, transferrin, members of the interleukin-6 family, neureglin, Nogo and Nogo receptors, zinc finger protein 191 (ZFP191), oligodendrocyte marker 1 (O1), Sox10, myelin regulatory factor, and the Notch, Wnt and BMP signalling pathways (Volpe, 2008; Emery, 2010a; Emery, 2010b). Furthermore, programmed cell death is an important feature of oligodendroglial development and leads to a 50% reduction in oligodendrocyte numbers (Barres et al., 1992).

A number of molecular markers can be used to identify oligodendrocytes at different stages of development (Figure 1.5). In the progenitor phase of the lineage, the oligodendrocytes are in a

mitotically active migratory cell state, which can be identified by immunoreactivity against the monoclonal antibodies A2B5, NG-2 chondrotin sulphate proteoglycan (NG2), Vimentin, PDGF receptor α, oligodendrocyte transcription factor 2 (Olig2) and Sox10 (Back and Volpe, 1997; Emery, 2010b). These progenitor cells are generally generated from around mid-gestation to the early postnatal period (Rivkin et al., 1995; Back et al., 2001; Back and Volpe, 1997; Back et al., 2002). As migration of these progenitor cells continues into the cerebral WM, oligodendroglial differentiation continues into the pre-oligodendrocyte phase, a multipolar, mitotically active cell type identified primarily by reactivity to the monoclonal antibody oligodendrocyte marker 4 (O4); this antibody recognises the cell surface glycolipids, sulfatide and seminolipid (Back and Volpe, 1997; Emery, 2010b). This pre-oligodendrocyte phase gives rise to immature oligodendrocytes or pre-myelinating oligodendrocytes that are postmitotic and have more extensive branching (Volpe, 2008; Back and Volpe, 1997). At this stage, cells are recognised primarily by monoclonal antibodies to 2' 3'-cyclic-nucleotide 3'-phosphodiesterase (CNP), O1 and O4 (Back and Volpe, 1997). Although these markers are not specific for only oligodendrocytes, they are useful in combination to define a given stage of oligodendrocyte development (Back and Volpe, 1997). For example pre-oligodendrocytes are O4+/O1-, while pre-myelinating oligodendrocytes are O4+/O1+. Once oligodendrocytes have progressed to their pre-myelinating form, the extensions of the cell wrap around axons in preparation for myelination. These cells then differentiate into mature or myelinating oligodendrocytes, a multipolar cell with membrane sheets recognised by antibodies to myelin basic protein (MBP), proteolipid protein (PLP), myelin associated glycoprotein (MAG) and myelin oligodendrocyte glycoprotein (MOG) amongst others (refer to Figure 1.5) (Back and Volpe, 1997; Emery, 2010b). Oligodendrocytes at this stage of the cycle give rise to myelin and become the predominant oligodendroglial stage in the months following birth (Semple et al., 2013; Rice and Barone, 2000). Interruptions and/or damage at any stage of the oligodendrocyte lineage can lead to detrimental consequences for myelination, with immature oligodendrocytes being more susceptible to injury than mature oligodendrocytes (Back et al., 2002; Back et al., 2001).

In the CNS, myelination is initiated at approximately 29 weeks of gestation (Figure 1.1), with the brainstem and cerebellum being myelinated first (Inder and Huppi, 2000). This is then followed by myelination within the cerebral hemispheres, particularly in regions involved in higher level associative functions and sensory discriminations (Semple et al., 2013). Myelin in these regions is present at around 37 - 40 weeks of gestation and myelination continues over the following decades of life (Inder and Huppi, 2000; Volpe, 2008). Myelination within the CNS is highly organised, with

proximal pathways myelinating before distal pathways, sensory pathways myelinating before motor pathways, projection pathways myelinating before cerebral associative pathways, central cerebral sites myelinating before cerebral poles and lastly occipital poles myelinating before fronto-temporal poles (Kinney et al., 1988; Semple et al., 2013). This organised myelination of pathways is important in relation to the manifestation of neurological deficits involving primary motor pathways, such as that seen in periventricular WM injury, a major form of brain injury associated with preterm birth and the leading cause of cerebral palsy (Volpe, 2008; Semple et al., 2013).

1.3 Preterm birth

Preterm birth is defined as birth before 37 weeks of gestation, where 40 completed weeks of gestation is regarded as full term. Preterm birth can be further divided into late preterm birth (<37 weeks of gestation) very preterm birth (<32 weeks of gestation) and extreme preterm birth (<28 weeks of gestation (Tucker and McGuire, 2004). Preterm birth is a major clinical problem worldwide. In Australia, preterm birth accounts for 8.3% of all births (25,113 babies in 2011) (Li et al., 2011). Most preterm births (80%) occur between 32 and 36 completed weeks of gestation and account for 6.6% of all live births (Li et al., 2011). Of particular concern however are those babies born before 32 weeks of gestation (i.e. very and extremely preterm babies). In 2011, approximately 0.8% of all babies were born very preterm (28 to 31 weeks of gestation) and 0.9% of babies were born extremely preterm (20 to 27 weeks of gestation) (Li et al., 2011). When combined, preterm birth prior to 32 weeks of gestation equated to over 5,000 babies born in Australia alone. Worldwide 15 million babies are born preterm every year, with preterm birth rates ranging from 5-18% of all births across 184 countries in 2010 (Howson et al., 2012).

The timing of preterm birth influences the risk of morbidity and mortality; that is, as gestational age at birth decreases the risk of morbidity and mortality increases (Kramer et al., 2000). There are also marked differences between the three categories of preterm birth in terms of the need for, and cost of, intensive care, as well as in long-term health and disability outcomes (Lumley, 2003). Due to recent advances in perinatal care, the rate of survival and long-term outcomes for infants born preterm have significantly improved (Iams et al., 2008). However, of concern are those infants born very preterm or extremely preterm because the incidence of neonatal illness and long-term morbidity in this population remains high (Fanaroff et al., 2007; Doyle et al., 2010b). Diseases such

as neonatal infection, necrotizing enterocolitis, bronchopulmonary dysplasia (BPD) and major brain lesions such as periventricular leukomalacia and intraventricular haemorrhage (IVH) have been shown to influence both the short- and long-term outcomes of these preterm infants (Ralser et al., 2012; Bassler et al., 2009; Stoll et al., 2004).

1.3.1 Causes and consequences of preterm birth

Most preterm births follow spontaneous, unexplained preterm labour or spontaneous preterm pre-labour rupture of the amniotic membrane (Tucker and McGuire, 2004). Some important predictors of spontaneous preterm delivery include intrauterine infections, a history of preterm birth, stress, endocrine and immune disorders, as well as genetic risk factors (Tucker and McGuire, 2004; Voltolini et al., 2013). Maternal or fetal complications during pregnancy such as uteroplacental insufficiency, uterine overdistention or cervical insufficiency can also lead to preterm birth (Voltolini et al., 2013).

The developmental outcomes of preterm infants not only depend on the cause of early delivery but also the interventions required to support them during the neonatal period. Depending on gestational age at birth, preterm infants are subject to serious physiological challenges due to the immature development of critical organs. The successful transition from fetal to neonatal life requires infants to establish independent gas exchange. However, one of the major problems that preterm infants face is lung immaturity, due to immaturity of the surfactant system, and immaturity of respiratory control (Martin et al., 2004; Di Fiore et al., 2013). Both of these contribute to poor ventilation of the lung and hence poor gas exchange, which can result in AOP. AOP is common in very preterm infants, occurring in 85% of infants born prior to 34 weeks (Barrington and Finer, 1991). The incidence of AOP is inversely correlated with gestational age, occurring in 7% of infants born between 34-35 weeks, 15% in 32-34 weeks, 54% in 30-31 weeks and nearly all infants born prior to 29 weeks or of a birth weight of less than 1000g (Henderson-Smart, 1981; Robertson et al., 2009). While AOP is a common developmental disorder associated with severe prematurity, its pathogenesis is still poorly understood.

1.4 Apnea of prematurity

AOP has been defined as a condition where there is a cessation in breathing that lasts for more 15 to 20 seconds and is commonly associated with hypoxia or bradycardia (Barrington and Finer, 1991; Miller and Martin, 2011). Three main categories of apnea are recognised: i) central apnea, characterized by a total cessation of inspiratory efforts in the absence of airway obstruction, ii) obstructive apnea, when the infant tries to breath against an obstructed upper airway and is most common in preterm infants and iii) mixed apnea, when there are obstructed respiratory efforts usually following periods of central apnea (Martin et al., 2004; Milner et al., 1980). Although the pathogenesis of AOP is still not well understood it has been attributed to a number of factors that affect the central respiratory network, which consists of peripheral receptors, chemoreceptors and mechanoreceptors (Di Fiore et al., 2013; Mathew, 2011). Any effects on the central respiratory network ultimately leads to a reduction in the output to the muscles of respiration, including the chest wall and soft tissues of the upper airway, which are quite compliant in preterm infants and predisposes them to upper and lower airway collapse and obstruction (Di Fiore et al., 2013). Furthermore, a stable respiratory pattern that is dynamic and responds to metabolic needs is dependent upon the correct balance of excitatory and inhibitory inputs from higher brain centres, mechanoreceptors in the upper airway and lungs, peripheral chemoreceptors in the carotid body and chemoreceptors in ventral medullary surface (Di Fiore et al., 2013; Mathew, 2011; Martin and Wilson, 2012).

1.4.1 Pathogenesis of apnea of prematurity

1.4.1.1 Central chemoreceptors and impaired ventilatory response to hypercapnia

Sensed near the ventral surface of the medulla oblongata, carbon dioxide (CO_2) is considered to be the major chemical driver of respiration at all ages (Forster and Smith, 2010; Blain et al., 2009; Blain et al., 2010). This is supported by the observation that the ventilatory response to hypercapnia is primarily mediated by central chemoreceptors (Forster and Smith, 2010). The central chemoreceptors that are thought to modulate breathing in response to changes in arterial partial pressure of CO_2 ($PaCO_2$) are serotonergic (5-HT) neurons, located near the rostral ventrolateral medulla (Mathew, 2011; Nattie and Li, 2012; Di Fiore et al., 2013). However, there is now evidence to suggest that chemoreceptors in the carotid body also respond to changes in $PaCO_2$ (Nattie and Li, 2012; Khan et al., 2005a). The inhibition of central chemoreceptor function at, or

near the ventral medullary surface can simulate many of the physiologic characteristics evident in apneic preterm infants (Martin and Abu-Shaweesh, 2005). This suggests that defective responsiveness to CO_2 underlies AOP.

Additionally, preterm infants have a diminished ventilatory response to CO_2 when compared to infants born at term (Gerhardt and Bancalari, 1984; Krauss et al., 1975; Martin and Wilson, 2012). Both adults and term neonates tend to increase ventilation via an increase in tidal volume and breathing frequency in response to increasing CO_2, whereas preterm infants do not show an increase in breathing frequency, presumably due to a reduced sensitivity to CO_2 (Krauss et al., 1975; Gerhardt and Bancalari, 1984; Mathew, 2011). Instead, preterm neonates tend to prolong their expiratory duration in response to CO_2 and this poor hypercapnic ventilatory response is more pronounced in preterm infants with apnea than those without apnea (Zhao et al., 2011; Martin and Abu-Shaweesh, 2005). The lack of difference in pulmonary mechanics (tidal volume, alveolar ventilation and oesophageal pressure change), between healthy preterm infants and preterm infants with apnea, suggests that there is a central origin for the observed difference (Gerhardt and Bancalari, 1984).

1.4.1.2 Peripheral chemoreceptors and impaired ventilatory response to hypoxia

Peripheral chemoreceptors are considered to be responsible for stimulating breathing in response to hypoxia (Martin and Wilson, 2012; Mathew, 2011; Di Fiore et al., 2013). These peripheral chemoreceptors are primarily located in the glomus cells of the carotid body, located in the bifurcation of the carotid artery (Mathew, 2011; Di Fiore et al., 2013). Glomus cells release various neurotransmitters in response to hypoxia, hypercapnia and acidosis, leading to an increase in the afferent discharge of the carotid sinus nerve terminals (Carroll et al., 2012). In particular, dopamine, acetylcholine and 5'adenosine triphosphate have been proposed to be the excitatory transmitters released by the carotid body (Kumar and Prabhakar, 2012). The local release of these excitatory transmitters causes an instant and rapid rise in ventilation by stimulating afferent fibres to the brainstem (Kumar and Prabhakar, 2012; Gonzalez et al., 1994). Furthermore it has been proposed that both enhanced and reduced peripheral chemoreceptor function can contribute to AOP (Al-Matary et al., 2004).

During fetal life, peripheral chemoreceptors are active at very low levels of oxygen (PaO_2 ~23-27 mmHg); however, after birth there is an increase in PaO_2 to around 50-70 mmHg following the establishment of breathing. When this relative hyperoxia occurs the peripheral chemoreceptors are essentially silenced (Hertzberg and Lagercrantz, 1987). Following this there is a gradual increase in hypoxic chemosensitivity and once peripheral chemosensitivity is established, the carotid chemoreceptor drive is significant at relatively hypoxic levels of 50–70 mmHg (Hertzberg and Lagercrantz, 1987; Kholwadwala and Donnelly, 1992; Jansen et al., 1981). Infants may become prone to apnea when their inspired oxygen is increased enough to produce a physiologic denervation of peripheral chemoreceptors (Williams et al., 1991).

Additionally, in response to hypoxia in the neonatal period, preterm infants show an initial increase in ventilation for approximately 1 min; following this there is a post-hypoxia decline in breathing frequency, which is not sustained beyond 1-3 min, termed hypoxic ventilatory depression (Rigatto et al., 1975). In contrast, adults show a sustained increase in ventilation in response to hypoxia (Mathew, 2011). This characteristic ventilatory response to hypoxia seen in preterm infants can persist for up to 4-6 weeks after birth (Martin et al., 1998). Hypoxic ventilatory depression appears to be centrally mediated with major inhibitory projections arising from the pons (Yan et al., 1995). Specifically, hypoxic ventilatory depression involves inhibitory neuromodulators, one of which is adenosine (Easton and Anthonisen, 1988; Koos et al., 2005; Yan et al., 1995). This may explain why there is an improvement in central respiratory drive after adenosine receptors are blocked with methylxanthine treatment in preterm infants (Henderson-Smart and De Paoli, 2010). So it is possible that this hypoxic depression, as opposed to a decline in peripheral chemoreceptor firing alone, is also contributing to apnea of prematurity, although the role of hypoxic depression in apnea is still relatively unclear (Martin and Wilson, 2012).

1.4.1.3 Mechanoreceptors and laryngeal afferents

Mechanoreceptors in the lungs and upper airway play a pivotal role in the pathogenesis of AOP (Di Fiore et al., 2013). In particular, studies have shown that stimulation of the laryngeal mucosa, either chemically or mechanically, leads to an inhibition of breathing in both preterm and term infants, subsequently leading to a host of events including, apnea, bradycardia, hypotension, closure of upper airways and swallowing movements (Pickens et al., 1988; Martin and Wilson, 2012). This

laryngeal chemoreflex-induced apnea is thought to be mediated via, the laryngeal mucosal receptors and afferent inputs into the brainstem via the superior laryngeal nerve (Goding et al., 1987; Lee et al., 1977). Animal studies suggest that reflex apnea is due to inhibition of the central respiratory pattern generator, and may also involve the contraction of the thyroarytenoid (laryngeal adductor) muscle, which closes the glottis (Harding, 1984; Goding et al., 1987; Lee et al., 1977). During laryngeal stimulation, the resulting apnea and laryngeal adduction is a fundamental protective reflex to protect the lungs from aspiration, however, an exaggerated response has been implicated as the cause of apnea in preterm infants (Martin and Abu-Shaweesh, 2005). Although the basis of the greater ventilatory sensitivity to the inhibitory effects of laryngeal stimulation early in development is still unclear, it has been attributed to the maturation of central chemosensitivity (Martin and Abu-Shaweesh, 2005).

Furthermore during apnea, preterm infants may exhibit either a pharyngeal or laryngeal obstruction; thus research on the causes of apnea have focused on the interactions between muscle groups involved in maintaining upper airway patency (Miller and Martin, 2011). While many upper airway muscles are actively involved in modulating airway patency, failure of genioglossus muscle activation in particular has been widely implicated in both mixed and obstructive apnea in infants (Miller and Martin, 2011). Carlo et al (1990; 1988) found that activation of the genioglossus muscle in preterm infants following CO_2 rebreathing was delayed for approximately 1 min and activation occurred only after an arterial $PaCO_2$ of 45 mmHg was reached. Thus it is believed that an absent or delayed upper airway muscle response to hypercapnia may lead to upper airway instability, which may predispose affected infants to obstructed inspiratory efforts after a period of central apnea (Miller and Martin, 2011).

1.5 Treatment of apnea of prematurity

The current treatments of AOP include interventions that have proven to be effective and also interventions with unclear efficacy. Treatments with unclear efficacy include administration of doxapram (Prins et al., 2013), prone sleeping position (Bhat et al., 2006), CO_2 inhalation (Joseph et al., 2009), sensory stimulation (Edraki et al., 2013), “Kangaroo care” (Gathwala et al., 2010) and maintaining the infant in a thermoneutral temperature range (Rieger-Fackeldey et al., 2003). Interventions that have been shown to be effective in reducing the frequency and severity of AOP

include the use of a continuous positive airway pressure (CPAP) (Abu-Shaweesh and Martin, 2008) and administration of methylxanthines (Henderson-Smart and De Paoli, 2010).

1.5.1 Doxapram

Doxapram is a known non-specific analeptic agent (stimulant of the CNS) that is used in treating AOP when apnea persists under methylxanthines (Prins et al., 2013; Yost, 2006). Doxapram is a respiratory stimulant that acts by increasing tidal volume and minute ventilation and although its direct effects on the CNS are relatively unclear, its effect is thought to be mediated through the stimulation of both central and peripheral chemoreceptors (Barrington et al., 1986; Prins et al., 2013; Yost, 2006). Although doxapram is just as effective as some methylxanthines in reducing apnea frequency and severity in the short-term, treatment involves a continuous intravenous infusion due to poor absorption from the gastrointestinal tract (Barrington et al., 1986; Brion et al., 1991; Peliowski and Finer, 1990). Additionally, doxapram is not routinely recommended for the treatment of apnea, in Australia, as it has been reported to have a number of adverse side effects, including increased blood pressure, abdominal distension and irritability (Prins et al., 2013). Furthermore, some of the more devastating negative side effects of doxapram include reduced cerebral oxygenation (Dani et al., 2006) and long-term neurodevelopmental delay (Lando et al., 2005; Sreenan et al., 2001).

1.5.2 Prone sleeping position

Arousal from sleep is an important survival response to life-threatening events such as prolonged apnea, as arousal from sleep increases ventilation (Bhat et al., 2006). Furthermore, positioning is an important factor related to ventilation in preterm infants (Chang et al., 2002). Prior to neonatal discharge, preterm infants sleep for longer periods with less arousals and more central apneas while sleeping in the prone position compared to the supine position (Bhat et al., 2006). It has been proposed that the prone sleeping position reduces AOP by improving thoracoabdominal synchrony during breathing and by stabilizing the chest wall without affecting the breathing pattern, which is linked to an increase in arterial oxygenation (Oliveira et al., 2009; Chang et al., 2002). However, the effects of prone sleeping position on oxygenation remains controversial as studies have shown that prone position sleeping in preterm infants can either increase oxygen saturation and decrease the number of desaturation episodes (Chang et al., 2002) or have no effect at all on oxygenation

when compared to the supine position (Keene et al., 2000). Furthermore two prone positions have currently been investigated, one of which includes a head-elevated tilt position, involving extending the neck 15 degrees from the prone position, and the other a "three-stair-position" which maintains the head and abdomen in a horizontal position. The head-elevated tilt position decreases episodes of oxygen desaturation by 48.5% (Sher, 2002), while the three-stair-position decreases apnea, bradycardic events and desaturation (Bauschatz et al., 2008). However, Reher et al (2008) found no significant advantage of the head elevated tilt position or the three-stair-position over the standard horizontal position in preterm infants. Thus, these two prone sleeping positions still require further investigation.

1.5.3 Carbon dioxide inhalation

Carbon dioxide is the major physiological stimulus for breathing (Zhao et al., 2011). In preterm infants, apnea commonly occurs when the CO_2 baseline decreases below the apnea threshold, thus a small rise in CO_2 above the apnea threshold will act to reduce or abolish apnea (Joseph et al., 2009; Khan et al., 2005b). Inhaled carbon dioxide ranging from as little as 0.5 to 1.5% is effective in reducing the number of apneic episodes while improving oxygenation in preterm infants with apnea (Al-Aif et al., 2001). Inhaled CO_2 leads to a decrease in the amount of periodic breathing and works largely by regularising breathing in order to reduce apnea (Mathew, 2011). A randomized controlled trial assessing the effects of theophylline versus inhaled CO_2 (0.8%) found that inhaled CO_2 is just as effective as theophylline in reducing apnea (Al-Saif et al., 2008). However, it is likely that infants will accommodate to a sustained increase in inhaled CO_2 (Al-Saif et al., 2008); the effectiveness of long-term exposure to CO_2 is currently unknown. Although the use of inhaled CO_2 is not associated with any apparent side effects (Mathew, 2011), further research is required before it is ready for clinical use.

1.5.4 Sensory stimulation

Studies suggest that tactile and olfactory stimulation are useful in the treatment of AOP. Although there is limited information on how sensory stimulation impacts upon the respiratory control networks it most likely operates by generating non-specific, neuronal excitation within brainstem centres, subsequently leading to the stimulation of respiratory activity (Zhao et al., 2011). Tactile stimulation has been shown to reduce apnea by 35% (Kattwinkel et al., 1975). Furthermore, a novel

therapy - olfactory stimulation - has also been assessed in preterm infants suffering from apnea. Exposure of apneic preterm newborns to a pleasant odour (vanillin) for 5 days in an incubator led to a reduction in the number of apneic episodes (Edraki et al., 2013). Vanillin exposure in apneic preterm newborns for as little as 24 hours in an incubator also led to a reduction in the frequency and severity of apnea in a small group of infants who were not responding to caffeine (Marlier et al., 2005). Vanillin is a known olfactory nerve stimulant, leading to improved regulation of respiratory patterns (Marlier et al., 2005). Although vanillin lacks side effects in the short-term, the effects of longer periods of treatment are yet to be determined.

1.5.5 Kangaroo care

Kangaroo care, also known as skin-to-skin care, has reported beneficial effects on a preterm infant's clinical status and vital signs (Gathwala et al., 2010). The effectiveness of the approach is however, controversial. A study by Ludington-Hoe et al (2004) found that kangaroo care led to fewer apneic and bradycardic events via an increase in regular breathing compared to those not receiving kangaroo care. On the other hand, bradycardic events and desaturation increased in response to kangaroo care in apneic preterm infants (Bohnhorst et al., 2004). Furthermore the improvements reported in some studies in response to kangaroo care appear the same as the improvements seen with prone position sleeping (Heimann et al., 2010). Thus the effects of kangaroo care for the treatment of apnea remains controversial.

1.5.6 Maintaining a thermoneutral ambient temperature range

It has been proposed that overheating may be a factor in AOP. A mild increase in body temperature of infants suffering from AOP enhanced the instability of the breathing pattern (Rieger-Fackeldey et al., 2003); specifically, incubator temperatures set at 30.4°C led to less apnea when compared to an incubator temperature of 32.5°C (Tourneux et al., 2008). While a number of factors play a role in incubator and infant body temperature, the specific environmental temperature required to reduce AOP is still not known and it appears that apneic events may be more closely related to the loss of body heat than to body temperature *per se* (Tourneux et al., 2008).

1.5.7 Continuous positive airway pressure

CPAP at a pressure of 4-6cm H_2O, has been proven effective in reducing the frequency and severity of AOP. Specifically, CPAP has been shown to eliminate longer episodes of apnea which are often associated with upper airway obstruction (Abu-Shaweesh and Martin, 2008). This is believed to be a result of CPAP delivering a continuous distending pressure via the infant's pharynx to the upper airway, which stabilises the upper airway thereby preventing pharyngeal or laryngeal collapse and hence improves airway patency (Zhao et al., 2011; Abu-Shaweesh and Martin, 2008; Martin and Wilson, 2012). This continuous distending pressure increases the functional residual capacity and reduces the work of breathing, which leads to a decrease in bradycardia and improved oxygenation (Abu-Shaweesh and Martin, 2008; Zhao et al., 2011; Martin and Wilson, 2012). However, CPAP does not appear effective in eliminating central apnea in which there is no obstruction of the airway; hence CPAP is more effective for reducing obstructive and mixed apnea (Miller et al., 1985). While CPAP alone (delivered via a face mask) has been proven effective in some infants, infants with severe apnea usually require placement of an endotracheal tube and mechanical ventilation (Martin et al., 2004). It has been suggested that infants receiving assisted ventilation should be maintained on minimal ventilator settings to allow spontaneous ventilatory efforts and to decrease the risk of barotrauma (Martin et al., 2004).

1.5.8 Methylxanthines

Methylxanthines, including caffeine, theophylline and aminophylline (the ethylenediamine salt of theophylline) have long been administered to preterm infants for the treatment of AOP. They have been, and remain the most commonly prescribed drugs in neonatal medicine (Millar and Schmidt, 2004). These agents reduce apnea via a range of pathways. In preterm infants, methylxanthines have the effect of increasing minute ventilation, improving CO_2 sensitivity, decreasing hypoxic depression of breathing while also improving diaphragmatic contraction and respiratory muscle function (Abu-Shaweesh and Martin, 2008; Miller and Martin, 2011). These effects of methylxanthines are mediated by an increase in neural output, but the pharmacological basis of this effect is still unclear. The hydrophobic properties of caffeine allows it to pass through all biological membranes, including the blood-brain barrier (Lachance et al., 1983). The ability of methylxanthines to competitively antagonise adenosine receptors within the CNS, in particular adenosine receptors A_1 and A_{2a}, has been proposed as the mechanism by which these agents stimulate breathing (Fredholm, 1995). A secondary mechanism may be via their effects on gamma-

aminobutyric acid (GABA) receptors, inhibition of phosphodiesterase (PDE) and calcium (Ca^{2+}) release (See section 1.6).

There have been numerous studies comparing the benefits and risks of the three major methylxanthines. Although aminophylline and theophylline are just as effective in treating AOP as caffeine, they are associated with far more adverse effects than caffeine (Henderson-Smart and Steer, 2010; Larsen et al., 1995). Some of the well documented side-effects of methylxanthines include tachycardia, cardiac dysrhythmias, food intolerance, increased metabolic rate, increased O_2 consumption and less frequently, seizures, all of which are less commonly observed with current therapeutic doses of caffeine (Abu-Shaweesh and Martin, 2008). Furthermore, in comparison to aminophylline and theophylline, caffeine is more easily absorbed, has a wider therapeutic range and a longer half-life, allowing for once-a-day dosing (Henderson-Smart and De Paoli, 2010; Millar and Schmidt, 2004; Henderson-Smart and Steer, 2010). This has made caffeine the methylxanthine of choice when treating AOP. However, the dosing regime of caffeine used in treating preterm infants tends to vary in neonatal intensive care units around the world (Scanlon et al., 1992; Steer et al., 2003).

1.6 Mechanism of action of caffeine

Although the precise mechanism by which caffeine reduces AOP is still under investigation, it is clear that caffeine has a wide range of molecular targets within the CNS. The most common molecular targets of caffeine within the CNS include, antagonism of adenosine receptors (AR), particularly A_1AR and $A_{2a}AR$, inhibition of PDE, intracellular Ca^{2+} release and antagonism of $GABA_A$ receptors. Although caffeine affects $GABA_A$ receptors, PDE and Ca^{2+} release, the effects of caffeine on arousal and breathing cannot be attributed to its ability to regulate these molecular targets (Fredholm et al., 1999). This is because the regulation of these molecular targets requires concentrations of caffeine that are rarely consumed or administered (Fredholm et al., 1999). Concentrations of caffeine in the millimolar (mM) range are required to regulate these molecular targets and hence to attain a significant physiological effect (Figure 1.6) (Fisone et al., 2004; Fredholm et al., 1999). Furthermore, a blood caffeine concentration of 500 μM is sufficient to cause lethal intoxication; even ingestion of 3 cups of coffee (300 mg of caffeine) results in a peak plasma caffeine concentration of 30 μM (Dews, 1982; Bonati et al., 1982), while administration of standard

doses of caffeine (loading 20 mg/kg; maintenance 5-10 mg/kg) leads to a peak plasma caffeine concentration of 84 to 128 μM (Aranda et al., 1979b; Charles et al., 2008). Thus the consensus is that the respiratory stimulatory action of caffeine and its effects on the CNS are due to antagonism of adenosine receptors, rather than the above-mentioned molecular targets (Abu-Shaweesh, 2007; Herlenius and Lagercrantz, 1999; Fredholm, 1995).

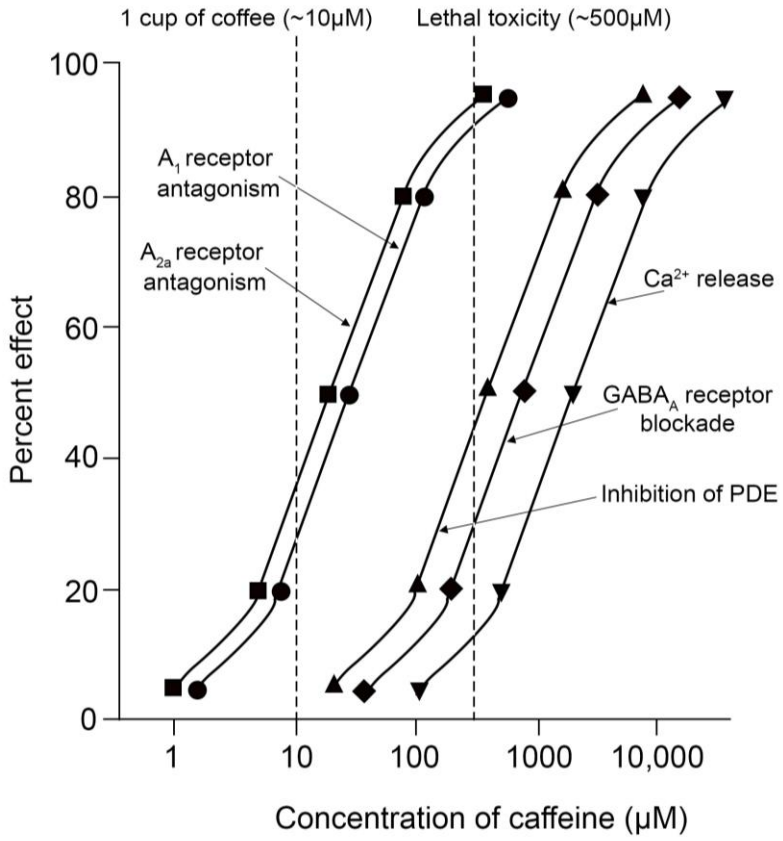


Figure 1.6 Concentration of circulating caffeine required to activate various molecular targets in relation to human caffeine consumption

One cup of coffee, leading to a circulating caffeine concentration of ~10μM, is enough to have a significant effect on adenosine receptors A_1 and A_{2a}. However, 20-times higher concentrations of caffeine are required to inhibit PDE, 40 times higher concentrations are needed to block $GABA_A$ receptors, and 100 times higher concentrations are required to lead to Ca^{2+} release. A significant effect on these later mentioned molecular targets occurs at approximately millimolar concentrations of caffeine, however 500μM of caffeine is sufficient to cause lethal intoxication. Figure adapted from (Dews, 1982; Bonati et al., 1982; Fredholm et al., 1999).

1.6.1 The role of adenosine receptors

Adenosine is a normal cellular constituent and a balance of numerous enzymes regulates its concentration. It is formed by the action of adenosine monophosphate (AMP) selective 5'-nucleotidase, with the rate of formation modulated by the amount of AMP (Fredholm et al., 1999). Thus the rate of AMP breakdown and synthesis, which is modulated by the rate of energy utilisation and availability of substrate that is able to be metabolised, determines the rate of adenosine formation via this pathway (Fredholm et al., 1999). Furthermore adenosine triphosphate (ATP) is rapidly hydrolysed to adenosine and other metabolites (Fredholm et al., 1999). Adenosine serves many diverse roles in normal physiology, including promotion or maintenance of sleep, regulating the general state of arousal as well as local neuronal excitability and the coupling of cerebral blood flow to energy demand (Dunwiddie and Masino, 2001). An increase in adenosine formation leading to high intracellular levels of adenosine due to events such as hypoxia (Zetterstrom et al., 1982) and ischemia (Andine et al., 1990; Dux et al., 1990), can lead to AR activation. There are currently four distinct adenosine receptors that have been cloned and characterised; they include A_1AR, A_{2A}AR, A_{2B}AR and A_3AR. Unlike A_1 and A_{2A}, A_3 and A_{2B} ARs require much higher concentrations of adenosine for activation, whereas A_1 and A_{2A} ARs are activated at low basal concentrations of adenosine (Fredholm et al., 1999). These receptors are likely to be the major targets for caffeine, which has a similar molecular structure to adenosine (Figure 1.7); thus the majority of the research on caffeine's effect on adenosine receptors has focussed on adenosine receptors A_1 and A_{2A}. The remainder of this section will therefore concentrate on studies assessing caffeine's effects on these two receptors.

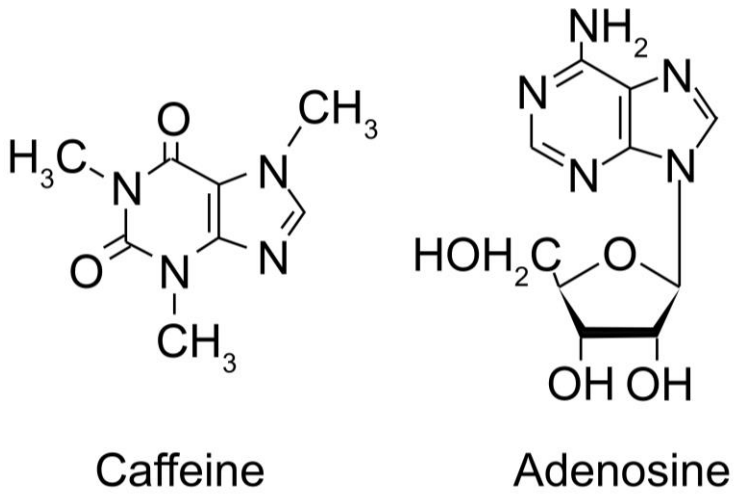


Figure 1.7 Molecular structures of caffeine and adenosine

A comparison of the molecular structures of caffeine and adenosine shows that both compounds share a similar double ring structure. Figure adapted from (Fredholm, 2011).

Adenosine receptors A_1 and A_{2A} are G protein coupled receptors with opposing actions at the cellular level. A_1AR activation leads to the inhibition of adenylyl cyclase and some types of voltage sensitive Ca^{2+} channels (eg. N- and Q- channels) and activation of several types of potassium channels such as phospholipase C and D, followed by a host of different cellular effects (Fredholm et al., 1999). On the other hand, A_{2A}AR activation activates adenylyl cyclase and some types of voltage sensitive Ca^{2+} channels, especially L-channels (Fredholm et al., 1999). Thus these two receptors tend to have partly opposing actions at the cellular level (Fredholm et al., 1999). A_1AR appear to be quite widespread and present in almost all brain areas, with highest abundance in the hippocampus, cerebral and cerebellar cortex and certain thalamic nuclei (Goodman and Synder, 1982; Fastbom et al., 1987a), and moderate expression in the striatum (Fredholm et al., 1999). Some messenger ribonucleic acid (mRNA) studies suggest that A_1ARs may be located on nerve terminals rather than cell bodies (Johansson et al., 1993), however, these receptors have been detected on neuronal cell bodies, oligodendrocytes, microglia, astrocytes and axons with abundant expression in WM tracts (Reppert et al., 1991; Swanson et al., 1995; Cunha, 2005). A_{2A}ARs are expressed only in a few regions of the brain; they are mainly concentrated in the dopamine-rich regions, including the striatum, nucleus accumbens, with very little mRNA detection in the hippocampus, cortex and medulla oblongata (Fredholm et al., 1999; Dunwiddie and Masino, 2001). A_{2a}ARs appear to be located in cell bodies of GABAergic output neurons and are also co-localised

with dopamine D_2 receptors on medium-sized spiny neurons in the dorsal striatum (Schiffmann et al., 1991; Fink et al., 1992; Johansson et al., 1993), as well as microglia and astrocytes (Cunha, 2005).

Previously, respiratory arousal and hence the effect of caffeine on arousal had been attributed to its effects on the A_1AR, as proteins for this receptor are expressed in the brainstem and adenosine-induced activation of A_1AR exerts inhibitory effects on inspiratory neurons (Herlenius et al., 2002; Herlenius and Lagercrantz, 1999). This inhibitory effect is induced via pathways that include inhibition of excitatory glutamatergic synaptic transmission, decreased Ca^{2+} current, inhibition of intracellular cyclic adenosine monophosphate (cAMP) production and facilitation of potassium conductance (Zaidi et al., 2006). However, studies now suggest that A_{2A}ARs may also be involved, and that the arousal effects of caffeine may be mediated by a combination of its effects on both the A_1 and A_{2A} receptors (Dunwiddie and Worth, 1982; Satoh et al., 1999). Using gene deletion strategies in rats to silence the expression of A_{2A}AR, Lazarus et al (2011) found that A_{2A}ARs in the shell region of the nucleus accumbens is responsible for the arousal effects of caffeine. This is because caffeine-induced arousal was absent in rats when A_{2A}ARs were focally removed from the core of the nucleus accumbens or other A_{2A}AR-positive areas of the basal ganglia (Lazarus et al., 2011). Furthermore, a subpopulation of GABAergic neurons that possess A_{2A}AR mRNA has been located in the medulla oblongata (Wilson et al., 2004; Zaidi et al., 2006) and it is known that GABAergic pathways contribute greatly to the inhibition of inspiration that characterises some respiratory reflexes in early postnatal life (Abu-Shaweesh et al., 2001; Mayer et al., 2006). Thus, adenosine and GABA may work synergistically to modulate respiratory neural output.

In the adult brain A_1AR activation exerts protective effects against ischemic and excitotoxic injury, and these effects are blocked by caffeine (Fredholm, 1995). While caffeine blocks the acute neuroprotective effects of adenosine, chronic caffeine consumption has a protective neurological effect in adults (Fredholm, 1995). On the other hand, during development A_1ARs are expressed in the CNS during neurogenesis, neuronal migration and axonal sprouting (Rivkees, 1995) and increased activation during the neonatal period can cause robust cell death during the early postnatal periods (Turner et al., 2002a). Additionally, neonatal rats treated with A_1AR agonists showed marked reductions in WM and GM volume with secondary ventriculomegaly, along with a reduction in total axonal volume and reduced expression of myelin basic protein (Turner et al.,

2002b). Thus, rather than exerting a positive neurological effect, adenosine acts via A_1ARs to induce WM injury in the developing brain, which is blocked by caffeine (Rivkees and Wendler, 2011). Given oligodendrocytes express A_1ARs, the negative effects on myelin resulting from A_1AR agonism or sustained activation is most likely a consequence of alterations to oligodendrocytes (Rivkees and Wendler, 2011). Sustained activation of A_1ARs leads to the inhibition of oligodendrocyte progenitor cell proliferation, resulting in a reduction in the number of oligodendrocytes, which could subsequently affect myelination (Stevens et al., 2002; Rivkees and Wendler, 2011). Alternatively activation of A_{2A}ARs contributes to ischemic tissue damage; however, when blocked, A_{2A}ARs seem to be neuroprotective (Cunha, 2005; Chen et al., 2007). This may be because A_{2A}AR play a key role in the control of BDNF secretion upon microglial activation, as well as on BDNF-induced proliferation of microglial cells (Gomes et al., 2013). Furthermore, A_{2A}AR stimulation by adenosine promotes activated microglia to assume their characteristic amoeboid morphology during brain inflammation (Orr et al., 2009). Hence, an A_{2A}AR antagonist such as caffeine prevents this effect and subsequently prevents neuroinflammation (Rebola et al., 2011). However, the mechanisms by which A_{2A}AR protects the developing brain against damage are still unknown (Rebola et al., 2011).

1.6.2 Effects on $GABA_A$ receptors

$GABA_A$ receptors are chloride channels, which upon activation by GABA permit an inward chloride current, resulting in the hyperpolarization of the cell (Shi et al., 2003). They are expressed ubiquitously throughout the CNS and are the most common receptor type at synaptic sites (Hines et al., 2012). $GABA_A$ receptors appear to cycle between membrane and intracellular compartments rather than being expressed on a cell surface (Hines et al., 2012) and are responsible for rapid neuronal transmission in the CNS (Davies, 2003). The receptor consists of various modulatory sites which most importantly include a benzodiazepine site which enhances GABA binding and responses, and a picrotoxin site, where picrotoxin non-competitively blocks the channel (Shi et al., 2003). Since 1979 there have been limited studies on the interaction of methylxanthines on $GABA_A$ receptors, which showed the inhibition of [^{3}H] diazepam binding to the benzodiazepine site on the $GABA_A$ receptor by caffeine (Marangos et al., 1979). Later studies have confirmed this finding and have also found that compared to other methylxanthines, caffeine is not a potent inhibitor of $GABA_A$ receptors (Shi et al., 2003). Furthermore, it has been suggested that caffeine may affect $GABA_A$ receptors indirectly, since adenosine acting via adenosine receptors can regulate the release of many different neurotransmitters including GABA (Fredholm et al., 1999). Additionally, data

suggests that caffeine may act as an antagonist or a reverse agonist at benzodiazepine and GABA sites and may also block picrotoxin sites, which consequently may be responsible for the convulsive effects of caffeine (Shi et al., 2003).

1.6.3 Inhibition of phosphodiesterase

Although little is known about the PDE inhibitory potency of caffeine, it has been shown that millimolar concentrations of caffeine are needed for the non-specific inhibition of PDE, an enzyme that degrades cAMP (Daly, 2007). The cAMP cascade is one of the most important intracellular signalling pathways and plays a key role in the modulation and expression of neural function in the CNS (Yoshimura, 2005). Caffeine leads to a depression in PDE activity thereby increasing cAMP, which consequently leads to an enhancement of cAMP signalling (Yoshimura, 2005; Daly, 2007). This enhancement in cAMP is thought to mediate some of caffeine's pharmacological actions (Daly, 2007).

1.6.4 Calcium release

Another biological action of caffeine includes promotion of Ca^{2+} release from intracellular stores via ryanodine-sensitive Ca^{2+} release channels. Caffeine, a ryanodine receptor agonist (Resende et al., 2010), acts by enhancing the binding of ryanodine to its Ca^{2+} release channel, leading to an increase in Ca^{2+} release (Daly, 2007). Changes to intracellular Ca^{2+} concentration play a pivotal role in neuronal differentiation and it has been shown that activation of ryanodine-sensitive Ca^{2+} channels by caffeine promotes neurogenesis (Resende et al., 2010). This is most likely due to the involvement of Ca^{2+} channels in regulating nestin, a filament protein expressed mostly in nerve cells where it is important in mediating axonal growth (Resende et al., 2010). Additionally, caffeine leads to an increase in nestin-positive cells in culture (Resende et al., 2010). However, caffeine at the millimolar concentrations is required in order to activate ryanodine-sensitive Ca^{2+} channels. (Daly, 2007; Shi et al., 2003).

1.7 Effects of caffeine in the CNS

1.7.1 Human studies

The "Caffeine for AOP" (CAP) clinical trial was one of the largest international randomized control trials in preterm neonates. This trial showed that preterm infants exposed to caffeine citrate (20 mg/kg loading dose; 5-10 mg/kg maintenance dose) had improved rates of survival without neurodevelopmental disability, and lower rates of cerebral palsy and cognitive delay compared to placebo treated control infants, both prior to discharge and at 18-21 months of age (Schmidt et al., 2006; Schmidt et al., 2007). However, when these infants were assessed at 5 years of age, neonatal caffeine therapy was no longer associated with significantly improved rates of survival without disability (Schmidt et al., 2012). The caffeine-treated preterm infants also showed improved cerebral WM development with a more mature cerebral WM organization, when brain magnetic resonance imaging (MRI) was conducted at term-equivalent age (Doyle et al., 2010a). In regards to caffeine's effect on behaviour, neonatal caffeine citrate administration (80 mg/kg loading dose; 20 mg/kg maintenance dose) did not result in adverse outcomes for development, temperament and behaviour, when infants were assessed at 1 year and at 2 years of age (Gray et al., 2011).

Studies have also assessed brain haemodynamics in preterm infants following caffeine administration. Specifically, acute low-dose caffeine citrate administration (2.5 mg/kg/day) to preterm infants had no affect on brain haemodynamics, measured by near-infrared spectroscopy on the third day of caffeine administration with measurements taken 30 min before to 60 min post caffeine (Dani et al., 2000). However, when brain haemodynamics were assessed in preterm infants following a loading dose of caffeine citrate (10 mg/kg), caffeine increased cortical activity during the first 2 hours (h) after intravenous administration (Supcun et al., 2010). Additionally, caffeine citrate administration (10 mg/kg) to preterm infants reduced cerebral oxygenation and cerebral blood flow velocity at 1 h post caffeine, with a partial recovery at 4 h post dose (Tracy et al., 2010). These differences regarding the outcomes of caffeine treatment may be attributed to differences in dosing, the age of infants at the time of assessment, as well as the time of assessment in relation to the administration of caffeine.

1.7.2 Animal studies

Several studies have used various animal models to assess the effects of caffeine on the developing brain; however these studies show both beneficial and detrimental effects. Thus the effects of caffeine on the developing brain at the cellular level remain controversial.

1.7.2.1 Beneficial effects

Chronic administration of a high dose of caffeine citrate (50 mg/kg/day) in rats from postnatal days (P) 1-P12 increased total dendritic length and arborization of layer III pyramidal neurons of the prefrontal cortex at P35 and this effect persisted after puberty (P70) (Juarez-Mendez et al., 2006). The authors suggest that such changes in dendritic arborization caused by caffeine could explain the improvement in cognitive function reported in both children and rats. Caffeine citrate exposure (15-20 mg/kg/day) from P2-P6 in rats has also been shown to reduce seizure susceptibility to some chemo-convulsants in both juvenile and adult rats (Guillet and Dunham, 1995). It is also evident from models of perinatal brain injury, that caffeine exerts some neuroprotective effects; in hypoxic-ischemic rat pups treated with caffeine via lactating dams that were provided water containing caffeine citrate (300 mg/L) from P0 to P12, myelination was enhanced and ventriculomegaly reduced (Back et al., 2006a). Another study following a similar method to the study above, showed that low dose caffeine administration to hypoxic-ischemic rat pups via lactating dams (300 mg/L) during the first week of life (P1-7), significantly reduced hypoxic-ischemic brain damage via a reduction in neuronal necrosis and infarction (Bona et al., 1995). Similarly, caffeine administered postnatally to Swiss mouse pups (loading dose 10mg/kg, maintenance dose 5mg/kg caffeine base) from P0 to P3 following induced brain damage, did not exacerbate excitotoxic lesions of the periventricular WM (Bahi et al., 2001).

The neurodevelopmental benefits of caffeine have been attributed to its effects on Ca^{2+}/cAMP response element binding protein (CREB), which mediates transcription of genes essential for the development and function of the CNS such as BDNF (Connolly and Kingsbury, 2010). More specifically, BDNF is involved in neuronal survival and in the maturation of developing neurons (Connolly and Kingsbury, 2010; Cunha et al., 2010). It has been shown that transcripts derived from endogenous CREB target genes, such as the gene encoding BDNF are increased in cell cultures following exposure to clinically relevant concentrations of caffeine (100-200 μM)

(Connolly and Kingsbury, 2010). The authors believe that the observed alterations in BDNF as a result of caffeine may contribute to the neurological benefits that have been observed in infants receiving caffeine (Connolly and Kingsbury, 2010).

1.7.2.2 Detrimental effects

A number of studies have shown that perinatal caffeine exposure can have detrimental effects on brain structure and function. One such study showed that daily administration of high-dose caffeine (40 mg/kg or 80 mg/kg caffeine base) from P1-17 resulted in a dose-dependent, transient decrease in myelin concentration at P30 with full recovery and overshoot by P70 (Fuller et al., 1982). Other studies have shown a reduction in cell proliferation in the subventricular zone and dentate gyrus in mice at P7 following daily caffeine citrate administration (10 mg/kg loading dose; 2.5 mg/kg maintenance dose) from P3 to P10 (Desfrere et al., 2007). This same study also found a decrease in astrocytogenesis in a number of brain regions including the cerebral cortex and WM and the authors suggested that this caffeine-induced reduction in astrocytogenesis is linked to the A_{2a}AR antagonist properties of caffeine, as these results could also be mimicked using an A_{2a}AR antagonist (Desfrere et al., 2007).

A study in newborn rats showed that acute high-dose caffeine administration (3 doses, 50 mg/kg; base) induces apoptosis throughout the cerebral hemispheres via caspase-3-dependent mechanisms (Kang et al., 2002). Similarly, administration of acute high-dose caffeine (100 mg/kg; base) at P3 in rats resulted in an increase in apoptosis in various brain regions including the cerebral cortex and the caudate nucleus (Black et al., 2008). Caffeine exposure during development has also been shown to interfere with cholinergic neurotransmission in the brain as well as causing behavioural deficits such as impaired learning in rats (da Silva et al., 2008; Pan and Chen, 2007; Zimmerberg et al., 1991). Additionally, low dose, acute maternal caffeine exposure at 130 DG in the ovine fetus decreases cerebral oxygenation without any effects on fetal systemic oxygenation (Tomimatsu et al., 2007).

It is important to note that the studies described in this section have been conducted mainly in rats and mice, a species in which brain development occurs predominantly postnatally. Furthermore,

these studies have used varied caffeine doses and regimes, making it difficult to draw conclusions on the safety of high dose caffeine. Thus it is important to conduct studies that assess the neuropathological effects of high dose caffeine in a suitable animal model in order to determine if high dose caffeine is safe for the developing brain. Furthermore, there is a lack of data on the long-term outcomes of neonatal caffeine treatment, thus highlighting the need to assess the long-term neuropathological outcomes of caffeine to determine if alterations in brain development in the short-term persists or take longer to manifest.

1.8 Use of ovine models

In order to address the aims of this project, I have chosen to use the sheep to investigate the effects of caffeine exposure on the developing brain. The sheep provides an excellent animal model in which to assess brain development and injury for several reasons. Sheep have a long gestation (147 days), making it possible to study the response of treatments over an extended period of brain development. A major advantage is that, unlike altrical species such as rats and mice, which have short gestation periods, multiple pregnancies and relative immaturity at the time of birth, the ovine fetus has a relatively long gestation and is relatively mature at birth; the offspring are large (similar to the human fetus) and singleton or twin pregnancies are the most common. Another major advantage over smaller species such as laboratory rodents is that, in sheep, both the mother and fetus can be instrumented, enabling us to monitor physiological and immunological responses, which can be later correlated with structural alterations in the brain. This also allows for the monitoring of fetal wellbeing and ensures that there are no adverse effects on fetal physiology. Importantly the ovine brain has a more appropriate ratio of WM to GM and extensive gyral folding as opposed to rats or mice. Furthermore, major events in brain development such as WM development occur *in utero* in sheep as they do in humans, unlike rats or mice. This is an important factor when aiming to replicate neuropathologies seen in human infants.

1.8.1 Comparison of human and sheep fetal brain development

Although the stages of brain development are similar in most mammals, there are some differences, most of which arise from differences in the developmental timing of the stages of brain development. At birth, brain development appears to be more advanced in sheep when compared to

humans. Table 1.1 presents the timing of the major events in brain development with regard to gestational ages in humans and sheep.

Table 1.1 Timing of the major events in brain development in humans (Rice and Barone, 2000; Herschkowitz et al., 1997; Semple et al., 2013; Lenroot and Giedd, 2006) and sheep (Cowan, 1979; Reynolds and Mollgard, 1985; Astrom, 1967; Brus et al., 2013)

Major developmental event	***Human*** ***(term = 40 weeks)***	***Sheep*** ***(term = 147days)***
Primary neurulation	18 to 28 days (0.1 of term)	<18 to 21 days (0.1 of term)
Neurogenesis	28 days (0.1 of term) to years postnatally	21 days (0.1 of term) to years postnatally
Neuronal migration and differentiation	12 to 20 weeks (0.3-0.5 of term)	30 to 80 days (0.2-0.5 of term)
Synaptogenesis, cell death and synaptic rearrangement	20 weeks (0.5 of term) to years postnatally	66 days (0.4 of term) - months postnatally
Gliogenesis and myelination	18 weeks (0.5 of term) to years postnatally	95 days (0.6 of term) to 2 months postnatally

In sheep the closure of the neural tube and appearance of the 5 vesicles occurs by approximately 21 days of gestation (DG; 0.1 of term) (Reynolds and Mollgard, 1985). In the cerebral hemispheres, neurogenesis has commenced by 21 DG (0.1 of term) (Reynolds and Mollgard, 1985) and is ongoing postnatally and into adulthood (Brus et al., 2013). Neuroblast migration and neuronal differentiation in the cerebral hemispheres commence by 30 DG (0.2 of term) (Barlow, 1969; Reynolds and Mollgard, 1985; Astrom, 1967). At this time, neurons in the cerebellum start to differentiate; however in the hippocampus, differentiation commences at 40 DG (0.3 of term) in sheep (Barlow, 1969). Myelination in the cerebral hemispheres begins by 100 DG (0.6 of term) and in the cerebellum and hippocampus by 80 DG (0.5 of term) (Barlow, 1969; Romanes, 1947). In sheep there are two periods in which fetal development results in a significant increase in brain size,

with the cerebral hemispheres being the most advanced segment of the brain in terms of development (McIntosh et al., 1979). The first period, from 30-80 DG (0.2-0.5 of term), sees the establishment of cortical activity, beginning with the proliferation of neurons, particularly in the cerebral hemispheres (Astrom, 1967) and brainstem (McIntosh et al., 1979). The appearance of neocortical layers occurs by 50 DG (0.3 of term) with the establishment of cortical layers apparent by 80 DG (0.5 of term) (Reynolds and Mollgard, 1985) and process outgrowth is observed at 66 DG (0.4 of term) (Astrom, 1967). The second period of growth is from 95-130 DG (0.6-0.9 of term), during which gliogenesis and the initiation of myelination occurs (McIntosh et al., 1979; Patterson et al., 1971). In sheep, as in humans, myelination and synaptogenesis continue well into the postnatal period (McIntosh et al., 1979). These two periods highlight the possible time points at which the brain is particularly vulnerable to injury.

Experiments in this thesis have been performed during the second period of brain growth in sheep. Thus any adverse effects on the brain during this time can lead to alterations in WM development or injury to the cortical GM. Specifically caffeine was administered to fetal sheep from 104-118 DG (0.7-0.8 of term). At this age, brain development in fetal sheep is equivalent to about 27-34 weeks postmenstrual age in humans (Rees et al., 2010; Back et al., 2002; Back et al., 2006b), which is a time when preterm infants are exposed to caffeine (Schmidt et al., 2007) and when the brain is still developing (Back et al., 2002).

1.9 Summary and aims of the thesis

Preterm birth is a major clinical problem worldwide and despite the advances in obstetric care, preterm infants, particularly those born extremely preterm, have high rates of cerebral palsy and neurodisability (Saigal and Doyle, 2008). Furthermore these infants are at a higher risk of AOP, with around 85% of infants born prior to 34 weeks developing this condition (Barrington and Finer, 1991). Caffeine is one of the few treatments available for preterm newborns associated with improved neurodevelopmental outcomes (Schmidt et al., 2006; Schmidt et al., 2007). However, of concern are those infants that do not respond to the current clinical doses of caffeine; thus these infants may be treated with higher doses of caffeine (Scanlon et al., 1992; Steer et al., 2003) in order to reduce the need for mechanical ventilation (Donn and Sinha, 2006). Yet, little is known regarding the effects of higher doses of caffeine on the immature brain. While several studies have

assessed the effects of high-dose caffeine on the developing brain, these studies show both beneficial (Juarez-Mendez et al., 2006; Connolly and Kingsbury, 2010) and detrimental (Fuller et al., 1982; Desfrere et al., 2007) effects. These previous studies have also been mainly conducted in rats and mice (Juarez-Mendez et al., 2006; Connolly and Kingsbury, 2010; Fuller et al., 1982; Desfrere et al., 2007), species in which WM to GM ratio is not comparable to humans and in which brain development occurs predominantly postnatally. Additionally previous studies have used widely differing dosing regimens. Given the conflicting experimental data, it is difficult to draw unequivocal conclusions about the likely effects of high-dose caffeine on the developing brain.

The **global aim** of this thesis was to determine if administration of high-dose caffeine adversely affects the immature ovine brain. To address this aim, high-dose caffeine was administered to the ovine fetus via the maternal circulation at a stage of brain development that is similar to that of very preterm infants. As caffeine was administered *in utero*, it was important to first assess whether caffeine would exert its potential effects on the brain directly or indirectly by influencing maternal or fetal physiology. Thus in Chapter 3, I have assessed the effects of high-dose caffeine administration on fetal physiology. Maternal physiological status was also assessed in order to determine if changes in fetal physiological status were related to altered maternal physiology.

The effect of high-dose caffeine on the immature brain was then investigated. Particular interest was placed on regions such as the WM and GM as these regions are connected and thus any adverse effects of caffeine on the developing WM is likely to translate into alterations within the GM and vice versa. The developing WM is a region that is particularly vulnerable in preterm infants. Thus in Chapter 4, the WM was assessed for any adverse effects of high-dose caffeine using known markers of brain injury. Caffeine's effect on myelination and axons was also assessed. Until recently, damage to the GM has been a relatively neglected aspect of preterm brain injury. This region is critical to cognition, memory and learning, thus in Chapter 5 analysis of the effect of high-dose caffeine was focused on major GM regions, including the cortical GM and striatum. Analysis in these regions included assessment of neurons and neuronal populations, which included, subcerebral projection neurons and GABAergic interneurons. Glial cells were also assessed in the cortical GM and connectivity was assessed via the analysis of dendritic spine density. It is important to determine if any neuropathological alterations present in the short-term (Chapter 3-5) as a result of high-dose caffeine treatment persist later in life, or in the absence of alterations in the

short-term, whether alterations are established later in life. Owing to the uncertainties and the lack of studies surrounding the neuropathological effects of high-dose caffeine treatment for AOP in the long-term, in Chapter 6 I have investigated the effects of prenatal caffeine exposure on the WM using MRI as well as on the postnatal growth of lambs. These studies will aid in increasing our understanding of the physiological and neurodevelopmental effects of high-dose caffeine.

2 General methods

2.1 Ethics clearance and animal welfare

Experimental procedures were approved by the Animal Ethics Committee of Monash University. All animal handling, use and care followed the National Health and Medical Research Council (NHMRC) Australia Code of Practice for the Care and Use of Animals for Scientific Purposes.

Date-mated pregnant cross-bred ewes (Merino X Border Leicester) carrying single or twin fetuses were obtained from an approved animal supplier and transported to the animal housing facility one week prior to surgery, giving the animals adequate time to adapt to the new environment. The pregnant ewes were housed in individual pens prior to surgery, after which they were moved into individual mobile cages during the study period. Each ewe was fed daily with ~1 kg Lucerne chaff and had access to water at all times. Food intake, as well as the general wellbeing of the animals were monitored and recorded daily throughout the experimental period and any abnormal physical or physiological symptoms were inspected by a veterinary specialist and treated accordingly. All ewes were housed at a temperature of 22°C and 45-55% humidity with a 12 h light/dark cycle (lights on at 0700 h and off at 1900 h). Ewes that did not make a rapid recovery following surgery or showed signs of discomfort were monitored closely over the next 24 – 48 h, and treated as determined by the veterinary specialist.

2.2 Experimental design

Experiments were undertaken in sheep, a species in which the timing of major developmental events in the brain, including WM and cortical GM development, aligns with human brain development. To study the effects of caffeine at a stage of brain development that is equivalent to the stage at which human preterm infants are treated with caffeine, it was necessary to perform these experiments *in utero*, when the ovine fetal brain is at a similar stage of development as that of preterm infants. In order to expose the developing brain to caffeine at a similar stage of brain development as in preterm infants we have used fetal sheep at 0.7–0.8 of term; at this age, WM and cortical GM development is similar to that of preterm infants at about 27-34 weeks postmenstrual age (Rees et al., 2010; Back et al., 2002; Back et al., 2006b), and thus represents the typical age at which preterm babies are exposed to caffeine (Schmidt et al., 2007) and when the brain is still developing (Back et al., 2002).

The preterm postnatal lamb was not suitable for this study. In order to determine the effects of caffeine on very preterm (postnatal) lambs, lambs would need to be delivered and provided with life support before 110 DG (term ~147 DG), which is currently not possible. It is possible to maintain preterm lambs born after 131 DG with essential life support (De Matteo et al., 2010); caffeine exposure during this age would not target the appropriate stages of brain development. By using fetal sheep, the effects of caffeine on the developing brain could be studied in the absence of potentially confounding factors that are associated with maintaining very preterm infants or animals; these include positive pressure ventilation, high levels of oxygen, providing adequate nutrition and preventing infection.

In order to administer caffeine to the ovine fetus *in utero* it was considered appropriate to administer caffeine to the mother (based on maternal body weight). Pilot experiments revealed that caffeine administered directly to the fetus was rapidly lost to the ewe's circulation via placental transport. Intravenous infusion of caffeine base (10 mg/kg followed by 5 mg/kg caffeine for 2 days) directly to the fetus via a femoral vein, increased fetal plasma caffeine concentrations by less than 0.1 mg/L. However, when the same dose of caffeine was administered to the pregnant ewe (based on maternal weight) the median concentration of caffeine reached 8.4 mg/L, which is comparable to

a median concentration of ~10.7 mg/L in preterm neonates (Natarajan et al., 2007; Leon et al., 2007).

The current treatment for preterm infants with apnea of prematurity (AOP) involves administration of 20mg/kg caffeine citrate on day 1 as a loading dose, followed by a single daily (maintenance) dose of 5-10 mg/kg of caffeine citrate. A high-dose caffeine exposure model was chosen to represent higher than normal doses, which are similar to those currently being used in some neonatal intensive care units (Steer et al., 2003; Gray et al., 2011); these doses were chosen in consultation with clinicians involved in the project.

Ewes were administered a loading dose of 25 mg/kg caffeine base (equivalent to 50 mg/kg caffeine citrate) followed by a daily maintenance dose of 20 mg/kg (equivalent to 40mg/kg caffeine citrate) dissolved in 25 mL saline. While preterm infants suffering from AOP are commonly treated with caffeine citrate, caffeine base is used in neonatal nurseries when caffeine citrate cannot be sourced. Here caffeine base was used, due to the large volumes required to appropriately dose the pregnant ewe. Caffeine citrate contains 50% anhydrous caffeine base and anhydrous citric acid. As each ml of caffeine citrate solution (20 mg/ml) contains the equivalent of 9.7 mg/ml caffeine base, the dose of caffeine base is half that of caffeine citrate. The control ewes and fetuses received an equal volume of sterile saline each day for the duration of the experiment. Throughout this thesis the term “caffeine” will be used to indicate caffeine base rather then caffeine citrate.

2.2.1 Experimental groups

Ewes were randomly assigned to either a short-term survival study cohort or a long-term survival study cohort; each of these cohorts contained caffeine-treated and control groups (Figure 2.1).

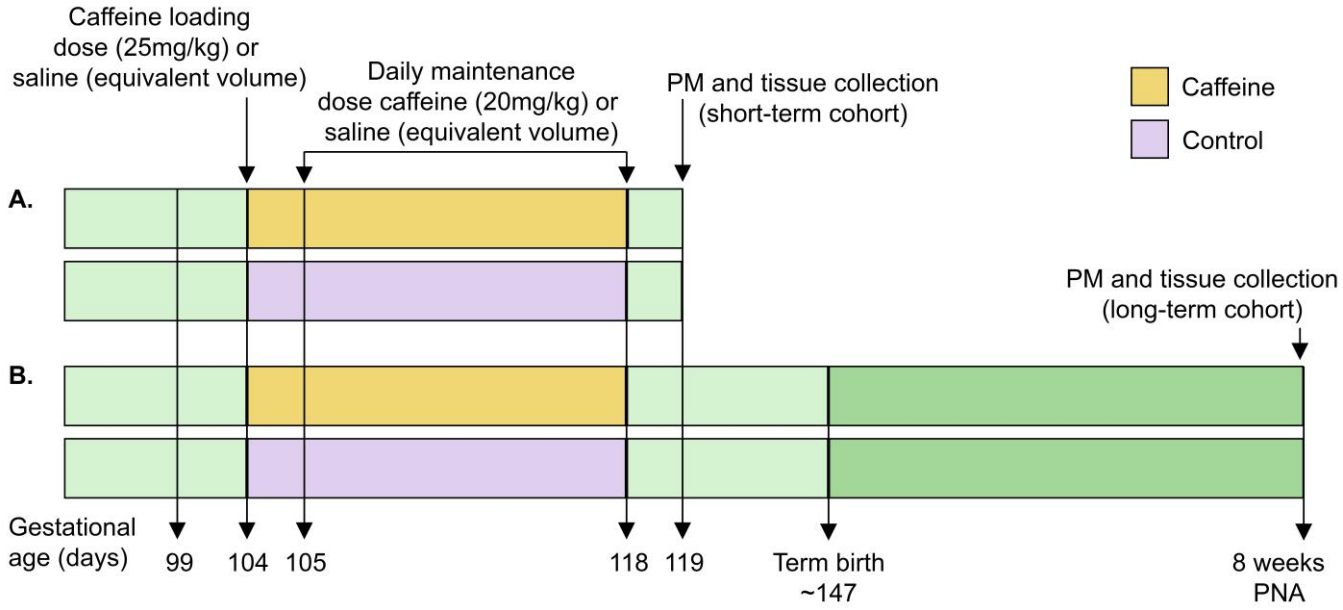


Figure 2.1 Short-term and long-term survival study protocols

All ewes underwent surgery at 99 DG for the implantation of maternal and fetal catheters (for the short-term survival study) or only maternal catheters (for the long-term survival study). Ewes were administered either a loading dose of caffeine (25 mg/kg) at 104 DG followed by daily maintenance doses of caffeine (20 mg/kg) from 105 DG to 118 DG (shown in yellow) or saline (shown as controls in purple). Fetuses from the short-term survival group (A) underwent necropsy at 119 DG while those from the long-term survival group (B) underwent necropsy at 8 weeks postnatal age (PNA). PM, post-mortem.

2.2.1.1 Short-term survival study group

At 99 DG, surgery was performed on both the ewe and fetus for catheterisation (see Section 2.4). Fetuses assigned to caffeine treatment were administered a loading dose of 25 mg/kg caffeine (Sigma, St Louis, Missouri) at 104 DG, followed by a daily maintenance dose of 20 mg/kg from 105 DG to 118 DG. Fetuses assigned to the control group were administered an equivalent volume of saline (25 mL) from 104 DG to 118 DG. At 119 DG all fetuses underwent necropsy.

2.2.1.2 Long-term survival study group

Ewes underwent surgery at 99 DG for the implantation of maternal catheters. Caffeine or saline was then administered daily, beginning with a loading dose (25 mg/kg) at 104 DG followed by daily maintenance doses (20 mg/kg) from 105 DG to 118 DG. The pregnancy continued to term and lambs were born spontaneously and lived with their mothers until 8 weeks postnatal age (PNA). At 8 weeks PNA all lambs underwent necropsy and magnetic resonance imaging was conducted on all brains prior to processing (Chapter 6).

2.3 Catheter preparations

Polyvinyl tubing (I.D. 0.86 mm; O.D. 1.52 mm, Microtube Extrusions, Australia) was cut to lengths of 1.5 m for use as fetal catheters. A 20 gauge Kendall Monoject aluminium hub blunt needle (Covidien, Australia) was inserted at one end of the catheter, for attachment to a syringe or pressure transducer. Catheters for implantation into maternal blood vessels and the amniotic sac were prepared the same way using thicker polyvinyl tubing (I.D. 1.5 mm; O.D. 2.7 mm) and a 15 gauge Kendall Monoject aluminium hub blunt needle. All catheters were packed in heat sealed sterilization bags and sterilized by ethylene oxide (Steritech, Australia) prior to surgery.

2.4 Animal surgery

2.4.1 Preparation for surgery

Aseptic surgery was conducted on pregnant ewes at 99 DG (term is ~147 DG). Food was withheld for 24 h prior to surgery, whereas access to water remained unrestricted. Prophylactic antibiotics [Engemycin (500 mg/5mL i.v., Coopers Animal Health, Australia) and Ampicillin (1 g/5mL i.v., Aspen Pharmcare Australia Pty Ltd)] were administered intravenously to the ewe immediately before induction of anaesthesia. Following antibiotic administration, both ewe and fetus were anaesthetised by a bolus (20 mL) injection of thiopentone sodium in water (50 mg/mL; Jurox Pty Ltd, Australia) into the ewe's jugular vein. Once anaesthesia was established the ewe was placed in a supine position and intubated with an endotracheal tube (size 8; Covidien, Australia). Anaesthesia was maintained throughout the period of surgery with 1.5 – 2% Halothane (Abbott, Australasia) or Isoflurane (Isoflo; Abbott, Australasia) in oxygen, delivered through a closed circuit system. The animal was ventilated using a positive pressure ventilator (Ohmeda 7000 ventilator; DRE

veterinary, USA). The wool on the abdomen, right flank and ventral neck was shorn. These areas were then cleaned with povidone-iodine solution (7.5% w/v Povidone-Iodine, Orion Lab Pty Ltd, Australia) and Betadine antiseptic solution (10% w/v povidone-iodine; Orion Lab Pty Ltd, Australia) prior to surgery.

All surgical drapes, towels, gowns and instruments were sterilised in an autoclave prior to surgery. Hands and arms of each surgeon were thoroughly scrubbed with chlorhexidine antiseptic skin cleanser (10% w/v chlorohexidine gluconate, 10% w/v alcohol; Orion Lab Pty Ltd, Australia). All surgeons wore hairnets, masks, sterile gowns and sterile latex gloves (Ansell International, Australia).

2.4.2 Surgical procedures

A midline incision was made on the abdominal skin from the umbilicus to the margin of the udder avoiding the superficial mammary veins. Subcutaneous adipose tissue was cleared in order to expose the *linea alba*. A second incision was then made in the *linea alba* and the peritoneum to expose the uterus. Fetuses were identified by palpitation to determine single or twin pregnancies. In the case of twin pregnancy, only one fetus was exposed and catheterised. Fetal orientation was noted via external palpitation and an incision was made in the uterus, taking care to avoid major uterine vessels and placental cotyledons. The fetal hindquarters were then exposed and both hind legs were brought out of the uterus through the incision site. The uterine muscle and membranes (amnion and chorion) were clamped close to the fetal skin to minimise loss of amniotic fluid. Fetal skin was kept moist with sterile saline (0.9% NaCl, pH 7.4; Baxter, Australia) during the surgery. Fetal hind limb length was measured in order to estimate GA at the time of surgery using a growth chart for fetal Merino sheep (Cloete, 1939). All fetuses from the short-term survival cohort were catheterised (see Section 2.4.2.1). Fetuses for the long-term survival cohort (lambs grown to 8 weeks of age) were not catheterised to prevent catheter tangling during spontaneous term birth.

2.4.2.1 Fetal and maternal catheterisation

In one of the exteriorised fetal hind legs, a small incision was made through the skin overlying the femoral artery. Blunt dissection was used to locate and expose the femoral artery for 10-15mm with

care taken to avoid damage to adjacent vessels. The catheter was then inserted and tied in place with silk (size 2-0; Dynek Pty Ltd, Australia). Another catheter was placed into a femoral vein, using the same method as for the femoral artery. The skin incision was sutured closed (size 3-0; Dynek Pty Ltd, Australia). Upon completion of fetal catheterisation, all catheters were secured to the skin to prevent catheter tangling and blockage. The hindquarters of the fetus were then gently placed back into the uterus, avoiding loss of any amniotic fluid. A large diameter catheter (I.D. 1.5 mm; O.D. 2.7 mm) was tied into the uterus for later administration of antibiotics and pressure measurements. The amniotic catheter was then secured to the uterine wall using a silk suture, avoiding any major vessels and placental cotyledons.

For the insertion of maternal catheters, an incision was made in the ventro-lateral surface of the ewe's neck below the level of the larynx. Using the same technique as for fetal catheter insertion, a polyvinyl catheter (I.D. 1.5 mm; O.D. 2.7 mm) was inserted into the maternal carotid artery and jugular vein and advanced 10-15 cm toward the heart. The skin incision on the neck was then closed using a continuous suturing pattern with a silk suture.

2.4.2.2 Exteriorising the fetal catheters

Following insertion of the amniotic catheter, the uterus and uterine membranes were closed using absorbable suture (size 2-0; Chromic Surgical Gut, Dynek Pty Ltd, Australia) and interlocking stiches to prevent leakage of amniotic fluid. The exteriorised part of the uterus was returned to the abdominal cavity. The fetal catheters were exteriorised by passing them through a small incision in the ewe's flank. The *linea alba* was closed using individual sutures placed approximately 1 cm apart, with an absorbable suture (size 2; Chromic Surgical Gut, Dynek Pty Ltd, Australia). The subcutaneous tissue and abdominal skin were then sutured closed with polyamide monofilament non-absorbable thread (size 2/0, Superamid White; Braun, Australia). The small incision on the upper right flank was also closed with stitches between the catheters to prevent leakage of abdominal fluids. Following wound closure, the incision sites were irrigated with Bupivacaine (Marcaine; 0.5% with 2-5 ml adrenalin; Astra Zeneca, Australia), a long lasting local anaesthetic with analgesic effects. Fentanyl (Durogesic 75; Janssen-Cilag Pty Ltd, NSW, Australia) was also administered via a transdermal patch adhered to the skin on the inner hind limb groin (delivering

7.5 mg over 3 days). Sterile three-way stopcocks (Becton and Dickson, Australia) were attached to the exposed end of each of the three catheters.

2.4.2.3 End of surgery

At the completion of surgery, the anaesthetic gas was terminated to allow recovery from anaesthesia. All incisions were sprayed with povidone-iodine (10% w/v povidone-iodine; Orion Lab Pty Ltd, Australia) and the abdominal incision covered with sterile cotton pads. Elastic netting (size 7; Tubular net, Sutherland Medical, Australia) was placed over the ewe's abdomen and chest to secure and to prevent damage to the catheters. Mechanical ventilation was disconnected once the ewe commenced spontaneous breathing and the endotracheal tube was removed once laryngeal reflexes had returned. Once the ewe was breathing independently, she was returned to the animal house and placed in an individual pen.

2.4.3 Post-surgical care

After recovering from surgery ewes were provided with food immediately, and water when the ewe was able to stand. The ewe was closely monitored during the recovery period. Ewes recovered for at least 5 days following surgery before any experiments were conducted. Following surgery and during experiments, fetal arterial blood samples were obtained each day for monitoring of fetal well-being and all catheters were flushed with heparinised saline to prevent blood clotting.

2.5 Necropsy and tissue collection

2.5.1 Necropsy

Following the completion of the short-term survival studies, each ewe and its fetus (single or twin) was humanely killed with sodium pentobarbitone (130 mg/kg i.v; Lethabarb, Virbac Pty. Ltd., Peakhurst, NSW, Australia). At necropsy the fetus was removed from the uterus and its condition noted. The sex, body weight and body morphometric measurements of each fetus were determined. This was then followed by tissue collection. Postnatal lambs were euthanized at 8 weeks of age by an intra-venous injection of sodium pentobarbitone. Each ewe or lamb was examined for the absence of pupillary and somatic reflexes before proceeding with tissue collection. Tissue collection

and processing were the same for long-term survival lambs as for the fetuses in the short-term survival study

2.5.2 Tissue collection

2.5.2.1 Fixation of brains

The fetus or lamb was placed in a supine position on a wire rack and the forelimb and the heart exposed through a midline thoracotomy. The pericardium and connective tissue around the aorta were removed and a piece of twine was threaded under the ascending aorta and left untied. A small incision was made at the apex of the heart and a 20 gauge Kendall Monoject aluminium hub blunt needle (Covidien, Australia) attached to a catheter was then inserted into the left ventricle and guided up through the ascending aorta to the aortic arch. The catheter was secured in the aorta by tying the twine around the artery. The other end of the catheter was attached to a peristaltic pump with a sphygmomanometer (Accoson, Harlow, UK), attached in-line to enable the perfusion pressure to be maintained at 40-60 mmHg for fetal lambs and at 60-80 mmHg for postnatal lambs; this is similar to the arterial pressure measured in the fetus and lamb (De Matteo et al., 2008). The right atrium was cut to allow blood and perfusate to escape. Prior to perfusion the descending aorta was clamped to avoid fixation of the lower extremities. The brain was first perfused with 500-1000 ml of 0.9% NaCL solution to clear the cerebral circulation of blood. Once the perfusate exiting the right atrium was clear the brain was perfused with 1500-2000 ml of 4% paraformaldehyde (PFA; Scharlau, Barcelona, Spain) in 0.1M phosphate buffer (PB, pH 7.4;).

When perfusion was complete, the fetal and lamb brain with upper spinal cord attached was removed from the skull, weighed and post-fixed for 72 h in the same fixative; lamb brains from the long-term survival cohort underwent MRI overnight following post-mortem (see chapter 6) and prior to tissue processing. Fetal and lamb organs, including, the heart, lung, liver, kidneys, adrenal glands and gut were weighed following the perfusion of the brain.

2.5.2.2 Brain tissue processing

Following the post-fixation of brains, the spinal cord was dissected from the medulla at the level of the obex, transferred into cassettes (Tissue-Tek, Sakura, USA) and processed to paraffin. The cerebellum was then detached from the pons, weighed and hemisected along the sagittal plane. The left-hand side of the cerebellum was further separated into lateral hemisphere and vermis; the lateral hemisphere was transferred into 20% sucrose in 0.1M PB for 72h then processed for cryostat sectioning, while the vermis was immersed into 4% PFA in 0.1M PB for the Golgi analysis. The right-hand side of the cerebellum immersed into 4% PFA in 0.1M PB and processed to paraffin. An incision was then made just below the middle cerebellar peduncle to separate the medulla from the pons and the medulla was weighed. The pons was then separated from the midbrain via an incision in front of the inferior colliculus and weighed. Both the pons and medulla were processed to paraffin. The left and right hemispheres of the cerebrum was then separated and blocked separately. Using a custom made brain-blocking device, the entire right hemisphere was cut into 0.5 cm blocks, producing 9 to 10 blocks, with the first cut made at the ansate sulcus (Figure 2.2); blocks were processed to paraffin. The left hemisphere followed the same protocol, except that blocks 1, 4 and 7 were processed to paraffin, blocks 2, 5 and 8 were transferred into 20% sucrose in 0.1M PB for 72 h then processed for cryostat sectioning and blocks 3, 6 and 9 were left in 4% PFA in 0.1M PB, until processing for Golgi-cox staining. Methodology for the Golgi-cox staining is described in more detail in Chapter 5 (Section 5.2.2.2). All blocks, beginning from the frontal lobe were placed caudal face down for processing with the exception of the last block from the occipital lobe, which was placed rostral face down.

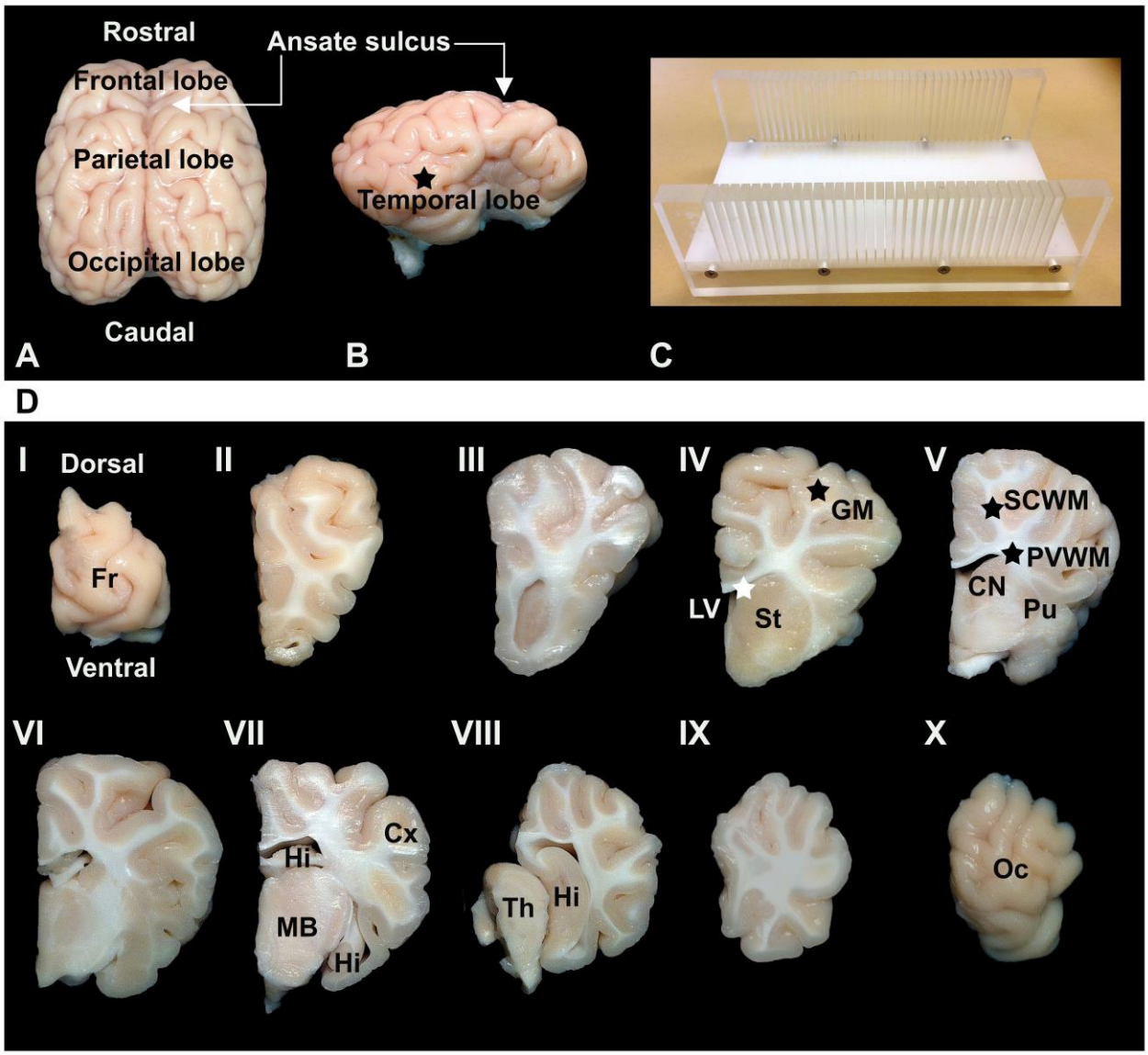


Figure 2.2 Brain tissue processing

A) Superior view of a fetal sheep brain indicating the frontal, parietal and occipital lobes, as well as the ansate sulcus. **B)** Lateral view of the right hemisphere, indicating the temporal lobe location and the ansate sulcus. **C)** A micrograph showing the custom-made brain mould where the right and left hemisphere was placed; coronal sections were obtained by passing a skin grafting blade at 0.5 cm intervals through the left and right hemisphere. **D)** Ten continuous blocks were obtained. These are positioned from rostral (I) to caudal (X). Fr = frontal cortex, LV = lateral ventricle, GM = grey matter, St = striatum, SCWM = subcortical white matter, PVWM = periventricular white matter, CN = caudate nucleus, Pu = putamen, Cx = cortex, Hi = hippocampus, MB = midbrain, Th = thalamus and Oc = occipital cortex.

2.6 Brain tissue processing for histology

Samples of brain tissue allocated to paraffin processing were processed using an enclosed vacuum automatic tissue processor (Leica ASP300, Leica Microsystems Pty Ltd, Australia). The fixed brain tissue was first thoroughly dehydrated using three solutions of increasing ethanol concentration, beginning at 70%, 90% then four repetitions at 100% ethanol (Chem-Supply Pty Ltd, Australia) for 45 mins in 70% and 90% ethanol and 1 h 40 mins in each repetition of 100% ethanol. This was followed by a clearing process, where the tissues were immersed in histolene four times for 1 h 40 mins each repetition, in order to ensure the removal of ethanol from the tissue. The tissues were then passed through 3 heated paraffin baths for 1 h 40 mins each, to replace the clearing agent. Upon completion of tissue processing, brain tissue were set in moulds containing fresh paraffin and chilled on a cold plate (Thermoline Scientific, Australia). Tissue was sectioned at a thickness of 8 μm using rotary microtome (Microtec Cut4060, Germany); sections were then mounted on Superfrost Plus slides (Mezel-Glaser, Germany). The slides were first dried at room temperature, then at 40°C for 24 h and finally at 60°C prior to histological and immunohistochemical staining.

The tissue samples immersed in 20% sucrose in 0.1M PB for cryostat sectioning were embedded in plastic moulds (Tissue-Tek, Sakura, USA) using optimal cutting temperature (O.C.T) compound (Tissue-Tek, Sakura, USA). The plastic moulds were then placed in a silver bowl containing Iso-Pentane (Chem-Supply Pty Ltd, Australia), which was placed in a larger bowl containing liquid nitrogen. This allowed the O.C.T compound to solidify and subsequently secure the tissue in place for cryostat sectioning. Once embedded the tissue moulds were stored in -80°C until required for staining. Blocks were cut at a thickness of 8 μm using a cryostat (Leica CM3050, Leica Microsystems Pty Ltd, Australia) and mounted on Superfrost Plus slides (Mezel-Glaser, Germany) for immunohistochemical staining.

2.7 Brain histology

Brain sections stained with either 0.1% thionin or Haematoxylin and Eosin (H&E) were used to examine the gross morphology of the brain, including the presence (or absence) of haemorrhages, lesions or infarcts. Sections were de-waxed in histolene and re-hydrated in ethanol prior to being immersed in Mayers Haematoxylin to stain the nuclei of cells and counterstained with eosin, which stains the cytoplasm, muscle, collagen and red blood cells pink/red. Section were then de-hydrated

and cleared in and coverslipped using a mixture of DPX mount (Merck, Germany). The thionin stain followed a similar protocol, however sections were immersed in 0.1% thionin in acetate buffer (pH 4.4) and taken through graded ethanol and histolene then coverslipped.

2.7.1 Immunohistochemistry stains

Immunohistochemical staining was performed on paraffin sections obtained from the right cerebral hemispheres as indicated in Table 2.1. These procedures were used to identify specific cell types or neural structures.

Table 2.1 Immunohistochemistry of brain tissue

Primary antibody	Localises	Supplier	Dilution	Secondary antibody
Rabbit anti-ionized calcium binding adaptor molecule-1 (Iba-1)	Macrophages and microglia	WAKO, Osaka, Japan	1:1500#	Biotinylated anti-rabbit IgG
Rabbit anti-glial fibrillary acidic protein (GFAP)	Reactive astrocytes	DAKO, CA, USA	1:1000#	Biotinylated anti-rabbit IgG
Rat anti-myelin basic protein (MBP)	Mature myelin and oligodendrocytes	Millipore, MA, USA	1:100#	Biotinylated anti-rat IgG
Mouse anti-pan-axonal neurofilament marker (SMI-312)	Axons	Convance Inc., New Jersey, USA	1:1000*	Biotinylated anti-mouse IgG
Rabbit anti-oligodendrocyte transcription factor 2 (Olig2)	Oligodendrocytes	Millipore, MA, USA	1:500#	Biotinylated anti-rabbit IgG
Mouse anti-neuronal nuclei (NeuN)	Neurons	Millipore, MA, USA	1:500#	Biotinylated anti-mouse IgG
Rat anti-ovalbumin upstream promoter transcription factor-interacting protein 2 (Ctip2)	Subcerebral projection neurons	Abcam, MA, USA	1:500#	Biotinylated anti-rat IgG
Rabbit anti-somatostatin (SST)	Subpopulation of GABAergic interneurons	DAKO, CA, USA	1:500*	Biotinylated anti-rabbit IgG
Rabbit anti-adenosine A_1 receptor (A_1AR)	A_1 adenosine receptors	Millipore, MA, USA	1:100#	Biotinylated anti-rabbit IgG
Rabbit anti-adenosine A_{2a} receptor (A_{2a}AR)	A_{2a} adenosine receptors	Millipore, MA, USA	1:500	Biotinylated anti-mouse IgG
Sections pre-treated with *0.02% proteinase K (30min/37°C) or #citrate buffer (pH 6; microwave) prior to incubation with the primary antibody				

In general, prior to immunohistochemical staining, sections of brain tissue were de-waxed through two changes of histolene solvent, and re-hydrated through graded solutions of ethanol to distilled water. An antigen retrieval procedure was used to recover antigen immunoreactivity from PFA-fixed, paraffin-embedded tissue. The two types of antigen retrieval procedures used were: a heat-induced epitope retrieval method in citric acid buffer (pH of 6.0) and a proteolytic enzyme-induced epitope retrieval method in 0.02% proteinase K at 37°C. This was followed by a 45 min cooling period in the antigen retrieval solution (citric acid buffer only). Sections were then washed three times in phosphate buffer solution (PBS) for 5 min per wash. Endogenous peroxidases were then blocked, by incubating sections in 1% hydrogen peroxide. Sections were then washed three times in PBS for 5 min per wash. Non-specific binding was blocked by incubating sections for 30 min in 4% bovine serum albumin (BSA) made in PBS. The relevant primary antibody diluted in a primary diluent (1 g BSA, 150 µl triton X-100 and 49 ml PBS) was then applied to each slides and incubated in 4°C overnight in a humidified chamber. Specificity of staining for each antibody was confirmed by omission of the primary antibody. Following incubation, sections were washed three times in PBS for 5min per wash. The secondary antibody (Vector Laboratories, Burlingame, CA) was then applied to each slide (1:200 dilution in 2% BSA in PBS) and slides were incubated for 90 min at room temperature in a humidified chamber. Following incubation slides were washed three times in PBS for 5min per wash followed by incubation with avidin-biotin peroxidase complex kit for 90 min at room temperature in a humidified chamber. Sections were then washed three times for 5min each. 3,3'-diaminobenzidine solution (DAB) was then applied to each slide and incubated at room temperature for ~7 min. The DAB solution consisted of 1 tablet of DAB (MP Biomedicals, Australia) in 10ml of PBS and 3 µl of 30% hydrogen peroxide. Sections were then counterstained (except MBP and SMI-312) with 0.1% thionin (Sigma, St Louis, Missouri) for 4 min followed by a rinse with tap water and de-hydration through graded ethanol solutions. The ethanol was then removed using two changes of histolene and slides were mounted with glass coverslips using DPX and allowed to dry at room temperature.

2.7.2 TUNEL staining

Apoptotic cells in sections of brain tissue from the right hemisphere were detected using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay kit (DeadEnd™ Colorimetric TUNEL system; Promega, USA); all buffers were provided in the kit. The TUNEL

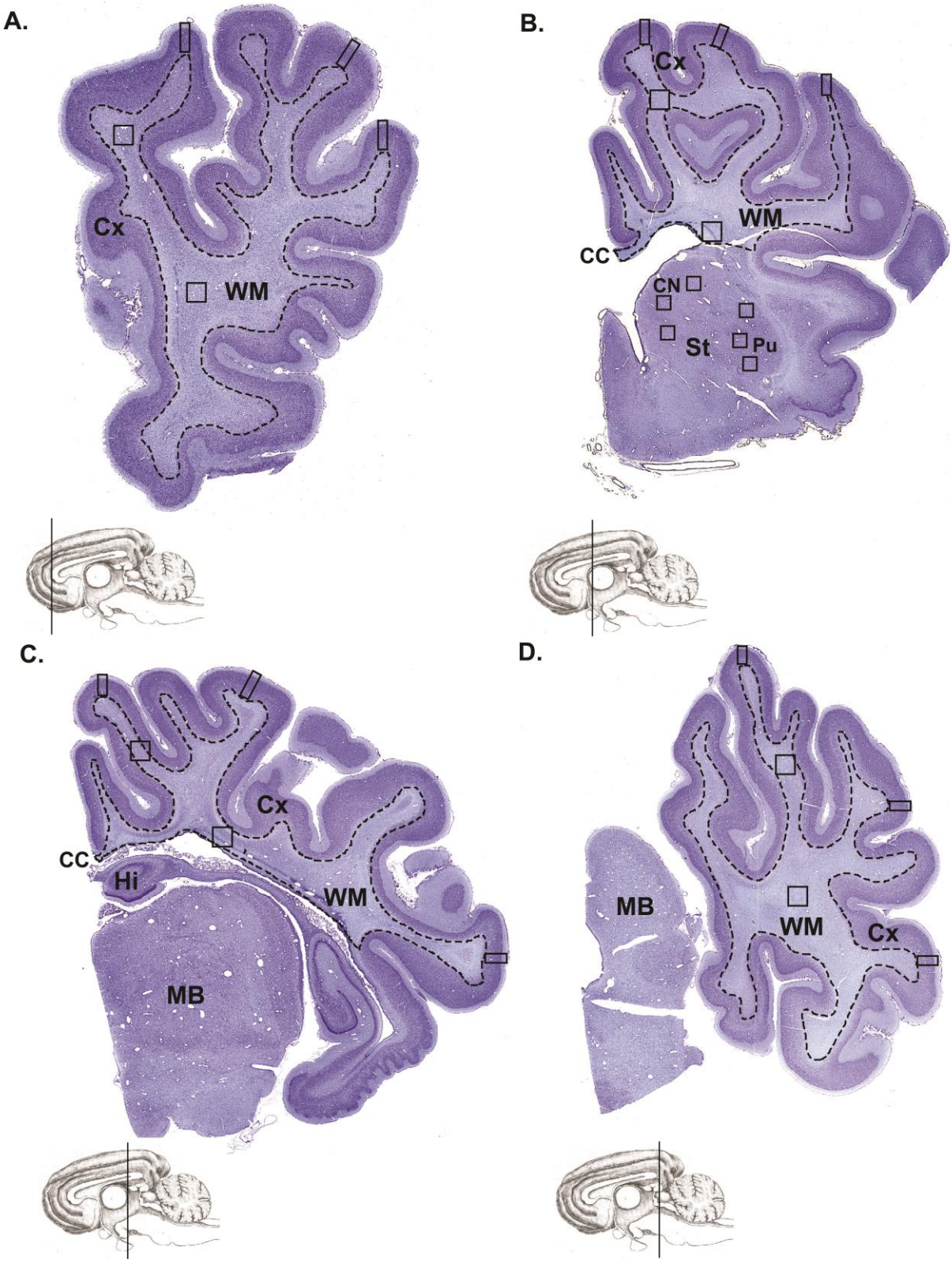
assay identifies apoptotic cells by enzymatically labelling DNA fragments, which are only present in cells undergoing apoptosis (Stadelmann and Lassmann, 2000). Thus the TUNEL assay was used to measure the density of apoptotic cells within the WM, cortical GM and striatum (See Section 2.7.3.2). Sections of brain tissue were de-waxed and re-hydrated as described in Section 2.7.1, followed by one 5 min wash in 0.85% sodium chloride and one 5 min wash in PBS. DNases and RNases were inactivated by treating sections with Proteinase K (20 μg/ml) for 30 min at 37°C, followed by 30 min at room temperature. Slides were then washed twice in PBS for 5 min each. Sections were then treated with equilibration buffer for a minimum of 5min before being incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber for 60 min at 37°C. The reaction was stopped by washing slides in stop-wash buffer, for 15 min at room temperature; this was followed by three washes in PBS for 5min each. Endogenous peroxidases were then blocked using 0.3% hydrogen peroxide in PBS for 5 min at room temperature, followed by three washes in PBS for 5min each. Sections were then incubated with streptavidin HRP in a humidified chamber for 30min at room temperature. Slides were washed in three changes of PBS for 5 min per wash, were treated with DAB for 10 min and washed in distilled water several times. Sections were then dehydrated and coverslipped as described in Section 2.7.1.

2.7.3 Quantitative analysis

Volumetric and immunohistochemical analyses were performed on coded slides so that the observer was blind to the treatment group. One section from each of the frontal, parietal, temporal and occipital lobe from the right hemisphere of each fetus at equivalent areas within the cerebral WM, cortical GM and striatum were analysed (Figure 2.3). Analysis was performed using a light microscope (Leitz diaplan, Leica Microsystems, Australia) where colour images were captured using a digital camera (AxioCam HR, Carl Zeiss, NSW, Australia) linked from the microscope to an image analysis program (Image-Pro Plus v6.2, Media Cybernetics, Maryland, USA). All images for analysis were captured using a x20 objective to measure areal cell density or for optical density measurements. Mean values were calculated for each animal and a mean of means for control and caffeine-treated groups determined.

Figure 2.3 Brain regions analysed

Coronal, thionin-stained, hemi-sections of the cerebral hemisphere at the level of the (A) frontal, (B) parietal, (C) temporal and (D) occipital lobe. White matter (WM; dashed line) and cortical grey matter (GM; regions outside the dashed line) was examined for all histological and immunohistochemical analysis. Measurements were made in the subcortical WM, periventricular WM and corpus callosum (squares indicate fields of view for subcortical and periventricular WM; 2-3 fields analysed/square). Measurements were also made in the cortical GM (squares indicate fields of view; 4 bins analysed/square for NeuN; 3 bins analysed/square for Ctip2; whole square without bins analysed for SST). A total of 6 fields of view was analysed from the striatum, with 3 fields of view selected from the caudate nucleus and 3 fields from the putamen (B). Cx, cortex; CC, corpus callosum; Hi, hippocampus; St, striatum; CN, caudate nucleus; Pu, putamen; MB, midbrain.


A.
Cx
WM
B.
Cx
WM
CC
CN
St
Pu
C.
Cx
CC
Hi
WM
MB
D.
MB
WM
Cx

2.7.3.1 Volumetric analysis of the brain

The cross-sectional area of the WM and cortical GM (from the frontal to occipital cortex) was measured in every 625th H&E stained section (1 section per block; n=8 per animal) with a digitizer tablet (Bamboo; Wacom Co. Ltd, Tokyo, Japan) interfaced to the image analysis software. Sections were first projected onto a microfiche (Carl Zeiss, NSW, Australia) and then the area of WM, cortical GM and striatum was then traced onto A4 paper. The traces were placed on the tablet and retraced using a pen linked to an analysis software program (SigmaScan Pro v5.0, Systat, San Jose, California) which then calculated the area of WM and GM. The total volume of each region was then estimated according to the Cavalieri principle (Gundersen et al., 1999) using the formula $V=\Sigma APt$, where V is the total volume (cm^3), ΣA is the sum of the areas measured, P is the inverse of the sampling fraction and t is the section thickness.

2.7.3.2 Areal density of microglia, astrocytes, oligodendrocytes and apoptotic cells

Resting (ramified) and activated (amoeboid) Iba-1-IR microglia (distinguished by morphology), GFAP-IR astrocytes, Olig2-IR oligodendrocytes and MBP-IR mature myelinating oligodendrocytes (counted in cortical GM only) were counted using image analysis software (Image J v1.44, National Institutes of Health, USA) in 2-3 fields from both the subcortical and periventricular WM, with a total of 4-6 fields per section (field, 0.093 mm^2; Figure 2.3); these cells were also counted in 6 fields from the cortical GM, with a total of 12 fields per section (field, 0.093 mm^2; Figure 2.3). For the analysis of TUNEL-positive apoptotic cells, cells were counted by scanning stained slides via a slide scanner (ScanScope XT, Aperio, CA, USA) linked to an image analysis software system (ImageScope, Aperio, CA, USA). Cells were counted throughout the subcortical WM, periventricular WM, cortical GM and striatum; the area of each region was also measured to determine the areal cell density.

2.8 Statistical analysis of data

Power analysis was performed using the software package G*Power3. Using data from a previous study (Rees et al., 2010), it was determined that 7 animals/group would enable the detection of 1.8 standard deviation difference between group means (80% power, 5% type 1 error rate). All data were analysed using SPSS software (Version 20, SPSS Inc., Chicago, IL, USA). For the short-term

cohort, differences between groups in body morphometry, organ weights and all histological data were analysed by the Student's *t*-test for parametric data or a Mann-Whitney *U*-test for nonparametric data. For blood chemistry data, separate one-way repeated measures ANOVAs (factors: treatment and time of day (repeated factor)) were used for each day (days 1-3), as time points for blood sampling was consistent throughout the first 3 days. For blood chemistry data on days 4-15, a single two-way repeated measures ANOVA (factors: day, treatment and time of day (repeated factor)) was used, as the time points for blood sampling were the same from day 4 to 15. For cardiovascular data two separate one-way repeated measures ANOVAs (factors: treatment and time of day (repeated factor)) were used for each day (days 1-3), one between basal recordings and 1 h post-caffeine and one between 2-6 h post-caffeine. Data are presented as mean of means ± SEM for all histological data and as mean ± SEM for all other data, with $p<0.05$ considered significant.

For the long-term cohort, differences between treatment groups in birth weight, gestational age at birth and organ weights were analysed by the Student's *t*-test for parametric data or a Mann-Whitney *U*-test (non-parametric data) if data failed a variance test (F-test). For maternal physiological data (days 1-15) and age-related changes in body morphometry, a single one-way repeated measures ANOVA (factors: treatment and day (repeated factor)) was used. Data are presented as mean ± SEM with $p<0.05$ considered significant. Statistical analysis on the results obtained from MRI data was performed using randomise from the Functional MRI of the Brain (FMRIB) software library (FSL), as described in Section 6.2.5.1.

3 Effects of high-dose caffeine administration on maternal and fetal physiology and growth

3.1 Introduction

Apnea of prematurity (AOP) occurs in approximately 85% of infants born prior to 34 weeks (Martin et al., 2004) and its incidence is inversely related to gestational age (Henderson-Smart, 1981). Typically AOP is treated with caffeine (or other methylxanthines) for 4-6 weeks, until at least 32-34 weeks postmenstrual age, or until the apnea has abated (Abu-Shaweesh and Martin, 2008). An international randomized-controlled trial has confirmed that caffeine is an effective treatment for AOP, leading to reduced morbidity and mortality (Schmidt et al., 2006; Schmidt et al., 2007). In treating AOP, the standard dosing regimen of caffeine is a loading dose of 20mg/kg (caffeine citrate) followed by a daily maintenance dose of 5-10mg/kg (Schmidt et al., 2006).

As the standard clinical dose of caffeine is not always sufficient to abolish AOP (Scanlon et al., 1992; Steer et al., 2003), higher doses are currently being trialled in some centres. However, little is known regarding the effects of higher doses of caffeine on the developing preterm infant. There are very few studies that have assessed the physiological effects of high-dose caffeine and those studies have used widely differing dosing regimens. Given the conflicting experimental data on the physiological effects of caffeine, it is difficult to draw unequivocal conclusions about the likely physiological effects of high-dose caffeine administered in early life. It is important to determine if high-dose caffeine leads to physiological alterations as caffeine may have an indirect effect on the

developing brain through alterations in arterial pressure (AP), blood chemistry or factors affecting growth.

The primary aim of this thesis was to determine if high-dose caffeine adversely affects the developing brain, the results for which are discussed in Chapters 4 and 5. Thus we have undertaken experiments in sheep, a species in which the timing of major developmental events in the brain, including WM and GM development, aligns with human brain development. In order to expose the developing brain to caffeine at a similar stage of brain development as in preterm infants we have used fetal sheep at 0.7–0.8 of term; at this age, WM and GM development is similar to that of preterm infants at about 27-34 weeks postmenstrual age (Rees et al., 2010; Back et al., 2002; Back et al., 2006b); this is the typical age-range at which preterm babies are exposed to caffeine (Schmidt et al., 2007) and when the brain is still developing (Back et al., 2002).

As the effects of caffeine on the developing brain could be influenced by indirect actions of caffeine on physiological status, there is a need to understand the physiological effects of caffeine during development. Thus, the aim of the study reported in this chapter was to determine whether or not repeated daily high-dose caffeine administration causes physiological alterations in the developing fetus, as well as its effects on organ and body growth. Potential physiological effects of high-dose caffeine were determined by assessing AP and heart rate (HR) and also blood chemistry. In addition, maternal physiological status was assessed to determine whether changes in fetal physiological status were related to alterations in maternal physiological status.

3.2 Methods

All experimental procedures were approved by the Monash University Animal Ethics Committee.

3.2.1 Surgical preparation

Using established techniques (Rees et al., 2010) aseptic surgery was conducted at 99 DG (term is approximately 147 DG) on 14 date-mated ewes (Merino x Border Leicester). Catheters were chronically implanted into a fetal femoral artery for blood sampling and pressure recording, and

into a fetal femoral vein and the amniotic sac for the administration of antibiotics. Catheters were also implanted into a maternal jugular vein for drug infusions and a maternal carotid artery for monitoring maternal physiological status including AP and HR. Antibiotics [Engemycin (100 mg/mL, i.v.; fetus: 0.2 mL and ewe: 4.8 mL ewe) and ampicillin (1 g/5 mL; fetus: 1 mL, i.v. and amniotic sac: 4mL)] were administered for 3 days after surgery. Postoperatively, sheep were held in individual pens with access to food and water. Detailed surgical methods are discussed in Section 2.4.

3.2.2 Experimental protocol

As described previously in Section 2.2, a daily bolus dose (loading dose, 25 mg/kg; maintenance dose, 20 mg/kg) of caffeine base (Sigma-Aldrich, St. Louis, MO, USA) (n=9) or an equivalent volume of saline (n=8) was administered intravenously to the ewe between 104 – 118 DG (0.7 - 0.8 of term). We administered caffeine base rather than caffeine citrate to minimise the volume required; as caffeine citrate contains anhydrous citric acid and 50% anhydrous caffeine base the volume of a dose of caffeine base is approximately half that of caffeine citrate (Aranda et al., 1979b). Caffeine base is used in neonatal nurseries when caffeine citrate is unavailable.

3.2.3 Recording fetal and maternal arterial pressure and heart rate

On the first day of caffeine administration, fetal and maternal arterial catheters and the amniotic catheter were each connected to a sterilised pressure transducer (Becton and Dickson, USA) for monitoring of fetal and maternal AP, HR and amniotic pressure. Each transducer was then connected to a “quad-bridge” amplifier (ML118, ADInstruments, Castle Hill, NSW, Australia), with signals passed to an analogue-to-digital converter (PowerLab 8/50, ML870, ADInstruments, Castle Hill, NSW, Australia) and recorded on a computer, using Chart 5 software (ADInstruments, Castle Hill, NSW, Australia).

Signals from the pressure transducers were calibrated via Chart 5 software to known calibration pressures. The pressure transducers for AP were calibrated from 0 to 100 mmHg using a sphygmomanometer (Accoson, Harlow, UK) and from 0 to 50 mmHg for amniotic pressure. Following calibration, transducers for amniotic and fetal AP recordings were secured to the outside

of the sheep cage at approximately the same level as the fetal heart when the ewe was standing. The fetal AP recording was corrected by subtracting amniotic fluid pressure. The pressure transducer used for maternal AP recording was secured on the ewes back under elastic netting. As the maternal pressure transducer was placed on the ewe's back and not at the level of the heart, recordings of maternal AP were corrected for the effect of hydrostatic pressure. The vertical distance from the ewe's heart to the transducer on the ewe's back was measured in centimetres (cm) and then the hydrostatic pressure difference was converted to a pressure in millimetres of mercury (mmHg) with the formula of 1 cm of water is equal to 0.7 mmHg. The resulting pressure was then added to the recorded AP.

Once calibration of the pressure transducers was completed, they were connected to the catheters. Fetal and maternal HR was calculated from the relevant AP signals using the Chart software. Mean arterial pressure (MAP) was calculated using the predefined Chart software formula: ie. diastolic pressure plus one third of the pulse pressure (systolic pressure minus diastolic pressure). Fetal and maternal APs were recorded continuously for 6 h per day for the first 3 days of caffeine administration, with basal recordings conducted for 30 min prior to caffeine administration. Written comments were made on the recordings when caffeine was administered as well as when blood samples were obtained.

3.2.3.1 Analysis of recordings

Any artefacts in the recorded traces of HR or AP were excluded prior to analysis of the chart files; artefacts primarily occurred during daily cleaning of sheep cages, blood sampling or blockage of catheters. Files were then digitally analysed using Chart 5 software. During baseline recordings, prior to caffeine administration, AP and HR data were averaged over 4 min. Following caffeine administration and up to 5 min post-caffeine, data were averaged every minute. Between 5 and 30 min post-caffeine, data were averaged over every 4 min; between 30 and 60 min, data were averaged over every 5 min and finally between 60 and 300 min, data were averaged over every 10 min. Analysis of AP and HR was performed in more detail in the first hour following caffeine administration, as this is when the greatest response to caffeine is generally observed. Data were transferred into an Excel file and grouped during specific time periods and then averaged across all animals within the same treatment groups.

3.2.4 Blood sampling

Fetal and maternal blood samples (0.5 ml for blood gases and 2.5 ml for plasma caffeine measurements) were collected at five different time points during the first three days (104-106 DG) of caffeine administration, at 0 h (prior to caffeine administration), then at 1, 2, 4 and 6 h post-caffeine administration. Subsequently, fetal and maternal blood samples were collected at three different time points for the remainder of the study (107-119 DG) which included a blood sample just prior to caffeine administration, 1 h after (peak of caffeine in plasma) and 6 h after in order to obtain data on the caffeine profile and rate of caffeine clearance.

Fetal blood chemistry, including the partial pressure of oxygen (PaO_2), partial pressure of carbon dioxide ($PaCO_2$), oxygen saturation (SaO_2), pH and arterial concentrations of total haemoglobin, glucose and lactate were analysed (see Section 3.2.4.3) using the 0.5 ml samples of arterial blood. The 2.5 ml sample was centrifuged at 3000 rpm for 10 min at 4°C, and the plasma divided into 4 aliquots of equal volume and frozen at -80°C until required for analysis (see Section 3.2.4.1).

3.2.4.1 Measurement of plasma caffeine concentration

Caffeine concentrations in plasma from each ewe and fetus were measured at Monash Health Pathology, Melbourne, Australia. Caffeine was measured by a homogeneous enzyme multiplied immunoassay (Emit® Caffeine Assay; Beckman and Coulter, California, USA) using a Beckman Coulter DxC 800 Analyser (California, USA), with reagents and calibrating solutions supplied by Siemens Diagnostics Australia. The assay is based on competition for antibody binding sites between the caffeine in the sample and the caffeine labelled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH) (Curtis and Patel, 1978). The enzyme activity decreases upon binding to the antibody, so the drug concentration in the sample can be measured in terms of enzyme activity. The active enzyme converts oxidized nicotinamide adenine dinucleotide (NAD) to NADH, resulting in an absorbance change that can be measured spectrophotometrically. Endogenous serum G6PDH does not interfere as the coenzyme functions only with the bacterial (Leuconostoc mesenteroides) enzyme employed in the assay.

The assay was performed using reagents supplied in the Emit caffeine assay kit. Reagent 1 included antibodies to a caffeine derivative, glucose-6-phosphate, bovine serum albumin, stabilizers and preservatives. Reagent 2 contained caffeine chemically coupled to G6PDH, bovine serum albumin, tris buffer, preservatives and stabilizers. Prior to use, reagents 1 and 2 were reconstituted according to the manufacturer's instructions. Each 50 μL of the plasma caffeine samples was first diluted with 250 μL of Emit buffer then diluted further with another 250 μL of buffer. This was followed by the addition of reagent 1 (50 μL) and 250 μL of buffer followed by the addition of Reagent 2 (50 μL) and 250 μL of buffer. The mixture was then aspirated into the flow cells of the spectrophotometer. After a 15 sec delay, the change in absorbance during 30 sec was measured. The change in absorbance was then compared to the change in absorbance of an unknown sample with the standard curve automatically generated with the six calibrators by the Beckman Coulter DxC 800 Analyser.

3.2.4.2 Calculation of plasma caffeine clearance

Once plasma caffeine concentrations were obtained, total fetal plasma caffeine clearance (Cl_{tot}) was calculated for days 1-15 of caffeine administration. This was calculated in order to compare clearance rates in fetal sheep with published data obtained from preterm infants. Clearance of a drug is defined as the volume of blood/plasma from which a drug is completely removed, or cleared, in a given time period and reflects the overall ability of the living organism to eliminate a drug (Toutain and Bousquet-Melou, 2004). Calculation of total plasma caffeine clearance was determined using the formula: Cl_{tot} (L/h/kg) = Dose (mg/kg)/AUC (mg.h.L^{-1}), as described by (Toutain and Bousquet-Melou, 2004). The "Dose" is the dose of caffeine base administered (Section 3.2.2), which is either 25 or 20 mg/kg. The AUC is the area under the curve, which was calculated between 1 and 24h after caffeine administration (Figure 3.1), using GraphPad Prism (GraphPad prism v6.0e, GraphPad Software Inc, La Jolla, USA). Cl_{tot} was calculated on each of the experimental days and then averaged to obtain a mean clearance rate.

3.2.4.3 Measurement of blood chemistry

Arterial blood gases (PaO_2, $PaCO_2$, SaO_2) and pH and arterial concentrations of total haemoglobin, glucose and lactate were determined using an automated analyser (Radiometer Pacific, Australia,

model ABL-800). For fetal blood measurements, readings were adjusted to 39°C, the expected fetal body temperature *in utero*.

3.2.5 Necropsy

At 119 DG (0.8 of term), the ewe and fetus were euthanized using an overdose of sodium pentobarbitone delivered to the ewe (130 mg/kg i.v.) and the fetus delivered via Caesarean section (see Section 2.5). The fetus was then weighed and body dimensions measured, including crown-to-rump length (CRL), thoracic girth (TG), forelimb length (FLL). We calculated fetal ponderal index (PI), which characterises the relationship of body length to body weight, using the formula: $PI = (body\ weight\ (g)/CRL^3)$. Following these measurements, the fetuses (control, n=8 and caffeine, n=9) were transcardially perfused with isotonic saline and 4% PFA in 0.1M PB (pH 7.4), see Section 2.5.2.1. The brain and other organs (lung, heart, kidneys, adrenals, liver and spleen) were then weighed.

3.2.6 Statistical analysis

Differences between treatment groups in body morphometry and organ weights were analysed by the Student's t test for parametric data or a Mann-Whitney U test (non-parametric data) if data failed a variance test (F-test). For blood chemistry data, separate one-way repeated measures ANOVAs (factors: treatment and time of day (repeated factor)) were used for each day (days 1-3). For blood chemistry data on days 4-15 a single two-way repeated measures ANOVA (factors: day, treatment and time of day (repeated factor)) was used. For cardiovascular data two separate one-way repeated measures ANOVAs (factors: treatment and time of day (repeated factor)) were used for each day (days 1-3), one between basal recordings and 1 h post-caffeine and one between 2-6 h post-caffeine. Data are presented as mean ± SEM with $p<0.05$ considered significant.

3.3 Results

3.3.1 Plasma caffeine concentration

On the first day of caffeine administration, fetal plasma caffeine concentration reached 32 ± 1 mg/L at 1 h, decreasing to 19 ± 3 mg/L at 6h and 5 ± 2 mg/L at 24 h (prior to the next injection, Figure

3.1). Maternal plasma caffeine concentration reached 43 ± 9 mg/L at 1 h, decreasing to 24 ± 5 mg/L at 6 h and 5 ± 1 mg/L at 24 h (Figure 3.1). This plasma caffeine concentration profile was similar to that seen throughout the remainder of the experiment (data not shown for days 4-15). The plasma caffeine concentration achieved in the ewe and fetus, as well as the profile of plasma caffeine concentration over time, was similar in the ewe and fetus.

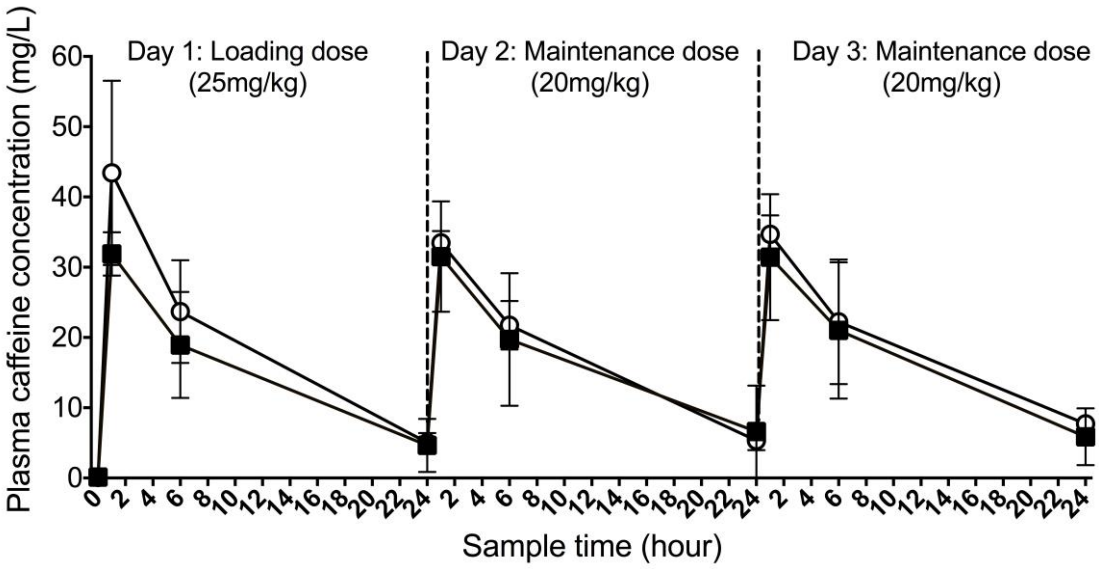


Figure 3.1 Maternal and fetal plasma caffeine concentrations

Maternal (open circles; n=2) and fetal (closed squares; n=6) plasma caffeine concentrations on days 1-3 of caffeine administration. Caffeine concentration was measured immediately prior to caffeine administration (0 h), at 1 h, 6 h and at 24 h post-caffeine administration, from day 1 (loading dose: 25 mg/kg) to day 3 (maintenance dose: 20 mg/kg). Data are mean ± SEM.

3.3.2 Plasma caffeine clearance

The average total plasma caffeine clearance in the ovine fetus, calculated for days 1 to 15 of caffeine administration was 1.1 ± 0.2 mL/min/kg.

3.3.3 Fetal cardiovascular data

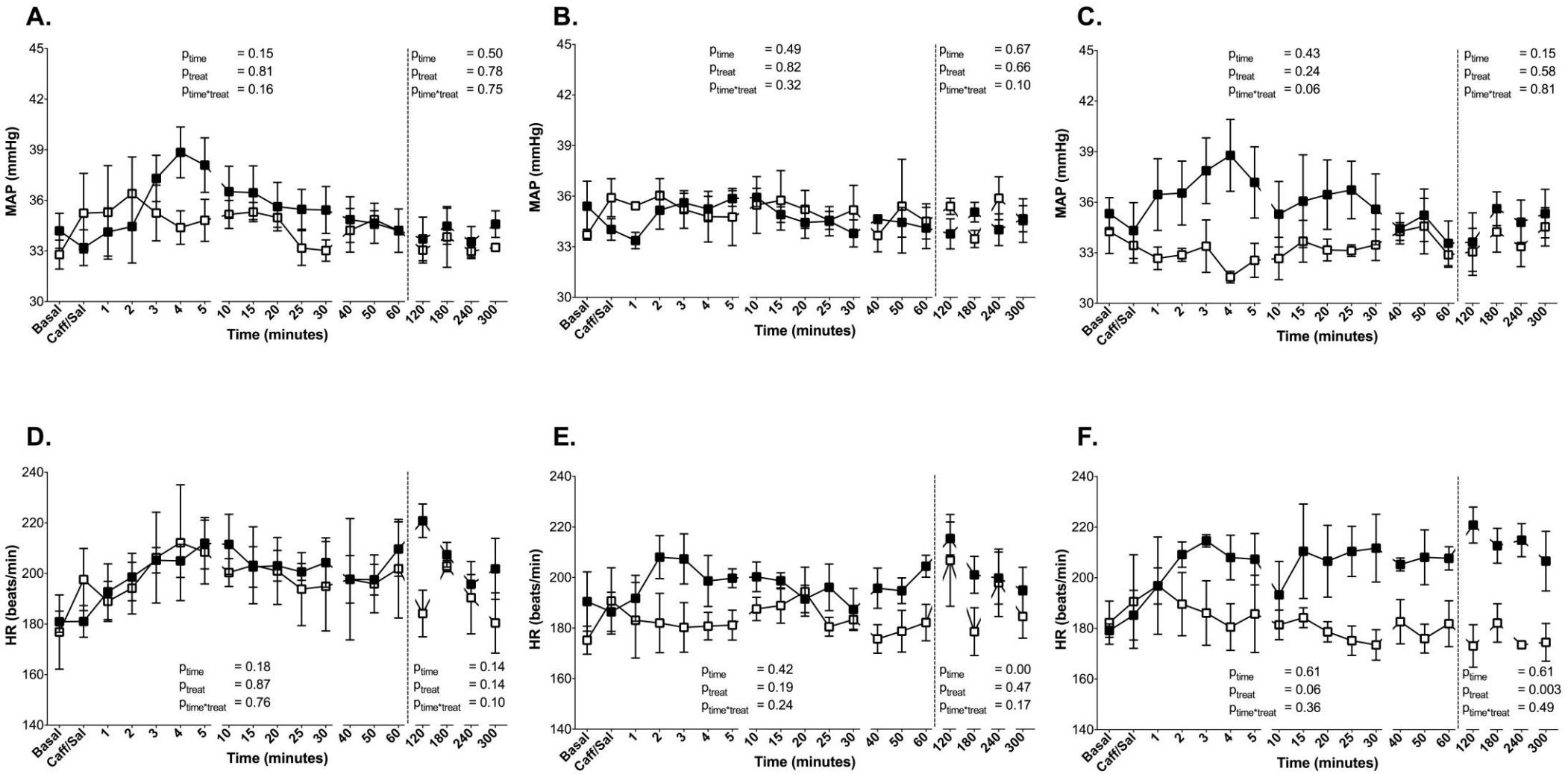
On day 1 and day 2 of the treatment period (104-105 DG) fetal MAP was similar between caffeine-treated and control fetuses (Figure 3.2A,B). On day 3 (106 DG) there was a tendency for an

interaction effect between treatment and time on fetal MAP between the basal recordings and 60 min post-caffeine ($p_{time\ x\ treat}$=0.06; Figure 3.2C); during this period, caffeine-treated fetuses appeared to have a higher MAP compared to controls within the first 5 min.

On days 1 and 2 of the treatment period (104-105 DG) HR was similar in control and caffeine-treated fetuses (Figure 3.2D,E). However, on day 3 (106 DG) there was a tendency for HR to be elevated in caffeine-treated fetuses within the first hour (p_{treat}=0.06; Figure 3.2F), which became significant between 120 min and 300 min post-caffeine treatment (p_{treat}=0.003; Figure 3.2F).

Figure 3.2 Effects of caffeine on fetal mean arterial pressure and heart rate

Fetal mean arterial pressure on day 1 (A), day 2 (B) and day 3 (C) and heart rate on day 1 (D), day 2 (E) and day 3 (F) in control (open squares; n=3) and caffeine treated (closed squares; n=4) fetuses. Data are mean ± SEM.

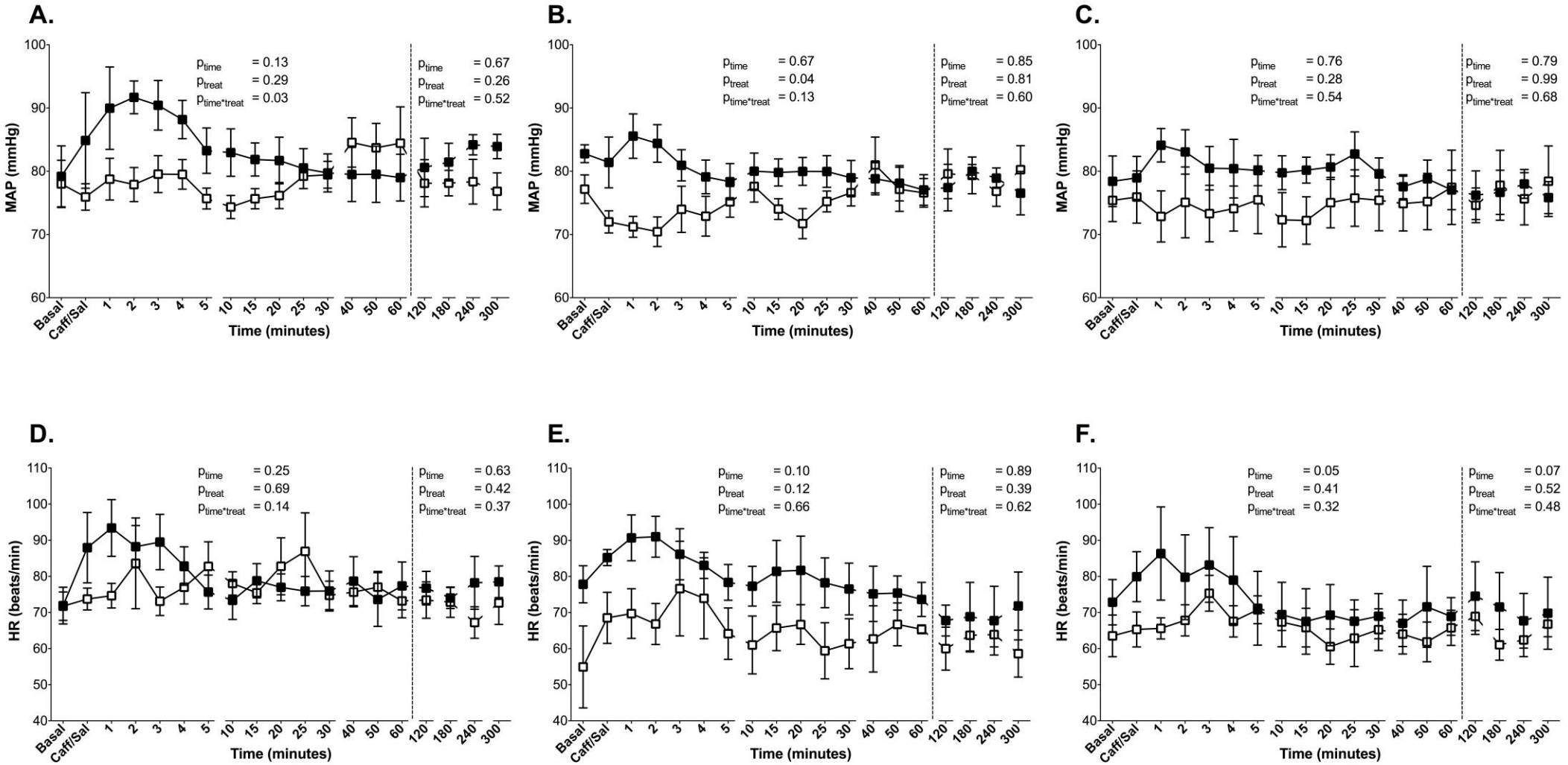

A.
B.
C.
D.
E.
F.
MAP (mmHg)
HR (beats/min)
Time (minutes)
Basal
Caff/Sal
p_{time} = 0.15
p_{treat} = 0.81
$p_{time*treat}$ = 0.16
p_{time} = 0.50
p_{treat} = 0.78
$p_{time*treat}$ = 0.75
p_{time} = 0.49
p_{treat} = 0.82
$p_{time*treat}$ = 0.32
p_{time} = 0.67
p_{treat} = 0.66
$p_{time*treat}$ = 0.10
p_{time} = 0.43
p_{treat} = 0.24
$p_{time*treat}$ = 0.06
p_{time} = 0.15
p_{treat} = 0.58
$p_{time*treat}$ = 0.81
p_{time} = 0.18
p_{treat} = 0.87
$p_{time*treat}$ = 0.76
p_{time} = 0.14
p_{treat} = 0.14
$p_{time*treat}$ = 0.10
p_{time} = 0.42
p_{treat} = 0.19
$p_{time*treat}$ = 0.24
p_{time} = 0.00
p_{treat} = 0.47
$p_{time*treat}$ = 0.17
p_{time} = 0.61
p_{treat} = 0.06
$p_{time*treat}$ = 0.36
p_{time} = 0.61
p_{treat} = 0.003
$p_{time*treat}$ = 0.49

3.3.4 Maternal cardiovascular data

On day 1 (104 DG) there was a significant interaction effect between treatment and time on maternal MAP during the first hour following caffeine administration ($p_{time\ x\ treat}$ = 0.03; Figure 3.3A); ewes administered caffeine appeared to have a higher MAP within the first 15 min when compared to controls. Between 2-6 h post-caffeine, maternal MAP was similar between groups (Figure 3.3A). On day 2 (105 DG) ewes had a higher MAP during the first hour following caffeine administration when compared to controls (p_{treat}=0.04; Figure 3.3B); however between 2-6 h, maternal MAP was similar between treatment groups (Figure 3.3B). On day 3 of the treatment period (106 DG) there were no significant differences in MAP between control and caffeine-treated fetuses (Figure 3.3C). Maternal HR was not significantly affected by caffeine on days 1 to 3 of the treatment period (Figure 3.3D-F).

Figure 3.3 Effects of caffeine on maternal mean arterial pressure and heart rate

Maternal mean arterial pressure on day 1 (A), day 2 (B) and day 3 (C) and heart rate on day 1 (D), day 2 (E) and day 3 (F) in control (open squares; n=5) and caffeine treated (closed squares; n=4) fetuses. Data are mean ± SEM.

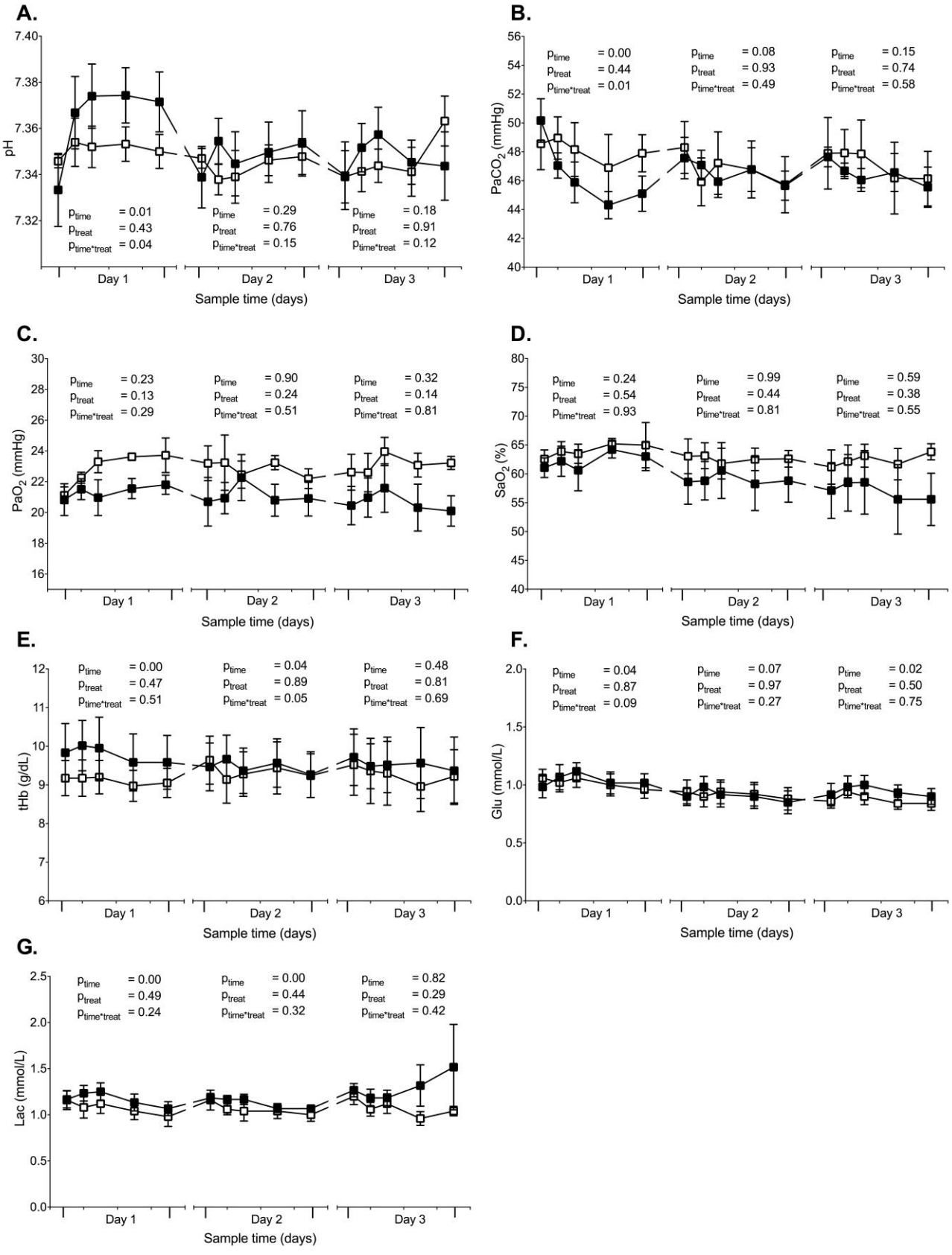

A.
B.
C.
D.
E.
F.
MAP (mmHg)
HR (beats/min)
Time (minutes)
Basal
Caff/Sal
1
2
3
4
5
10
15
20
25
30
40
50
60
120
180
240
300
p_time = 0.13
p_treat = 0.29
p_time*treat = 0.03
p_time = 0.67
p_treat = 0.26
p_time*treat = 0.52
p_time = 0.67
p_treat = 0.04
p_time*treat = 0.13
p_time = 0.85
p_treat = 0.81
p_time*treat = 0.60
p_time = 0.76
p_treat = 0.28
p_time*treat = 0.54
p_time = 0.79
p_treat = 0.99
p_time*treat = 0.68
p_time = 0.25
p_treat = 0.69
p_time*treat = 0.14
p_time = 0.63
p_treat = 0.42
p_time*treat = 0.37
p_time = 0.10
p_treat = 0.12
p_time*treat = 0.66
p_time = 0.89
p_treat = 0.39
p_time*treat = 0.62
p_time = 0.05
p_treat = 0.41
p_time*treat = 0.32
p_time = 0.07
p_treat = 0.52
p_time*treat = 0.48

3.3.5 Fetal blood chemistry

There was a small but significant interaction effect between treatment and time in fetal arterial pH ($p_{time\ x\ treat}$=0.04) and $PaCO_2$ ($p_{time\ x\ treat}$=0.01) on day 1 of the treatment period (104 DG); caffeine-treated fetuses appeared to have a transiently higher pH and lower $PaCO_2$ (Figure 3.4A,B) compared to control fetuses. On days 2 and 3 there were no significant differences between groups in fetal pH and $PaCO_2$. On days 1 to 3 there were no significant effects of caffeine on fetal PaO_2 (Figure 3.4C), arterial O_2 saturation (SaO_2; Figure 3.4D) total haemoglobin (Figure 3.4E), glucose (Figure 3.4F) or lactate (Figure 3.4G) concentrations. From days 4-15 there were no significant differences between groups in any measured fetal blood variable (data not shown).

Figure 3.4 Fetal blood chemistry

Fetal arterial pH (A), partial pressure of carbon dioxide ($PaCO_2$; B), partial pressure of oxygen (PaO_2; C), oxygen saturation (SaO_2; D), total haemoglobin (tHb; E), glucose (Glu; F) and lactate (Lac; G) concentrations in control (open squares; n=5) and caffeine treated (closed squares; n=6) fetuses. Data are presented as mean ± SEM.

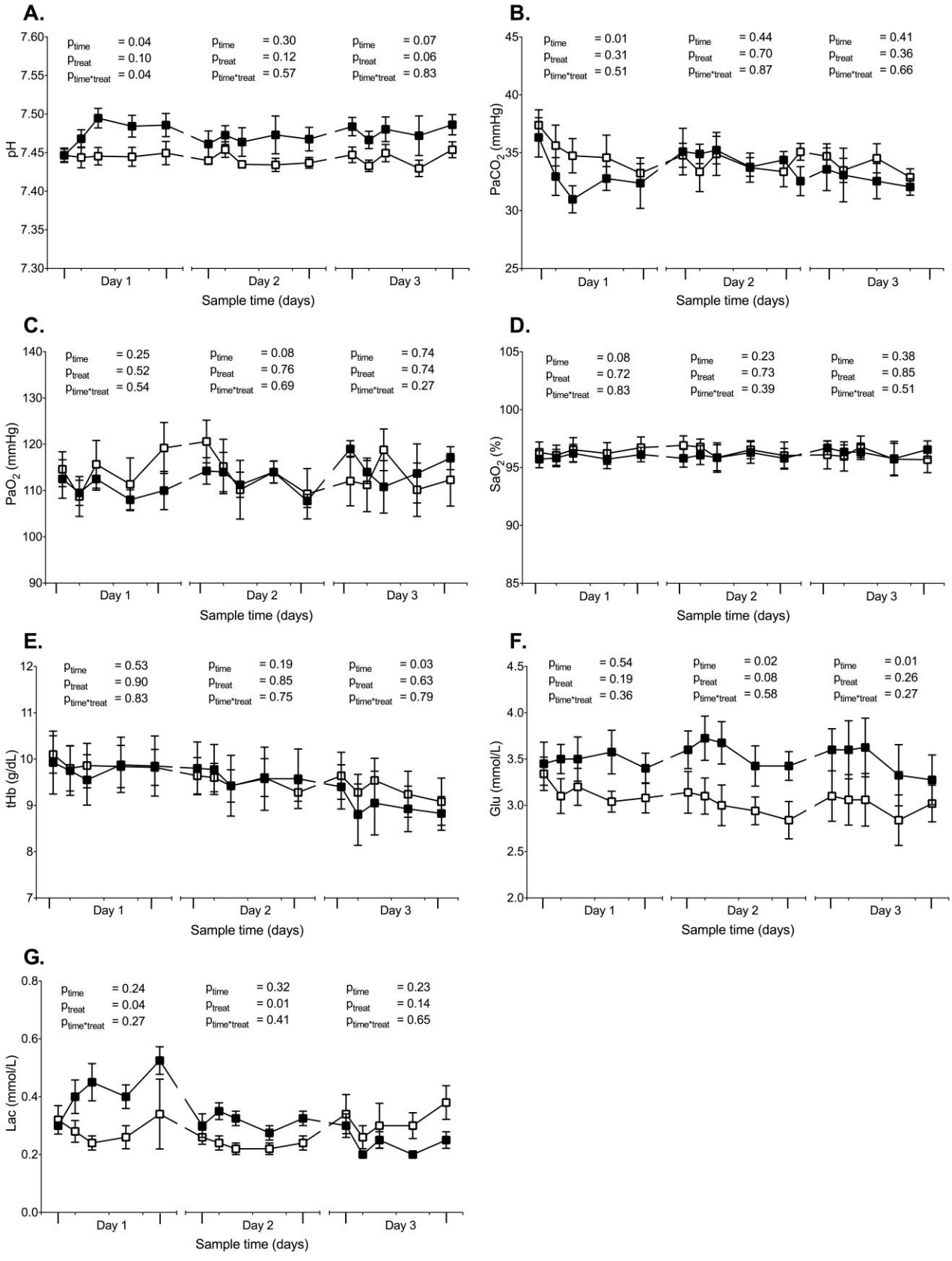

A.
pH
7.40
7.38
7.36
7.34
7.32
p_{time} = 0.01
p_{treat} = 0.43
$p_{time*treat}$ = 0.04
p_{time} = 0.29
p_{treat} = 0.76
$p_{time*treat}$ = 0.15
p_{time} = 0.18
p_{treat} = 0.91
$p_{time*treat}$ = 0.12
Day 1
Day 2
Day 3
Sample time (days)
B.
$PaCO_2$ (mmHg)
56
54
52
50
48
46
44
42
40
p_{time} = 0.00
p_{treat} = 0.44
$p_{time*treat}$ = 0.01
p_{time} = 0.08
p_{treat} = 0.93
$p_{time*treat}$ = 0.49
p_{time} = 0.15
p_{treat} = 0.74
$p_{time*treat}$ = 0.58
Day 1
Day 2
Day 3
Sample time (days)
C.
PaO_2 (mmHg)
30
28
26
24
22
20
18
16
p_{time} = 0.23
p_{treat} = 0.13
$p_{time*treat}$ = 0.29
p_{time} = 0.90
p_{treat} = 0.24
$p_{time*treat}$ = 0.51
p_{time} = 0.32
p_{treat} = 0.14
$p_{time*treat}$ = 0.81
Day 1
Day 2
Day 3
Sample time (days)
D.
SaO_2 (%)
80
75
70
65
60
55
50
45
40
p_{time} = 0.24
p_{treat} = 0.54
$p_{time*treat}$ = 0.93
p_{time} = 0.99
p_{treat} = 0.44
$p_{time*treat}$ = 0.81
p_{time} = 0.59
p_{treat} = 0.38
$p_{time*treat}$ = 0.55
Day 1
Day 2
Day 3
Sample time (days)
E.
tHb (g/dL)
12
11
10
9
8
7
6
p_{time} = 0.00
p_{treat} = 0.47
$p_{time*treat}$ = 0.51
p_{time} = 0.04
p_{treat} = 0.89
$p_{time*treat}$ = 0.05
p_{time} = 0.48
p_{treat} = 0.81
$p_{time*treat}$ = 0.69
Day 1
Day 2
Day 3
Sample time (days)
F.
Glu (mmol/L)
2.0
1.5
1.0
0.5
0.0
p_{time} = 0.04
p_{treat} = 0.87
$p_{time*treat}$ = 0.09
p_{time} = 0.07
p_{treat} = 0.97
$p_{time*treat}$ = 0.27
p_{time} = 0.02
p_{treat} = 0.50
$p_{time*treat}$ = 0.75
Day 1
Day 2
Day 3
Sample time (days)
G.
Lac (mmol/L)
2.5
2.0
1.5
1.0
0.5
0.0
p_{time} = 0.00
p_{treat} = 0.49
$p_{time*treat}$ = 0.24
p_{time} = 0.00
p_{treat} = 0.44
$p_{time*treat}$ = 0.32
p_{time} = 0.82
p_{treat} = 0.29
$p_{time*treat}$ = 0.42
Day 1
Day 2
Day 3
Sample time (days)

3.3.6 Maternal blood chemistry

For maternal pH, on day 1 of the treatment period (104 DG), there was an interaction effect between treatment and time; caffeine administration appeared to cause a transient increase in maternal arterial pH ($p_{time\ x\ treat}$=0.04; Figure 3.5A), with no significant effects on days 2 to 15 (data not shown for days 4-15). From days 1-15 of the treatment period (104-118 DG), there were no significant effects of caffeine on maternal $PaCO_2$, PaO_2, SaO_2, blood glucose or total haemoglobin concentrations (Figure 3.5; day 1-3 only, day 4-15 data not shown). However, there was an interaction effect between caffeine and time on lactate concentration on day 1 and day 2 of caffeine administration ($p_{time\ x\ treat}$=0.04 and 0.01 respectively; Figure 3.5G), with no significant effects from day 3-15 (data not shown for days 4-15).

Figure 3.5 Maternal blood chemistry

Maternal arterial pH (A), partial pressure of carbon dioxide ($PaCO_2$; B), partial pressure of oxygen (PaO_2; C), oxygen saturation (SaO_2; D), total haemoglobin (tHb; E), glucose (Glu; F) and lactate (Lac; G) concentrations in control (open squares; n=5) and caffeine treated (closed squares; n=4) ewe. Data are presented as mean ± SEM).


A.
p_{time} = 0.04
p_{treat} = 0.10
$p_{time*treat}$ = 0.04
p_{time} = 0.30
p_{treat} = 0.12
$p_{time*treat}$ = 0.57
p_{time} = 0.07
p_{treat} = 0.06
$p_{time*treat}$ = 0.83
pH
7.60
7.55
7.50
7.45
7.40
7.35
7.30
Day 1
Day 2
Day 3
Sample time (days)
B.
p_{time} = 0.01
p_{treat} = 0.31
$p_{time*treat}$ = 0.51
p_{time} = 0.44
p_{treat} = 0.70
$p_{time*treat}$ = 0.87
p_{time} = 0.41
p_{treat} = 0.36
$p_{time*treat}$ = 0.66
$PaCO_2$ (mmHg)
45
40
35
30
25
Day 1
Day 2
Day 3
Sample time (days)
C.
p_{time} = 0.25
p_{treat} = 0.52
$p_{time*treat}$ = 0.54
p_{time} = 0.08
p_{treat} = 0.76
$p_{time*treat}$ = 0.69
p_{time} = 0.74
p_{treat} = 0.74
$p_{time*treat}$ = 0.27
PaO_2 (mmHg)
140
130
120
110
100
90
Day 1
Day 2
Day 3
Sample time (days)
D.
p_{time} = 0.08
p_{treat} = 0.72
$p_{time*treat}$ = 0.83
p_{time} = 0.23
p_{treat} = 0.73
$p_{time*treat}$ = 0.39
p_{time} = 0.38
p_{treat} = 0.85
$p_{time*treat}$ = 0.51
SaO_2 (%)
105
100
95
90
85
Day 1
Day 2
Day 3
Sample time (days)
E.
p_{time} = 0.53
p_{treat} = 0.90
$p_{time*treat}$ = 0.83
p_{time} = 0.19
p_{treat} = 0.85
$p_{time*treat}$ = 0.75
p_{time} = 0.03
p_{treat} = 0.63
$p_{time*treat}$ = 0.79
tHb (g/dL)
12
11
10
9
8
7
Day 1
Day 2
Day 3
Sample time (days)
F.
p_{time} = 0.54
p_{treat} = 0.19
$p_{time*treat}$ = 0.36
p_{time} = 0.02
p_{treat} = 0.08
$p_{time*treat}$ = 0.58
p_{time} = 0.01
p_{treat} = 0.26
$p_{time*treat}$ = 0.27
Glu (mmol/L)
4.5
4.0
3.5
3.0
2.5
2.0
Day 1
Day 2
Day 3
Sample time (days)
G.
p_{time} = 0.24
p_{treat} = 0.04
$p_{time*treat}$ = 0.27
p_{time} = 0.32
p_{treat} = 0.01
$p_{time*treat}$ = 0.41
p_{time} = 0.23
p_{treat} = 0.14
$p_{time*treat}$ = 0.65
Lac (mmol/L)
0.8
0.6
0.4
0.2
0.0
Day 1
Day 2
Day 3
Sample time (days)

3.3.7 Fetal body and organ weights

At necropsy (119 DG) there were no significant differences between control and caffeine-treated fetuses in body weight, brain weight, brain-to-body-weight ratio, crown-to-rump length, thoracic girth, hind limb length, head length, ponderal index and other organ weights (absolute or relative to body weight) (Table 3.1).

Table 3.1 Necropsy data for control and caffeine-treated fetuses

	Control fetuses *(n=8)*	Caffeine-treated fetuses *(n=9)*
Body wt, kg	2.48 ± 0.13	2.36 ± 0.14
CRL, mm	429 ± 7	418 ± 9
TG, mm	282 ± 7	285 ± 6
HLL, mm	342 ± 9	350 ± 7
HL, mm	113 ± 1	113 ± 1
PI, g/mm^3	$3.13 \times 10^{-5} \pm 1.08 \times 10^{-6}$	$3.22 \times 10^{-5} \pm 1.15 \times 10^{-6}$
Brain, g	38.1 ± 0.4	42.2 ± 1.7
Brain/body wt, g/kg	15.7 ± 0.9	18.3 ± 0.9
Lung, g	90.8 ± 5.5	82.8 ± 4.9
Lung/body wt, g/kg	36.7 ± 1.5	35.4 ± 1.6
Heart, g	17.8 ± 0.9	16.5 ± 1.7
Heart/body wt, g/kg	7.21 ± 0.23	6.93 ± 0.59
Kidney, g	16.3 ± 1.0	16.6 ± 0.9
Kidney/body wt, g/kg	6.61 ± 0.35	7.11 ± 0.23
Adrenals, g	0.28 ± 0.04	0.33 ± 0.03
Adrenals/body wt, g/kg	0.11 ± 0.01	0.14 ± 0.01
Liver, g	91.5 ± 8.5	83.7 ± 7.0
Liver/body wt, g/kg	36.6 ± 1.9	35.3 ± 2.0
Spleen, g	5.73 ± 0.48	4.65 ± 0.49
Spleen, g/kg	2.30 ± 0.11	1.97 ± 0.16

WT, weight; CRL, crown-to-rump length; TG, thoracic girth; HLL, hind limb length; HL, head length; PI, ponderal index. Organ weights are expressed as absolute weights and adjusted for body weight. All data are mean ± SEM.

3.4 Discussion

Use of a catheterised fetal sheep model allowed us to assess the physiological effects of daily high-dose caffeine at a stage of brain development that is similar to that of the very preterm human infants (Back et al., 2001; Roessmann and Gambetti, 1986a; Haynes et al., 2005). This study shows that the daily administration of high-dose caffeine results in a high fetal plasma caffeine concentration. Additionally, administration of high-dose caffeine leads to small transient changes in fetal and maternal MAP and HR with no persistent changes in fetal or maternal blood chemistry or fetal growth. Thus, any effects of caffeine on the developing central nervous system are unlikely to be secondary to metabolic or cardiovascular changes induced by caffeine.

3.4.1 Plasma caffeine concentration

Daily administration of high-dose caffeine via the maternal circulation exposed the fetus to high blood concentrations of caffeine, with a maximal fetal plasma caffeine concentration of 32 mg/L. Concentrations of caffeine in the maternal and fetal circulations were similar, which confirms that caffeine readily crosses the placenta (Wilson et al., 1983; Ikeda et al., 1982). The maximal blood concentrations achieved in our study are high relative to the range of concentrations measured in preterm infants treated with a standard dose of caffeine (20 mg/kg caffeine citrate loading dose; 5-10 mg/kg maintenance dose), where serum caffeine concentrations typically range from 4.8-25.1 mg/L (Natarajan et al., 2007; Leon et al., 2007; Charles et al., 2008). A study in preterm infants treated with high dose caffeine (50 mg/kg caffeine citrate loading dose; 12 mg/kg maintenance dose) similar to those used in the present study showed that plasma caffeine concentration varied between 30 and 33 mg/L between day 1 to day 5 of caffeine treatment (Scanlon et al., 1992). These concentrations are similar to those I have measured here in the ovine fetus.

It is important to note that, although the plasma caffeine concentration achieved in the present study is high relative to what has been measured in preterm neonates, the rate of plasma caffeine clearance is slower in preterm neonates compared to fetal sheep. Plasma caffeine clearance rates in preterm neonates ranges from 0.08 to 0.14 mL/min/kg (Charles et al., 2008; Lee et al., 1997). In comparison, plasma caffeine clearance rate calculated for fetal sheep in the present study was 1.1 ± 0.2 mL/min/kg, approximately 10-fold greater than in the human infant. This suggests that preterm

infants may be exposed to higher doses of caffeine for a longer period of time compared to fetal sheep.

In preterm infants, the peak plasma caffeine concentration appears to be maintained until the administration of the next dose (Lee et al., 1997; Charles et al., 2008; Aranda et al., 1979b), as the half-life of caffeine can be over 100 h (Fredholm et al., 1999) due to the immaturity of the liver, leading to a lower cytochrome P-450 activity, an enzyme involved in the metabolism of caffeine (Aranda et al., 1979a). However the fetal plasma caffeine profile is different in fetal sheep. In the present study the peak fetal caffeine concentration gradually declines by the time the next dose of caffeine is administered. In the fetus, drug clearance occurs primarily via the placenta, as well as by urinary excretion into the amniotic sac and fetal metabolism by the liver (Ring et al., 1999). Thus caffeine clearance in fetal sheep is likely to be affected by placental permeability to caffeine, as caffeine readily crosses the placenta (Wilson et al., 1983) and the ability of the fetal kidney and liver to eliminate the drug (Ring et al., 1999; Pretheeban et al., 2012). As a result of being functionally connected to the maternal circulation, the fetus is able to eliminate caffeine more rapidly than preterm infants. Thus in order to mimic the plasma caffeine concentration profile and clearance seen in preterm infants, fetal sheep may require more than one dose of caffeine per day or may require a continuous steady infusion of caffeine in order to expose fetal sheep to higher doses of caffeine for a longer duration. Regardless, it is important to note that in the present study fetal sheep were exposed to high levels of caffeine for 15 consecutive days with a peak plasma caffeine level of ~30 mg/l/d which is equivalent to 154 μmol/l of caffeine. The concentration at which caffeine completely saturates A_1 and A_{2a} adenosine receptors is 100 μmol/l (Fredholm, 1995); therefore, these receptors are likely to have been activated by caffeine. However, the measurement of caffeine concentrations within the fetal brain would be beneficial to further confirm that the adenosine receptors are being completely saturated.

3.4.2 Effects of high-dose caffeine on fetal and maternal cardiovascular physiology

Daily administration of high-dose caffeine led to transient increases in fetal MAP and HR on the third day of caffeine administration and a transient increase in maternal MAP and HR on the first 2 days of caffeine administration. It is important to note that tachycardia is a commonly seen clinical

side-effect in preterm infants treated with standard doses of caffeine with plasma caffeine concentrations ranging from 16.6-34.4 mg/L (Francart et al., 2013; Scanlon et al., 1992; Aden, 2011), concentrations similar to that seen in the present study. Thus the changes in cardiovascular physiology measured in fetal and maternal sheep in this study are not uncommon.

Although few studies have assessed the cardiovascular effects of high-dose caffeine in preterm infants, a study using doses similar to that used in the present study (25 mg/kg loading dose caffeine base given in 2 doses of 12.5 mg/kg 4 h apart; 5 mg/kg maintenance dose given 24 h after the first dose) assessed AP and HR in preterm infants, 1 h after the first dose of caffeine and then 1 and 20 h after the second dose of caffeine (Hoecker et al., 2006). Here they reported that there was an increase in AP at 1 and 20 h after the second dose of caffeine and an increase in heart rate at 20 h post-caffeine (Hoecker et al., 2006). Contrary to what I have shown here, administration of high-dose caffeine (40 mg/kg caffeine base) to sedated preterm (~126 DG) ventilated lambs increased HR 10 min after caffeine administration and HR remained elevated for the duration of the experiment (2 h), with no effect on MAP throughout the experimental protocol (Crossley et al., 2012).

When the effects of standard doses of caffeine administered to preterm infants (20 mg/kg caffeine citrate loading; 5 mg/kg citrate maintenance dose) were compared to data in control infants, MAP increased on the first 3 days of administration; no differences in HR were seen (Walther et al., 1990). Additionally, caffeine administration significantly increased left ventricular output and stroke volume from day 1 to day 7 of caffeine administration (Walther et al., 1990). Similarly, caffeine administration in preterm neonates increased AP, HR and stroke volume and cardiac output in comparison to cardiovascular assessments conducted prior to caffeine administration (Soloveychik et al., 2009). Differences between studies are likely a result of differences in caffeine doses and regimens used and also differences in the time of assessment following caffeine administration. Although the long-term clinical significance of the cardiovascular effects of caffeine remain uncertain due to a lack of long-term follow-up data of cardiorespiratory function in humans, it is possible that early exposure to caffeine may have long-lasting cardiovascular effects (Aden, 2011).

Caffeine has been shown to alter the cardiovascular function through centrally mediated and local actions. In adults, caffeine has been shown to increase MAP by a sympathetically driven increase in total peripheral resistance without increasing cardiac output (Casiglia et al., 1991; Farag et al., 2005; Pincomb et al., 1985). However, studies in preterm infants suggest that caffeine administration is associated with an increase in cardiac output due to an increase in HR and stroke volume (Soloveychik et al., 2009; Walther et al., 1990). Moreover, the cardiovascular effects of caffeine have also been attributed to its effects on blood vessels where caffeine has been shown to attenuate adenosine-induced vasodilation in humans (Smits et al., 1990). Although we did not assess cardiovascular physiology after the first 3 days of caffeine administration, if these cardiovascular changes were to persist, they may have a detrimental effect on heart muscle structure and function. Thus a prolonged investigation of the effect of caffeine on cardiovascular physiology would be valuable in order to determine if alterations persist after the first 3 days of caffeine administration. A limitation of the present study is that the number of animals analysed per group was low, and therefore the analysis may lack statistical power; more fetuses may be required in order to accurately describe the actions of high-dose caffeine on cardiovascular physiology.

3.4.3 High-dose caffeine does not significantly alter fetal and maternal blood chemistry

High-dose caffeine led to a mild, transient increase in fetal arterial pH and decrease in arterial $PaCO_2$ on day 1 of treatment, with no differences in all other physiological variables. The small transient alkalosis is most likely secondary to the observed maternal hyperventilation. Consistent with our findings, acute high-dose caffeine (40 mg/kg caffeine base) administration to preterm (~126 DG), mechanically ventilated lambs did not alter blood chemistry, nor did it alter renal function (Crossley et al., 2012). The short-term effects of high-dose caffeine administration on blood chemistry have not been thoroughly assessed in preterm infants. One study, however, has reported an increase in O_2 consumption in preterm infants born at 28-33 weeks of gestation when treated with standard doses of caffeine citrate, with no differences in SaO_2 four weeks after caffeine therapy (Bauer et al., 2001). Another, study assessing the effects of both standard doses of caffeine and doses similar to that used in this study (50 mg/kg caffeine citrate loading; 25 mg/kg citrate maintenance dose) reported no glucose intolerance in any infant (Scanlon et al., 1992). Although it was not the focus of this study, there is a need to assess long-term respiratory outcomes in infants treated with caffeine, as experimental evidence suggests that neonatal caffeine treatment in rats,

with standard doses of caffeine, alters the ventilatory response to hypercapnia and hypoxia in adolescence and adulthood (Montandon et al., 2008).

3.4.4 Unaltered body and organ growth following high dose caffeine

High-dose caffeine administration did not lead to alterations in fetal body weight, body morphometry or organ weights (both absolute and relative to body weight). In agreement with the findings from this study, caffeine treated (20 mg/kg caffeine base loading; 15 mg/kg maintenance dose) rat pups showed no difference in body weight throughout the treatment period (postnatal day 2-6) compared to controls (Pan and Chen, 2007). However caffeine treatment has been shown to reduce weight gain in preterm neonates. Administration of caffeine (10 mg/kg caffeine citrate loading; 5 mg/kg maintenance dose) led to a reduction in weight gain during the four weeks of caffeine therapy in preterm neonates (Bauer et al., 2001). The CAP trial followed infants up to discharge and found that, although weight gain was temporarily reduced (first 3 weeks only) in infants treated with caffeine, the reduced growth did not persist until discharge, by which time the weights of caffeine-treated infants were similar to those of controls (Schmidt et al., 2006). Although we did not observe any changes in body or organ growth directly after caffeine administration, the potential long-term consequences of caffeine administration were assessed in this thesis and are reported in Chapter 6.

3.5 Conclusions

The daily dosing regimen used in this study produced high plasma caffeine levels, which are comparable to plasma caffeine concentrations measured in preterm infants administered doses of caffeine that are higher than standard doses. By exposing fetal sheep to high-dose caffeine we have demonstrated that a daily high dose of caffeine does not adversely affect fetal blood chemistry, nor does it affect fetal growth. High-dose caffeine was shown to transiently alter fetal cardiovascular physiology; however, as cardiovascular assessment was only made for 3 days, further analysis on subsequent days of caffeine treatment is required to determine if changes in fetal MAP and HR persist after the first 3 days of treatment. As the changes in fetal physiology following caffeine administration appear to be transient, any observed effects of caffeine on the developing brain are likely to be direct effects, rather than being indirect or secondary effects resulting from alterations in fetal metabolic or cardiovascular physiology.

4 Impact of high-dose caffeine administration on the developing white matter

4.1 Introduction

It is now evident that the standard clinical dose of caffeine is not always sufficient to abolish AOP (Scanlon et al., 1992; Steer et al., 2003), potentially leading to the use of higher doses. However, little is known regarding the effects of higher doses of caffeine on the very immature brain, in particular its effects on the development of WM, a brain region that is very vulnerable in preterm infants (Steer et al., 2003; Gray et al., 2011). While several studies have assessed the effects of high levels of caffeine on the developing brain, the results of these studies are inconsistent, showing both beneficial (Juarez-Mendez et al., 2006; Connolly and Kingsbury, 2010) and detrimental (Fuller et al., 1982; Desfrere et al., 2007) effects. Because of these inconsistencies in the literature, it is difficult to draw unequivocal conclusions about the likely effects of high-dose caffeine on the developing human brain. It should also be taken in into consideration that a majority of previous studies have been conducted in rats and mice (Juarez-Mendez et al., 2006; Connolly and Kingsbury, 2010; Fuller et al., 1982; Desfrere et al., 2007), species in which white-to-grey matter ratio is not comparable to humans and in which brain development occurs predominantly postnatally; furthermore previous studies have used widely differing dosing regimens. Owing to current uncertainties about the effects of caffeine on the developing brain, there is a need for further experimentation in a species in which brain development, including WM development, is similar to that of humans.

The results from Chapter 3 indicate that caffeine is unlikely to have an indirect effect through altering fetal physiology, as we found no significant effect of high-dose caffeine on cardiovascular physiology, blood chemistry, fetal body growth or organ growth. Thus if any adverse alterations are detected in the fetal ovine brain following high-dose caffeine, they are likely due to a direct effect of caffeine on the brain. As in Chapter 3, caffeine was administered to fetal sheep from 0.7-0.8 of term which corresponds to about 27-34 weeks postmenstrual age in preterm infants, in terms of brain development (Rees et al., 2010; Back et al., 2002; Back et al., 2006b) and represents the typical age at which preterm babies are exposed to caffeine (Schmidt et al., 2007). In this study my aim was to determine whether or not repeated daily high-dose caffeine administration causes structural alterations to the developing WM and associated glial cells in the very immature ovine brain. The potential effects of caffeine on the developing WM were assessed using oligodendrocyte transcription factor 2 (Olig2) to identify the entire pool of oligodendrocytes, myelin basic protein (MBP) to identify mature myelin and SMI-312 for axonal integrity. The nature of the glial cell response (microgliosis and astrogliosis), an indicator of WM injury (Roessmann and Gambetti, 1986b; Billiards et al., 2006), was assessed using Iba-1 (ionized binding adaptor molecule-1) for microglia and GFAP (glial fibrillary acidic protein) for astrocytes. Lastly, apoptosis in the developing WM was assessed using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL).

4.2 Methods

All experimental procedures were approved by the Monash University Animal Ethics Committee.

4.2.1 Experimental protocol

As described in detail in Section 2.4, aseptic surgery was conducted at 99 DG (term is approximately 147 DG) on 14 date-mated ewes (Merino x Border Leicester). Catheters were chronically implanted into a fetal femoral artery, femoral vein and the amniotic sac. Catheters were also implanted into a maternal jugular vein for drug infusions and a maternal carotid artery. Antibiotics were administered for 3 days after surgery. Postoperatively, sheep were held in individual pens with access to food and water. In order to expose the fetal brain to caffeine, a daily bolus dose (loading dose, 25 mg/kg; maintenance dose, 20 mg/kg) of caffeine base (Sigma-Aldrich, St. Louis, MO, USA) (n=9) or an equivalent volume of saline (n=8) was administered intravenously

to the ewe between 104 and 118 DG (0.7 - 0.8 of term). At the conclusion of the experiment, the ewe and fetus were euthanized using an overdose of sodium pentobarbitone (130 mg/kg i.v.) and the fetus delivered via Caesarean section at 119 DG (0.8 of term). Fetuses (control, n=8 and caffeine, n=9) were transcardially perfused with isotonic saline and 4% PFA in 0.1M PB (pH 7.4) (see Section 2.5 for further details on necropsy).

4.2.2 Brain histology

The entire forebrain was cut coronally into blocks 5 mm thick (8-10/animal). Blocks of the entire right hemisphere were then post-fixed in 4% PFA (4 days, 4°C) and embedded in paraffin. Serial sections (8 μm thick) were cut from each block and 1 section/block stained with thionin and examined for haemorrhages and gross structural alterations (see Section 2.7).

4.2.2.1 Immunohistochemistry

Sections from equivalent sites from each lobe of the right cerebral hemisphere were reacted with antibodies to: rabbit anti-Olig2 to identify the entire population of oligodendrocytes, rat anti-MBP to identify mature oligodendrocytes and myelin, mouse anti-SMI-312 to identify axonal neurofilaments, rabbit anti-Iba-1 to identify microglia, and rabbit anti-GFAP to identify astrocytes (see Table 2.1 and Section 2.7.1 for immunohistochemistry protocol and antibody details). Sections from control and caffeine-treated animals were simultaneously reacted to reduce staining variability. There was no staining when the primary antibodies were omitted.

4.2.2.2 TUNEL staining

Sections from each lobe were stained with the DeadEnd^{TM} Colorimetric TUNEL system (Promega, Madison, WI, USA) to identify apoptotic and necrotic cell death (Stadelmann and Lassmann, 2000), as described in Section 2.7.2. For each antibody, sections from control and caffeine treated animals were simultaneously reacted to reduce staining variability. There was no staining when the primary antibodies were omitted.

4.2.2.3 Quantitative analysis

Analyses were performed on coded slides (I was therefore blinded to the group) from the right cerebral hemisphere using an image analysis system (Image-Pro Plus v6.2, Media Cybernetics, Rockville, MD). Immunohistochemical analyses were performed on one section from each of the frontal, parietal, temporal and occipital lobe from each fetus at equivalent areas within the WM. All areal densities are expressed as cell/mm^2 and optical density (OD) is expressed in arbitrary units. Further details are presented in Section 2.7.3.

4.2.2.3.1 Volumetric analysis

Cross-sectional area of the total WM was measured in every 625th thionin-stained section (1 section/block; n=8/animal) with a digitizer interfaced to the image analysis software (Tolcos et al., 2011) (see Section 2.7.3.1). The total volume of each brain region was estimated according to the Cavalieri principle using the formula $V=\Sigma APt$, where V is the total volume, ΣA is the sum of the areas measured, P is the inverse of the sampling fraction and t is the section thickness (Gundersen et al., 1999).

4.2.2.3.2 Optical density of MBP- and SMI-312-immunoreactivity (IR)

A total of 3 fields of view from each of SCWM, PVWM and corpus callosum (field 0.093 mm^2) per slide, with one section from the frontal lobe, two from the parietal and temporal lobe and one from the occipital lobe was obtained for the analysis of MBP- and SMI-312-IR. The intensity of MBP- and SMI-312-IR in the cerebral WM was determined using an OD analysis as validated in the Tolcos laboratory (Tolcos et al., 2011). Prior to measuring, the image analysis system was calibrated using an image of a blank section of the glass slide (incident light) and obscured section of the slide (infinite optical density). From each section, OD was assessed in 3 fields from equivalent regions of each of the subcortical WM, periventricular WM and corpus callosum, with a total of 6-9 fields from each section (field 0.093 mm^2). A correction was applied to each of these images by subtracting the OD measurement from a region of background staining. The mean OD was then calculated within each region, for each animal, and a mean of means determined for control and caffeine-treated animals. Imaging and analysis of each of the immunostains was performed in a single day using identical parameters to maintain consistency and eliminate error.

4.2.2.3.3 Percentage WM occupied by Iba-1-IR microglia

The proportion of WM area occupied by aggregations of Iba-1-IR amoeboid (activated) microglia (identified by a round soma and no processes) was assessed in every 625th section (1 section from each block; n=8-9 per animal) using a digitizer interfaced to the image analysis software for analysis as previously discussed in Section 2.7.3.1. The area of each aggregation within a section was totalled and divided by the area of the WM of that section; data are expressed as a percentage (%). The mean was then calculated for each animal and a mean of means for control and caffeine-treated groups determined.

4.2.2.3.4 Areal density of Iba-1-IR, GFAP-IR, Olig2-IR and TUNEL-positive cells

Resting (ramified) and activated (amoeboid) Iba-1-IR microglia (distinguished by morphology), GFAP-IR astrocytes and Olig2-IR oligodendrocytes were counted in 2-3 fields from both the subcortical and periventricular WM, with a total of 4-6 fields per section (field, 0.093 mm^2). TUNEL-positive cells were counted throughout the subcortical WM and periventricular WM; the area of each region was also measured to determine the areal cell density. See Section 2.7.3.2.

4.2.2.4 Qualitative analysis

All SMI-312-IR sections were qualitatively assessed for the presence of disrupted axons and axonal spheroids. The white matter tracts of all stained sections were first visually scanned under a microscope at x10 magnification to check for any obvious damage to axons, after which sections were visually scanned under x40 to further assess the tissue for the presence of disrupted axons.

4.2.3 Statistical analysis

All data were analysed using SPSS software (Version 20, SPSS Inc., Chicago, IL, USA). Differences between treatment groups were analysed by the Student's t test for parametric data or a Mann-Whitney U test for non-parametric data. Data are presented as mean of means ± SEM with $p<0.05$ considered significant.

4.3 Results

4.3.1 Cerebral white matter volume

There was no difference in the estimated volume of cerebral WM matter between control and caffeine-treated fetuses (Table 4.1, Figure 4.1 images).

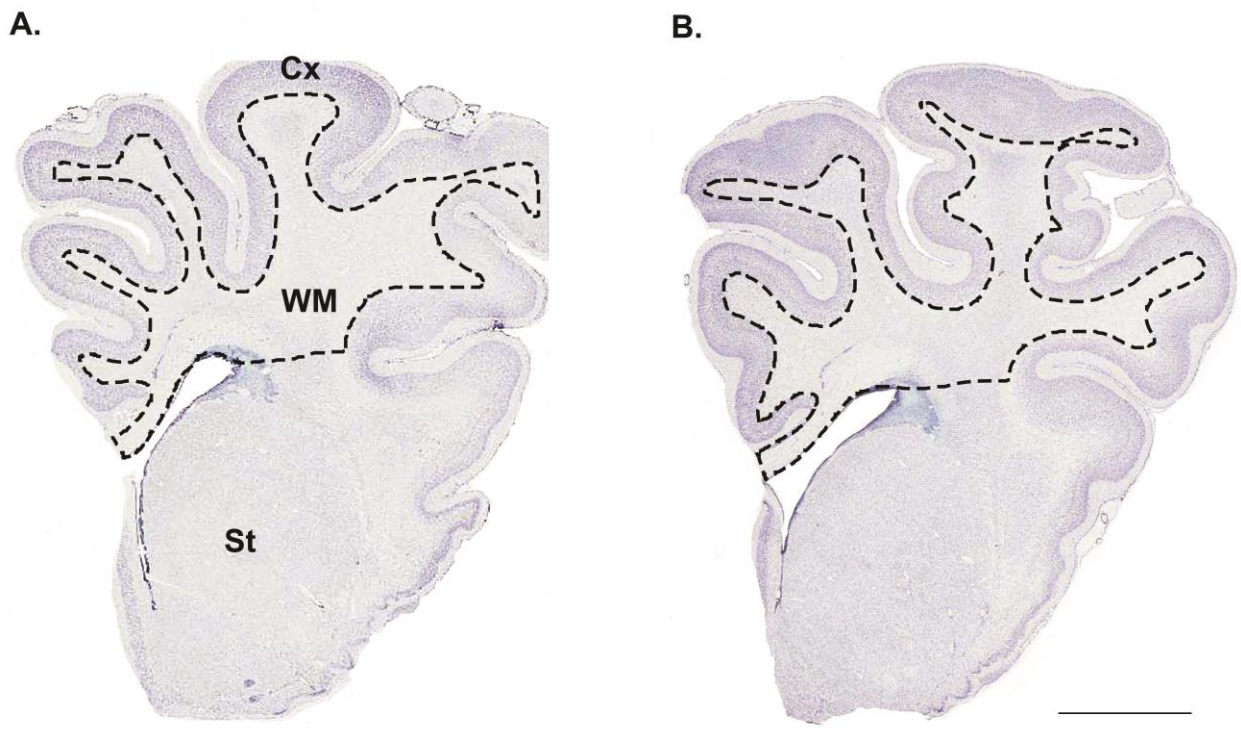


Figure 4.1 Cerebral white matter volume

There was no significant difference between groups in the estimated volume of white matter. This is illustrated by comparing photomicrographs of coronal sections from the cerebral hemispheres from control (A) and caffeine treated (B) fetuses at 119DG stained with thionin. The area of white matter used for analysis enclosed by the dashed line. Cx, cortex; WM, white matter; St, striatum. Scale bar = 4.5mm.

4.3.2 Myelination and axonal integrity

There was no significant difference between groups in the optical density of MBP-IR (Table 4.1) in the subcortical WM, periventricular WM or corpus callosum (Figure 4.2 images). Similarly, there was no significant difference between groups in the optical density of SMI-312-IR (Table 4.1) in the subcortical WM, periventricular WM or corpus callosum (Figure 4.3 images). There was also no evidence of axonal disruption or axonal spheroids in any of these regions as assessed in SMI-312 immunostained sections (Figure 4.3 C, D insets).

Figure 4.2 MBP-IR in the subcortical WM periventricular WM and corpus callosum of control and caffeine treated fetuses

There was no significant difference between groups in the optical density of MBP-IR in the subcortical WM, periventricular WM or corpus callosum. This is illustrated by comparing photomicrographs (A-B; scale bar = 4.5 mm) and high power images (C-H; x4; scale bar = 500 μm) of coronal sections from the cerebral hemispheres from control (A,C,E,G) and caffeine treated (B,D,F,H) fetuses at 119 DG immunohistochemically stained with MBP.

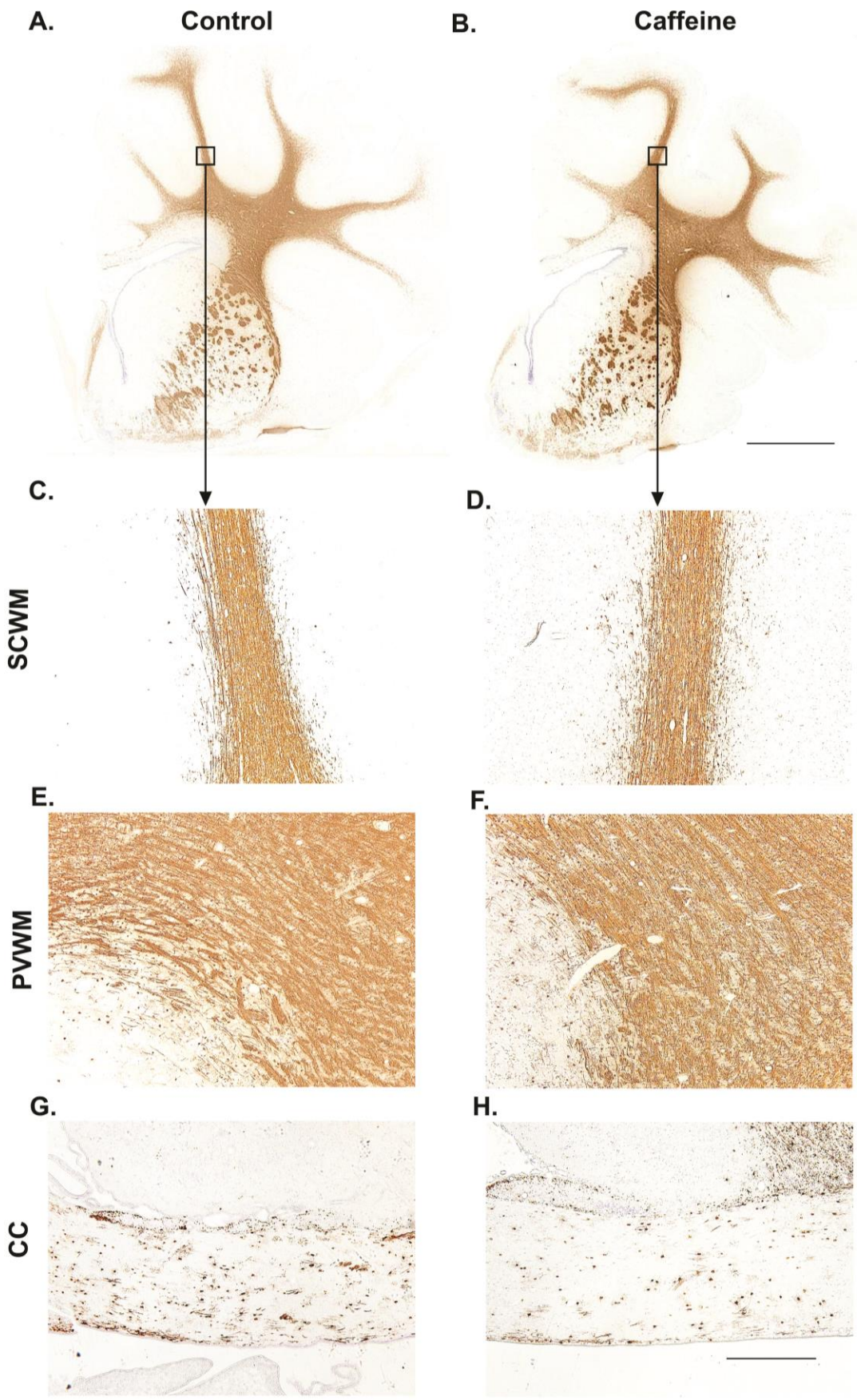
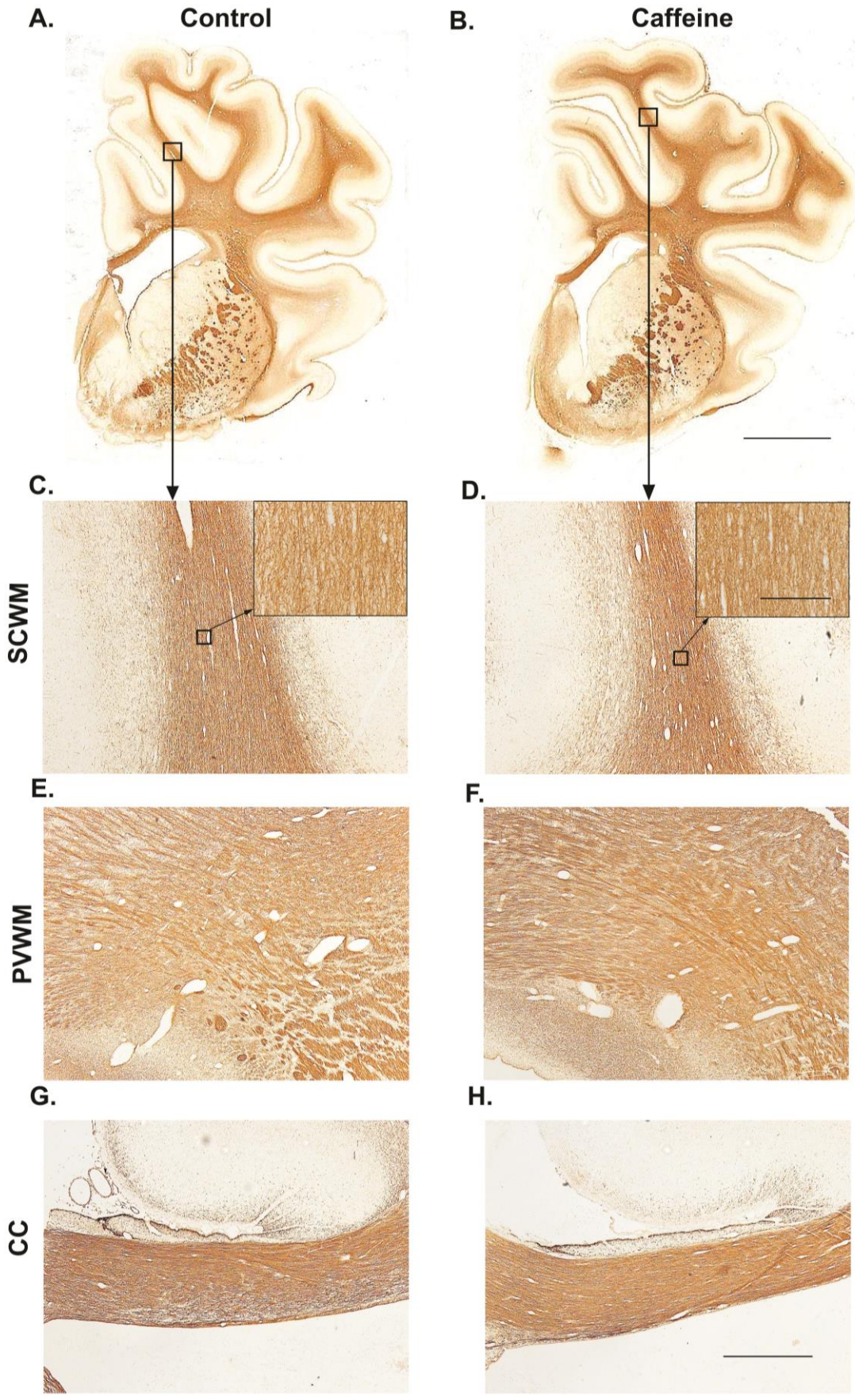

A.
Control
B.
Caffeine
C.
D.
SCWM
E.
F.
PVWM
G.
H.
CC

Figure 4.3 SMI-312-IR in the subcortical WM periventricular WM and corpus callosum of control and caffeine treated fetuses

There was no significant difference between groups in the optical density of SMI-312-IR in the subcortical WM, periventricular WM or corpus callosum. This is illustrated by comparing photomicrographs (A,B; scale bar = 4.5 mm) and high power images (C-H; x4; scale bar = 500 μm) of coronal sections from the cerebral hemispheres from control (A,C,E,G) and caffeine treated (B,D,F,H) fetuses at 119 DG immunohistochemically stained with SMI-312. In SMI-312-immunostained sections, we found no evidence of axonal disruption or axonal spheroids (black boxes in C-D represent high power inserts; x40; scale bar = 100 μm).


A.
Control
B.
Caffeine
C.
D.
SCWM
E.
F.
PVWM
G.
H.
CC

4.3.3 Oligodendrocytes

The density of oligodendrocytes in the subcortical and periventricular WM was similar between control and caffeine-treated fetuses (Table 4.1, Figure 4.4 images).

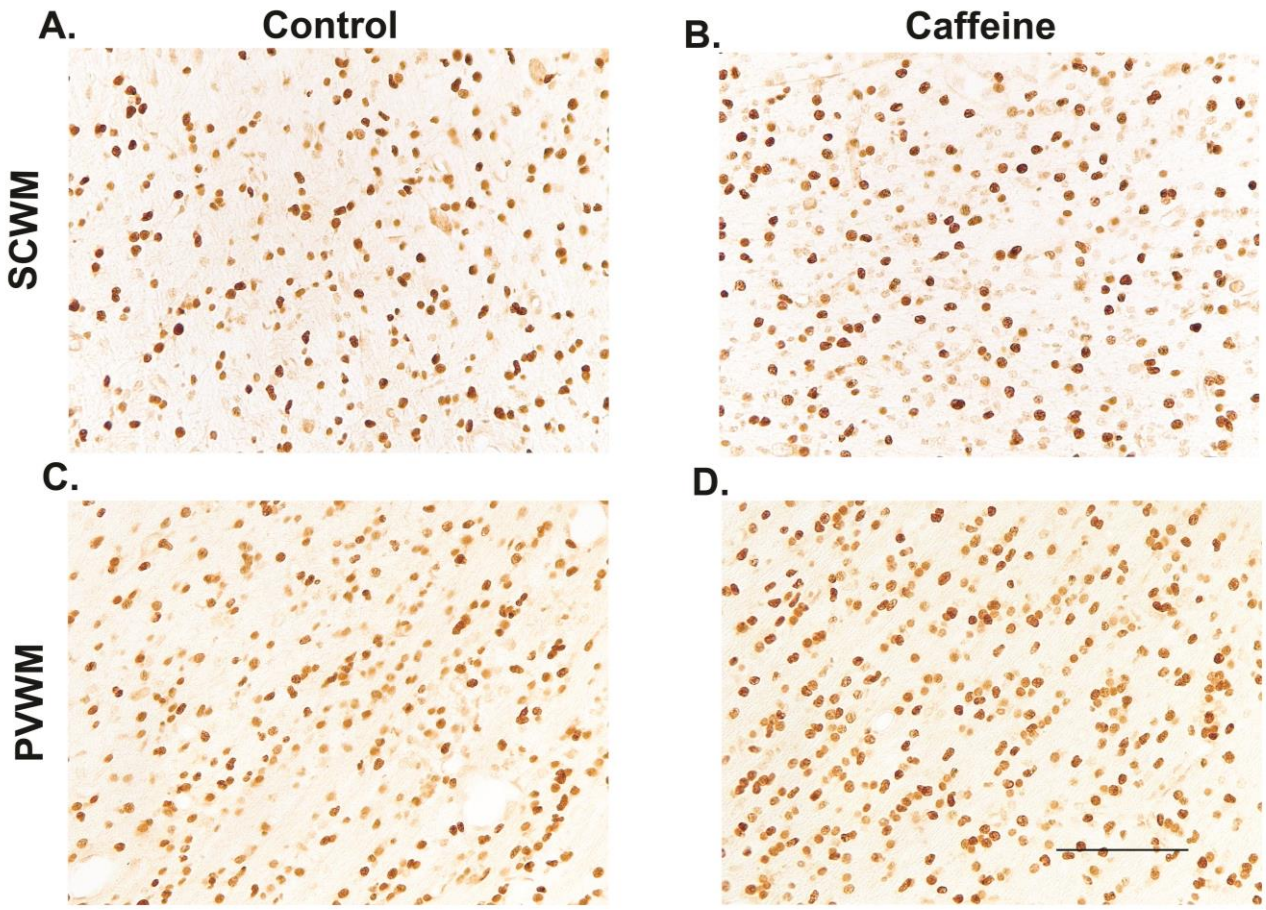


Figure 4.4 Olig2-IR oligodendrocytes in the subcortical and periventricular WM of control and caffeine treated fetuses

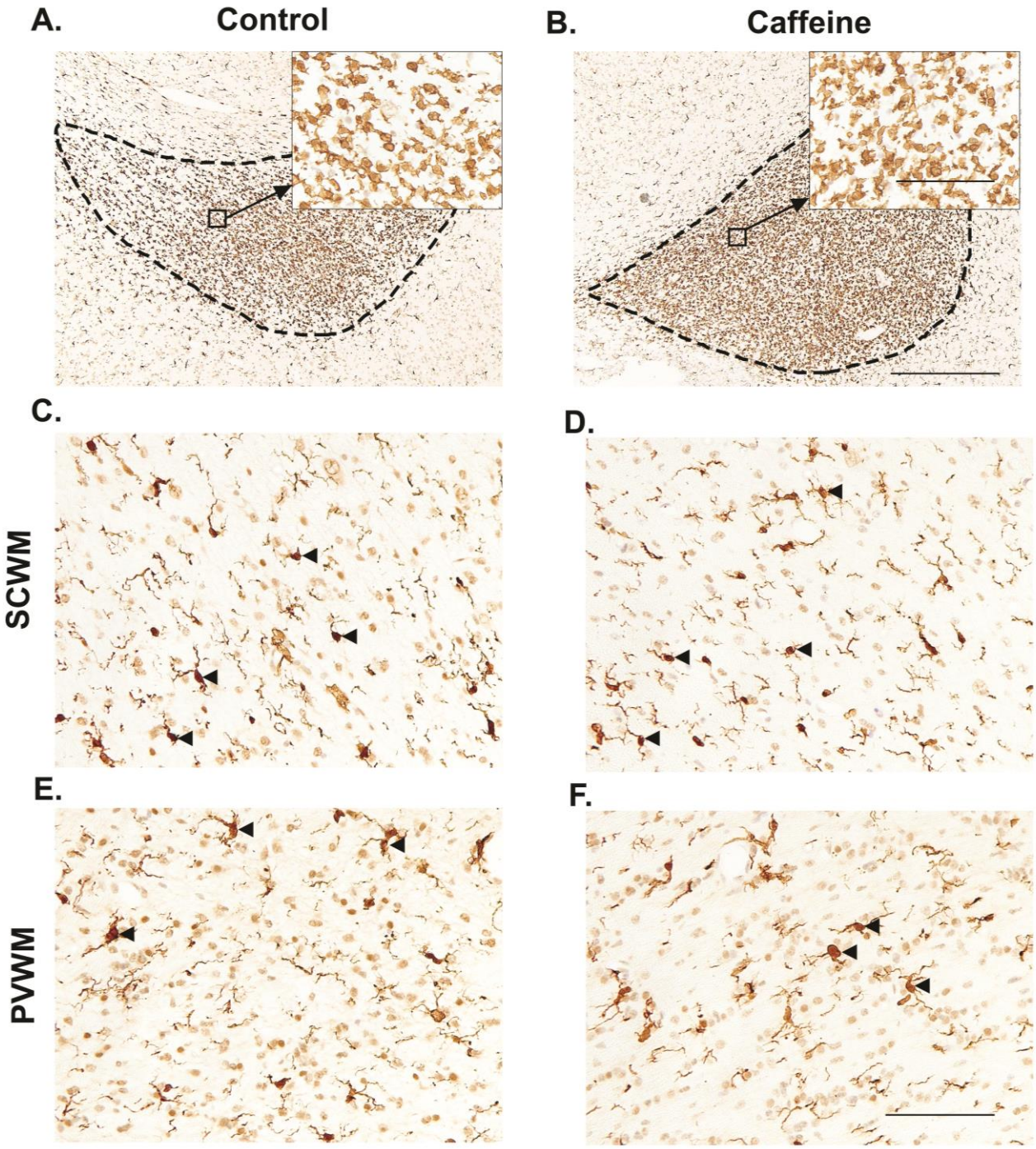
There was no significant difference between groups in the density of Olig2-IR in the subcortical WM and periventricular WM. This is illustrated by comparing high power images (A-D; x20; scale bar = 100 μm) of coronal sections from the cerebral hemispheres from control (A,C) and caffeine treated (B,D) fetuses at 119 DG immunohistochemically stained with Olig2 to show the entire population of oligodendrocytes.

4.3.4 Microglia

The percentage of WM occupied by microglia was not different between control and caffeine treated fetuses (Table 4.1, Figure 4.5 images). The density of activated and resting Iba-1-IR microglia was not significantly different between control and caffeine treated fetuses (Table 4.1) when assessed in the subcortical and periventricular WM (Figure 4.5 images).

Figure 4.5 Iba-1-IR microglia in the subcortical and periventricular WM of control and caffeine treated fetuses

The percentage of WM occupied by microglia was not different between control and caffeine treated fetuses, illustrated by comparing low power images (A,B; x4; scale bar = 500 μm) of microglial aggregations containing activated microglia (A,B inset; x40; scale bar = 100μm) in control (A) and caffeine treated (B) fetuses. There was also no significant difference between groups in the density of Iba-1-IR microglia (resting and activated) in the subcortical WM and periventricular WM. This is illustrated by comparing high power images (C-F; x20; scale bar = 100 μm) of coronal sections from the cerebral hemispheres from control (C,E) and caffeine treated (D,F) fetuses at 119 DG immunohistochemically stained with Iba-1 to show microglia. Arrowheads show resting microglia.


A.
Control
B.
Caffeine
C.
D.
SCWM
E.
F.
PVWM

4.3.5 Astrocytes

There was no significant difference between groups in the density of GFAP-IR astrocytes in the subcortical WM and periventricular WM (Table 4.1, Figure 4.6 images).

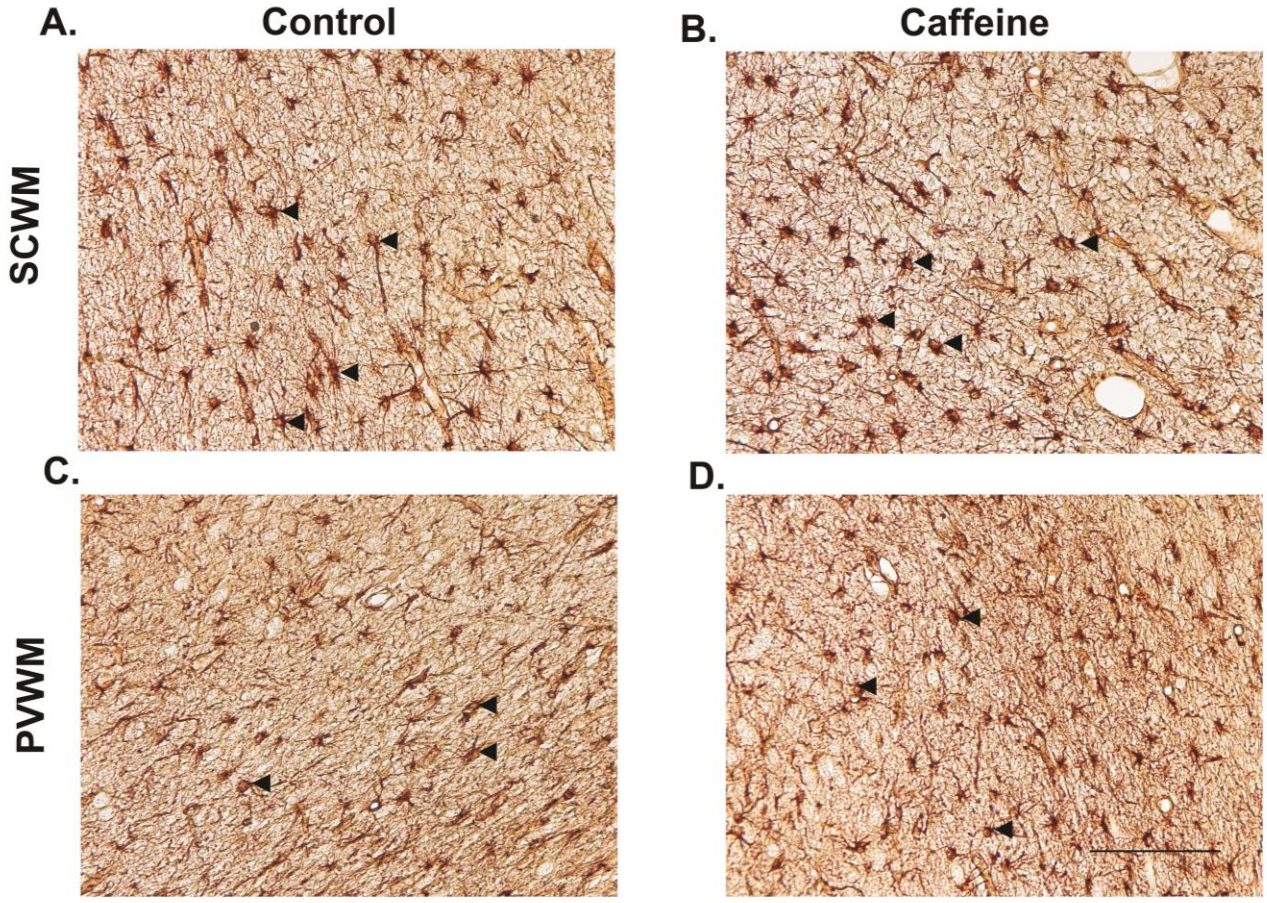


Figure 4.6 GFAP-IR astrocytes in the subcortical and periventricular WM of control and caffeine treated fetuses

There was no significant difference between groups in the density of GFAP-IR astrocytes in the subcortical WM and periventricular WM. This is illustrated by comparing high power images (A-D; x20; scale bar = 100 μm) of coronal sections from the cerebral hemispheres from control (A,C) and caffeine treated (B,D) fetuses at 119 DG immunohistochemically stained with GFAP to show astrocytes. Arrowheads show astrocytes.

4.3.6 Apoptotic cells

There was also no significant difference between groups in the density of TUNEL-positive apoptotic cells in the subcortical WM and periventricular WM (Table 4.1, Figure 4.7 images).

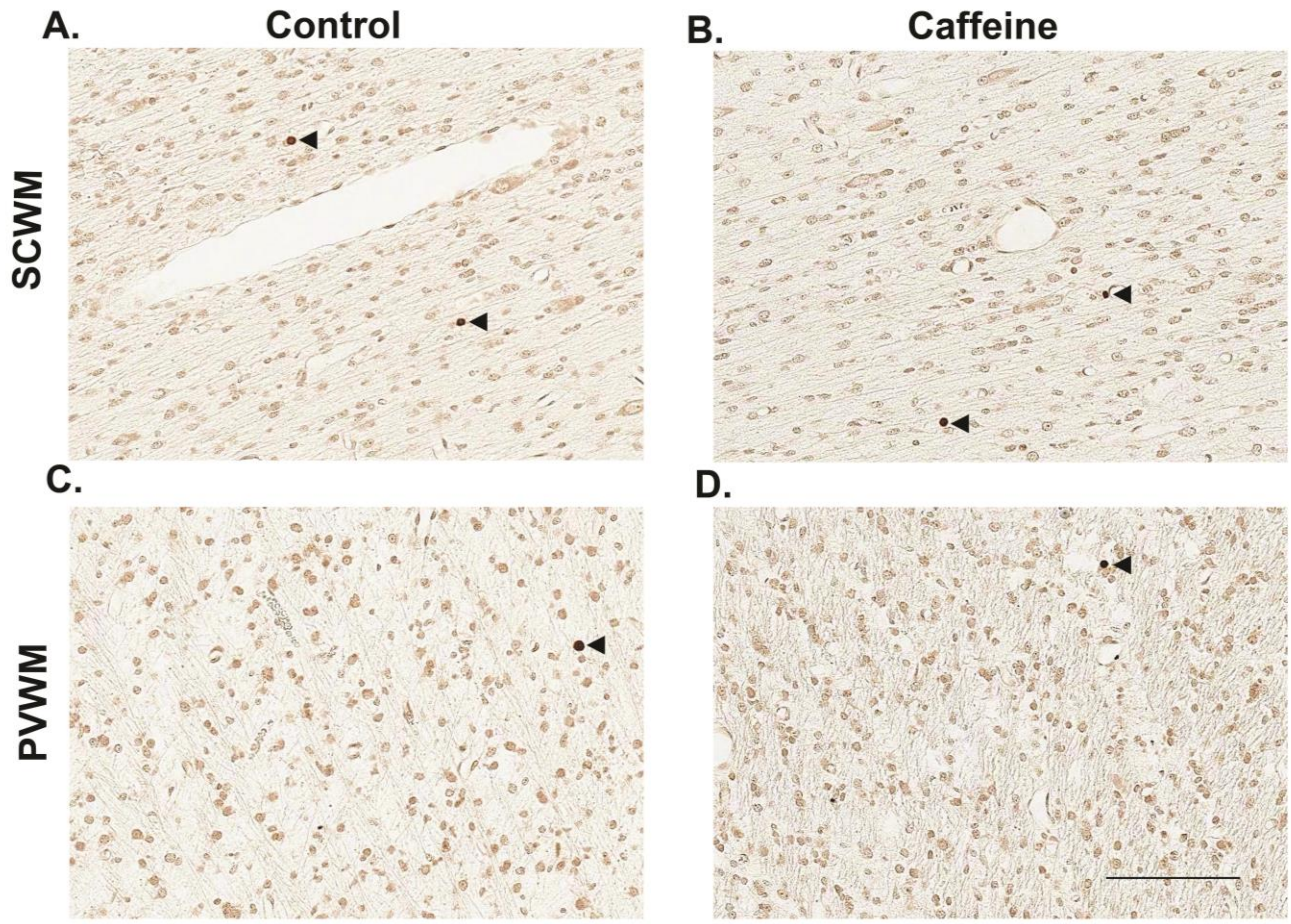


Figure 4.7 TUNEL-positive cells in the subcortical and periventricular WM of control and caffeine treated fetuses

There was no significant difference between groups in the density of TUNEL-positive apoptotic cells in the subcortical WM and periventricular WM. This is illustrated by comparing high power images (A-D; x20; scale bar = 100 μm) of coronal sections from the cerebral hemispheres from control (A,C) and caffeine treated (B,D) fetuses at 119 DG immunohistochemically stained with TUNEL to show apoptotic cells. Arrowheads show TUNEL-positive cells.

Table 4.1 Cerebral white matter volume and immunohistological analysis

Parameter	Control n=8	Caffeine n=9
WM volume, cm^3	4.96 ± 0.15	5.10 ± 0.38
MBP-IR optical density in subcortical WM	0.11 ± 0.01	0.13 ± 0.01
MBP-IR optical density in periventricular WM	0.10 ± 0.01	0.12 ± 0.01
MBP-IR optical density in corpus callosum	0.09 ± 0.01	0.09 ± 0.01
SMI-312-IR optical density in subcortical WM	0.07 ± 0.00	0.07 ± 0.00
SMI-312-IR optical density in periventricular WM	0.06 ± 0.00	0.05 ± 0.00
SMI-312-IR optical density in corpus callosum	0.05 ± 0.01	0.05 ± 0.01
Olig2-IR cells/mm^2 in subcortical WM	910 ± 31	933 ± 58
Olig2-IR cells/mm^2 in periventricular WM	1571 ± 55	1558 ± 58
Iba-1-IR microglial aggregations (% occupied in WM)	4.62 ± 0.96	5.31 ± 0.71
Iba-1-IR activated microglia/mm^2 in subcortical WM	20 ± 2	16 ± 2
Iba-1-IR activated microglia/mm^2 in periventricular WM	24 ± 3	19 ± 2
Iba-1-IR resting microglia/mm^2 in subcortical WM	77 ± 6	84 ± 7
Iba-1-IR resting microglia/mm^2 in periventricular WM	84 ± 5	100 ± 12
GFAP-IR astrocytes/mm^2 in subcortical WM	230 ± 24	258 ± 31
GFAP-IR astrocytes/mm^2 in periventricular WM	189 ± 14	240 ± 34
TUNEL-IR apoptotic cells/mm^2 in subcortical WM	1 ± 0	2 ± 0
TUNEL-IR apoptotic cells/mm^2 in periventricular WM	2 ± 0	2 ± 0

Values are mean of means ± SEM. For all parameters there were no significant differences between groups. IR, immunoreactive; MBP, myelin basic protein; SMI, pan axonal neurofilament; Olig2, oligodendrocyte transcription factor; Iba-1, ionized binding adaptor molecule-1; GFAP, glial fibrillary acidic protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

4.4 Discussion

This is the first study to examine the neuropathological response to high-dose caffeine in a long-gestation, clinically relevant animal model. Importantly the fetal sheep model allowed us to assess the effects of caffeine at a stage of brain development that is similar to that of the very preterm human infant with respect to the major periods of gliogenesis (Back et al., 2001; Roessmann and Gambetti, 1986a), axonal development (Haynes et al., 2005) and myelination (Back et al., 2001). Our findings suggest that daily high-dose caffeine treatment does not cause structural alterations or injure the developing cerebral WM. Specifically we found that high-dose caffeine does not significantly affect WM volume or alter markers of myelination and axonal structure, and it does not significantly affect the overall density of oligodendrocytes, microglia, astrocytes or apoptotic cells.

4.4.1 High dose caffeine and white matter volume and myelination

Cerebral WM volume was unaffected by high dose caffeine. Similarly, the caffeine for apnea of prematurity (CAP) trial (Doyle et al., 2010a) found no significant differences in white matter volume determined by MRI, between control and caffeine treated infants at the conclusion of treatment. We also found no significant effect of high dose caffeine on the areal density of oligodendrocytes or in the densitometry of myelin in the cerebral WM. Similarly, an *in vitro* study has shown that treatment with 20 mg/L caffeine (base), a concentration similar to the mean blood concentration achieved in our study (see Section 3.3.1), did not affect the density of oligodendroglial lineage cells (Marret et al., 1993). Furthermore this dose of caffeine did not affect the secretion of hyaluronic acid, a factor known to inhibit myelination (Marret et al., 1993). Our observed lack of effect on myelination is in contrast to a study in newborn mice, in which high-dose (40 mg/kg or 80 mg/kg; caffeine base) caffeine administration from postnatal days (P) 1-17 resulted in a dose-dependent and transient decrease in myelin concentration at P30 with full recovery and overshoot by P70 (Fuller et al., 1982). Here I used MBP, one of the markers of mature myelin, as a measure of myelination and Olig2 as a marker of the total population of oligodendrocytes. A future assessment of other markers of mature myelin (myelin-associated glycoprotein and myelin proteolipid protein), which are differentially affected by perinatal interventions (Tolcos et al., 2011), together with ultrastructural analysis of myelination, may offer insight into the effects of caffeine on WM development. Similarly, a future analysis of the oligodendroglial lineage using

stage-specific markers could determine whether differentiating, immature oligodendrocytes are more vulnerable to high-dose caffeine than mature oligodendrocytes.

4.4.2 Unaffected axonal integrity as a result of high dose caffeine

To date there have been no studies that have examined the effects of any doses of caffeine on axonal integrity. Here I show for the first time that high-dose caffeine treatment does not affect axonal integrity in the developing WM, at least not at the level of immunoreactivity for the axonal neurofilament protein SMI-312. Although this method permits assessment of overt axonal damage (axonal disruption and axonal spheroids) and densitometry of SMI-312-IR within the WM, it is not an accurate measure of axonal density; for that, electron microscopy would be required. Magnetic resonance imaging of preterm infants at term equivalent age, treated with standard doses of caffeine (citrate; 20 mg/kg loading; 5-10 mg/kg maintenance) revealed a reduction in axial diffusivity in treated infants (Doyle et al., 2010a), which may be explained by axonal preservation and/or a greater axonal density. However, the dose of caffeine used in that study is much lower than those used in my study; MRI scans were performed at a more advanced stage of brain development than in my study. Nevertheless, a thorough assessment of axonal density (myelinated and unmyelinated axons) in the WM at the ultrastructural level warrants further investigation.

4.4.3 High dose caffeine does not lead to WM gliosis

High-dose caffeine treatment did not result in cerebral haemorrhage, astrogliosis, or microgliosis in the cerebral WM indicating that this regimen is not overtly injurious to the developing ovine brain. Our finding of unaltered astrogliosis is consistent with *in vitro* studies showing that addition of caffeine (20 mg/L; base) to newborn rat primary glial cell cultures does not affect the number of type 1 astrocytes and O-2A lineage cells (Marret et al., 1993). Conversely, caffeine treatment from P3-P10 in mice resulted in a transient and dose-dependent reduction in astrocyte density in various brain regions including the cerebral cortex and WM (Desfrere et al., 2007). These differences between studies most likely reflect differences in the timing of the insult and the species used. While no previous studies have assessed the effects of caffeine on microglia, a common marker of injury (Roessmann and Gambetti, 1986b; Billiards et al., 2006) in the developing brain, we show that high-dose caffeine does not lead to increased activation or proliferation of microglia in the developing ovine WM.

4.4.4 High dose caffeine does not affect apoptosis

Consistent with our finding that microglia and astrocytes are unaffected by high-dose caffeine, high-dose caffeine did not result in increased apoptosis in the cerebral WM. Acute high-dose caffeine (3 doses, 50 mg/kg; base) induces apoptosis throughout the cerebral hemispheres via caspase-3-dependent mechanisms in newborn rats (Kang et al., 2002). Similarly, acute high-dose caffeine (100 mg/kg; base) administered to P3 rats resulted in increased apoptosis in various brain regions including the cerebral cortex and caudate nucleus (Black et al., 2008). Once again, differences between this and previous studies are likely due to the timing and dose of administration as well as the species used.

4.5 Conclusions

Using a clinically relevant animal model in which the degree of brain development resembles that of the very preterm infant, we have shown that daily administration of high-dose caffeine does not overtly injure the developing ovine WM. Before definitive conclusions can be drawn about the safety of high-dose caffeine for the treatment of AOP, the possible effects of caffeine on more subtle brain development parameters such as process growth and connectivity, known to be affected by caffeine (Juarez-Mendez et al., 2006), need further investigation. Furthermore, other regions of the brain (e.g. cerebral cortex and striatum) and the long-term consequences of high-dose caffeine treatment require further investigation. The next chapters will therefore investigate caffeine's effect the developing cerebral cortex and striatum, by not only assessing cells important in the development of the cortex but also connectivity (Chapter 5). Furthermore, I will explore the long-term outcomes of high dose caffeine administration (Chapter 6).

5 Effects of high-dose caffeine administration on the grey matter of the immature ovine brain

5.1 Introduction

In Chapter 4, I reported the effects of high-dose caffeine on the development of cerebral WM, and found no significant alterations. Specifically I found that high-dose caffeine did not alter the overall cell density of microglia, astrocytes, apoptotic cells or oligodendrocytes in cerebral WM; nor could I detect any effects on myelination or axonal integrity. In this chapter, my aim was to examine the cerebral GM, as it could be affected by high-dose caffeine, even in the absence of alterations to the WM.

Caffeine at high doses could contribute to injury or altered development of cerebral GM, as neurons and axons in the perinatal brain are known to be susceptible to injury (Bell et al., 2005; Kinney et al., 2005; Back and Miller, 2014; Dean et al., 2014; Dean et al., 2013; McClendon et al., 2014). Assessing the GM is important as any developmental alterations within the cerebral GM, in particular the cortical GM, have the potential to translate to alterations within the WM at a later time (Back and Miller, 2014). This is evident from studies showing that WM injury in the developing brain, in the form of periventricular leukomalacia, is associated with GM injury (Pierson et al., 2007). Detection of alterations within the cortical and subcortical GM is also important because, in the long-term, impairment of GM development is independently associated with functional impairment, after adjustment for WM injury (Woodward et al., 2005). Thus the objective

of the study reported in this chapter was to determine whether or not repeated daily high-dose caffeine administration causes structural alterations to the GM in the immature ovine brain. In particular I assessed the cortical GM and the striatum. Analysis was focussed on these specific regions because neuronal loss and/or gliosis in these regions, which are critical to cognition, memory and learning, play a role in cognitive defects in the long-term (Pierson et al., 2007). Assessment of these brain structures is important as neuroimaging studies on survivors of preterm birth show volumetric deficits in the basal ganglia and thalamus (Inder et al., 2003b; Inder et al., 2005b; Srinivasan et al., 2007), and to a lesser degree the cerebral cortex (Peterson et al., 2000) and hippocampus (Isaacs et al., 2000), indicating that these regions are just as vulnerable to injury as the WM.

Firstly, possible effects of high-dose caffeine on adenosine receptors located in the WM and cortical GM, in particular ARs A_1 and A_{2a} was investigated; this was considered important as it is evident that the neurodevelopmental outcomes related to caffeine exposure are due to its ability to block both the A_1 and A_{2a} receptors. The effects of caffeine on the cortical GM and striatum were then assessed using neuron-specific protein NeuN (NEUronal nuclei) to assess all neurons, chicken ovalbumin upstream promoter transcription factor-interacting protein 2 (Ctip2) to assess all subcerebral projection neurons, and somatostatin (SST) to assess a subpopulation of GABAergic interneurons. Analysis was focussed on Ctip2 as this transcription factor plays a critical role during axonal extension and pathfinding by subcerebral projection neurons of the cerebral cortex (Arlotta et al., 2005; Molyneaux et al., 2005). These projection neurons form the largest proportion of all cortical neurons (Leyva-Diaz and Lopez-Bendito, 2013). In the striatum Ctip2 is uniquely expressed by GABAergic medium-sized spiny neurons (MSN), the output projection neurons of the striatum, which account for 90-95% of all striatal neurons (Gerfen, 1992). The loss of Ctip2 function within the striatum results in failure of MSN differentiation, disruption of MSN organisation, as well as distinct changes in the expression of multiple genes (Arlotta et al., 2008). Assessment of the effect of caffeine on interneurons was focused on a subpopulation of GABAergic interneurons as these neurons play a vital role in the maintenance of neural circuitry and activity (Le Magueresse and Monyer, 2013). GABAergic interneurons account for 10 to 25% of the total number of cortical neurons (Le Magueresse and Monyer, 2013) and 5-10% of neurons in the striatum (Tepper and Bolam, 2004). Using the Golgi-cox stain, the effect of caffeine on neural connectivity was assessed by measuring the density of dendritic spines on cortical pyramidal neurons.

As an indicator of injury, the glial cell response (microgliosis and astrogliosis) to caffeine exposure was assessed in the cortical GM using Iba-1 (ionized binding adaptor molecule-1) for microglia and GFAP (glial fibrillary acidic protein) for astrocytes (Roessmann and Gambetti, 1986b; Billiards et al., 2006). The effect of caffeine on oligodendrocytes in cortical GM was assessed using oligodendrocyte transcription factor 2 (Olig2), which labels the entire pool of oligodendrocytes and myelin basic protein (MBP) to identify mature myelinating oligodendrocytes. Apoptosis in the cortical GM and striatum was assessed using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL).

5.2 Methods

All experimental procedures were approved by the Monash University Animal Ethics Committee.

5.2.1 Experimental protocol

As described in Chapters 3 and 4, a daily bolus dose of caffeine base (loading dose, 25 mg/kg; maintenance dose, 20 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) was administered to 9 pregnant ewes from 104 and 118 DG (0.7 - 0.8 of term). An equivalent volume of saline was administered intravenously to 8 pregnant ewes over the same gestational period. At the conclusion of the experiment (119 DG; 0.8 of term), the ewe and fetus were euthanized using an overdose of sodium pentobarbitone (130mg/kg i.v.) and the fetus delivered via Caesarean section (Section 2.5). Fetuses (control, n=8 and caffeine, n=9) were transcardially perfused with isotonic saline and 4% PFA in 0.1M PB (pH 7.4) (Section 2.5.2).

5.2.2 Brain histology

The entire forebrain was cut coronally into blocks 5 mm thick (8-10/animal). Blocks of the entire right hemisphere were then post-fixed in 4% PFA (4 days, 4°C) and embedded in paraffin. Serial sections (8 μm thick) were cut from each block and 1 section/block stained with thionin and examined for hemorrhages and gross structural alterations (Section 2.6).

5.2.2.1 Immunohistochemistry

Sections from equivalent sites from each lobe of the right cerebral hemisphere were reacted with antibodies to: rabbit anti-A_1AR to identify A_1 adenosine receptors, mouse anti-A_{2a}AR to identify A_{2a} adenosine receptors, mouse anti-NeuN to identify mature neurons, rat anti-Ctip2 to identify subcerebral projection neurons, rabbit anti-SST to identify a subpopulation of GABAergic interneurons, rabbit anti-Iba-1 to identify microglia, rabbit anti-GFAP to identify astrocytes, rabbit anti-Olig2 to identify the entire population of oligodendrocytes and rat anti-MBP to identify mature myelinating oligodendrocytes and myelin (see Table 2.1 for antibody details). Sections from each lobe were stained with DeadEnd^{TM} Colorimetric TUNEL system (Promega, Madison, WI, USA) to identify apoptotic and necrotic cell death (Stadelmann and Lassmann, 2000). For each antibody, sections from control and caffeine treated animals were simultaneously reacted to reduce staining variability. When the primary antibodies were omitted there was no staining, which validates the specificity of the primary antibody (refer to Section 2.7.1 for immunohistochemistry protocol and Section 2.7.2 for TUNEL protocol). Both A_1 and A_{2a} AR antibodies required optimization for sheep brain tissue. This was done using tissue sections from postnatal rat heart and kidney as positive controls (Reppert et al., 1991; Fink et al., 1992; Rivkees, 1995).

5.2.2.2 Golgi-cox staining

One block (at the level of the hippocampus) per animal from the left hemisphere (see Section 2.5.2.2 for brain blocking protocol) was processed for Golgi-cox impregnation with the FD Rapid Golgi Stain Kit (FD Neurotechnologies Inc. Columbia, MD); all buffers were provided in the kit. Prior to impregnation, a mixture of equal volumes of solution A and B was made 24h prior to use; the upper, precipitate-free portion of solution was used. Blocks were kept in the impregnation solution for 2 weeks and the solution replaced twice. Blocks were then transferred into solution C and stored at 4°C in the dark for 1 week; the solution was replaced after the first 24 h. Each block was serially sectioned at 100 μm in the coronal plane with a cryostat (Leica CM3050, Leica Microsystems Pty Ltd, Australia). Sections were then mounted onto gelatin-coated slides, dried for 48hrs and processed for Golgi visualisation using materials supplied in the kit. Sections were first rinsed twice (2 min each) in Milli-Q water then placed in a mixture consisting of 1 part solution D, 1 part solution E and 2 parts Milli-Q water for 10mins. This was followed by two rinses in Milli-Q

water (4 min each). Sections were then dehydrated in a series of graded alcohols, cleared in histolene and coverslipped.

5.2.2.3 Histological and immunohistochemical analysis

As described in Section 2.7.3, analyses were performed on coded slides (observer blinded to group) from the right cerebral hemisphere using an image analysis system (Image-Pro Plus v6.2, Media Cybernetics, Rockville, MD). Immunohistochemical analyses were performed on one section from each of the frontal, parietal, temporal and occipital lobes (A_1AR, parietal and temporal only) from each fetus; equivalent areas of the GM were assessed (see Section 2.7.3, Figure 2.3). Dendritic spine density was assessed in one Golgi-stained section from the level of the hippocampus (parietal lobe) per animal. All areal densities are expressed as cells/mm^2. Dendritic spine density is expressed as spines/10 μm.

5.2.2.3.1 Analysis of adenosine receptors A_1

Immunohistochemical analyses were performed on one section from each of the parietal and temporal lobe from each fetus; equivalent areas of the WM and GM were assessed. Due to the nature of A_1AR staining, only qualitative analysis of A_1AR-positive cells could be performed; as staining intensity varies between stained cells, counts of A_1AR-positive cells would not have been accurate. Thus A_1AR positive cells were assessed via qualitative assessment of staining intensity. Staining intensity was scored qualitatively (– no staining; + moderate intensity staining; ++ high intensity staining) based on the staining intensity of the cells that were stained. Stained sections from control animals were first assessed to determine which regions, both in the WM and GM, were positive for the A_1AR. Following this, controls were compared to caffeine-treated fetuses, and analysis was focussed on regions of the WM investigated in Chapter 4, including the subcortical white matter (SCWM) and periventricular white matter (PVWM). The cerebral GM was also assessed, which included assessment of the cortical GM and striatum. Due to the extremely low levels of A_{2a}AR staining in the fetal sheep brain (control and caffeine-treated), this receptor subtype was not assessed further. Sections from the postnatal rat heart and kidney showed high levels of A_1 and A_{2a} AR expression (data not shown).

5.2.2.3.2 Volumetric analysis

Cross-sectional area of the cortical GM and striatum was measured in every 625th thionin-stained section (1 section/5 mm block; n=8/animal) with a digitizer interfaced to the image analysis software (Tolcos et al., 2011) (see Section 2.7.3.1). The total volume of each brain region was estimated according to the Cavalieri principle using the formula $V=\Sigma APt$, where V is the total volume, ΣA is the sum of the areas measured, P is the inverse of the sampling fraction and t is the section thickness (Gundersen et al., 1999).

5.2.2.3.3 Areal density of neuronal, glial and apoptotic cells

Neurons stained for NeuN-IR, Ctip2-IR and SST-IR were analysed via a slide scanner (ScanScope XT, Aperio, CA, USA) linked to image analysis software (ImageScope, Aperio, CA, USA). NeuN-positive neurons in the cortical GM were counted in one field (field, ~0.56 mm^2) in 3 gyri, with a total of 3 fields of view analysed per section; each field was then divided into 4 bins (bin 1: cortical layer I; bin 2: layer II and III; bin 3: layer IV; bin 4: layer V and VI). NeuN-IR neurons were also counted in the striatum in a total of 6 fields of view (field, 0.40 mm^2) per section, with 3 fields analysed from the caudate and 3 from the putamen (Figure 2.3B). Similarly Ctip2-IR subcerebral projection neurons in the cortical GM were counted in one field (field, 0.56 mm^2) in 3 gyri, with a total of 3 fields of view analysed per section; each field was then divided into 3 bins (bin 1: cortical layer I; bin 2: layer II, III and IV; bin 3: layer V and VI). Ctip2-IR projection neurons were also counted in the striatum in a total of 6 fields of view (field, 0.40 mm^2; Figure 2.3B) per section. SST-IR interneurons were counted in the GM in 3 fields (field, ~0.56 mm^2) in 4 gyri, with a total of 12 fields analysed per section. SST-IR interneurons were also counted in the striatum in a total of 6 fields of view (field, 0.40 mm^2; Figure 2.3B) per section. TUNEL-positive cells were counted throughout the cortical GM and striatum; the area of each region was measured to determine the areal cell density (see Section 2.7.3.2 for more detailed methodology). Resting (ramified) and activated (amoeboid) Iba-1-IR microglia (distinguished by morphology), GFAP-IR astrocytes, Olig2-IR oligodendrocytes and MBP-IR mature myelinating oligodendrocytes were counted in 6 fields from the cortical GM (field, 0.093 mm^2). For each stain/immunostain, the mean cell density was calculated for each animal and for each region or bin, and a mean of means for control and caffeine-treated groups determined.

5.2.2.3.4 Analysis of dendritic spine density

To further define possible effects of high-dose caffeine on neurons, I analysed the density of spines on apical dendrites of pyramidal neurons in both treatment groups. Pyramidal neurons were chosen because of their unique morphology, which allowed them to be distinguished from other neuronal populations in the cortical GM, following Golgi staining. Importantly, cortical pyramidal neurons are glutamatergic neurons that transmit information to other major regions of the brain such as the striatum, thalamus, brainstem and spinal cord (Molyneaux et al., 2007).

To assess dendritic spine density of pyramidal cells, I used region-matched Golgi-stained tissue sections; dendritic spines were visualised at x100 magnification under oil immersion, using a light microscope (Leitz diaplan, Leica Microsystems, Australia). To analyse equivalent dendritic branches between animals and groups, spine density on first order dendritic branches was quantified. Pyramidal neurons were selected for analysis when they met the following criteria: (i) triangular-shaped soma with an apical dendrite perpendicular to the pial surface; (ii) complete impregnation of the cell with Golgi-stain that permitted visualisation of the entire arbour of dendrites and spines; and (iii) neuronal processes not obscured by other neurons, glia or the vasculature. No distinction was made between subtypes of pyramidal neurons or cortical layers. Five neurons were selected per section, per animal, and for each neuron a 50 μm apical dendrite segment was chosen for analysis. Spine density was determined by counting all visible spines on each apical dendrite segment. The mean number of spines per 10 μm segment of dendrite was calculated for each animal from the control (n=6) and caffeine (n=7) groups. Group means were obtained by averaging the mean values of each animal. Basal dendrites were excluded from analysis due to the large overlap with nearby stained dendrites, which obstructed the view of a single basal dendrite. Due to this overlap and obstructed view, dendritic length could not be measured. The length of spines was not assessed because of their dynamic nature.

5.2.3 Statistical analysis

All data were analysed using SPSS software (Version 20, SPSS Inc., Chicago, IL, USA). Differences between treatment groups were analysed by the Student's t test for parametric data; if data failed a variance test (F-test), a Mann-Whitney U test (non-parametric data) was used. Data are presented as mean of means ± SEM with $p<0.05$ considered significant.

5.3 Results

5.3.1 Grey matter volume

The estimated volumes of cortical GM and striatum were similar between groups (Table 5.1; Figure 5.1).

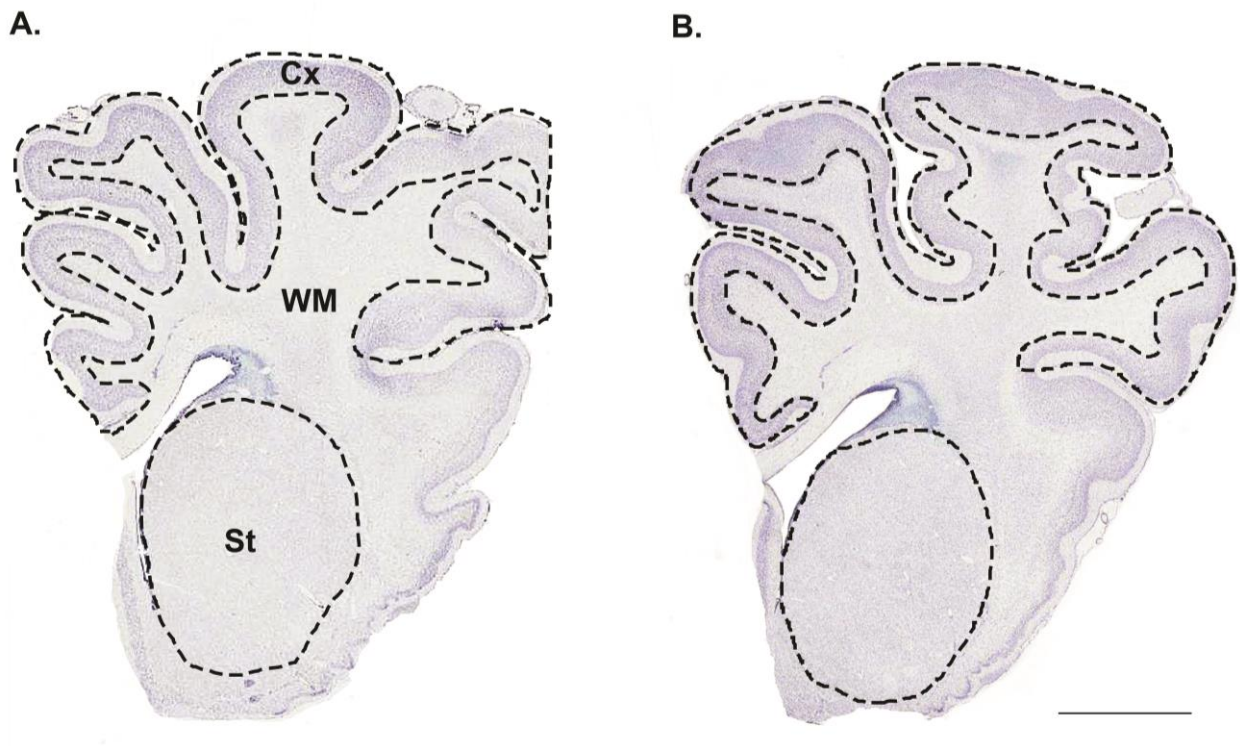


Figure 5.1 Grey matter volume

Photomicrographs of coronal sections from the right cerebral hemisphere from control (A) and caffeine-treated (B) fetuses at 119 DG stained with thionin. There was no significant difference between groups in the estimated volume of cortical GM or striatum. The dashed line encloses the area of cortical GM used for analysis. WM, white matter; St, striatum; Cx, cortical GM. Scale bar = 4.5 mm.

5.3.2 Adenosine receptor A_1

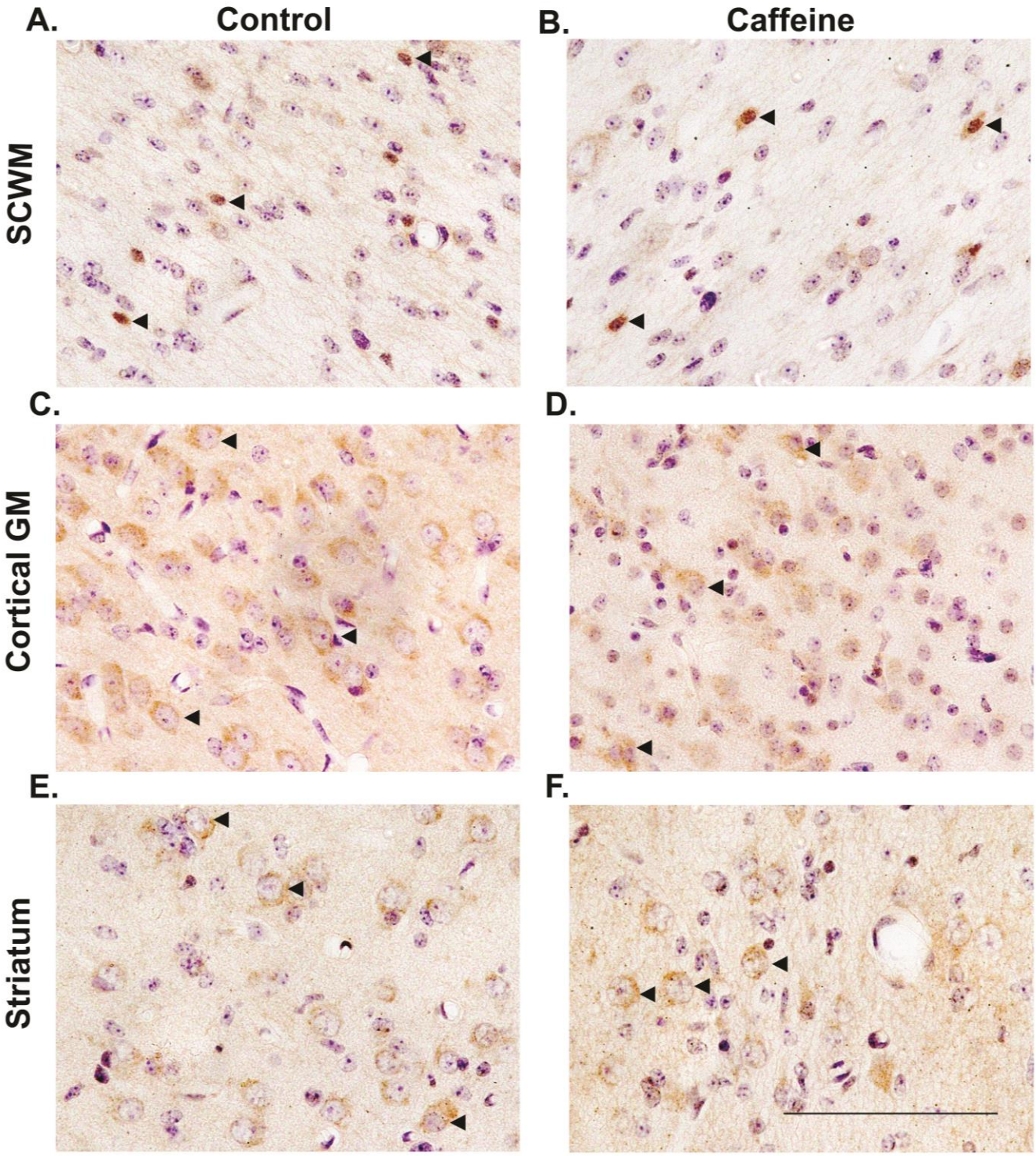
When the distribution of A_1AR staining was assessed in control fetuses, it was evident that A_1ARs were expressed at low intensities in the SCWM and PVWM. Staining in the WM was localised to

the cell body of what appeared to be oligodendrocytes, based on morphology. In comparison, in the cortical GM, low intensity A_1AR staining was only evident in cell bodies of neurons. This localisation of A_1AR staining was similar to that described in other studies (Othman et al., 2003; Rivkees et al., 1995). Higher intensity staining of A_1ARs was apparent in regions such as the midbrain, striatum and hippocampus. Within all of these regions, A_1AR staining was only present on the cell bodies of neurons. In the hippocampus, high intensity A_1AR staining was present in the CA1 and CA3 regions, with lower intensity A_1AR staining in the hilus and dentate gyrus.

In the SCWM and PVWM, 8/8 control and 7/9 caffeine-treated fetuses showed low intensity staining for A_1AR (Figure 5.2A,B). In the cortical GM, 8/8 control and 9/9 caffeine-treated fetuses showed positive A_1AR staining (Figure 5.2C,D). The intensity of the stain appeared lower in caffeine-treated fetuses compared to controls, but this effect could not be accurately assessed quantitatively. In the striatum, 7/8 control and 8/9 caffeine-treated fetuses showed positive A_1AR staining with similar (high) staining intensity (Figure 5.2E,F).

Figure 5.2 A_1 adenosine receptor-IR cells in the white mater, cortical grey matter and striatum of control and caffeine-treated fetuses

Photomicrographs of coronal sections from the WM (SCWM shown only; A,B), cortical GM (C,D) and striatum (E,F) in control (A,C,E) and caffeine-treated (B,D,F) fetuses at 119 DG immunohistochemically stained with A_1AR. In the SCWM and PVWM (A-B; SCWM shown only), positive A_1AR staining was localised on what appeared to be oligodendrocytes and was evident in 8/8 control fetuses (A) and only 7/9 caffeine-treated (B) fetuses. Positive A_1AR staining was evident in the cell bodies of neurons in the cortical GM (C-D) of all control (C) and caffeine-treated (D) fetuses, with a higher intensity staining in controls compared to caffeine-treated fetuses. In the striatum (E-F) 7/8 controls (E) and 8/9 caffeine-treated (F) fetuses showed high intensity A_1AR staining. Arrowheads show A_1AR-positive cells. Scale bar (A-F): 100 μm.


A.
Control
B.
Caffeine
SCWM
C.
D.
Cortical GM
E.
F.
Striatum

5.3.3 Neurons

There was no significant difference between groups in the density of NeuN-positive neurons in the cortical GM in individual or combined cortical bins (Table 5.1; Figure 5.3A,B). There was also no significant difference between groups in the density of NeuN-positive neurons in the striatum (Table 5.1; Figure 5.3C,D). Compared to controls the caffeine-treated group showed a significant increase in the density of Ctip2-positive subcerebral projection neurons in bin 3 (layer V and VI) within the cortical GM ($p=0.02$; Table 5.1; Figure 5.4A,B), with a tendency for an increase when all cortical bins were combined ($p=0.07$; Table 5.1). Caffeine exposure did not affect the density of Ctip2-positive projection neurons in the striatum compared to controls (Table 5.1; Figure 5.4C,D). There was also no significant difference between groups in the density of SST-positive GABAergic interneurons in the cortical GM or striatum (Table 5.1; Figure 5.5).

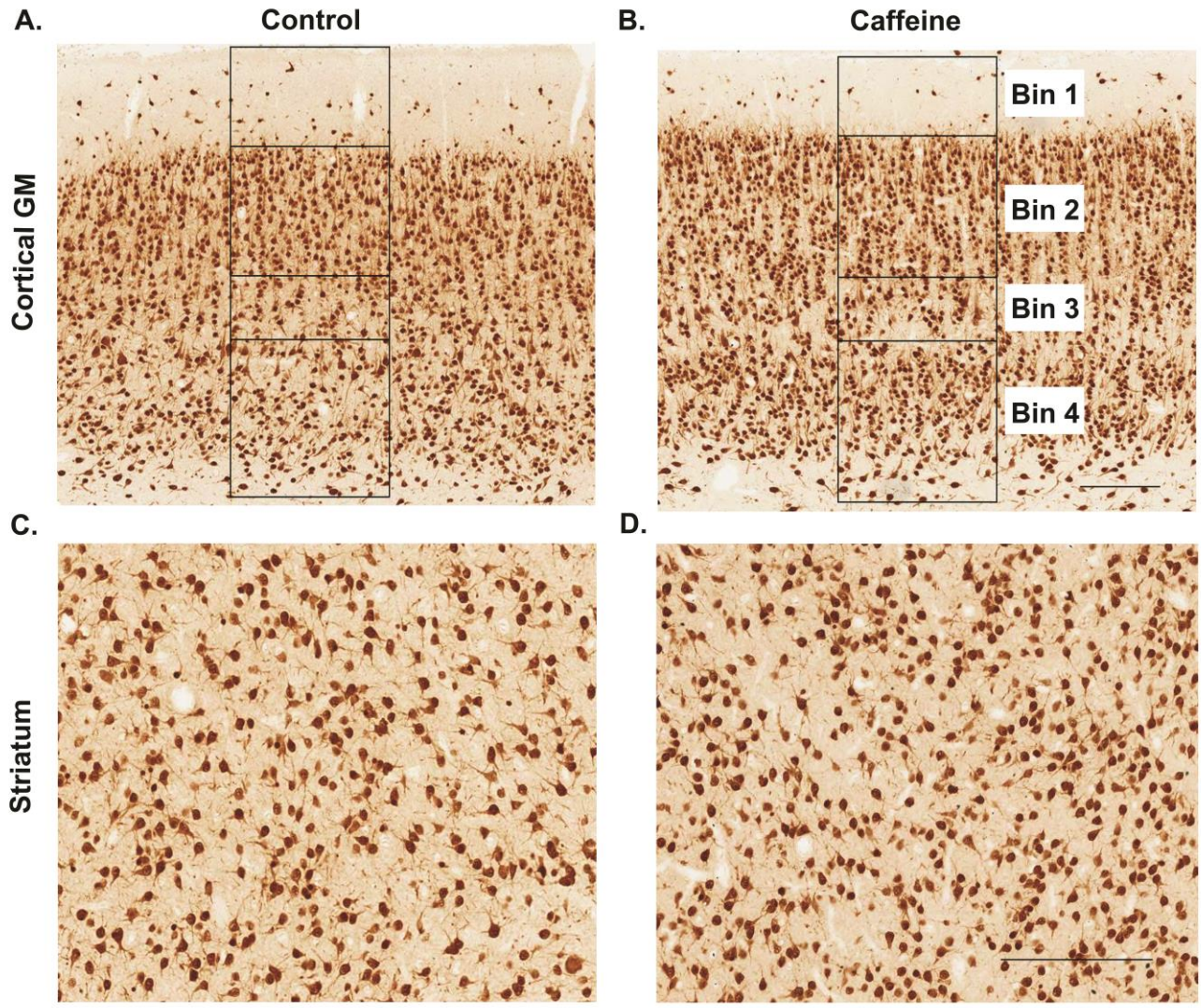
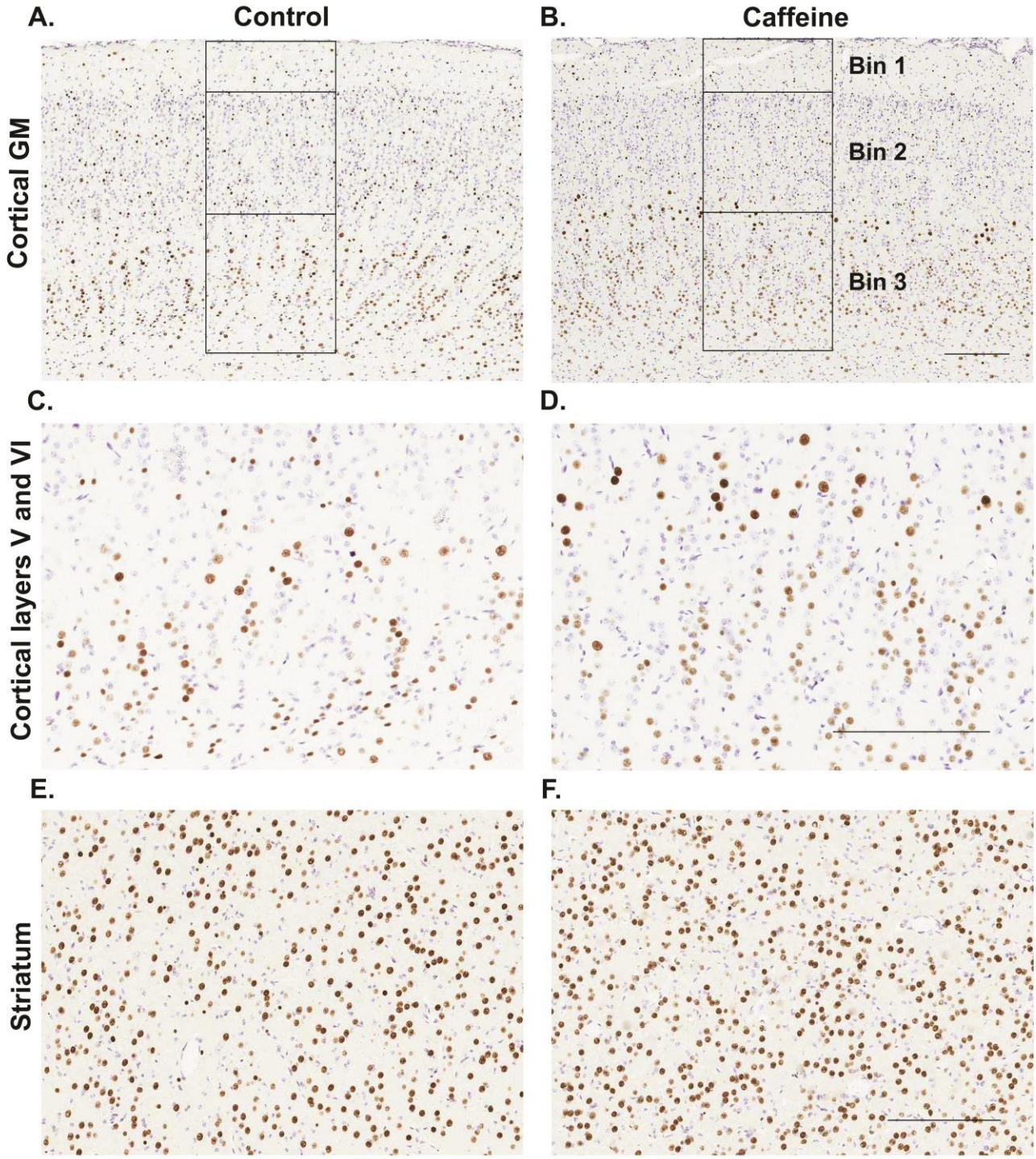


Figure 5.3 NeuN-IR neurons in the cortical grey matter and striatum of control and caffeine treated fetuses

Photomicrographs of coronal sections from the cortical GM (A,B) and striatum (C,D) in control (A,C) and caffeine-treated (B,D) fetuses at 119 DG, immunohistochemically stained with NeuN. NeuN-IR neuronal density in the cortical GM was not different between control (A) and caffeine-treated (B) fetuses in any bins analysed or when bins were combined. Bins were divided according to cortical layers (bin 1: cortical layer I; bin 2: layer II and III; bin 3: layer IV; bin 4: layer V and VI). There was also no significant difference in NeuN-IR neuronal density within the striatum between control (C) and caffeine-treated (D) fetuses. Scale bar (A-D): 200 μm.

Figure 5.4 Ctip2-IR projection neurons in the cortical grey matter and striatum of control and caffeine-treated fetuses

Photomicrographs of coronal sections from the cortical GM (A-D) and striatum (E,F) in control (A,C,D) and caffeine-treated (B,D) fetuses at 119 DG immunohistochemically stained with Ctip2. Bins were divided according to cortical layers (bin 1: cortical layer I; bin 2: layer II, III and IV; bin 3: layer V and VI) as shown in A and B. There was a significant increase in Ctip2-IR subcortical pyramidal neurons in bin 3 (layer V and VI) in the cortical grey matter, in the caffeine group (D) compared to controls (C), with no significant differences in all other bins. There was a tendency for an increase in Ctip2-IR projection neurons in the caffeine group (B) compared to controls (A) when all bins were combined. There was also no significant difference between control (E) and caffeine-treated (F) fetuses in Ctip2-IR neuronal density within the striatum. Scale bar (A-F): 200 μm.


A.
Control
B.
Caffeine
Bin 1
Bin 2
Bin 3
Cortical GM
C.
D.
Cortical layers V and VI
E.
F.
Striatum

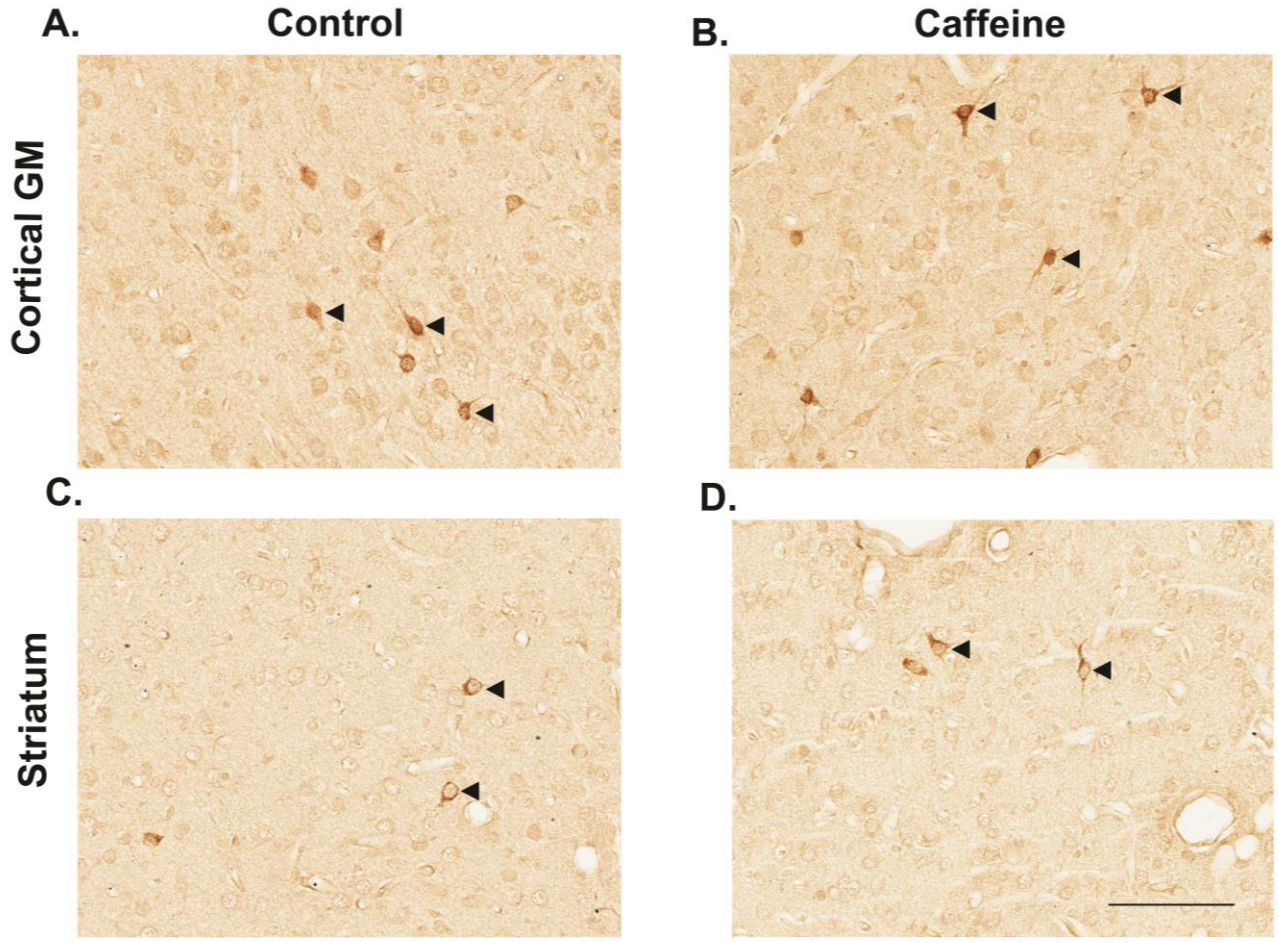


Figure 5.5 SST-IR interneurons in the cortical grey matter and striatum of control and caffeine treated fetuses

Photomicrographs of coronal sections from the cortical GM (A,B) and striatum (C,D) in control (A,C) and caffeine-treated (B,D) fetuses at 119 DG immunohistochemically stained with SST. SST-IR interneuron density was not affected by high dose caffeine compared to controls in the cortical GM or striatum. Arrowheads show SST-positive cells. Scale bar (A-D): 100 μm.

5.3.4 Dendritic spine density

There was no significant difference between groups in the linear density of pyramidal cell dendritic spines (Table 5.1; Figure 5.6).

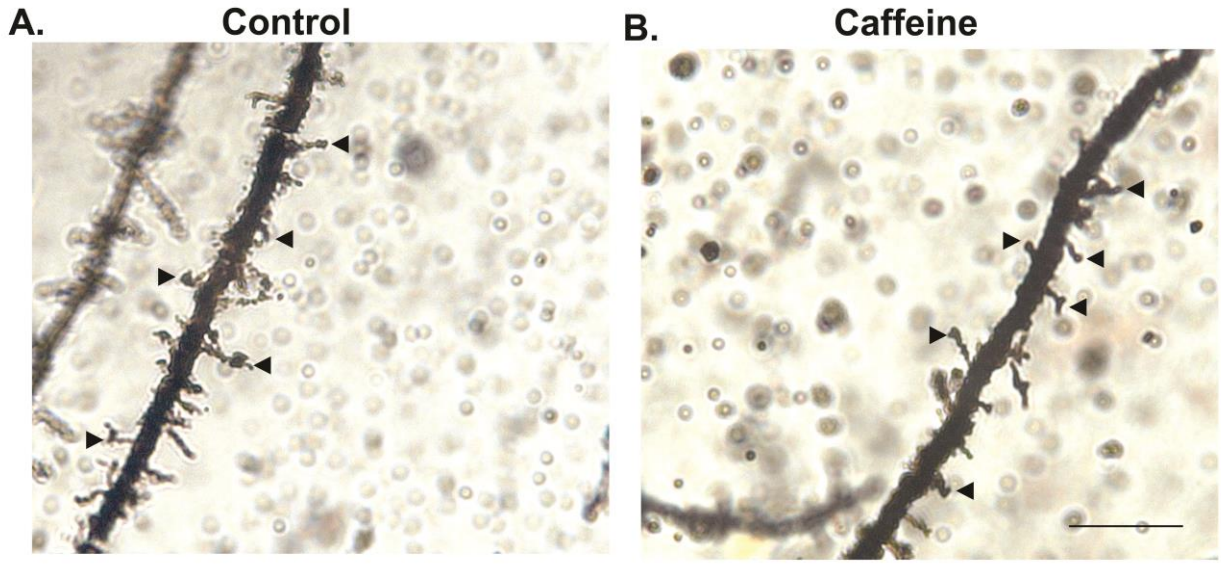


Figure 5.6 Dendritic spine density of pyramidal neurons

High power photomicrographs of coronal sections from the cortical GM in control (A) and caffeine-treated (B) fetuses at 119 DG stained with Golgi-cox stain. There was no significant difference between control and caffeine-treated fetuses in dendritic spine density per 10 μm of apical dendrite. Arrowheads show spines on apical dendrites. Scale bar=10 μm.

5.3.5 Microglia and astrocytes

The density of activated and resting Iba-1-IR microglia was combined due to the low numbers of activated microglia (control 3 ± 0; caffeine 3 ± 0) in both treatment groups. The density of Iba-1-IR microglia did not significantly differ between control and caffeine treated fetuses when assessed in the cortical GM ($p = 0.08$; Table 5.1; Figure 5.7A,B). Caffeine exposure did result in a 43% increase in the density of GFAP-IR astrocytes in the cortical GM of caffeine-treated fetuses compared to controls (Table 5.1; Figure 5.7C,D).

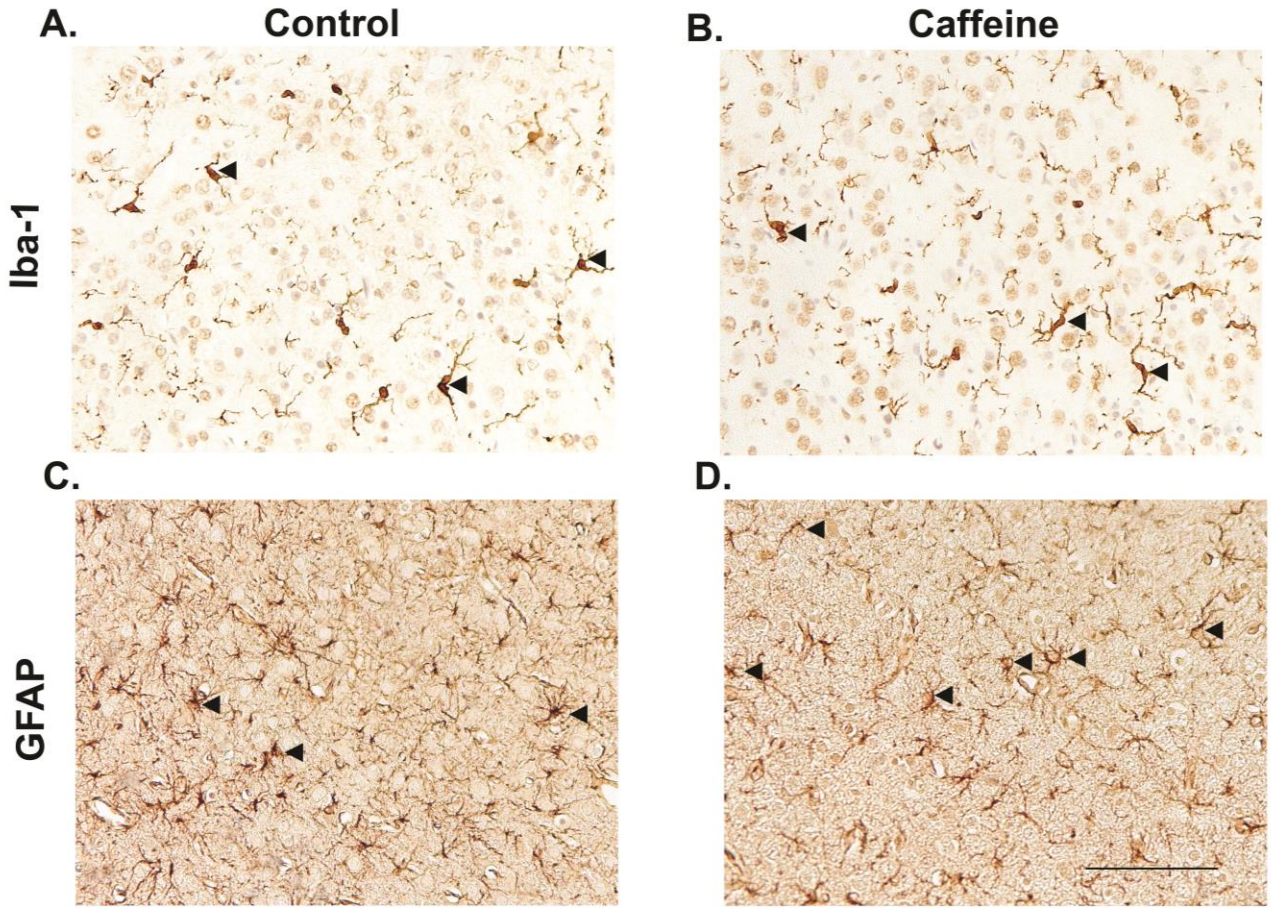


Figure 5.7 Iba-1-IR microglia and GFAP-IR astrocytes in the cortical grey matter of control and caffeine treated fetuses

Photomicrographs of coronal sections from the cortical GM in control (A,C) and caffeine-treated (B,D) fetuses at 119 DG immunohistochemically stained with Iba-1 (A,B) or GFAP (C, D). The density of Iba-1-IR microglia (resting and activated combined) was not different in caffeine-treated (B) and control (A) fetuses. There was a significant increase in the density of GFAP-IR astrocytes in caffeine-treated fetuses (D) compared to controls (C). In A and B arrowheads show resting microglia; in C and D arrowheads show astrocytes. Scale bar (A-D): 100 μm.

5.3.6 Oligodendrocytes

There was a significant increase (12%) in the density of Olig2-IR oligodendrocytes in the cortical GM following caffeine exposure compared to controls ($p=0.02$; Table 5.1; Figure 5.8A,B). The density of MBP-IR mature myelinating oligodendrocytes was similar between control and caffeine-treated fetuses (Table 5.1; Figure 5.8C,D).

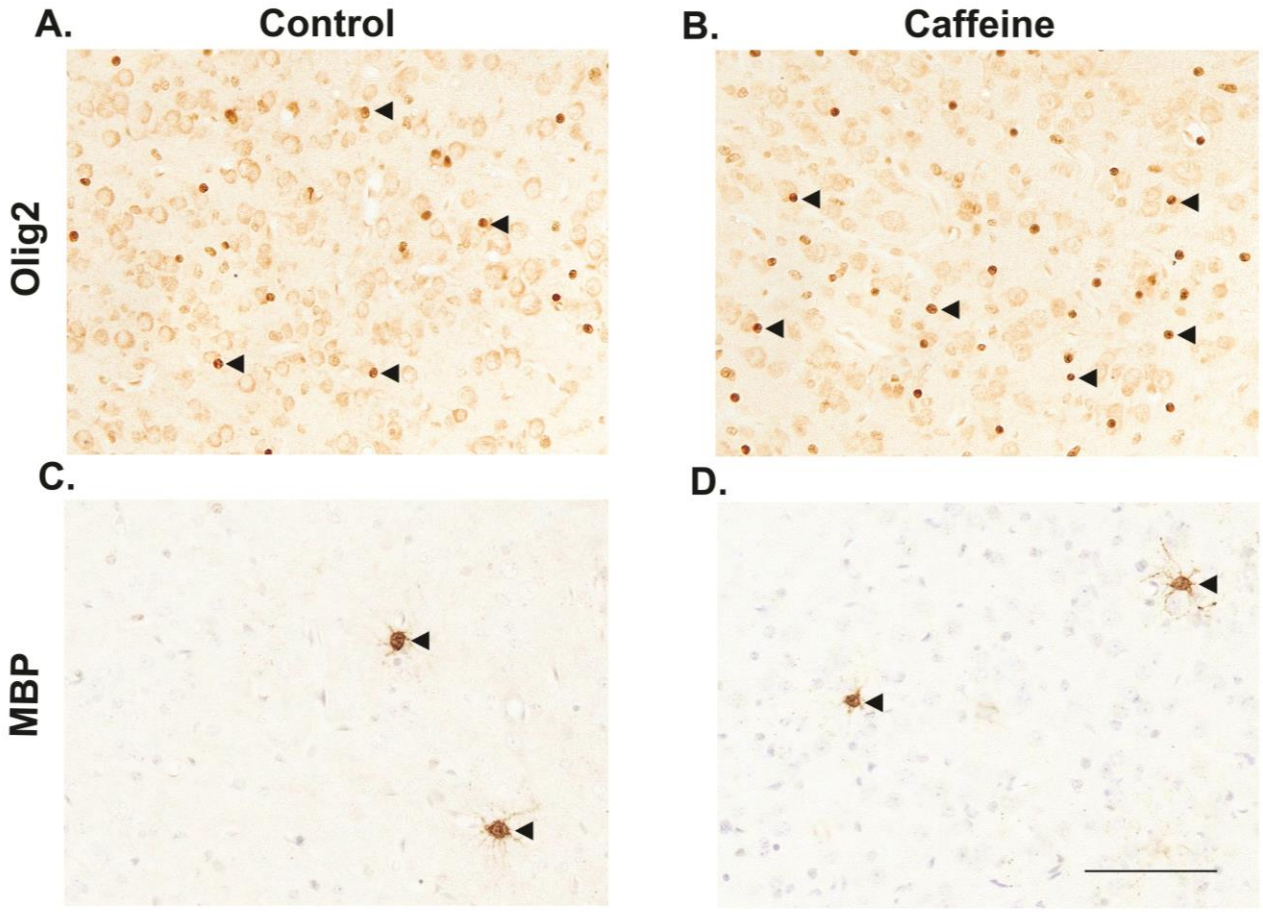


Figure 5.8 Olig2- and MBP-IR in the cortical grey matter of control and caffeine-treated fetuses

Photomicrographs of coronal sections from the cortical GM in control (A,C) and caffeine-treated (B,D) fetuses at 119 DG immunohistochemically stained with Olig2 (A,B) or MBP (C,D). There was a significant increase in the density of Olig2-IR oligodendrocytes in the cortical GM in caffeine-treated fetuses (B) compared to controls (A). The density of MBP-IR, mature myelinating oligodendrocytes was not different in the cortical GM of caffeine-treated (D) and control (C) fetuses. Arrowheads in A and B show Olig2-positive oligodendrocytes and in C and D show MBP-positive mature oligodendrocytes. Scale bar (A-D): 100 μm.

5.3.7 Apoptotic cells

There was no significant difference between groups in the density of TUNEL-positive (ie apoptotic cells) in the cortical GM (Table 5.1; Figure 5.9A,B) or striatum (Table 5.1; Figure 5.9C,D).

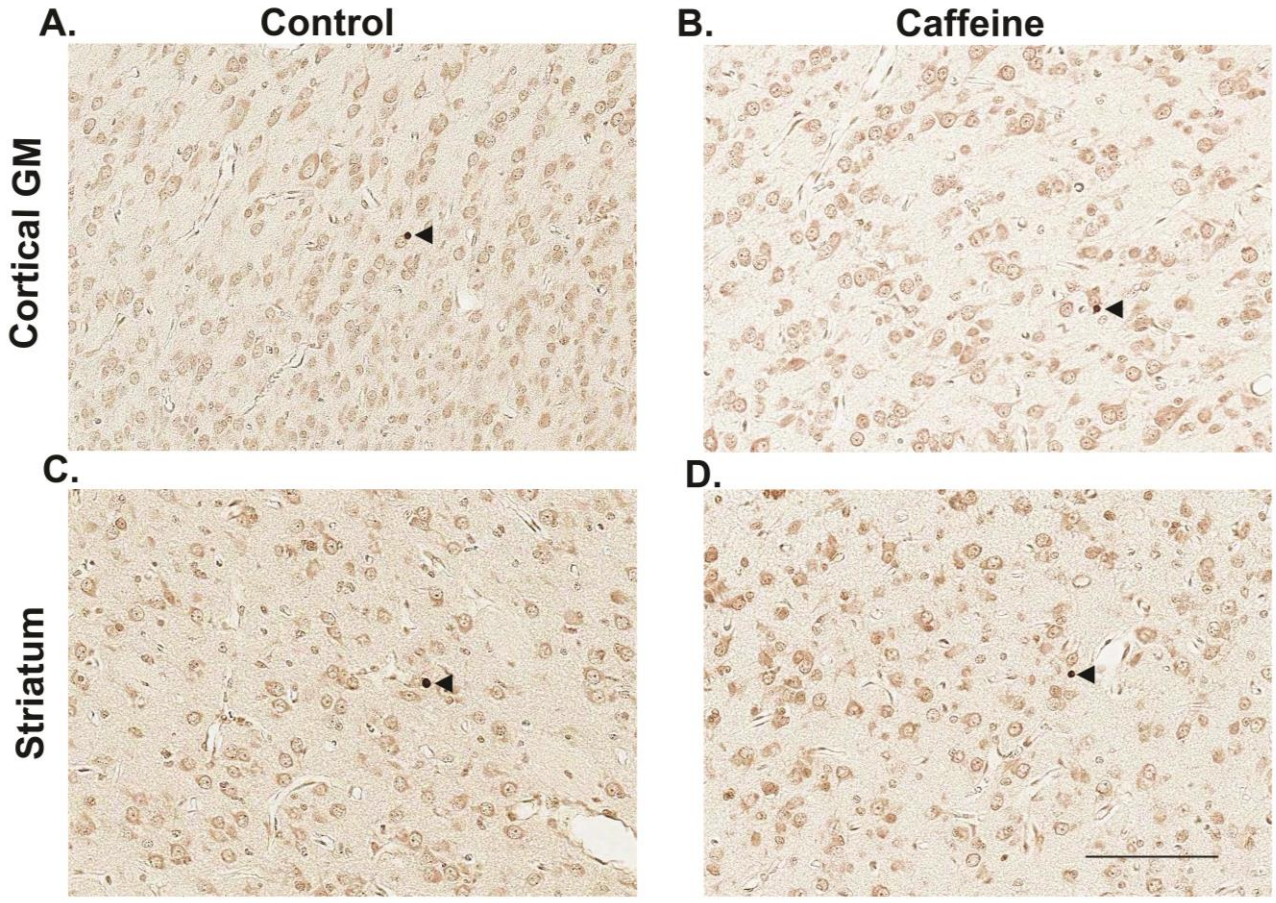


Figure 5.9 TUNEL-positive cells in the cortical grey matter and striatum of control and caffeine-treated fetuses

Photomicrographs of coronal sections from the cortical GM (A,B) and striatum (C,D) in control (A,C) and caffeine-treated (B,D) fetuses at 119 DG stained using the TUNEL assay. The density of TUNEL-positive cells in cortical GM or striatum did not differ between control (A,C) and caffeine-treated (B,D) fetuses. Arrowheads show TUNEL-positive cells. Scale bar (A-D): 100 μm.

Table 5.1 Grey matter volume and immunoreactive cell density assessment.

Parameter	Control n=8	Caffeine n=9
Cortical GM volume, cm^3	6.74 ± 0.25	7.16 ± 0.56
Striatal volume, cm^3	0.37 ± 0.05	0.41 ± 0.04
NeuN-IR neurons/mm^2 in bin 1 (cortical layer 1)	314 ± 28	334 ± 16
NeuN-IR neurons/mm^2 in bin 2 (cortical layers 2-3)	2080 ± 60	2218 ± 82
NeuN-IR neurons/mm^2 in bin 3 (cortical layer 4)	1602 ± 62	1657 ± 72
NeuN-IR neurons/mm^2 in bin 4 (cortical layers 5-6)	1419 ± 71	1502 ± 92
NeuN-IR neurons/mm^2 bins combined (all cortical layers)	1397 ± 45	1472 ± 51
NeuN-IR neurons/mm^2 in striatum	975 ± 44	1023 ± 65
Ctip2-IR neurons/mm^2 in bin 1 (cortical layer 1)	179 ± 8	195 ± 6
Ctip2-IR neurons/mm^2 in bin 2 (cortical layer 2-4)	287 ± 16	290 ± 16
Ctip2-IR neurons/mm^2 in bin 3 (cortical layer 5-6)	325 ± 24	419 ± 27*
Ctip2-IR neurons/mm^2 bins combined (all cortical layers)	285 ± 17	333 ± 16
Ctip2-IR neurons/mm^2 in striatum	704 ± 32	753 ± 33
SST-IR interneurons/mm^2 in cortical GM	15 ± 1	16 ± 1
SST-IR interneurons/mm^2 in striatum	11 ± 1	12 ± 1
Linear density of pyramidal cell dendritic spines/10 μm	7 ± 1	8 ± 1
Iba-1-IR microglia/mm^2 in cortical GM	69 ± 4	82 ± 5
GFAP-IR astrocytes/mm^2 in cortical GM	36 ± 5	63 ± 9*
Olig2-IR cells/mm^2 in cortical GM	223 ± 9	264 ± 13*
MBP-IR cells/mm^2 in cortical GM	12 ± 1	13 ± 1
TUNEL-IR apoptotic cells/10 mm^2 in cortical GM	3 ± 0	3 ± 1
TUNEL-IR apoptotic cells/10 mm^2 in striatum	12 ± 3	17 ± 3

Values are mean of means ± SEM; *$p<0.05$.

IR, immunoreactive; GM, grey matter; NeuN, NEUronal nuclei; Ctip2, chicken ovalbumin upstream promoter transcription factor interacting protein 2; SST, somatostatin; Iba-1, ionized binding adaptor molecule-1; GFAP, glial fibrillary acidic protein; Olig2, oligodendrocyte transcription factor 2; MBP, myelin basic protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

5.4 Discussion

In this chapter, the neuropathological response to high-dose caffeine was further investigated, with a particular focus on cerebral GM. Despite the absence of any alterations within the developing WM (Chapter 4) following high-dose caffeine exposure, it was considered important to determine whether there were alterations in the cerebral GM. The findings from this part of my study suggest that daily high-dose caffeine treatment does cause structural alterations in the cerebral GM. Specifically, I found that high-dose caffeine significantly increased the overall density of subcerebral projection neurons, astrocytes and the entire population of oligodendrocytes in the cortical GM, with no effect on GM volume or in the overall density of mature neurons, interneurons, microglia, mature oligodendrocytes, apoptotic cells or in the linear density of pyramidal cell dendritic spines.

5.4.1 Effect of caffeine on A_1 adenosine receptors

Here I have shown that A_1ARs are present within the fetal sheep brain at 119 DG. Specifically, A_1AR-positive cells were detected in the SCWM, PVWM, cortical GM, striatum, hippocampus and midbrain with higher intensity staining detected in the hippocampus and striatum, compared to the WM. Higher intensity staining was observed in the hippocampal CA1 and CA3 regions, with lower intensity staining in the dentate gyrus and hilus. This is consistent with findings in neonatal rats where the highest expression of A_1ARs was apparent in the hippocampus, specifically in the CA1 and CA3 regions (Etzel and Guillet, 1994; Guillet and Kellogg, 1991). My findings are consistent with previous studies showing that the A_1AR is quite widespread throughout the brain with highest abundance in the hippocampus, cerebral and cerebellar cortex and certain thalamic nuclei (Goodman and Synder, 1982; Fastbom et al., 1987a; Rivkees et al., 1995). Within the striatum, while A_1ARs have been detected at modest levels in rats (Fredholm et al., 1999; Rivkees et al., 1995), a high level of A_1AR expression within the striatum has been detected in adult humans (Fastbom et al., 1987b; Fastbom et al., 1986). As shown in humans (Fastbom et al., 1986; Fastbom et al., 1987b), I found that, in fetal sheep, the highest expression of A_1AR was in the striatum. This is likely due to species differences; the majority of AR expression studies have been conducted in rats or mice. While others have shown that the A_{2a}AR is expressed at low levels in the cortex, hippocampus and medulla, with high expression in the striatum (Fredholm et al., 1999; Dunwiddie and Masino, 2001), I have shown that, although cellular A_{2a}AR-positive staining was detected

within the fetal sheep brain, this was at extremely low levels. For this reason A_{2a}AR staining was not assessed further in my study.

When A_1AR expression was assessed in WM, high-dose caffeine was shown to cause a reduction in the number of fetuses that showed positive A_1AR staining. Within the cortical GM, the number of fetuses that showed positive A_1AR staining was similar between caffeine-treated and control fetuses; however, caffeine administration appeared to result in lower cellular intensity of A_1AR staining. High-dose caffeine did not affect A_1AR staining in the striatum of both caffeine-treated and control fetuses. While immunohistochemical analysis of the A_1AR is important in order to compare staining intensity in different regions, as well as to determine which cells express these receptors (Rivkees et al., 1995), the use of receptor autoradiography to assess caffeine's effect on A_1ARs may be beneficial as it is more sensitive to alterations in A_1AR expression (Greary and Wooten, 1989). However, this was not possible in the present study, as tissue was not collected appropriately for receptor autoradiography. Due to the greater sensitivity of receptor autoradiography (Greary and Wooten, 1989), its use may have allowed for better identification and thus analysis of A_{2a}ARs in the fetal sheep brain.

Previous studies assessing the effects of caffeine on both the A_1AR and A_{2a}AR have shown that caffeine either leads to down-regulation (Bairam et al., 2009), up-regulation (Bairam et al., 2009; Gaytan and Pasaro, 2012; Johansson et al., 1993; Gaytan et al., 2006; Marangos et al., 1984; Hunter et al., 1990) or no alteration of receptor expression within different regions of the brain (Johansson et al., 1993; Marangos et al., 1984; Etzel and Guillet, 1994). These results vary depending on the doses of caffeine administered, length of caffeine exposure and the age at the time of assessment. For example, the administration of caffeine to rats from postnatal day (P) 2 to P6 (20 mg/kg loading, 15 mg/kg maintenance dose) resulted in an increase in both A_1 and A_{2a}ARs mRNA expression within the hypothalamus at P6 (Gaytan and Pasaro, 2012). Using this same caffeine dosing regimen, with assessment at a later time point (P14), caffeine did not alter A_1AR expression in the hypothalamus, hippocampus, cortex, brainstem and cerebellum (Etzel and Guillet, 1994; Guillet and Kellogg, 1991); contrary to this finding, when rats were assessed at P28 caffeine administration was associated with an increased expression of A_1AR in the hippocampus, cortex and cerebellum, with no differences in the brainstem or hypothalamus (Guillet and Kellogg, 1991; Etzel and Guillet, 1994). Similarly, a longer duration of caffeine treatment (12-13 days) in rats,

from either P4-P27 (1g caffeine per L of drinking water) (Hunter et al., 1990) or P1-12 (50 mg/kg) (Johansson et al., 1993), increased A_1AR expression in the cortex, striatum and hippocampus. In contrast, 16 days of high-dose caffeine exposure (400 mg/kg caffeine supplementation in diet) in mice increased A_1AR expression in the cerebellum and brainstem, with no differences in the hippocampus, thalamus or cortex (Marangos et al., 1984). A longer duration (P1-23) of high-dose caffeine led to a sustained increase in A_1AR expression in the cerebellum and brainstem and additionally increased A_1AR expression in the thalamus and cortex (Marangos et al., 1984).

Taken together, these studies suggest that the effect of caffeine on adenosine receptors may be dependent on the dose of caffeine used, the duration of exposure and the interval between caffeine exposure and assessment of receptor expression. It is evident that regardless of these differences between previous studies, a consistent finding is that caffeine does affect A_1AR expression within the cerebellum, brainstem and hippocampus. Thus it would be valuable, in future studies, to assess A_1AR expression in these brain regions in tissue collected from the present study, to determine if similar results are seen within the caffeine-exposed fetal sheep brain and also to determine if high-dose caffeine leads to any alterations in neuropathology in these regions. The studies described above have concentrated mainly on A_1ARs as they are more widespread within the brain than A_{2a}ARs. However, it would be important to determine how A_{2a}ARs are affected by caffeine in order to differentiate which neurodevelopmental effects can be attributed to the A_1 or A_{2a}AR.

5.4.2 Caffeine induced alterations in neuronal cell density

The calculated volumes of the cortical GM and striatum were apparently unaffected by daily high-dose caffeine exposure. Similarly, I found no alterations in the density of mature neurons, in the cortical GM or striatum, following daily administration of high-dose caffeine. However, caffeine did lead to an increase in the number of Ctip2-positive subcerebral projection neurons in the cortical GM, with no effects in the striatum. This increase in Ctip2-expressing neurons was confined to layers V and VI, which contain neurons that project axons away from the cortex into the basal ganglia, thalamus, midbrain, hindbrain and spinal cord. Ctip2 is a transcription factor critical for axonal outgrowth and extension as well as for axonal pathfinding by cortical projection neurons (Leyva-Diaz and Lopez-Bendito, 2013; Arlotta et al., 2005). The increase in Ctip2 expressing neurons as a result of high-dose caffeine in my study may be due to either an up-regulation in Ctip2

in neurons that would normally express undetectable levels of Ctip2, an increase in the production of subcerebral projection neurons, or both.

Caffeine may also act via other transcription factors up-stream of Ctip2, such as zinc finger-like protein 2 (Fezf2), to regulate the increase in Ctip2 expression (Molyneaux et al., 2005; McKenna et al., 2011; Molyneaux et al., 2007). During development, Fezf2 is capable of instructing the birth of new neuronal subtypes from neural progenitor cells in the developing cortex and striatum (Leyva-Diaz and Lopez-Bendito, 2013; Chen et al., 2008). The expression of Fezf2 in cortical progenitor cells that are programmed to become upper layer neurons can lead to a fate change in these cells (Molyneaux et al., 2005; Chen et al., 2008). These cortical progenitor cells are then destined to become subcerebral projection neurons (Molyneaux et al., 2005; Chen et al., 2008); hence the over-expression of Fezf2 is associated with premature differentiation and overproduction of subcerebral projection neurons (Molyneaux et al., 2005). This overproduction of subcerebral projection neurons may explain the increase in Ctip2-positive cells in layers V and VI seen in this study. Since the observed increase in Ctip2-positive cells includes neurons from both layers V and VI, it is unclear whether an increase in a single layer is causing an overall increase. The future use of a layer marker such as T-box brain gene 1 (Tbr1), specific to layer VI, would help clarify if caffeine was having an effect on a specific cortical layer.

The Ctip2-positive subcerebral neurons are excitatory, glutamatergic neurons and cortical activity is dependent on a balance between excitation and inhibition (Yizhar et al., 2011). Caffeine may be causing an imbalance in this system, as I have shown in this study for the first time that daily high-dose caffeine administration during development does not affect the density of a subpopulation of GABAergic interneurons (inhibitory neurons), both in the cortical GM or striatum. It appears that, while high-dose caffeine does not lead to alterations in the inhibitory neurons of the fetal cortex, it does lead to an increase in the density of excitatory glutamatergic neurons in layer V/VI. This imbalance between excitation and inhibition could lead to both behavioural and cognitive deficits, such as those observed in disorders like autism (Yizhar et al., 2011; Kehrer et al., 2008; Rubenstein and Merzenich, 2003). Specifically, increased excitation with reduced inhibition (or no change in inhibition) can lead to a broad-range of abnormalities in perception, memory and cognition, and motor control (Rubenstein and Merzenich, 2003). It is important to determine if an imbalance between GABAergic and glutamatergic neuron density caused by caffeine persists in the long-term,

and whether this is associated with aberrant neural function. It should be noted that the inhibitory neurons assessed here (somatostatin-expressing interneurons) are only one subpopulation of GABAergic interneurons, comprising approximately 23% of the entire GABAergic interneuron population (Gonchar et al., 2007). Thus it would be beneficial to assess the effects of high-dose caffeine on other subpopulations of interneurons (e.g. calretinin, vasointestinal peptide, neuropeptide Y), as well as on a population that does not overlap with somatostatin such as parvalbumin (39% of population) (Gonchar et al., 2007) as each may be differentially affected.

5.4.3 Dendritic spines

Dendritic spines are key sites of synaptic activity (Nimchinsky et al., 2002). In the present study, high-dose caffeine did not alter the linear density of dendritic spines on cortical pyramidal cells, suggesting that high-dose caffeine may not adversely affect synapses or alter connectivity. Given that dendritic spines are a proxy measure for synapses, assessment at the ultrastructural level would offer more conclusive information on the effects of high-dose caffeine on synapse development. This assessment would be beneficial, particularly as high-dose caffeine exposure during development can cause a transient increase in synaptic proteins such as BDNF, which can influence morphology and synaptic connectivity of developing neurons (Mioranzza et al., 2014). However, it is important to note that in that study caffeine was given early in development and thus is not comparable to the ovine model used in this thesis. The changes in connectivity potentially induced by caffeine may also be reflected in the growth and complexity of dendritic processes. To date there has been only one other study that has assessed the effects of caffeine exposure on dendritic morphology. Caffeine, when administered to rat pups (50mg/kg), daily from P1-P12, led to increased dendritic length of pyramidal neurons of the prefrontal cortex with no differences in spine density when assessed at puberty (P35) and after puberty (P70) (Juarez-Mendez et al., 2006). These results align with our finding of unaltered dendritic spine density (albeit at a different developmental age), and highlight the need to assess dendritic growth in my model. Unfortunately this was not possible due to the large overlap in dendrites following the Golgi-cox stain, rendering the assessment of process outgrowth inaccurate.

5.4.4 Effects of high dose caffeine on apoptosis and astrogliosis

Administration of high-dose caffeine did not result in apoptosis within the cortical GM or striatum. Contrary to the present findings, a previous study showed that high-dose caffeine (3 doses, 50 mg/kg; base) administration in newborn rats induces apoptosis throughout the cerebral hemispheres in regions including the cortex, caudate nucleus and putamen, via caspase-3-dependent mechanisms (Kang et al., 2002). In the same study, caffeine administered to cortical cell cultures (150-1000 μM) also led to neuronal cell death in a dose-dependent manner (Kang et al., 2002). Similarly, acute high-dose caffeine (100 mg/kg; base) administered to rats at P3, resulted in increased apoptosis in various brain regions including the cortex and caudate nucleus (Black et al., 2008). The lack of apoptosis in the present study may reflect the faster clearance rate of caffeine from the fetal sheep circulation in comparison to the postnatal rodent circulation. Thus, differences between the findings of my study and previous studies are likely due to the timing and dose of caffeine administration as well as the species used.

I found that high-dose caffeine treatment did not lead to increased activation or proliferation of microglia in the ovine cortical GM. This finding of unaltered microglia and apoptosis is consistent with the observed absence of overt GM injury. While in the present study high-dose caffeine did not affect microglia it did result in astrogliosis, characterised by an increased density of GFAP-positive astrocytes. I am currently unable to discern whether the increase in astrocytes is due to increased proliferation of astrocytes or an up-regulation of GFAP expression in astrocytes that would not normally express detectable levels of GFAP. Caffeine may be inducing this increase in astrocytes via an A_{2a}AR-mediated increase in glial cell proliferation (Desfrere et al., 2007).

Previous studies that have assessed astrocyte density have shown either unaffected or reduced density. Specifically, addition of caffeine base (20 mg/L) to newborn rat primary glial cell cultures does not alter the number of type 1 astrocytes (Marret et al., 1993). On the other hand caffeine base (10-40 mg/kg) administered to mice from P3-P10 resulted in a transient and dose-dependent reduction in astrocyte density in various brain regions including the cerebral cortex (Desfrere et al., 2007). The authors suggest that this reduction in astrocyte density may have been due to the observed reduction in glial cell proliferation and not to increased programmed cell death assessed using cleaved caspase-3 immunostaining (Desfrere et al., 2007). The reduction in glial cell proliferation and astrocyte density was attributed to the antagonism of A_{2a}ARs, as opposed to

A_1ARs, as this effect of reduced proliferation and related reduction in astrocyte density could be mimicked with an A_{2a}AR antagonist and blocked with an agonist (Desfrere et al., 2007). In further support of the involvement of A_{2a}ARs in mediating astrocytogenesis, the antagonism of A_1ARs had no effect on astrocyte density (Desfrere et al., 2007).

While it is evident in previous studies that astrocytogenesis is influenced by the effect of caffeine on A_{2a}AR, this may not be the case in my model of caffeine exposure, as in fetal sheep extremely low levels of A_{2a}ARs were detected in the cortical GM of both control and caffeine-treated fetuses. Regardless, it is important to determine the cause of the astrogliosis in the present study, as astrogliosis is commonly associated with injury and the astrocytic response is thought to restore homeostasis in the damaged brain via glial scar formation, regulation of immune response, modulation of neuronal survival and neuron outgrowth (Sofroniew, 2009). Reactive astrocytes express proteins which are capable of both beneficial and detrimental effects on neuronal health, with the outcome dependent on the age as well as the type and extent of injury (Sofroniew and Vinters, 2010). It would be beneficial in future to assess the effect of high-dose caffeine on astrocytes later in life to determine if the alterations in astrocyte density persist in the long-term and what this means for neuronal health and subsequent brain function.

5.4.5 High-dose caffeine increases oligodendrocyte cell density

I have shown that daily administration of high-dose caffeine leads to an increase in the areal density of oligodendrocytes using the marker Olig2, which labels all cells of the oligodendrocyte lineage. Although the density of Olig2-IR oligodendrocytes was increased, there was no difference in the density of mature myelinating oligodendrocytes. This finding suggests that caffeine may either delay oligodendrocyte maturation, leading to an increased pool of oligodendrocyte progenitor cells (OPC) and immature pre-myelinating oligodendrocytes, or alternatively, may increase OPC proliferation. It has previously been shown that sustained activation of A_1AR leads to the inhibition of OPC proliferation, leading to a reduction in the number of oligodendrocytes (Stevens et al., 2002; Rivkees and Wendler, 2011; Back et al., 2006a). Thus antagonism of this receptor by caffeine may lead to increased OPC proliferation and an increased number of oligodendrocytes.

This effect of caffeine on oligodendrocytes is evident in models of WM injury induced by chronic hypoxia, where caffeine administration prevented the reduction in oligodendrocyte progenitors and thereby enhanced myelination (Back et al., 2006a). Although the effect of caffeine on oligodendrocytes in the absence of chronic hypoxia is still unclear, results from the caffeine for apnea of prematurity (CAP) trial suggest that standard dose caffeine is associated with improved cerebral WM development and subsequently improved neurodevelopmental outcomes (Doyle et al., 2010a). Further analysis of the oligodendrocyte lineage and cell proliferation in my model would help to determine at which developmental stage oligodendrocytes are being affected and if these effects persist in the long-term. It would also be beneficial to assess whether the alterations in oligodendrocyte density in the cortical GM are associated with changes in the WM in later life.

5.4.6 Could high-dose caffeine increase proliferation of Ctip2, GFAP and Olig2-positive cells?

The increase in subcerebral projection neurons, astrocytes and oligodendrocytes in response to high-dose caffeine may have been due to an increase in the proliferation of these cells. Previously, caffeine administered to adult mice has been shown to alter the proliferation of adult-born hippocampal neurons and this occurs in a dose dependent manner (Wentz and Magavi, 2009). The administration of 20-30mg/kg of caffeine depressed hippocampal neuron proliferation whereas a much higher dose (60mg/kg) increased proliferation (Wentz and Magavi, 2009). It is possible that there may also be a dose-response effect of caffeine on glial cell proliferation. This is apparent in studies that have shown that administration of low-dose caffeine does not alter glial proliferation (Marret et al., 1993) and moderate to high doses depress proliferation (Desfrere et al., 2007). In the present study it is possible that the high-dose caffeine may be causing an increase in proliferation of glial cells as well as subcerebral projection neurons.

It is unknown whether the alterations that I observed persist in the long-term, but evidence suggests that the increase in the proliferation of adult-born hippocampal neurons following high-dose caffeine is not sustained in the long-term (Wentz and Magavi, 2009). In my model, if the increase in subcerebral projection neurons (with no alteration in GABAergic interneurons) were to persist in the long-term this could have adverse consequences for cortical activity leading to deficits in cognition, memory and/or motor control. Additional presence of sustained alterations in glial cell

number may further hinder cognitive function (Pierson et al., 2007). However, the neurofunctional effects of the observed gliosis in the present study are still unclear. In light of these findings, the results in this chapter need to be followed up with double labelling immunofluorescence in order to determine if high-dose caffeine is causing alterations in cell proliferation and if so, in which cell types, both in the short- and long-term. Using immunohistochemical markers of proliferation such as Ki67 or proliferating cell nuclear antigen (PCNA) would also be valuable to determine if proliferation of any cell type is in fact altered in the cortical GM and more importantly within the highly proliferative zones of the brain such as, the sub-ventricular zone and dentate gyrus of the hippocampus. Due to time constraints this analysis could not be performed. It would also be important to determine what is driving this increase in cell proliferation and if it is, in fact, AR mediated.

5.5 Conclusions

Using an animal model in which the stage of brain development resembles that of the very preterm infant, I have shown that daily administration of high-dose caffeine adversely impacts on the structure of the cortical GM. Specifically, in the developing cerebral cortex, high-dose caffeine led to an increase in the density of Ctip2-positive subcortical projection neurons, GFAP-positive astrocytes and Olig2-positive oligodendrocytes, with no change in A_1AR expression in the WM or striatum and a qualitative decrease in A_1AR staining intensity in the cortical GM. An increase in Ctip2 expressing subcortical excitatory projection neurons coupled with no change in inhibitory GABAergic interneurons could cause an imbalance in cortical circuitry, the proper function of which is driven by a balance in excitation and inhibition. If this excitation/inhibition imbalance is maintained, it could lead to a range of cognitive abnormalities as well as abnormalities in motor control. High-dose caffeine could also be causing an increase in neuronal and glial cell proliferation and if these effects are sustained, they may lead to neurofunctional alterations (Sofroniew and Vinters, 2010; Le Magueresse and Monyer, 2013). This effect of caffeine on proliferation could be A_1 and A_{2a} AR meditated, and this warrants further investigation. Although I reported no adverse effects of caffeine on the developing WM (Chapter 4) it is possible that changes in the GM had not yet translated to changes in WM development at the time we assessed the brain tissue. Thus it would be valuable to assess the effects of high-dose caffeine both at a later time point following the conclusion of the experimental period and in the long-term.

6 Long-term consequences of prenatal caffeine exposure on postnatal growth and the developing ovine brain

6.1 Introduction

In the studies reported in Chapters 3 to 5, I investigated the short-term effects of high-dose caffeine on physiological changes and the development of the immature brain. Briefly, I showed that daily high-dose caffeine administration may have a direct effect on the brain rather than an indirect effect (Chapter 3) and that the developing WM was not adversely affected (Chapter 4). However, when GM development was assessed, high-dose caffeine led to an increase the density of subcerebral projection neurons, astrocytes and oligodendrocytes in the cortical GM (Chapter 5), which may have implications for cognitive function both in the short- and long-term. While most human and animal studies have concentrated on the short-term neurodevelopmental effects of high-dose caffeine, to my knowledge only two animal studies (Fuller et al., 1982; Juarez-Mendez et al., 2006) and only one human study (Gray et al., 2011) have assessed the long-term effects of high-dose caffeine on the brain following neonatal caffeine exposure. Thus a better understanding of the long-term effects of early life exposure to high-dose caffeine on body growth, and more importantly on neurodevelopmental outcomes, is required without the presence of confounding factors.

Animal studies have shown that neonatal high-dose caffeine administration has either a beneficial effect on the developing brain in the short-term and these effects persist in the long-term (Juarez-Mendez et al., 2006), or that the detrimental effects of high-dose caffeine seen in the short-term are not sustained into adulthood (Fuller et al., 1982). Thus the long-term neurodevelopmental effects of caffeine, based on animal models, remain unclear. High-dose caffeine treatment in preterm infants

has been shown to have no adverse effect on development, temperament, cognition and behaviour when infants were assessed at 1 year and 2 years of age (Gray et al., 2011). It is important to note that a thorough assessment of neurodevelopment was not performed in the Gray et al., 2011 study compared to neurodevelopmental assessments performed as part of the caffeine for apnea of prematurity (CAP) trial. When the effects of standard dose caffeine administration were assessed during the CAP trial, infants subject to neonatal caffeine treatment had improved rates of survival without neurodevelopmental disability, due to a reduced incidence of cerebral palsy and cognitive delay when assessed at 18-21 months (Schmidt et al., 2007). When these infants were assessed at 5 years of age, neonatal caffeine therapy was no longer associated with significantly improved rates of survival without disability (Schmidt et al., 2012). However, neonatal caffeine therapy led to a reduction in the rates of developmental coordination disorder (DCD), an assessment of motor dysfunction that is not associated with cerebral palsy or cognitive impairment (Doyle et al., 2014). The incidence of DCD has been associated with other problems, which include, learning disabilities and academic performance, behavioural problems, poor social skills and low self-esteem (Roberts et al., 2011; Davis et al., 2007; Dewey et al., 2002). At both follow-up ages, the studies found no significant differences in height, body weight or head circumference between infants who received neonatal caffeine therapy and those who were administered placebo (Schmidt et al., 2012; Schmidt et al., 2007). Similarly, in a separate study performed almost four decades earlier, infants who were treated with standard clinical doses of caffeine showed no differences in height, weight or head circumference at 6 or 12 months of age, compared to age matched controls (Gunn et al., 1979).

As in Chapters 3 to 5, the experiments described in this chapter were conducted in sheep, a species in which the timing of major developmental events in the brain aligns with human brain development (Rees et al., 2010; Back et al., 2002; Back et al., 2006b). In this study, my aim was to determine whether or not repeated daily high-dose caffeine administration causes long-term alterations in body and organ growth or in the structure of the developing brain. It is important to determine whether alterations to critical aspects of brain development following high-dose caffeine administration have long-term sequelae, as it is known from earlier studies in fetal sheep, that alterations in brain development can persist from fetal life to postnatal life or can become established after birth, while some changes that were present in the fetus do not persist to postnatal life (Duncan et al., 2004). In this study, the possible effects of high-dose caffeine on long-term growth was assessed by measuring body weight, body dimensions and organ weights from birth to 8 weeks of age, an age in sheep which corresponds to adolescence in humans.

Assessment of the neurodevelopmental effects of standard doses of caffeine in infants who were treated with caffeine as neonates showed that caffeine led to an improvement in cerebral WM development, with more mature cerebral WM organisation when MRI was conducted at term equivalent age (Doyle et al., 2010a); whether these positive effects are sustained into childhood or adolescence in ex-preterm infants will need to be assessed in the future. The aim of this study was to use MRI to determine the effect of high-dose caffeine on the structure of the adolescent brain focusing on the WM.

6.2 Methods

All experimental procedures were approved by the Monash University Animal Ethics Committee.

6.2.1 Surgical preparation

As for the animals used in my short-term study (Chapters 3 to 5), aseptic surgery was conducted at 99 DG (term is approximately 147 DG) on 13 date-mated ewes (Merino x Border Leicester), as described in Section 2.4. However, in the present study only maternal catheterisation was performed, which involved the chronic implantation of catheters into the maternal jugular vein for drug infusions and maternal carotid artery for monitoring maternal physiological status. Fetuses were not catheterised in order to avoid catheters obstructing labour during spontaneous birth at term. Antibiotics [Engemycin (100 mg/mL, i.v.; ewe: 5 mL) and ampicillin (1 g/5 mL; ewe: 5 mL)] were administered to each ewe for 3 days after surgery. Postoperatively, sheep were held in individual pens with access to food and water.

6.2.2 Experimental protocol

As in Chapters 3 to 5 a daily bolus dose of caffeine base (loading dose, 25 mg/kg; maintenance dose, 20 mg/kg; Sigma-Aldrich, St. Louis, MO, USA; n=9 ewes) or an equivalent volume of saline (n=8 ewes) was administered intravenously to each ewe between 104 DG and 118 DG (0.7 - 0.8 of term). Maternal blood samples were collected daily from 104-118 DG, just prior to caffeine administration, to assess maternal physiological status. This was done by measuring arterial pH, partial pressure of carbon dioxide ($PaCO_2$), partial pressure of oxygen (PaO_2), oxygen saturation

(SaO_2) and concentration of total haemoglobin (tHb), glucose (Glu) and lactate (Lac). We did not measure plasma caffeine concentrations in the ewe, as the plasma caffeine profile was previously determined in Chapter 3 (Section 3.3.1), using the same dose as in the present study.

Ewes were housed individually in large pens following the completion of caffeine administration. The ewe's carotid artery and jugular vein catheters were regularly flushed with heparinised saline (50 U/mL) to prevent blood clotting within the catheters. Ewes were placed under video surveillance for three days prior to expected delivery (term ~147 DG) in order to monitor delivery and lamb condition. After birth, lambs were closely monitored and allowed to bond with their mother. Once the lambs reached 7 days postnatal age (PNA) they were moved with their mother to a group animal holding facility at the Monash Animal Research Platform until necropsy. At 7 days PNA, the maternal jugular vein catheter was removed aseptically and the carotid artery catheter tied off as close as possible to the point of exit from the neck. The ewes and lambs were moved back to the animal housing facility 7 days prior to necropsy, allowing adequate time to adapt to their new environment.

6.2.3 Measurement of body weight and morphometry

At birth all lambs had their sex recorded and were weighed; morphometric measurements were made including crown-to-rump length (CRL), thoracic girth (TG), forelimb length (FLL). Lambs were weighed every day for the first 6 days following birth then weighed and measured weekly thereafter, with the last measurements taken just prior to necropsy. The ponderal index (PI), which characterises the relationship of body length to body weight, was calculated from morphometric measurements, using the formula $PI = body\ weight\ (g)/CRL\ (mm)^3$.

6.2.4 Necropsy

At 8 weeks PNA, each lamb was euthanized using an overdose of sodium pentobarbitone (130 mg/kg i.v.). The lambs (control, n=8 and caffeine, n=9), which included both singletons and twins, were transcardially perfused with isotonic saline and 4% PFA in 0.1M PB (pH 7.4; see Section 2.5). The brain and other organs (lung, heart, kidneys, adrenals, liver and spleen) were then weighed.

6.2.5 Magnetic resonance imaging

Following perfusion, the fixed brains underwent MRI examination using a 4.7 Tesla Bruker Avance III MRI scanner, with BGA12S2 gradient set and volume transmit/receive coil. Diffusion-weighted imaging was performed using an echo-planar imaging sequence with Stejskal-Tanner diffusion preparation (Stejskal and Tanner, 1965) and the following imaging parameters were used: repetition time =16,000 ms, echo time = 44 ms, 4 shots, acquisition matrix = 128 x 128, field of view = 7.68 x 7.68 cm^2, 64 slices with thickness 0.6 mm, 126 diffusion directions with b =1200 s/mm^2, δ = 4.5 ms, Δ = 15 ms, 10 b = 0 s/mm^2 images and 2 averages. MRI and analysis was conducted at the small animal MRI facility of The Florey Institute of Neuroscience and Mental Health (Melbourne, Australia) by a collaborator.

6.2.5.1 Magnetic resonance imaging analysis

Diffusion tensor images (DTIs) were created using the Functional MRI of the Brain (FMRIB) diffusion toolbox (FDT) (Smith, 2002) and processed with a diffusion tensor imaging-template construction (DTI-TK; (developed by Gary Zhang at Penn Image Computing and Science Laboratory, University of Pennsylvania, Philadelphia, PA, USA)), a neuroimaging, processing software that allows spatial normalization and atlas construction. Fractional anisotropy (FA) images were then generated from the aligned DTI volumes. FA, one of the measures most commonly derived from diffusion data, quantifies how strongly directional the local WM tract structure is. Furthermore, FA is a measure that is thought to reflect fibre density, axonal diameter and myelination within the white matter (Lazar et al., 2014). These FA images are then used in voxel-wise statistical analyses, in order to localise brain changes related to development, degeneration and disease. Here, voxel-wise statistical analysis of the FA data was carried out using tract-based spatial statistics (TBSS, (Smith et al., 2006)), which is part of the FMRIB, software library (FSL; developed by Analysis Group, FMRIB, University of Oxford, UK). The mean FA image was calculated and thinned to create a mean FA skeleton, which represents the centres of all tracts common to the group (Figure 6.1). Each subject's aligned FA data was then projected onto the skeleton and the resulting data were statistically analysed using the program randomise from FSL. A threshold-free cluster enhancement was applied which corrects for multiple comparisons (Smith and Nichols, 2009).

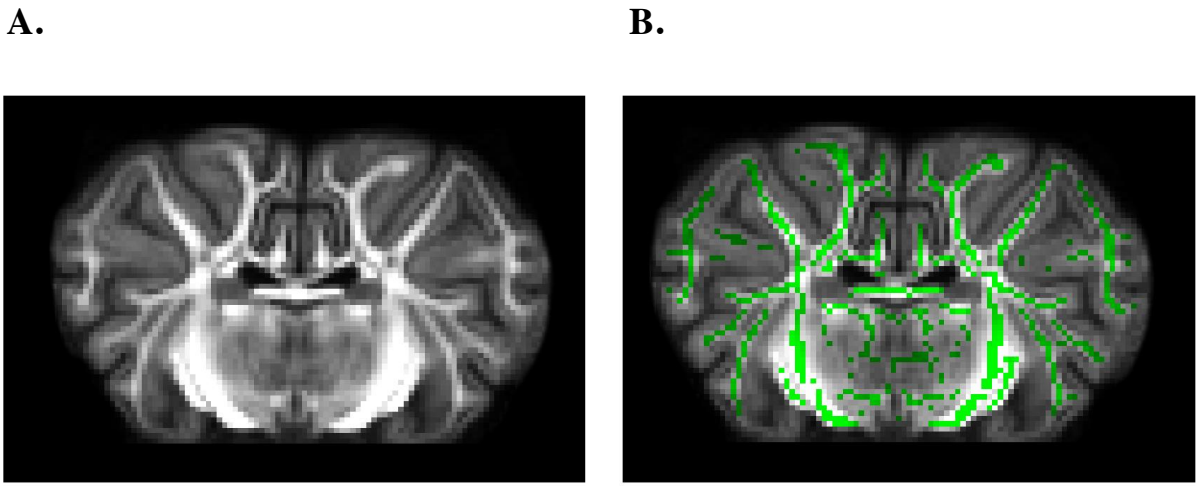


Figure 6.1 Voxel-wise analysis of the fractional anisotropy data using tract-based spatial statistics

Analysis of diffusion tensor images was performed using DTI-TK to construct an atlas or template (A) based on all animals within the study. After aligning all the Fractional anisotropy (FA) images to the common atlas, the mean FA image was calculated and thinned to make a mean FA skeleton using TBSS, which represents the centres of all the tracts common to the group (B). Each subject's FA is then projected onto the skeleton and statistical analysis using FSL was performed.

6.2.6 Statistical analysis of data

Differences between treatment groups in birth weight, gestational age at birth and organ weights were analysed by the Student's t test for parametric data or a Mann-Whitney U test (non-parametric data) if data failed a variance test (F-test). For maternal physiological data (days 1-15) and age-related changes in body morphometry, a single one-way repeated measures ANOVA (factors: treatment and day (repeated factor)) was used. Data are presented as mean ± SEM with $p<0.05$ considered significant. Statistical analysis on the results obtained from MRI data was performed using randomise from FSL, as described in Section 6.2.5.1.

6.3 Results

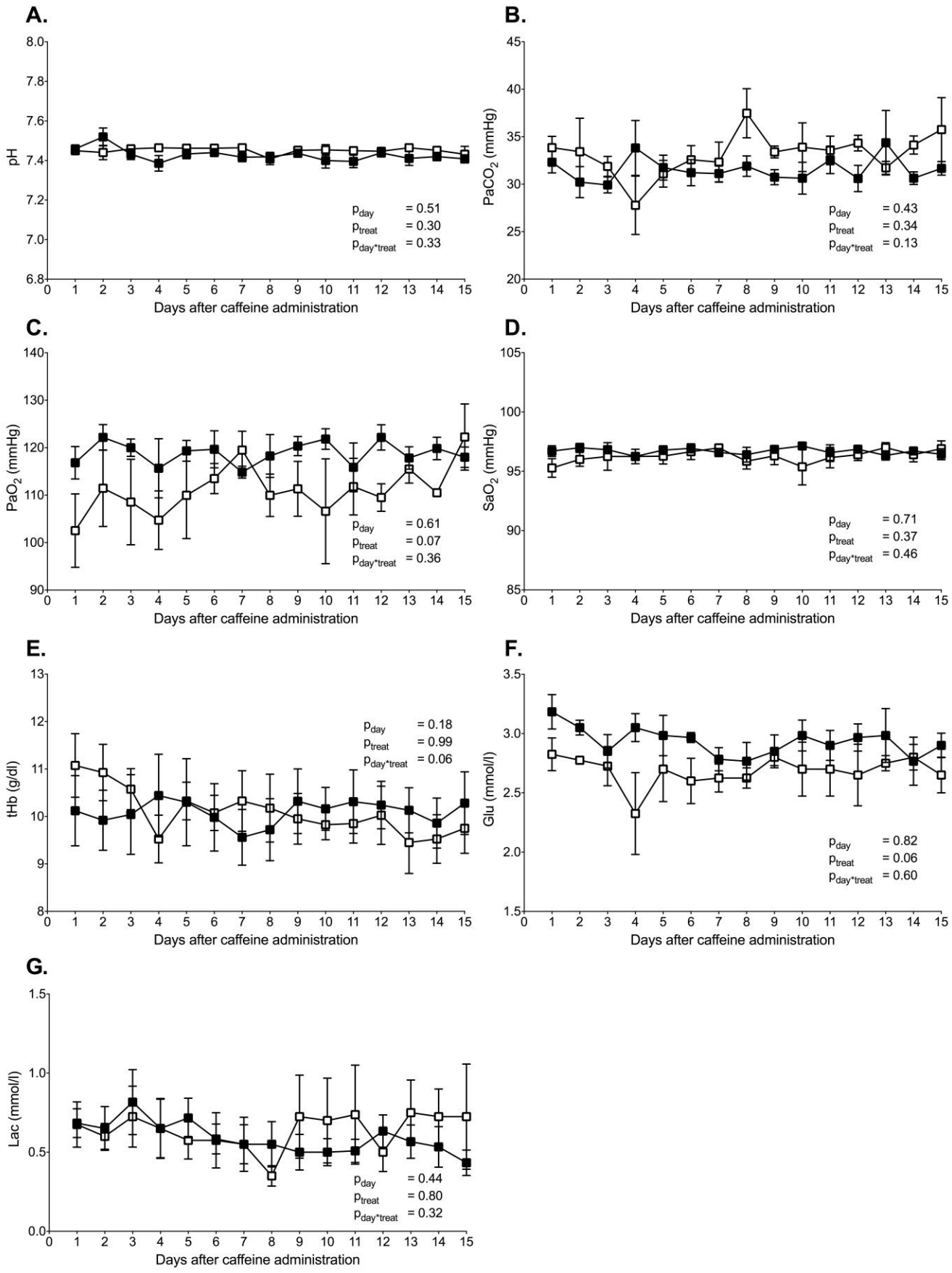
6.3.1 Maternal blood chemistry

There were no significant effects of high-dose caffeine on maternal arterial pH, $PaCO_2$, PaO_2, SaO_2, total haemoglobin, glucose or lactate concentrations, from day 1 to day 15 of caffeine administration (Figure 6.2)

.

Figure 6.2 Maternal blood chemistry

Maternal arterial pH (A), partial pressure of carbon dioxide ($PaCO_2$; B), partial pressure of oxygen (PaO_2; C), oxygen saturation (SaO_2; D), total haemoglobin (tHb; E), glucose (Glu; F) and lactate (Lac; G) from 104-118 DG between control (open squares; n=4) and caffeine-treated (closed squares; n=6) ewes. The caffeine administration was started on day 1 and the last dose of caffeine was given on day 14 with the last blood gas measurement taken on day 15, immediately prior to necropsy. Data are presented as mean ± SEM.


A.
pH
p_{day} = 0.51
p_{treat} = 0.30
$p_{day*treat}$ = 0.33
Days after caffeine administration
B.
$PaCO_2$ (mmHg)
p_{day} = 0.43
p_{treat} = 0.34
$p_{day*treat}$ = 0.13
Days after caffeine administration
C.
PaO_2 (mmHg)
p_{day} = 0.61
p_{treat} = 0.07
$p_{day*treat}$ = 0.36
Days after caffeine administration
D.
SaO_2 (mmHg)
p_{day} = 0.71
p_{treat} = 0.37
$p_{day*treat}$ = 0.46
Days after caffeine administration
E.
tHb (g/dl)
p_{day} = 0.18
p_{treat} = 0.99
$p_{day*treat}$ = 0.06
Days after caffeine administration
F.
Glu (mmol/l)
p_{day} = 0.82
p_{treat} = 0.06
$p_{day*treat}$ = 0.60
Days after caffeine administration
G.
Lac (mmol/l)
p_{day} = 0.44
p_{treat} = 0.80
$p_{day*treat}$ = 0.32
Days after caffeine administration

6.3.2 Birth weight and gestational age

There was no difference between control and caffeine-treated fetuses in birth weight (control: 5.34 ± 0.32 kg, caffeine: 5.51 ± 0.25 kg; Figure 6.3A) or gestational age after spontaneous birth at term (control: 148 ± 0 days, caffeine: 148 ± 1 days; Figure 6.3B).

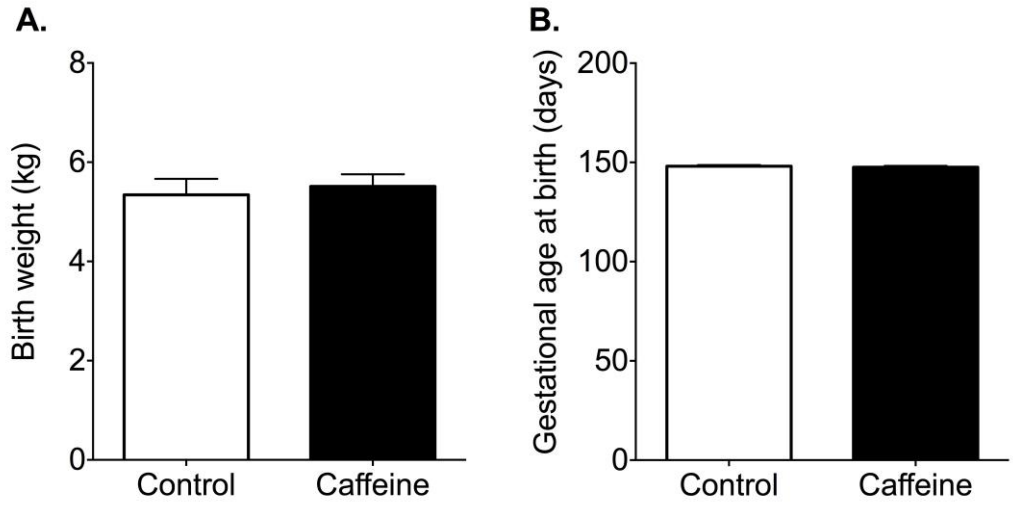


Figure 6.3 Weight and gestational age at birth in control and caffeine-treated lambs

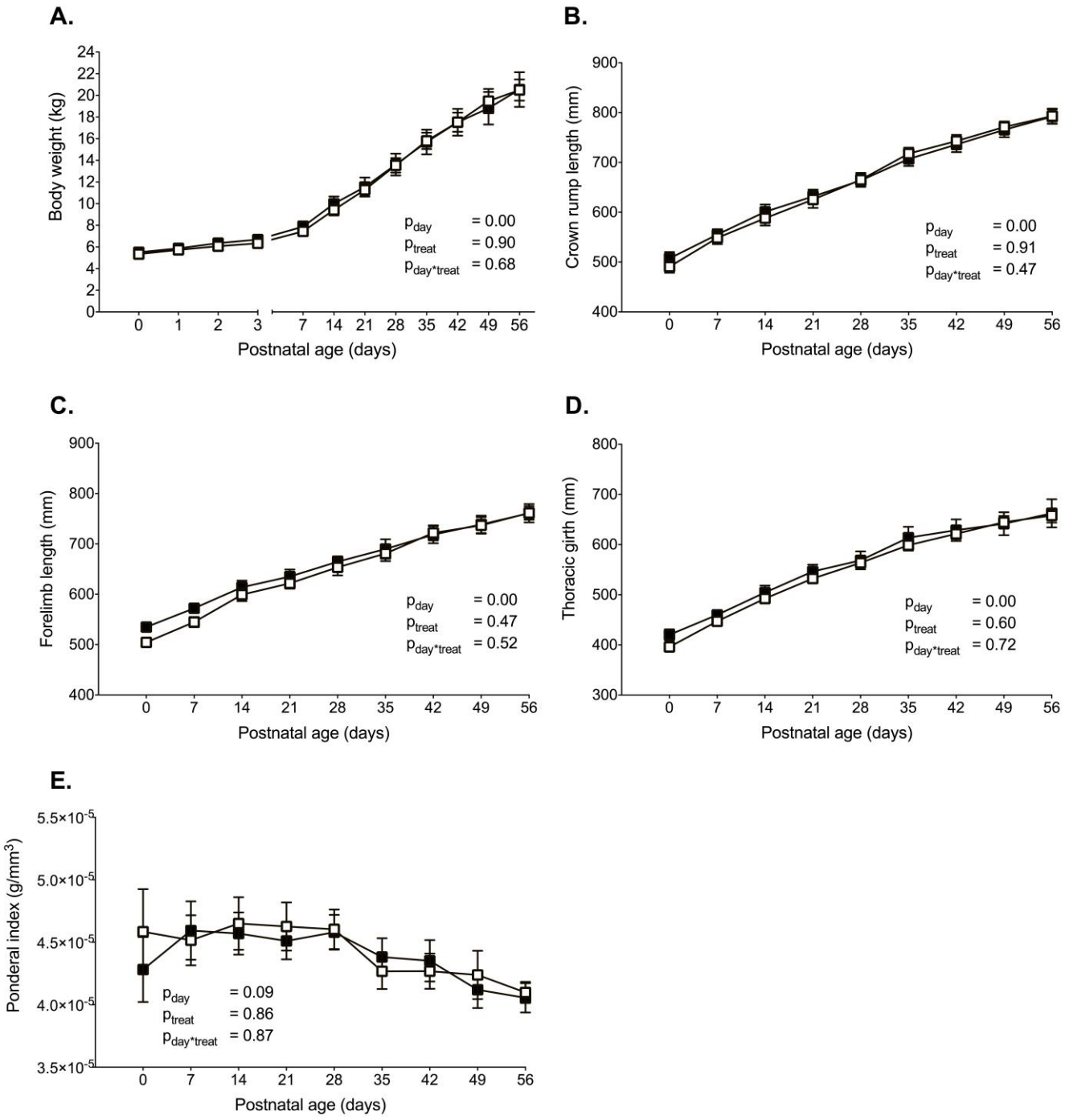
Weight (A) and gestational age (B) at birth between control (white columns; n=8) and caffeine-treated (black columns; n=9) lambs. Data are presented as mean ± SEM.

6.3.3 Body weight and morphometry

Between birth and necropsy (~56 days PNA), body weight, body dimensions (CRL, TG, FLL) and PI were similar between groups (Figure 6.4).

Figure 6.4 Age-related changes in body weight and body dimensions

Body weight (A) and body morphometry, including, crown to rump length (B), forelimb length (C), thoracic girth (D) and ponderal index (E), from birth to 8 weeks postnatal age in control (open squares; n=8) and caffeine-treated (closed squares; n=9) lambs. Lambs were exposed either to caffeine or saline from 104-118 DG, followed by term birth at ~148 DG. Data are presented as mean ± SEM.


A.
Body weight (kg)
Postnatal age (days)
p_{day} = 0.00
p_{treat} = 0.90
$p_{day*treat}$ = 0.68
B.
Crown rump length (mm)
Postnatal age (days)
p_{day} = 0.00
p_{treat} = 0.91
$p_{day*treat}$ = 0.47
C.
Forelimb length (mm)
Postnatal age (days)
p_{day} = 0.00
p_{treat} = 0.47
$p_{day*treat}$ = 0.52
D.
Thoracic girth (mm)
Postnatal age (days)
p_{day} = 0.00
p_{treat} = 0.60
$p_{day*treat}$ = 0.72
E.
Ponderal index (g/mm^3)
Postnatal age (days)
p_{day} = 0.09
p_{treat} = 0.86
$p_{day*treat}$ = 0.87

6.3.4 Organ weights

At necropsy there was a 14% reduction in total (combined) kidney weight relative to body weight in caffeine-exposed postnatal lambs compared to controls ($p=0.02$; Table 6.1). This reduction in relative kidney weight was a result of a reduction in both left ($p=0.03$; control: 2.43 ± 0.08 g/kg vs caffeine: 2.09 ± 0.12 g/kg) and right ($p=0.02$; control: 2.47 ± 0.12 g/kg vs caffeine: 2.10 ± 0.08 g/kg) kidneys relative to body weight. There was a tendency for a reduction in absolute total kidney weight in caffeine-exposed lambs compared to controls ($p=0.09$). There were no significant differences between control and caffeine-exposed lambs in all other organ weights (absolute or relative to body weight; Table 6.1).

Table 6.1 Absolute and relative organ weights at necropsy for control and caffeine-treated postnatal lambs

	Control lambs *(n=8)*	Caffeine-exposed lambs *(n=9)*
Brain, g	86.9 ± 1.4	88.0 ± 1.8
Brain/body wt, g/kg	4.39 ± 0.26	4.49 ± 0.34
Lung, g	270 ± 22	243 ± 22
Lung/body wt, g/kg	13.4 ± 1.1	12.3 ± 1.1
Heart, g	103 ± 8	101 ± 7
Heart/body wt, g/kg	5.01 ± 0.17	4.97 ± 0.14
Kidneys, g	102 ± 9	84.5 ± 4.9
Kidneys/body wt, g/kg	4.90 ± 0.19	4.20 ± 0.18*
Adrenals, g	1.62 ± 0.05	1.64 ± 0.06
Adrenals/body wt, g/kg	0.08 ± 0.00	0.09 ± 0.01
Liver, g	391 ± 20	371 ± 26
Liver/body wt, g/kg	19.2 ± 0.7	18.4 ± 0.8
Spleen, g	78.0 ± 8.2	72.0 ± 10.4
Spleen/body wt, g/kg	3.76 ± 0.27	3.49 ± 0.40

All data are mean ± SEM. *$p<0.05$, caffeine-treated compared with control lambs.

6.3.5 MRI analysis

There was no significant difference between groups in fractional anisotropy values (Figure 6.5 images). FA is a measure that is often used to reflect fibre density, axonal diameter and myelination within the white matter. Thus there appears to be no differences between groups, in white matter structure in the long-term as detected using diffusion MRI, following fetal high-dose caffeine administration.

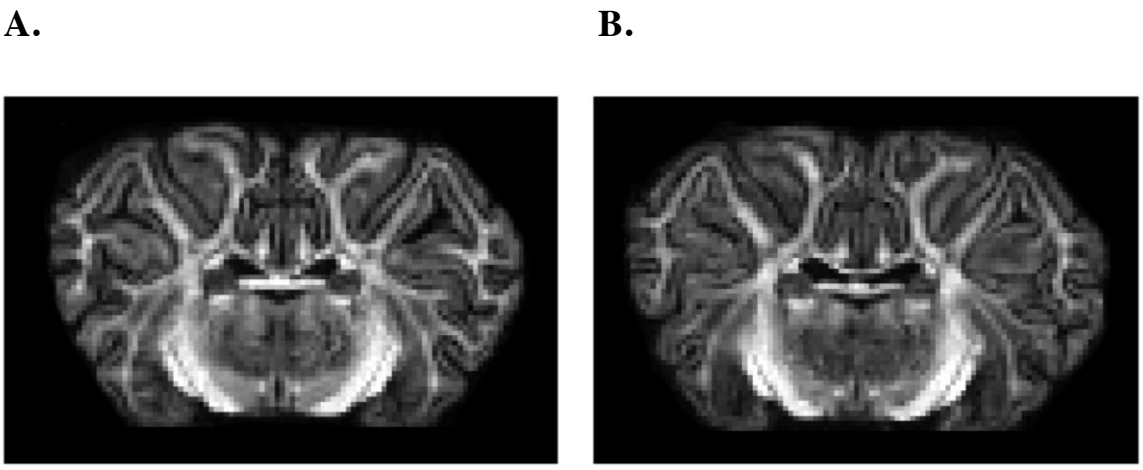


Figure 6.5 MR images obtained from control and caffeine-treated lambs

Images represent coronal MR images from equivalent areas within the brains of control (A) and caffeine-treated (B) lambs at 8 weeks postnatal age. MRI analysis was focussed on the white matter as shown in white in the image. There were no significant differences in white matter structure between control and caffeine-treated lambs.

6.4 Discussion

This is the first study to examine the long-term response to daily high-dose caffeine exposure in a long-gestation, clinically relevant animal model. The findings from this study suggest that caffeine does not alter growth postnatally or cause structural alterations in the WM as detected using diffusion MRI. Specifically we found that high-dose caffeine administration does not affect maternal blood chemistry during the administration period (104-118 DG) or the postnatal growth of caffeine-exposed offspring; nor does it alter the WM of 8 week-old offspring. However, caffeine administration did lead to a 14% reduction in combined kidney weight relative to body weight in postnatal lambs, with no significant effects on all other organs weights.

6.4.1 Effects of high dose caffeine on maternal blood chemistry

There were no significant differences in any maternal blood gas parameters over the 15 days of caffeine administration. These results are not consistent with the findings from the short-term studies (Chapter 3), where a transient increase in pH and lactate was noted, which did not persist past day 3 of caffeine administration. However, blood samples in this study were not taken as often as in the short-term studies (Chapter 3), during which more than one blood sample was analysed per day per ewe; therefore the effect of caffeine may not have been detected in this chapter. Moreover,

blood samples in this study were taken just prior to the next dose of caffeine administration each day; thus a physiological response to caffeine may not be noticeable 24 h after administration. A limitation of the present study is that the number of animals analysed per group was low due to catheter blockage in some ewes, and therefore the analysis may lack statistical power; more ewes may be required in order to accurately describe the actions of high-dose caffeine on blood chemistry. Our finding of unaltered maternal blood chemistry is consistent with the findings of a study in pregnant sheep which showed that intravenous caffeine infusion to the pregnant ewe (30 min infusion; 8 mg/kg caffeine) at 130 DG did not alter maternal blood chemistry (pH, PaO_2, $PaCO_2$, oxyhaemoglobin saturation, haemoglobin concentration or base excess) before, during or 40 min after caffeine administration (Tomimatsu et al., 2007). However, the doses used in that study were much lower than those used in the present study and the duration of caffeine administration was much shorter.

6.4.2 Effects of prenatal caffeine exposure on growth of offspring

High-dose caffeine treatment for 14 days during late gestation does not appear to adversely affect birth weight or the length of gestation. Owing to caffeine consumption by many pregnant women, the effect of caffeine on fetal growth remains of interest. The data are controversial as studies have shown that high caffeine consumption (>150 mg/day) during human pregnancy can either have no effect on birth weight (Kuzma and Sokol, 1982; Olsen et al., 1991; Fenster et al., 1991; Santos et al., 1998) or lead to lower birth weight (Fernandes et al., 1998; Furuhashi et al., 1985; Watkinson and Fried, 1985; Fortier et al., 1993). In accordance with findings of the present study, previous studies have shown that caffeine consumption (>150 mg/day) during human pregnancy does not have an effect on gestational age at birth or length of gestation (Bech et al., 2007; Jarosz et al., 2012). However, the high doses of caffeine consumed in these studies are much lower than doses used in the present study. Additionally, differences between studies may be due to differences in doses of caffeine consumed, differences the duration of high caffeine use (which are not known or estimated in some studies), and genetic risk factors.

Furthermore, we saw no effect of prenatal caffeine exposure on postnatal growth, including body weight and body dimensions. Similarly, neonatal caffeine treatment with standard doses of caffeine, did not lead to alterations in height, weight or head circumference at 6 or 12 months of age, compared to age matched controls (Gunn et al., 1979). The CAP trial found that infants who

received neonatal caffeine therapy (caffeine citrate; loading dose 20 mg/kg; maintenance 5 mg/kg) at doses lower than those used here, presented with reduced weight gain during the first 3 weeks only of caffeine treatment (Schmidt et al., 2006). At 18 to 21 months (Schmidt et al., 2007) and at 5 years of age (Schmidt et al., 2012), these infants were not different to controls in height, weight or head circumference. When assessing the postnatal effects of high-dose caffeine, studies in rats have found that pups treated from postnatal day 2 to 6 with caffeine base (loading dose 20 mg/kg; maintenance 15 mg/kg) with doses similar to that used in our study, showed similar body weight gain to controls, from birth to postnatal day 56 (Pan and Chen, 2007). Thus it appears that early caffeine exposure does not lead to alterations in long-term growth.

6.4.3 Caffeine induced changes in kidney weight

Glomerular filtration rate within the kidneys is controlled by a balance between vasodilation and vasoconstriction of renal and intra-renal blood vessels (Toth-Heyn et al., 2000); caffeine, a known renal vasodilator (Osswald and Schnermann, 2011), causes an imbalance in this system and thereby may alter renal perfusion (Osswald and Schnermann, 2011). This could lead to an increase in the functional demands of the kidney, which could potentially lead to alterations in kidney development or impaired nephrogenesis, leading to a reduction in kidney weight. To add to this, the period of caffeine administration coincides with nephrogenesis in sheep. In sheep nephrogenesis is first evident at 50 DG and after 120 DG no significant nephrogenesis occurs (Gimonet et al., 1998). By birth, kidney maturation in sheep, as in humans, is complete, with no morphological evidence of nephrogenesis detected at 140 DG in sheep (Gimonet et al., 1998; Robillard et al., 1981). Furthermore, drug treatments in preterm neonates can affect drug handling in the kidney, given the already immature drug handling system, and may alter postnatal kidney development, which could include a lower number of nephrons (Schreuder et al., 2013). Any nephron deficit at the time of nephrogenesis is likely to be permanent and a reduction in the final complement of nephrons could increase the risk of renal disease later in life (Rodriguez et al., 2005; Abitbol and Ingelfinger, 2009). Thus, the reduction in kidney weight seen in this study following caffeine administration could be the result of a reduction in the number of nephrons. However, histological assessment of the kidneys is required in order to confirm this hypothesis.

While there are no studies that have assessed the long-term effects of caffeine on kidney structure and function, there have been studies that have assessed the effects of caffeine on renal function in

the short-term. Specifically, when renal function was assessed in preterm infants prior to and after caffeine administration (15 mg/kg; caffeine citrate), there was a significant increase in urine flow rate, water output/input ratio and creatinine clearance following the administration of caffeine (Gillot et al., 1990). On the contrary, infants who were treated with lower doses of caffeine citrate (loading dose 10 mg/kg; maintenance dose 2.5 mg/kg) displayed no differences in urinary sodium excretion or creatinine clearance, but showed increased urinary calcium concentrations, urinary calcium/creatinine ratio and urinary calcium/sodium ratio after 5 days of caffeine treatment (Zanardo et al., 1995). The hypercalciuria could potentially lead to an increased risk of nephrocalcinosis and osteopenia later in life (Zanardo et al., 1995). These findings and the findings from the study presented here, highlight the need to assess the long-term renal effects of caffeine.

6.4.4 Long-term postnatal effects of caffeine on the WM

Results from our MRI analysis suggest that the WM is unaffected in the early postnatal period as a result of *in utero* caffeine exposure. Although these results are not consistent with the findings of the CAP trial, which showed improved cerebral WM development at term-equivalent age following neonatal caffeine treatment with standard doses of caffeine (Doyle et al., 2010a), in this chapter a higher dose of caffeine was administered than that used in the CAP trial and neurostructural assessment was performed much later in life. When these infants from the CAP trial were assessed at 18-21 months, caffeine treatment was associated with lower rates of neurodevelopmental disability, due to a reduced incidence of cerebral palsy and cognitive delay (Schmidt et al., 2007). Findings from the 5-year follow up of the CAP trial suggests that neonatal caffeine therapy was no longer associated with improved neurodevelopment compared to controls (Schmidt et al., 2012). Specifically, that study found no differences in the incidences of moderate or severe cognitive delay, motor impairment or behavioural problems between infants that received neonatal caffeine therapy and controls (Schmidt et al., 2012). However, new findings from the 5 year follow up of the CAP trial suggests that neonatal caffeine therapy is associated with reduced incidence of DCD, a more common motor impairment in the absence of cognitive impairment and cerebral palsy (Doyle et al., 2014). It is important to note that the neurological effects observed in these aforementioned studies were in response to standard doses of caffeine. The only study on the long-term effects of neonatal high-dose caffeine (80mg/kg loading; 20mg/kg maintenance dose caffeine citrate) administration, suggests that high-dose caffeine does not have any effect on development, temperament, cognition and behaviour (Gray et al., 2011). A limitation in this study was that a

thorough neurological assessment was not performed and the effect of caffeine on brain structure was not assessed.

Studies on the long-term neurological outcomes of high-dose caffeine are limited; thus more studies are required to further evaluate caffeine's effect on neurodevelopment in the absence of confounding factors. Moreover, the assessment of neurostructural outcome following prenatal caffeine administration in this study only included measurement of FA; thus a more thorough assessment of the WM using MRI is required. In addition, it would be important to assess the GM using MRI, as in our short-term studies we found alterations in GM development (Chapter 5) in the absence of alterations to WM (Chapter 4). The ex-vivo imaging performed in the present study has many benefits, which includes greater resolution and sensitivity due to the ability to image for longer times and lack of movement artifacts (Lerch et al., 2012). A limitation however, is that ex-vivo imaging prevents the longitudinal analysis of structural change and changes to absolute volume (Lerch et al., 2012). Thus, while it is important that we assess the brain macrostructurally using MRI, it is also important that these brains are assessed microstructurally, in particular using histology to determine if caffeine has an effect at this level. Thus the brains from this cohort have been collected and processed for further histological analysis.

6.5 Conclusions

Here I have shown that caffeine exposure during early life does not compromise birth weight or growth up to 8 weeks PNA but appears to decrease kidney weight. High-dose caffeine may be causing injury to the developing nephrons by increasing the functional demands of the kidney. Thus the long-term renal effect of caffeine in premature neonates needs to be further investigated. While high-dose caffeine exposure during early brain development does not appear to have long-term effects on WM structure of 8 week-old lambs, it is important to assess the effects of caffeine on the cerebral GM, as this brain component is critical to cognition, memory and learning. While we did not assess the brain at the microstructural level due to the absence of findings at the MRI level in the WM, the effect of caffeine on long-term brain development needs to be further evaluated to determine if its effect on the cortical GM, as shown in Chapter 5, persists postnatally and also whether there are any effects on the brain at the microstructural level. The data presented in this

chapter adds to the current literature and provides novel data regarding the effects of high-dose caffeine in the long-term.

7 General discussion

The use of high-dose caffeine to treat AOP in infants who do not respond to standard doses is now common in some neonatal units. However, despite many studies assessing the effects of standard doses of caffeine, the effects of high-dose caffeine on the developing brain remain unclear. Since adverse conditions during early postnatal life can result in altered brain development and in some cases brain injury, it is important to thoroughly assess the effects of high-dose caffeine. The studies reported in this thesis examined the impact of high-dose caffeine administration during a vulnerable stage of fetal brain development on the ovine brain. My studies focussed mainly on the cerebral hemispheres, in particular the WM, the cortical GM and striatum, as these regions are among those that are particularly vulnerable to compromise during development. Injury to, or altered development of these brain regions has the potential to lead to altered neurological function in later life, including deficits in memory and learning, motor coordination and cognition. The assessment of maternal and fetal physiological responses to high-dose caffeine administration has permitted investigation of potential mechanisms underlying any observed brain injury or altered development, as it is possible that caffeine may have an indirect effect on the brain through alterations in physiology or growth. Results from each study have been discussed in detail in Chapters 3-6; thus in this chapter I will focus on discussing the major conclusions to be drawn from these studies, strengths and weaknesses of the studies, how the major findings contribute to our understanding of the consequences of high-dose caffeine and my suggestions for future research in the field.

7.1 Our animal model for exposing the immature brain to caffeine

In order to better understand the effects of high-dose caffeine on the developing brain at a neuropathological level, studies were conducted in a species in which brain development closely resembles that of humans, and at a stage of neurodevelopment similar to that of preterm infants at risk of AOP. The stages of brain development in sheep are similar to those of humans, and importantly, the majority of brain development in sheep occurs *in utero* as it does in humans. Furthermore, unlike in rats and mice, the ratio of WM to GM is similar in sheep and humans. However, unlike the clinical setting where preterm infants receive caffeine postnatally, here caffeine was administered *in utero*. The use of fetal sheep was necessary so that the stage of brain development at which sheep were exposed to caffeine was similar to the stage of brain development at which preterm human infants are exposed to caffeine. This *in utero* model of caffeine administration has also allowed us to avoid the confounding effects of interventions that are usually required following preterm birth, such as mechanical ventilation, use of inspired high oxygen concentration and administration of glucocorticoids. To deliver caffeine to preterm lambs at the appropriate stage of brain development *ex utero*, lambs would need to be delivered as young as 104 DG (0.7 of term) and would not survive without interventions such as those stated above. Indeed, even with intensive care, lambs at this stage of gestation would not survive for sufficient time to address the aims of the study, owing to severe lung immaturity.

Nonetheless a limitation in the model used for the present study is that unlike preterm infants with apnea who experience intermittent hypoxia, fetal sheep in this study were not exposed to hypoxic episodes. Intermittent hypoxia has been described as having brief, repetitive cycles of decreases in hemoglobin oxygen saturation from a normoxic baseline, followed by reoxygenation and return to normoxia (Ruehland et al., 2009). Studies have shown that exposure to intermittent hypoxia alone can result in impairments to numerous physiological systems, such as cardiorespiratory control (Urlesberger et al., 1999; Poets, 2010; Mathew, 2011), sleep fragmentation, neuropathological and neurocognitive deficits (Ruehland et al., 2009; Nagata et al., 2000; Prabhakar, 2001; Feldman et al., 2003), decreased neuronal integrity (Douglas et al., 2007) and neuronal apoptosis (Machaalani and Waters, 2006; Ratner et al., 2007). While caffeine treatment has been shown to significantly decrease the frequency and severity of intermittent hypoxia (Jabr and Al Hazzani, 2014; Rhein et al., 2014), the combination of intermittent hypoxia and caffeine treatment may have a differential effect on the brain when compared to caffeine alone.

7.2 Caffeine's mechanism of action

Although the mechanism by which caffeine acts to reduce AOP is still largely unknown, its effects on the brain have been largely attributed to the antagonism of both A_1 and A_{2a} ARs. This is because, unlike caffeine's other molecular targets (PDE, $GABA_A$ receptors and Ca^{2+} release), antagonism of ARs requires much lower concentrations of caffeine (Fisone et al., 2004; Fredholm et al., 1999). For this reason, studies have focussed on caffeine's effects on ARs and how these receptors may act to cause changes in the developing brain. Although it is now clear how A_1 and A_{2a} AR activation and antagonism affects the adult brain, these processes may not necessarily apply to the developing brain (Cunha, 2005).

In the developing brain more is known about the neuropathological effects of A_1AR antagonism than A_{2a}ARs due to its high expression in various brain regions (Cunha, 2005). The effect of caffeine on AR expression remains controversial as studies have shown that caffeine either leads to down-regulation (Bairam et al., 2009), up-regulation (Bairam et al., 2009; Gaytan and Pasaro, 2012; Johansson et al., 1993; Gaytan et al., 2006; Marangos et al., 1984; Hunter et al., 1990) or has no effect on A_1 and A_{2a}AR expression within different regions of the brain (Johansson et al., 1993; Marangos et al., 1984; Etzel and Guillet, 1994). In this thesis I have shown that A_1 And A_{2a} ARs are expressed within the developing ovine brain, including the brain regions assessed throughout this thesis. Furthermore, while administration of high-dose caffeine did not affect the expression of A_1AR in the WM or striatum it appeared that caffeine administration resulted in lower intensity staining in the cortical GM (Chapter 5). While the use of immunohistochemistry to assess A_1AR is important for the comparison of staining intensity in different regions of the brain and to determine which cells express these receptors (Rivkees et al., 1995), the use of receptor autoradiography to assess caffeine's effect on A_1ARs may be beneficial as it is more sensitive to alterations in A_1AR expression (Greary and Wooten, 1989). A more sensitive method of detecting alterations in A_{2a}AR expression may also be beneficial, as this receptor subtype was expressed at extremely low levels in the fetal ovine brain using immunohistochemistry and thus could not be assessed (Chapter 5). The effect of caffeine on ARs appears to be dependent on the doses of caffeine administered, length of caffeine exposure and the age of assessment following caffeine administration.

7.3 Effects of exposing the developing brain to caffeine

Previous animal studies that have assessed the effects of both standard and higher doses of caffeine on the developing brain have produced conflicting results; in the short-term, the effect of high-dose caffeine can have either beneficial (Juarez-Mendez et al., 2006; Connolly and Kingsbury, 2010) or detrimental effects (Fuller et al., 1982; Desfrere et al., 2007). The beneficial effects of caffeine on the brain have been shown to persist in the long-term (Juarez-Mendez et al., 2006), while the detrimental effects of caffeine seen in the short-term do not persist in the long-term (Fuller et al., 1982). These differences are mainly related to the doses of caffeine administered, the duration of caffeine exposure and the model used. Data from human studies suggest that the standard dose of caffeine has beneficial effects in the short-term (Schmidt et al., 2006; Schmidt et al., 2007; Doyle et al., 2010a), which include lower rates of neurodevelopmental disability and cognitive delay. While these positive effects were not sustained in the long-term (Schmidt et al., 2012), standard-dose caffeine did lead to a reduction in the rates of developmental coordination disorder, a form of motor dysfunction not associated with cerebral palsy or cognitive impairment (Doyle et al., 2014). However, these long-term follow-up studies assessed the neurodevelopmental effects of caffeine using MRI or via assessment of cognitive function, and therefore cannot provide data at the cellular level. Thus animal studies assessing the neuropathological effects of caffeine exposure are highly valuable, as their findings will help guide the future imaging of the brains of preterm infants.

7.3.1 High-dose caffeine affects the cortical GM

In this thesis the fetal sheep was used as an animal model to further evaluate the neurodevelopmental effects of high-dose caffeine. I have shown that while high-dose caffeine does not adversely affect the developing WM, it does lead to changes within the cortical GM, a region critical for cognition, memory and learning (Pierson et al., 2007). Specifically administration of high-dose caffeine led to an increase in Ctip2-positive subcerebral projection neurons, GFAP-positive astrocytes and Olig2-positive oligodendrocytes. As discussed in Chapter 6, the caffeine-induced increase in Ctip2-positive subcerebral projection neurons could occur because of an increase in Ctip2 expression (possibly regulated via up-stream transcription factors) or an increase in the number of Ctip2-positive cells via increased cell proliferation. Likewise, increased proliferation could also account for the increased density of astrocytes and oligodendrocytes following caffeine exposure. It has previously been shown that caffeine does have an effect on the proliferation of neurons and glia and that this effect is dose dependent (Marret et al., 1993; Wentz

and Magavi, 2009; Desfrere et al., 2007). The effect of caffeine on neural cell proliferation has largely been attributed to activation of ARs, however it is possible that caffeine is acting on other known molecular targets such as $GABA_A$ receptors at higher than standard doses.

Caffeine may mediate its effects on $GABA_A$ receptors indirectly via neuroactive steroids such as pregnenolone, progesterone and allopregnanolone. Allopregnanolone in particular is a potent positive allosteric modulator of $GABA_A$ receptors (Lambert et al., 1995; Majewska, 1992). The administration of caffeine at doses ranging from 25 to 100 mg/kg in adult rats results in a dose dependent increase in allopregnanolone as well as pregnenolone and progesterone in both the cerebral cortex and plasma (Concas et al., 2000). An increase in allopregnanolone has been associated with a significant increase in the proliferation of cortical cells in vitro, specifically rat hippocampal neuroprogenitor cells and human neural stem cells derived from the cerebral cortex (Wang et al., 2005). Thus it is possible that high-dose caffeine is acting via allopregnanolone to increase cell proliferation in the brain. Further analysis of plasma and brain levels of neuroactive steroids, as well as neural cell proliferation, in the short-term cohort (control vs high-dose caffeine) would be of interest.

Following high-dose caffeine treatment, the density of Ctip2-positive subcerebral projection neurons increased; however there was no change in the density of somatostatin-positive GABAergic interneurons. This imbalance in the excitatory and inhibitory neurons in the developing cortical GM could potentially result in cortical dysconnectivity which may subsequently lead to behavioural and cognitive deficits (Yizhar et al., 2011; Kehrer et al., 2008; Rubenstein and Merzenich, 2003). Specifically, an increase in excitation with no change in inhibition can lead to a broad range of abnormalities in perception, memory and cognition, and motor control (Rubenstein and Merzenich, 2003). However, whether this effect of caffeine on excitatory and inhibitory neurons persists in the long-term is currently unknown. Regardless, any alteration in the normal population of neuronal or glial cells (e.g. astrocytes, oligodendrocytes) during critical periods of development could conceivably affect connectivity and potentially contribute to altered brain function in the long-term.

7.4 The physiological response to high-dose caffeine

It is known that caffeine readily crosses the blood-brain barrier (Lachance et al., 1983) and acts directly on the brain via the antagonism of adenosine receptors. Caffeine may also induce its effects indirectly by altering physiological status, specifically cardiovascular physiology and blood chemistry to result in altered nutrient availability. If high-dose caffeine does adversely affect any of these physiological parameters this could lead to alterations in cerebral blood flow, cellular metabolism, oxidative stress or neuroinflammation (Back et al., 2006b), which could have detrimental consequences for the developing brain. In this thesis, some non-neurological effects of high-dose caffeine were detected, possibly highlighting the potential for indirect effects of high-dose caffeine on the preterm infants, some of which are discussed below.

7.4.1 Metabolic effects of caffeine

Although the metabolic consequences of standard or high-dose caffeine have not been thoroughly assessed, administration of standard-doses of caffeine in preterm infant is associated with increased oxygen consumption, in the absence of changes in arterial oxygen saturation (Bauer et al., 2001). The authors suggest that the rise in oxygen consumption and energy expenditure may have contributed to the temporary reduction of weight gain seen in preterm infants treated with standard-dose caffeine (Bauer et al., 2001; Schmidt et al., 2006). Although I did not measure oxygen consumption in my studies, I have shown that high-dose caffeine administration in the ovine fetus is associated with a transient increase in fetal arterial pH and decrease in $PaCO_2$ on the first day of treatment with no alterations in body weight or morphometry (Chapter 3). While it appears that high-dose caffeine does not have adverse metabolic effects in the short-term, it is still unknown if caffeine has long-term metabolic effects. Thus further studies are required to examine both the short- and long-term metabolic effects of high-dose caffeine in preterm infants.

7.4.2 Effects on the heart

The effects of caffeine on cardiac function have been attributed to the antagonism of A_1AR located in the atrial and ventricular myocardium and sinoatrial/atrioventricular nodes (Norton et al., 1999). Pharmacological studies suggest that AR antagonism is the single most important mechanism underlying the effects of caffeine on cardiac function (Fredholm, 1984; Fredholm et al., 2001).

Caffeine antagonises the effects of adenosine on the cardiac rhythm and conduction; thus it is believed that caffeine can increase firing rate in the sinus node and increase atrioventricular nodal conduction velocity, but this has not yet been demonstrated (Fredholm, 2011). The effect of caffeine on cardiac rhythm and conductance via A_1AR antagonism is thought to be responsible for the tachycardia observed in preterm infants treated with standard doses of caffeine (Francart et al., 2013; Scanlon et al., 1992; Aden, 2011). In accordance with this finding, I have shown that high-dose caffeine led to a transient increase in heart rate on the third day of caffeine administration (Chapter 3). As heart rate was not measured after the third day of the treatment period, it is unknown if this increase in heart rate persisted. Thus further investigation of caffeine's effects on cardiac function may be beneficial, especially given that a persistent increase in heart rate could have detrimental effects on heart muscle structure and function.

7.4.3 Effects on the kidneys

Within the kidney, adenosine plays a complex role in vascular control. In afferent arterioles of the glomerulus, adenosine induces vasoconstriction which is A_1AR mediated, however in efferent arterioles, adenosine induces vasodilation via an A_{2a}AR mediated mechanism (Fredholm, 2011). Thus the blockade of ARs by caffeine can cause disturbances in glomerular circulation leading to a disrupted tubuloglomerular feedback system and increased glomerular filtration fraction (Persson, 2001; Gouyon and Guignard, 1987). This increase in glomerular filtration fraction is evident in preterm infants during a 12 h period following the administration of a single oral dose of caffeine (15 mg/kg) (Gillot et al., 1990). If these alterations in kidney function persist, they could lead to alterations in kidney development or impaired nephrogenesis. Given that very preterm infants are already born with an immature kidney, as nephrogenesis is still ongoing, caffeine may further affect their development, especially given the already immature drug handling ability (Schreuder et al., 2013). Of interest, I found that, although kidney growth was not affected in the short-term (Chapter 3), high-dose caffeine administration did lead to a reduction in kidney weight in the long-term (Chapter 6); this is suggestive of altered kidney development, although histological assessment would be required to confirm this. At the time of caffeine administration (from 104-118 DG), nephrogenesis is ongoing in the fetal sheep (Gimonet et al., 1998). As with humans, nephrogenesis in sheep is complete during the last third of gestation, at around 120 DG (Gimonet et al., 1998). This makes sheep a suitable species to study the effects of caffeine on renal development. The findings from this thesis suggest that investigations of the long-term renal effects of caffeine administration in preterm infants are warranted. As caffeine has been, and will remain, the

treatment of choice for AOP, the assessment of a dose-effect relationship between caffeine and kidney development is required.

7.5 Limitations

As discussed previously it was important to administer caffeine *in utero* to fetal sheep to assess effects on neurodevelopment at an early stage of brain development in the absence of confounding factors such as mechanical ventilation, use of inspired high oxygen concentration and administration of glucocorticoids. However, a disadvantage of *in utero* caffeine administration is that it may result in a different level of exposure compared to postnatal caffeine administration. Specifically, the presence of the placenta and maternal circulation led to more rapid clearance of caffeine from the circulation. Nevertheless, in this thesis fetal sheep were exposed to high levels of caffeine for 15 consecutive days with a peak plasma level of ~30 mg/l/d (equivalent to 154 μmol/l of caffeine). The concentration at which caffeine completely saturates A_1 and A_{2a} ARs is 100 μmol/l; thus, these receptors are likely to have been blocked by caffeine in my study. Ideally, it would be valuable to also measure caffeine concentrations in the fetal sheep brain following caffeine administration.

7.5.1 Caffeine clearance

In Chapter 3, the daily profile of plasma caffeine concentration was different in fetal sheep compared to preterm infants; this was attributed to different half-lives of caffeine in fetal sheep and the *ex utero* human infant. Fetal sheep clear caffeine from blood approximately 10 times faster than preterm infants, which would explain the different plasma caffeine concentration profiles in the two species (Chapter 3). The most likely reason for this difference is that caffeine clearance from the fetus is largely determined by maternal excretion and/or metabolism of caffeine, as blood levels of caffeine in the fetus are a reflection of maternal blood concentrations owing to the high permeability of the placenta to caffeine (Wilson et al., 1983).

In adult humans, the half-life of caffeine ranges from 2.5-4.5 h (Aranda et al., 1979a), with a plasma caffeine clearance rate of 155 ml/kg/h (Morgan et al., 1982); cytochrome P-450 is the hepatic enzyme responsible for caffeine metabolism (Shimada et al., 1994). In comparison, in the newborn

infant, clearance of caffeine occurs primarily via the kidneys and to a lesser extent by the liver; however, with increasing maturity there is a shift towards increased metabolism by the liver (Aranda et al., 1979a). In neonates the lower activity of cytochrome P-450 and the relative immaturity of some demethylation and acetylation pathways leads to a longer half-life of caffeine (Aranda et al., 1979a). Maturational changes in these enzymes result in the half-life of caffeine in full-term newborn infants being about 80 h compared to 100 h in premature infants (Aranda et al., 1979a).

In the fetus, drug clearance can occur via the placenta (and hence the mother), fetal urinary excretion and by the fetal liver, leading to a shorter half-life of caffeine and faster caffeine clearance relative to preterm neonates (Ring et al., 1999); the placental-maternal route is likely to be the most significant. It is difficult to determine whether non-maternal clearance is due to metabolism by the placenta or the fetus or both, and additionally, which fetal organs are involved. In the fetus, drug clearance is influenced by the molecular structure of the drug, the route and rate of drug administration, rate of placental transfer of the drug, rate of drug elimination and the degree of drug binding to plasma proteins (Ring et al., 1999). If drug clearance were to occur to some extent via the fetal liver, this requires the activity of cytochrome P-450. As in preterm infants, cytochrome P-450 activity has been detected at low levels in the fetal sheep liver at the same gestational age that caffeine was administered in my studies, and the activity of this enzyme has been shown to increase with gestational age (Kaddouri et al., 1990). Thus it is evident that caffeine clearance in fetal sheep is influenced by a number of factors. In order to develop a model of fetal caffeine exposure that mimics what is seen in preterm neonates, fetal sheep may require more than a single daily dose of caffeine or a continuous infusion, which would result in a sustained and longer duration of exposure.

7.5.2 Timing of post-mortem and neuropathological assessment

For the short-term survival studies reported in this thesis, fetuses were euthanized and organs collected the day after the last dose of caffeine (119 DG). In the long-term survival studies, lambs were assessed approximately 84 days after the conclusion of caffeine treatment as they were grown to 8 weeks PNA after term birth. In this long-term cohort we did not see any alterations in body or brain growth (Chapter 6); however, there was a long interval between the end of caffeine exposure

and assessment of the cerebrum. Thus it is possible that caffeine could have affected brain development between 119 DG and 8 weeks PNA and that this effect was no longer evident by 8 weeks PNA because of on going growth and development. In future studies, it will be important to assess brain development and growth between these two time points; this is relevant because findings of the CAP trial suggest that the beneficial effects of standard doses of caffeine are evident from birth to 21 months of age in infants (Doyle et al., 2010a; Schmidt et al., 2006; Schmidt et al., 2007) and that caffeine was no longer associated with improved neurodevelopmental outcomes by 5 years of age (Schmidt et al., 2012).

7.6 Future directions

7.6.1 Potential effects of different doses of caffeine or increased caffeine exposure

As discussed above, the half-life of caffeine, a reflection of plasma caffeine clearance, is different in human neonates (preterm and term) compared to fetal sheep. Thus the establishment of an alternative ovine model, with sustained caffeine exposure, is warranted. Others in our laboratory are currently assessing the effects of continuous high-dose caffeine infusion on the development of the immature brain. Results from this study will provide valuable data on how sustained high-dose caffeine exposure, similar to what is seen in preterm neonates, will affect the brain.

Previous studies that have assessed the effects of caffeine on the developing brain have done so using different doses and have produced conflicting results. In my studies, it is possible that the changes I have shown in the cortical GM as a result of high-dose caffeine are not apparent at standard or moderate doses of caffeine, and these doses may also have different effects on the developing WM. Thus it would be valuable to assess the effect of escalating doses of caffeine on neurodevelopment, particularly using sheep as an animal model. Studies like this will allow us to determine the range of doses that are safe for the developing brain.

The results of this thesis suggest that high-dose caffeine may have adverse effects on long-term heart and kidney development, indicated by altered cardiovascular function and kidney growth.

Thus more studies are required to assess the effects of high-dose caffeine exposure on the long-term development of these organs. It is possible that caffeine affects these organs and the brain differently, at different times in life, likely due to a disparity in the timing of organogenesis. Previous studies using animal models have shown that caffeine can have a beneficial or detrimental effect on the brain, and that these changes can persist (Juarez-Mendez et al., 2006) or resolve with further development (Fuller et al., 1982). Thus it would be valuable to assess the neuropathology of the brain at different time points to determine when the adverse effect of caffeine is greatest. It is also important to assess neuropathology in brain regions other than the WM and cortical GM, such as the hippocampus, thalamus, brainstem and cerebellum, as these regions may be differentially affected by high-dose caffeine.

7.6.2 Potential effects of caffeine on brain regions not investigated

A brain region that has not been thoroughly examined following high-dose caffeine exposure is the cerebellum. This is an important region of the brain as cerebellar injury or altered cerebellar development can lead to long-term neurodevelopmental deficits such as impairments in motor function (fine motor co-ordination and ataxia), cognition (verbal fluency, reading, memory and learning), as well as attention and socialisation (Powls et al., 1995; Goyen et al., 1998; Messerschmidt et al., 2008; Tavano et al., 2007; Volpe, 2009). The impact of preterm birth on cerebellar development is now being increasingly recognised (Messerschmidt et al., 2008; Pierson et al., 2007; Nosarti et al., 2008; Parker et al., 2008; Tam et al., 2009; Tam et al., 2011b; Tam et al., 2011a). However, the effects of standard or high-dose caffeine administration on the development of the cerebellum are still unknown. This understanding of how, or if, caffeine can affect the development of the cerebellum is important as studies have shown that AR expression in the cerebellum is up-regulated or unaffected by caffeine depending on the dose of caffeine administered, length of caffeine exposure and the age of assessment following caffeine administration (Guillet and Kellogg, 1991; Etzel and Guillet, 1994; Marangos et al., 1984).

In order to determine the effects of high-dose caffeine on the developing cerebellum, the cerebellum of each control and caffeine-treated fetus from the short-term cohort has been analysed as part of an Honours project in the Tolcos laboratory. The preliminary findings from this analysis suggest that high-dose caffeine may have developmental consequences for the cerebellum (Boomgardt et al.,

2014 Journal of Paediatrics and Child Health 50 (Suppl 1) A292, p35). Specifically, administration of high-dose caffeine resulted in a 10% reduction in Purkinje cell body size, with no alteration in the areas of the cerebellum, molecular layer, internal granular layer or WM. In addition, high-dose caffeine did not alter the width of the external granular layer or the surface foliation index. Similarly, the areal density of microglia, astrocytes and oligodendrocyte and the optical density of myelin basic protein or neurofilament in the WM were not adversely affected. Although high-dose caffeine did not appear to affect overall growth or overtly injure the developing cerebellum, it may have disrupted the development of Purkinje cells, the primary output cells of the cerebellum. Thus further analysis to determine whether arborisation of the Purkinje cells is affected and whether caffeine alters cerebellar connectivity is currently underway. These preliminary results suggest that future studies should assess the cerebellum, as well as other brain regions vulnerable to injury in preterm infants such as the hippocampus and thalamus (Inder et al., 2005a; Inder et al., 2003a; Peterson et al., 2000; Isaacs et al., 2000).

7.7 Conclusions

The use of fetal sheep as an animal model has allowed the effects of high-dose caffeine on brain development to be assessed in a clinically relevant species in which brain development is comparable to human brain development. The results from this thesis indicate that fetal or maternal physiology is not adversely affected by high-dose caffeine and that any effect of caffeine on the brain is likely a direct effect possibly mediated via A_1ARs. High-dose caffeine did not cause any structural alterations to the WM but did so to the GM. Here high-dose caffeine significantly increased the density of Ctip2-positive subcerebral projection neurons, GFAP-positive astrocytes and Olig2-positive oligodendrocytes in the cortical GM; there was no effect on somatostatin-positive GABAergic interneurons. It is possible that high-dose caffeine affects proliferation of these cells, thus further analysis is required to determine if this is the case. An alteration in the density of excitatory neurons (subcerebral projection neurons) with no effect on inhibitory neurons (GABAergic interneurons) may have adverse neurofunctional outcomes; further analysis is required to determine if these alterations in the cortical GM persist in the long-term. While I only investigated the WM in the long-time using diffusion MRI and found no adverse neurostructural effects of caffeine, assessment of both WM and GM structures at the cellular level is still required. Prenatal high-dose caffeine administration did not affect long-term growth, however it did significantly affect kidney weight, the cause of which is unknown but may be associated with

alterations in kidney growth particularly nephrogenesis. These results add to the current literature on the safety of high-dose caffeine and provide substantial evidence that daily administration of high-dose caffeine can subtly affect the cerebral cortex (and the cerebellum) of the immature brain. We can never mimic the exact situation in very preterm infants being treated for AOP, nevertheless this thesis provides a model that may help us better understand the effects of high-dose caffeine; an ovine model for sustained caffeine exposure is also currently being established in our laboratories. It is clear that caffeine is less harmful and more effective than other methylxanthines (Henderson-Smart and Steer, 2010) thus it will remain the drug of choice when treating AOP. Moreover, infants will continue to need high-dose caffeine if they do not respond to standard doses and to minimise their time spent on mechanical ventilation, which alone is associated with adverse neurodevelopmental outcomes (Henderson-Smart and Steer, 2001; Donn and Sinha, 2006; Schmidt et al., 2003). Thus the results from this thesis have provided the first step towards understanding what doses of caffeine are safe for the preterm brain; future studies must now focus on determining the maximum dose of caffeine administration with minimal, or better still, no adverse effects.

8 References

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Appendix 1 **Atik A.,** Cheong J., Harding R., Rees S., De Matteo & Tolcos M. (2014). Impact of daily high-dose caffeine exposure on developing white matter of the immature ovine brain. *Pediatric Research* **76**, 54-63.

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Impact of daily high-dose caffeine exposure on developing white matter of the immature ovine brain

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BACKGROUND: Caffeine is widely used to treat apnea of prematurity, but the standard dosing regimen is not always sufficient to prevent apnea. Before higher doses of caffeine can be used, their effects on the immature brain need to be carefully evaluated. Our aim was to determine the impact of daily high-dose caffeine administration on the developing white matter of the immature ovine brain.

METHODS: High-dose caffeine (25 mg/kg caffeine base loading dose; 20 mg/kg daily maintenance dose; $n = 9$) or saline ($n = 8$) were administered to pregnant sheep from 0.7 to 0.8 of term, equivalent to approximately 27–34 wk in humans. At 0.8 of term, the white and gray matter were assessed histologically and immunohistochemically.

RESULTS: Daily caffeine administration led to peak caffeine concentration of 32 mg/l in fetal plasma at 1 h, followed by a gradual decline, with no effects on mean arterial pressure and heart rate. Initial caffeine exposure led to transient, mild alkalosis in the fetus but did not alter oxygenation. At necropsy, there was no effect of daily high-dose caffeine on brain weight, oligodendrocyte density, myelination, axonal integrity, microgliosis, astrogliosis, apoptosis, or neuronal density.

CONCLUSION: Daily high-dose caffeine administration does not appear to adversely affect the developing white matter at the microstructural level.

Caffeine is used to stimulate breathing in very preterm infants, especially those who develop apnea of prematurity (AOP). AOP occurs in approximately 85% of infants born prior to 34 wk (1), and its incidence is inversely related to gestational age (2). Typically, AOP is treated with caffeine (or other methylxanthines) for 4–6 wk, until at least 32–34-wk postmenstrual age, or until the apnea has abated (3). An international randomized-controlled trial has confirmed that caffeine is an effective treatment for AOP, leading to reduced morbidity and mortality (4,5). In treating AOP, the standard dosing regimen of caffeine is a loading dose of 20 mg/kg (caffeine citrate) followed by a daily maintenance dose of 5–10 mg/kg (4).

The standard clinical dose of caffeine is not always sufficient to abolish AOP (6,7), potentially leading to the use of higher doses. However, little is known regarding the effects of higher doses of caffeine on the immature brain, in particular its effects on the development of white matter (WM), a brain component that is very vulnerable to injury in preterm infants (7,8). Although several studies have assessed the effects of high-dose caffeine on the developing brain, these studies show both beneficial (9,10) and detrimental (11,12) effects. The majority of previous studies have been conducted in rats and mice (9–12), species in which white to gray matter (GM) ratio is not comparable to humans and in which brain development occurs predominantly postnatally. Furthermore, previous studies have used widely differing dosing regimens. Given the conflicting experimental data on the effects of caffeine on the developing brain, it is difficult to draw unequivocal conclusions about the likely effects of high-dose caffeine on the developing WM of preterm infants.

We have undertaken experiments in sheep, a species in which the timing of major developmental events in the brain, including WM development, aligns with human brain development. In order to expose the developing brain to caffeine at a similar stage of WM development as in preterm infants, we have used fetal sheep at 0.7–0.8 of term; at this age, WM development is similar to that of preterm infants at about 27–34-wks postmenstrual age (13–15), and thus represents the typical age at which preterm babies are exposed to caffeine (5) and when the cerebral WM is still developing (14). Our objective was to determine whether or not repeated daily high-dose caffeine administration causes structural alterations to the developing WM and GM in the immature ovine brain; we also assessed the effects on organ growth and physiological status. Potential effects of caffeine on the developing WM were assessed using oligodendrocyte transcription factor 2 (Olig2) to identify the entire pool of oligodendrocytes, myelin basic protein (MBP) to identify mature myelin, and the pan-axonal neurofilament marker (SMI-312) for axonal integrity. The nature of the glial cell response (microgliosis and astrogliosis), an indicator of

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WM injury (16,17), was assessed using ionized binding adaptor molecule-1 (Iba-1) for microglia and glial fibrillary acidic protein (GFAP) for astrocytes. Apoptosis in the developing WM, cortical GM, and striatum was assessed using terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling (TUNEL). Finally, we assessed neuronal density in the cortical GM using the neuron-specific protein NEUronal nuclei (NeuN).

RESULTS

Plasma Caffeine Concentration and Fetal Cardiovascular Data

On the first day of caffeine administration, fetal plasma caffeine concentration reached 32 ± 1 mg/l at 1 h, decreasing to 19 ± 3 mg/l at 6 h and 5 ± 2 mg/l at 24 h (prior to the next injection, Figure 1a). Maternal plasma caffeine concentration reached 43 ± 9 mg/l at 1 h, decreasing to 24 ± 5 mg/l at 6 h and 5 ± 1 mg/l at 24 h (Figure 1a). This concentration profile was similar for the next 2 d of caffeine administration (only days 1–2 shown). On days 1–3 of caffeine administration, there were no significant differences in plasma caffeine concentrations between the maternal and fetal circulation at 1, 6, or 24 h ($P_{treatment}$ = 0.43).

Fetal mean arterial pressure remained stable following caffeine administration and was not significantly different at 1, 6, or 24 h postcaffeine administration between the control and the caffeine-treated groups from day 1 to 3 ($P_{treatment}$ = 0.61; only days 1 and 2 shown; Figure 1b). Caffeine administration did not affect fetal heart rate at 1, 6, or 24 h postcaffeine administration from day 1 to 3 ($P_{treatment}$ = 0.18; only days 1 and 2 shown; Figure 1c).

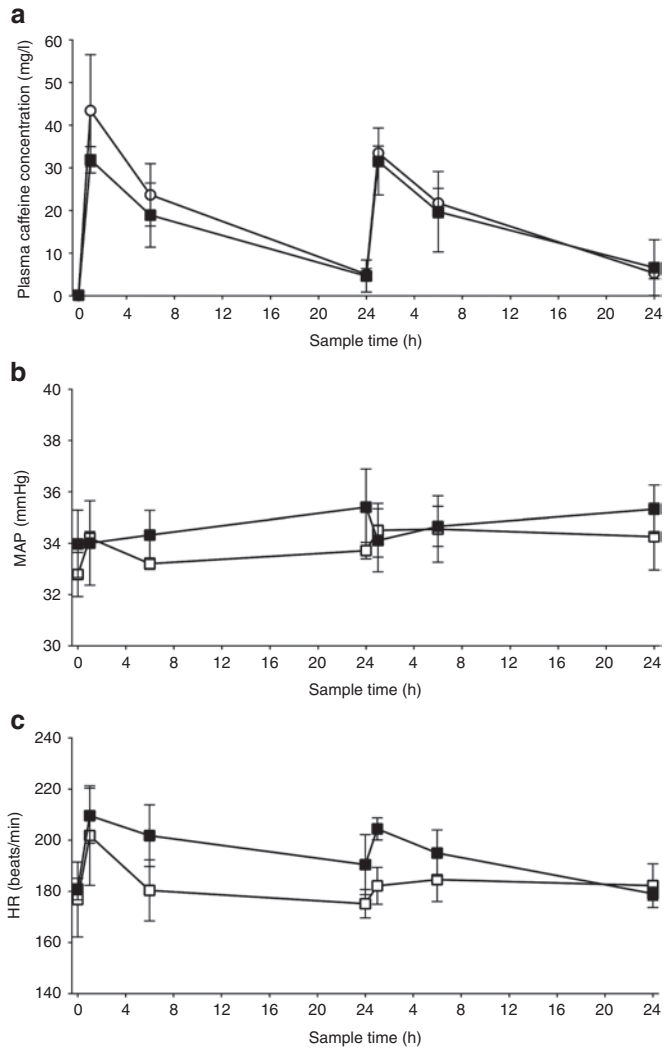

Figure 1. Maternal and fetal plasma caffeine concentrations (**a**), fetal mean arterial pressure (MAP) (**b**), and heart rate (**c**) on day 1–2 of caffeine administration. There were no significant differences in plasma caffeine concentrations between the maternal (open circles; $n = 2$) and fetal (closed squares; $n = 6$) circulation at 1, 6, or 24 h after caffeine administration from day 1 (loading dose: 25 mg/kg) to day 2 (maintenance dose: 20 mg/kg). There was also no significant difference in fetal arterial pressure or heart rate at 0, 1, or 6 h between the control (open squares; $n = 3$) and caffeine-treated (closed squares; $n = 4$) fetuses from day 1 to 2.

Fetal and Maternal Blood Chemistry

On day 1 of the treatment period (104 d of gestation (DG)), there was a small but significant effect of caffeine over time in fetal arterial pH ($P_{time \times treatment}$ = 0.04) and $PaCO_2$ ($P_{time \times treatment}$ = 0.005); caffeine-treated fetuses had a transiently higher pH and lower $PaCO_2$ (Figure 2a,b) than control fetuses. On days 2 and 3, there were no significant differences between groups in pH (day 2, $P_{treatment}$ = 0.76; day 3, $P_{treatment}$ = 0.90) and $PaCO_2$ (day 2, $P_{treatment}$ = 0.90; day 3, $P_{treatment}$ = 0.74) measured just prior to caffeine administration (0 h) and at 1-, 2-, 4-, and 6-h postcaffeine. Between days 1 and 3, there were no significant effects of caffeine on fetal PaO_2 ($P_{treatment}$ = 0.14; Figure 2c), arterial oxygen saturation ($P_{treatment}$ = 0.42; Figure 2d) or hemoglobin ($P_{treatment}$ = 0.70; Figure 2e), glucose ($P_{treatment}$ = 0.80; Figure 2f), and lactate ($P_{treatment}$ = 0.32; Figure 2g) concentrations. From days 4 to 15, there were no significant differences between groups in any measured fetal blood variable (data not shown).

Caffeine administration led to a transient increase in maternal arterial pH ($P_{time \times treatment}$ = 0.04) on day 1 of the treatment period (104 DG), with no significant effects thereafter (data not shown). From 104 to 118 DG, there were no significant effects of caffeine on maternal $PaCO_2$, PaO_2, arterial oxygen saturation or glucose, and hemoglobin concentrations. Caffeine administration led to a significant increase in maternal lactate concentration ($P_{day \times treatment}$ = 0.008) from day 1 to 3 of treatment, with no significant effects subsequently.

Body and Organ Weights

At necropsy, there were no significant differences between control and caffeine-treated fetuses in body weight, brain weight, brain-to-body-weight ratio, crown-to-rump length, thoracic girth, hind limb length, head length, ponderal index, and other organ weights (absolute or relative to body weight; Table 1).

Structural Analysis of Cerebral WM

There was no evidence of intracerebral or intraventricular hemorrhages in control or caffeine-treated fetuses. The density of oligodendrocytes was similar in control and caffeine-treated fetuses when assessed in the subcortical (P = 0.92) and

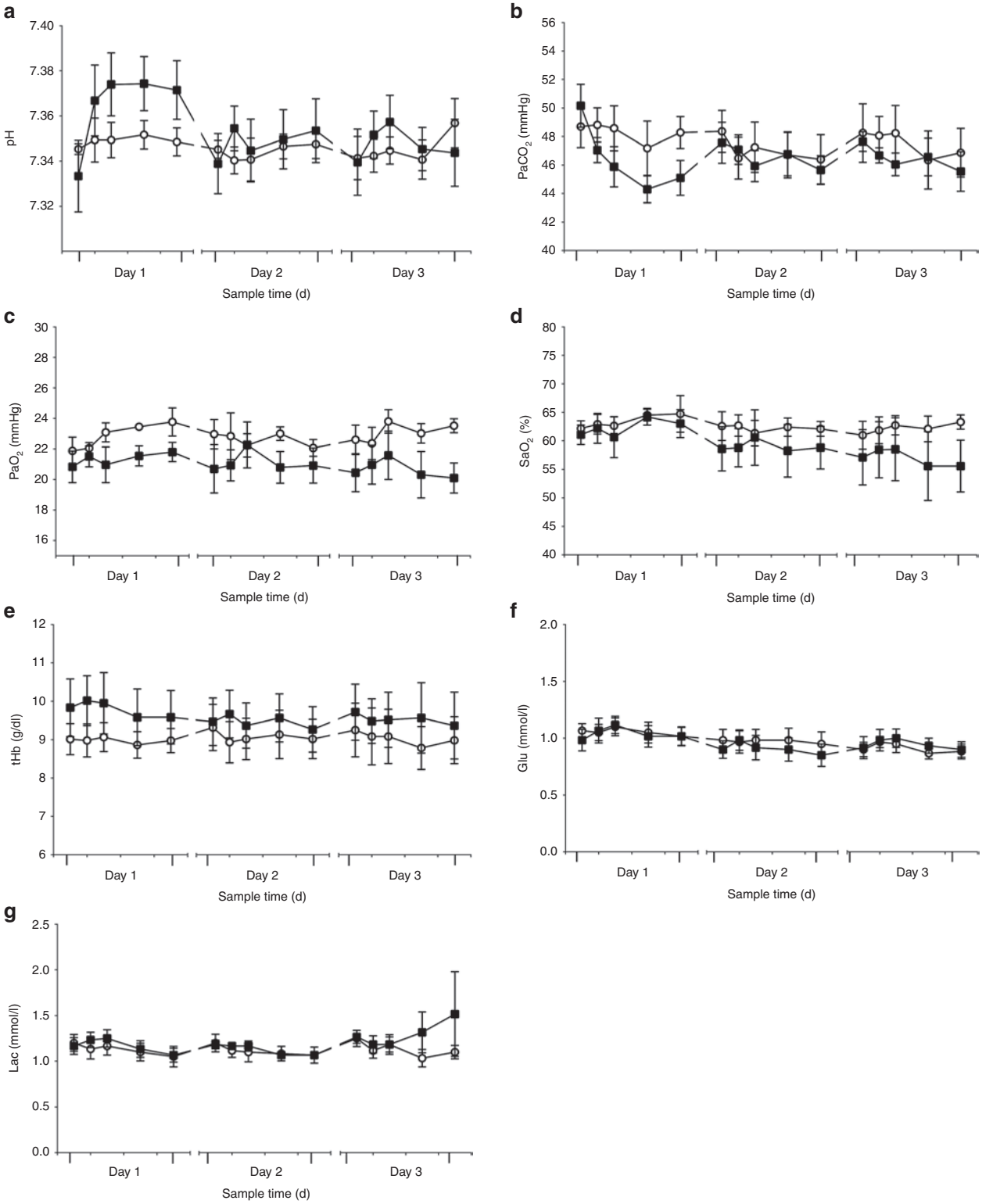

Figure 2. Arterial pH (**a**), $PaCO_2$ (**b**), PaO_2 (**c**), arterial oxygen saturation (SaO_2; **d**), total hemoglobin (tHb; **e**), glucose (Glu; **f**), and lactate (Lac; **g**) in control (open circles; $n = 5$) and caffeine-treated (closed squares; $n = 6$) fetuses. On day 1 of treatment (104 d of gestation (DG)), there was an effect of caffeine over time for arterial pH ($P_{time \times treatment} = 0.04$); caffeine-treated fetuses had a transiently higher pH, with no significant differences from 105 to 118 DG. There was a significant effect of caffeine over time for arterial $PaCO_2$ ($P_{time \times treatment} = 0.005$) on day 1 of treatment (104 DG); caffeine-treated fetuses had a lower $PaCO_2$ compared with controls, with no significant differences from 105 to 118 DG. There were no significant differences in the fetal PaO_2, SaO_2, tHb, Glu, and Lac concentrations from 104 to 118 DG.

Table 1. Necropsy data for control and caffeine-treated fetuses

	Control ($n = 8$)	Caffeine ($n = 9$)
Body weight, kg	2.48 ± 0.13	2.36 ± 0.14
CRL, mm	429 ± 7	418 ± 9
TG, mm	282 ± 7	285 ± 6
HLL, mm	342 ± 9	350 ± 7
HL, mm	113 ± 1	113 ± 1
PI, g/mm^3	$3.13 \times 10^{-5} \pm 1.08 \times 10^{-6}$	$3.22 \times 10^{-5} \pm 1.15 \times 10^{-6}$
Brain, g	38.1 ± 0.4	42.2 ± 1.7
Brain/body weight, g/kg	15.7 ± 0.9	18.3 ± 0.9
Lung, g	90.8 ± 5.5	82.8 ± 4.9
Lung/body weight, g/kg	36.7 ± 1.5	35.4 ± 1.6
Heart, g	17.8 ± 0.9	16.5 ± 1.7
Heart/body weight, g/kg	7.21 ± 0.23	6.93 ± 0.59
Kidney, g	16.3 ± 1.0	16.6 ± 0.9
Kidney/body weight, g/kg	6.61 ± 0.35	7.11 ± 0.23
Adrenals, g	0.28 ± 0.04	0.33 ± 0.03
Adrenals/body weight, g/kg	0.11 ± 0.01	0.14 ± 0.01
Liver, g	91.5 ± 8.5	83.7 ± 7.0
Liver/body weight, g/kg	36.6 ± 1.9	35.3 ± 2.0
Spleen, g	5.73 ± 0.48	4.65 ± 0.49
Spleen/body weight, g/kg	2.30 ± 0.11	1.97 ± 0.16

CRL, crown-to-rump length; HL, head length; HLL, hind limb length; PI, ponderal index; TG, thoracic girth.
All data are mean ± SEM.

periventricular WM ($P = 0.88$; **Figure 3a–c**). There was also no significant difference between control and caffeine-treated groups in the optical density (OD) of MBP-immunoreactivity (IR) (**Figure 3d**) or SMI-312-IR (**Figure 3g**), in the subcortical WM ($P = 0.27$ and 0.68, respectively; **Figure 3e,f,h,i**), periventricular WM ($P = 0.31$ and 0.15, respectively; images not shown), or corpus callosum ($P = 0.89$ and 0.84, respectively; images not shown). There was no evidence of axonal disruption or axonal spheroids in any of these regions as assessed in SMI-312-immunoreactive sections (**Figure 3h–i** insets).

The percentage of WM occupied by microglia was not different between control and caffeine-treated ($P = 0.57$) fetuses (**Figure 4a–c**). The density of activated and resting Iba-1-immunoreactive microglia was not significantly different between control and caffeine-treated fetuses (**Figure 4d–f**) when assessed in the subcortical and periventricular WM individually (data not shown) or combined ($P = 0.14$ activated and $P = 0.29$ resting).

There was no significant difference between groups in the density of GFAP-immunoreactive astrocytes in the subcortical WM ($P = 0.50$) and periventricular WM ($P = 0.39$; **Figure 4g–i**). There was also no significant difference between groups in the density of TUNEL-positive cells in the subcortical WM ($P = 0.21$) and periventricular WM ($P = 0.46$; **Figure 5a–c**).

Structural Analysis of Cortical GM and Striatum

There was no significant difference between groups in the density of TUNEL-positive cells in the cortical GM ($P = 0.63$) or striatum ($P = 0.32$; **Figure 5a–c**). There was also no significant difference between groups in the density of NeuN-positive neurons in the cortical GM in individual (**Figure 6a,c**) or combined cortical bins ($P = 0.30$; **Figure 6b,c**).

DISCUSSION

This is the first study to examine the neuropathological and physiologic responses to high-dose caffeine in a long-gestation, clinically relevant animal model. Importantly, the ovine model allowed us to assess the effects of caffeine at a stage of brain development that is similar to that of the very preterm human infant with respect to the major periods of gliogenesis (18,19), axonal development (20), and myelination (18). Our findings suggest that daily high-dose caffeine treatment does not cause structural alterations or injury in the developing cerebral WM or GM. Specifically, we found that high-dose caffeine does not appear to significantly alter markers of myelination and axonal structure, and it does not significantly affect the overall density of oligodendrocytes, microglia, astrocytes, apoptotic cells, or neurons. We also found that high-dose caffeine does not affect growth or physiological status, apart from a transient mild alkalosis on the first day of treatment.

Administration of high-dose caffeine via the maternal circulation exposed the fetus to high blood concentrations of caffeine, with a maximal fetal plasma caffeine concentration of 32 mg/l. Concentrations of caffeine in the maternal and fetal circulations were similar, confirming that caffeine readily crosses the placenta (21,22). The maximal concentration achieved in this study is high relative to the range of concentrations measured in preterm infants treated with a standard dose of caffeine (20 mg/kg caffeine citrate loading dose; 5–10 mg/kg maintenance dose), where serum caffeine concentrations typically range from 11 to 33 mg/l (23,24). However, the published concentrations cannot be considered definitive as the interval between caffeine administration and blood sampling varies between studies and in some cases is unknown or unreported; measurement of serum caffeine in the preterm human newborn is not routinely performed in clinical practice.

High-dose caffeine led to a mild, transient increase in fetal arterial pH and decrease in arterial $PaCO_2$ on day 1 of treatment and an increase in total hemoglobin concentration on day 2, with no differences in all other physiological variables. The small transient alkalosis is most likely secondary to the observed maternal hyperventilation. In agreement with our findings, acute high-dose caffeine (40 mg/kg caffeine base) administration to preterm (~126 DG) ventilated lambs did not affect blood chemistry, renal function, or cardiopulmonary function (25). The short-term physiological actions of high-dose caffeine administration have not been thoroughly assessed in preterm infants. One study, however, has reported an increase in O_2

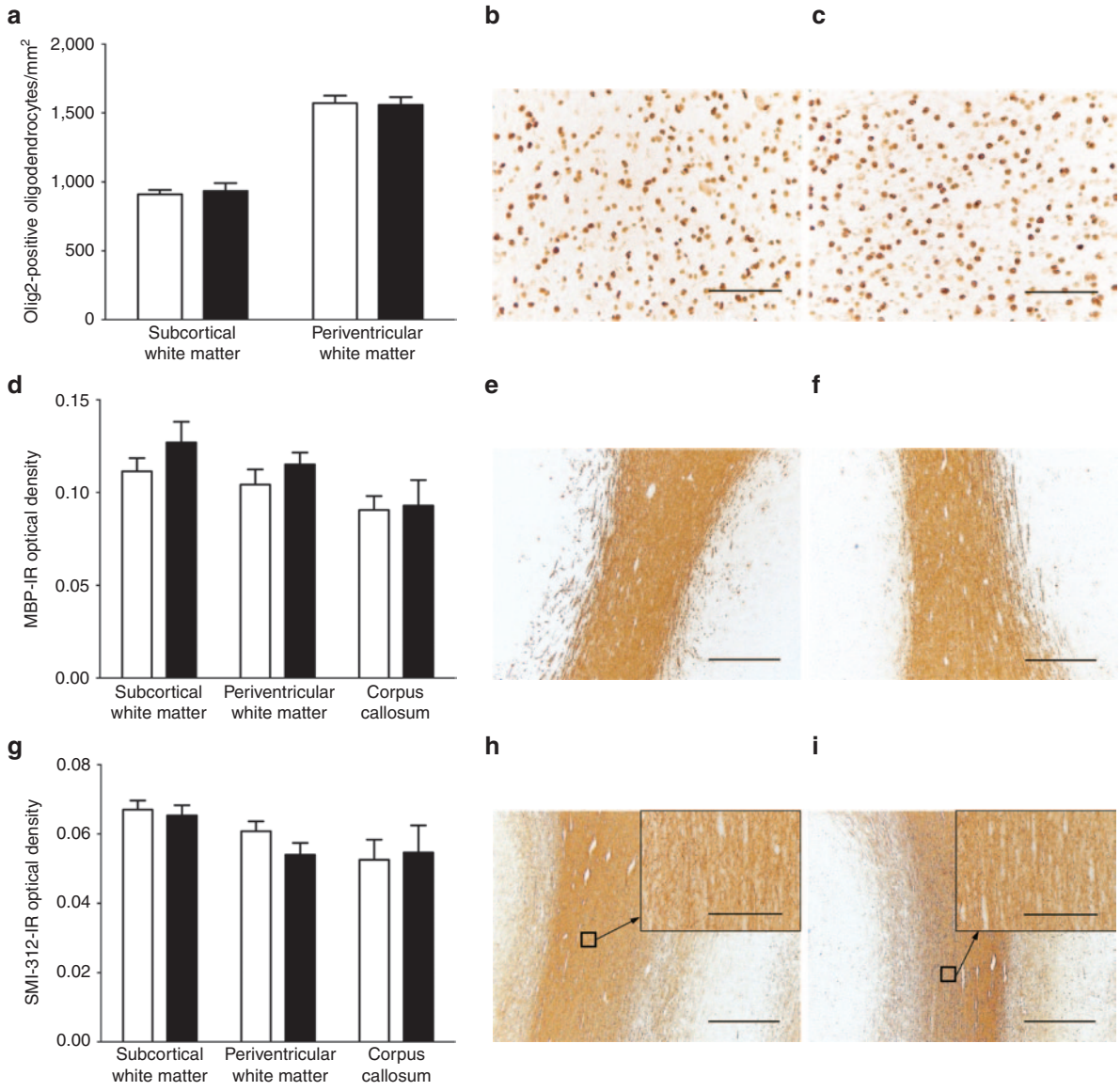

Figure 3. Oligodendrocyte transcription factor 2 (Olig2-), myelin basic protein (MBP-), and SMI-312-IR in the WM and corpus callosum of control (white columns; $n = 8$) and caffeine-treated (black columns; $n = 9$) fetuses. There was no significant difference between groups in the density of Olig2-immunoreactive oligodendrocytes (**a**), optical density (OD) of MBP-IR (**d**), or OD of SMI-312-IR (**g**) in the subcortical WM, periventricular WM, or corpus callosum. For the subcortical WM, this is illustrated by comparing images of Olig2-, MBP-, and SMI-312-IR in control (**b**, **e**, and **h**) and caffeine-treated fetuses (**c**, **f**, and **i**). In SMI-312-immunostained sections, we found no evidence of axonal disruption or axonal spheroids (black boxes in **h–i** represent high-power insets). Scale bars: **b** and **c** = 100 µm; **e**, **f**, **h**, and **i** = 500 µm; inset (**h** and **i**) = 100 µm.

consumption and a reduction in weight gain in preterm infants born at 28–33 wk of gestation when treated with standard doses of caffeine citrate, with no differences in arterial oxygen saturation 4 wk after caffeine therapy (26). Furthermore, Hoecker *et al* (27) reported an increase in arterial pressure in preterm infants, 1 and 20 h after day 2 of caffeine administration, and an increase in heart rate 20 h after day 2 of caffeine exposure. However, these increases were compared with measurements taken just prior to caffeine administration and not to a control group. When caffeine-treated preterm infants were compared with controls, mean arterial pressure increased on the first 3 days of administration; no differences in heart rate were found for measurements taken 1 h before and 1 h after caffeine (28).

High-dose caffeine treatment did not affect myelination in the brain; more specifically, there was no change in the areal density of oligodendrocytes or densitometry of MBP-IR in the cerebral WM. Our findings are consistent with an *in vitro* study, which showed that treatment with 20 mg/l of caffeine (base), a concentration similar to the mean blood concentration achieved in the present study, did not affect the density of oligodendroglial lineage cells or the secretion of hyaluronic acid (29), a factor known to inhibit myelination. This is in contrast to a study in postnatal mice, in which high-dose caffeine administration (40 or 80 mg/kg; base) from postnatal days (P) 1–17 resulted in a dose dependent and transient decrease in myelin concentration at P30 with full recovery and overshoot

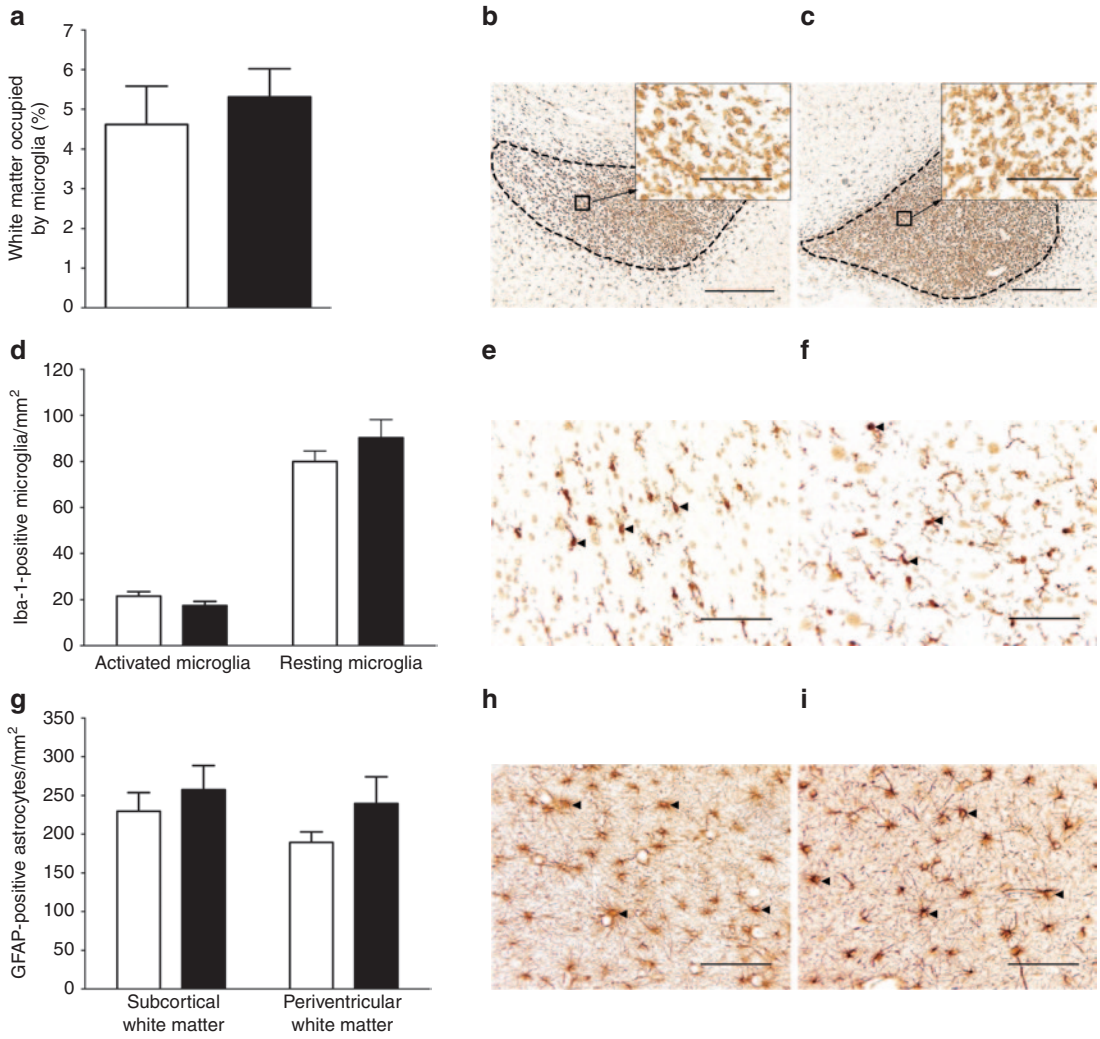

Figure 4. Ionized binding adaptor molecule-1 (Iba-1)-positive microglia (**a–f**) and glial fibrillary acidic protein (GFAP)-positive astrocytes (**g–i**) in the white matter (WM) in control (white columns; $n = 8$) and caffeine-treated (black columns; $n = 9$) fetuses. The percentage of WM occupied by microglia was not different between control and caffeine-treated fetuses (**a**). Iba-1-immunoreactive microglial aggregations containing activated microglia (inset) in control (**b**) and caffeine-treated (**c**) fetuses. There was no difference in the density of Iba-1-positive microglia (activated and resting) in the WM of control and caffeine-treated fetuses (**d**), illustrated by comparing images of Iba-1-positive microglia (arrowheads showing resting microglia) in the subcortical WM in control (**e**) and caffeine-treated (**f**) fetuses. (**g**) The density of GFAP-positive astrocytes in the subcortical and periventricular WM was not different between the control and caffeine-treated fetuses; illustrated by comparing images of GFAP-positive astrocytes (arrowheads) in the subcortical WM in control (**h**) and caffeine-treated (**i**) fetuses. Scale bars: **b** and **c** = 500 μm; inset (**b** and **c**) = 100 μm; **e**, **f**, **h**, and **i** = 100 μm.

by P70 (11). Here, we have used MBP, one of the markers of mature myelin, as a measure of myelination and Olig2 as a marker of the total population of oligodendrocytes. An assessment of other markers of mature myelin (myelin-associated glycoprotein and myelin proteolipid protein), which are differentially affected by perinatal interventions (30), together with ultrastructural analysis of myelination, may offer insight into the effects of caffeine on WM development. Furthermore, analysis of oligodendroglial lineage cells using stage-specific markers could determine whether differentiating, immature oligodendrocytes are more vulnerable to high-dose caffeine than mature oligodendrocytes.

Although a few studies have assessed the effect of caffeine on myelination, there appear to be no animal studies that have examined its effects on axons. Thus, we show for the first time that high-dose caffeine treatment does not affect axonal integrity in the developing WM, at least not at the level of IR for the axonal neurofilament protein SMI-312. Although this method permits assessment of overt axonal damage (axonal disruption and axonal spheroids) and densitometry of SMI-312-IR within the WM, it is not an accurate measure of axonal density; for that, electron microscopy would be required. Magnetic resonance imaging of preterm infants at term equivalent age, treated with standard doses of caffeine revealed a reduction in axial diffusivity in treated infants (31), which may be explained by axonal preservation and/or a greater axonal density. However, it should be noted that this was following doses of caffeine lower than that used in the present study, and MRI scans were performed at a more advanced stage of brain development than in the present study.

High-dose caffeine treatment did not result in cerebral hemorrhage, astrogliosis, or microgliosis in the cerebral WM

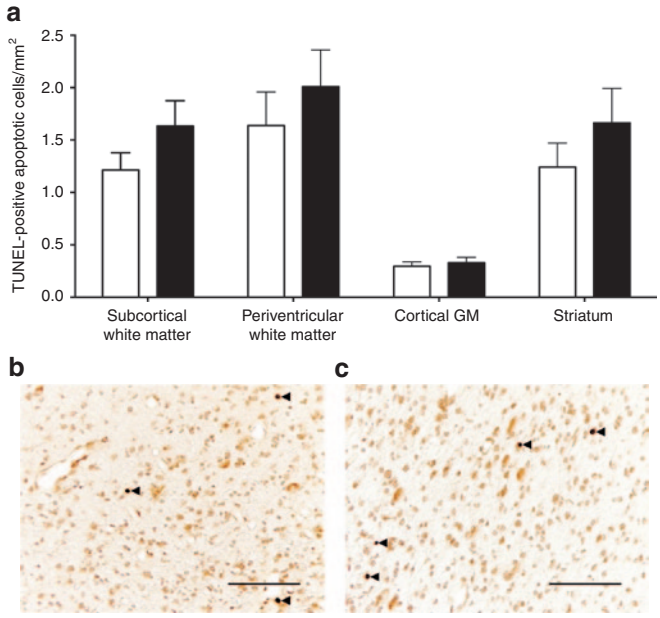

Figure 5. Terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling (TUNEL)-positive cells in the subcortical white matter (WM), periventricular WM, cortical gray matter (GM), and striatum of control (white columns; $n = 8$) and caffeine-treated (black columns; $n = 9$) fetuses. TUNEL-positive cell density in the WM, cortical GM, and striatum was not different between control and caffeine-treated fetuses (**a**), as illustrated by comparing images of TUNEL-positive cells (arrowheads) in the subcortical WM in controls (**b**) and caffeine-treated (**c**) fetuses. Scale bar = 100 µm.

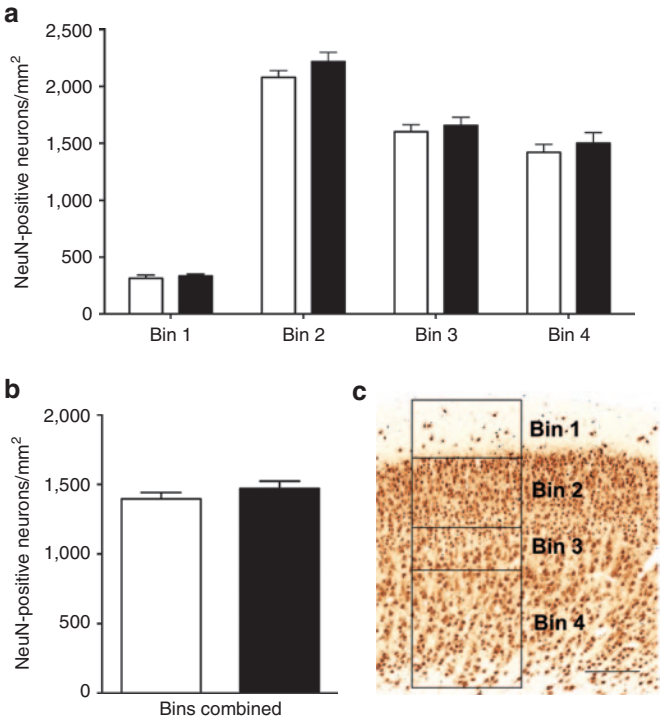

Figure 6. NEUronal nuclei (NeuN)-positive neurons in the cortical gray matter (GM; white columns; $n = 8$) and caffeine-treated (black columns; $n = 9$) fetuses. NeuN-positive neuronal density in the cortical GM was not different between control and caffeine-treated fetuses in any bins analyzed (**a**) or when bins were combined (**b**). (**c**) Bins were divided according to cortical layers (bin 1: cortical layer I; bin 2: layers II and III; bin 3: layer IV; bin 4: layers V and VI). Scale bar = 200 µm.

suggesting that this regimen is not overtly injurious to the developing ovine brain. The lack of finding of astrogliosis is consistent with *in vitro* studies showing that addition of caffeine (20 mg/l; base) to newborn rat primary glial cell cultures does not affect the number of type 1 astrocytes and O-2A lineage cells (29). Conversely, caffeine treatment from P3 to P10 in mice resulted in a transient and dose-dependent reduction in astrocyte density in various brain regions including the cerebral cortex and WM (12). This difference most likely reflects differences in the timing of the insult and also the species used. Although no previous studies have assessed the effects of caffeine on microglia in the developing brain, we show that high-dose caffeine does not lead to activation or proliferation of microglia in the developing ovine WM. Although this finding likely reflects the lack of overt WM injury, it is possible that caffeine may act directly on microglia via adenosine receptors to influence the production of neurotrophins (e.g., nerve growth factor (32)) and suppress pro-inflammatory mediators (33) as seen in the adult brain.

Consistent with our finding that myelination and axonal integrity are unaffected by high-dose caffeine, apoptosis was not increased in the cerebral WM, cortex, or striatum. Furthermore, we found no difference in neuronal density in the cerebral cortex. Previously, acute high-dose caffeine (three doses, 50 mg/kg; base) has been proposed to induce apoptosis throughout the cerebral hemispheres via caspase-3-dependant mechanisms in newborn rats (34). Similarly, acute high-dose caffeine (100 mg/kg; base) administered to P3 rats resulted in increased apoptosis in various brain regions including the cortex and caudate nucleus (35). Once again, differences between this and previous studies are likely due to the timing and dose of administration as well as the species used.

A limitation of our study is that the half-life of caffeine, a reflection of plasma caffeine clearance, is different in human neonates (preterm and term) compared with fetal sheep. In preterm neonates, the half-life of caffeine ranges from 80 to 100 h (36), which is greater than we observed in fetal sheep. Regardless, fetal sheep were exposed to high levels of caffeine for 15 consecutive days with a peak plasma level of ~30 mg/l/d (equivalent to 154 µmol/l of caffeine). The IC_{50} value at which caffeine completely saturates A_1 and A_{2a} adenosine receptors is 100 µmol/l (37); thus, these receptors are likely to have been activated by caffeine in this study and warrant investigation. A further limitation is that the number of animals analyzed per group for the physiological data was low, and thus, additional studies are required to validate this data. It is also likely that apparent increases in some histological parameters (e.g., astrocyte, microglia, and apoptotic cell density) in the caffeine group may be due to modest animal numbers; these numbers, however, were appropriate given prior power analysis. In addition, we assessed measures of WM development and markers suggestive of injury at only one timepoint after caffeine administration (0.8 of term). Future studies will need to address the

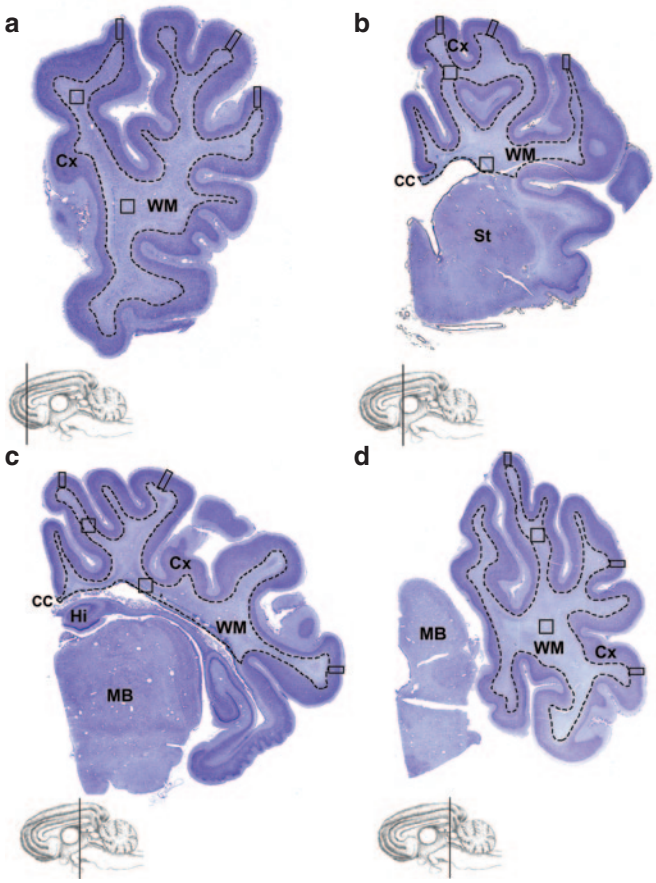

Figure 7. Brain regions analyzed. Coronal, thionin-stained, hemisections of the cerebral hemisphere at the level of the frontal (**a**), parietal (**b**), temporal (**c**), and occipital (**d**) lobe. White matter (WM; dashed line) was examined for immunohistochemical analysis. Measurements were made in the subcortical WM, periventricular WM, and corpus callosum (squares indicate fields of view for subcortical and periventricular WM; 2–3 fields analyzed/square). Measurements of neuronal number were made in the cortical gray matter (GM; squares indicate fields of view; 4 bins analyzed/square). CC, corpus callosum; Cx, cortex; Hi, hippocampus; St, striatum; MB, midbrain.

effects of daily high-dose caffeine treatment on the ultrastructure of the WM, both in the short- and long-term.

Conclusion

Daily high-dose caffeine does not overtly injure the developing ovine WM or GM, or affect physiological status or growth at a stage of brain development that is similar to that of preterm infants receiving caffeine for AOP. Before definitive conclusions can be drawn about the safety of high-dose caffeine for the treatment of AOP, the possible effects of caffeine on other parameters of brain development such as process growth and connectivity, previously shown to be affected by caffeine (9), need to be studied. Furthermore, the potential impact of high-dose caffeine on other brain regions and the long-term consequences of high-dose caffeine treatment require further investigation.

METHODS

All experimental procedures were approved by the Monash University Animal Ethics Committee.

Surgery

Using established techniques, aseptic surgery was conducted at 99 DG (term is ~147 DG) on 14 date-mated ewes (Merino × Border Leicester (13)). Catheters were chronically implanted into a fetal femoral artery for blood sampling and recording arterial pressure, a fetal femoral vein and the amniotic sac for the administration of antibiotics, a maternal jugular vein for drug infusions, and a maternal carotid artery for monitoring maternal physiological status including arterial pressure and heart rate. Antibiotics (Engemycin (100 mg/ml, i.v.; fetus: 0.2 ml and ewe: 4.8 ml) and ampicillin (1 g/5 ml; fetus: 1 ml, intravenous and amniotic sac: 4 ml)) were administered for 3 d after surgery. Postoperatively, sheep were held in individual pens with access to food and water.

Experimental Protocol

A daily bolus dose (loading dose, 25 mg/kg; maintenance dose, 20 mg/kg) of caffeine base (Sigma-Aldrich, St Louis, MO; $n = 9$) or an equivalent volume of saline ($n = 8$) was administered intravenously to the ewe between 104 and 118 DG (0.7–0.8 of term). We administered caffeine base rather than caffeine citrate to minimize the volume required. Caffeine citrate contains anhydrous citric acid and 50% anhydrous caffeine base; thus, the dose of caffeine base is approximately half that of caffeine citrate (36). On the first 3 d of the caffeine administration (104–106 DG), fetal and maternal arterial pressures were monitored for 1 h before and for 5 h after each daily caffeine administration. Blood samples were collected hourly for 6 h from 104–106 DG and then 3 times/d (just prior to caffeine administration (0 h), 1 and 6 h postcaffeine administration) until 118 DG; these were used to measure plasma caffeine concentrations (maternal and fetal) and to assess physiological status.

Necropsy

At 119 DG (0.8 of term), the ewe and fetus were euthanized using sodium pentobarbitone (130 mg/kg intravenous), and the fetus was delivered via cesarean section. Fetal weight and body dimensions were measured, and the ponderal index was calculated (body weight/crown-to-rump length^3). The fetuses (control, $n = 8$ and caffeine, $n = 9$) were transcardially perfused with isotonic saline and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Cerebral hemisphere, whole brain, and major organs were weighed.

Histology

The entire forebrain was cut coronally into blocks 5 mm thick (8–10/animal). Blocks of the entire right hemisphere were then postfixed in 4% paraformaldehyde (4 d, 4 °C) and embedded in paraffin. Serial sections (8 μm thick) were cut from each block, and 1 section/block stained with thionin and examined for hemorrhages and gross structural alterations.

Immunohistochemistry

Sections from equivalent sites from each lobe of the right cerebral hemisphere were reacted with the following antibodies: rabbit anti-Olig2 (1:500, AB9610; Millipore, Billerica, MA) to identify the entire population of oligodendrocytes, rat anti-MBP (1:100, MAB395; Millipore) to identify mature oligodendrocytes and myelin, mouse anti-SMI-312 (1:1,000, SMI-312; Covance, Princeton, NJ) to identify axonal neurofilaments, rabbit anti-Iba-1 (1:1,500, 019-19741; WAKO Pure Chemical Industries, Osaka, Japan) to identify microglia, rabbit anti-GFAP (1:1,000, ZO2334; DAKO, Carpinteria, CA) to identify astrocytes, and mouse anti-NeuN (1:200, MAB377; Millipore) to identify neurons. Sections were incubated in the appropriate biotinylated secondary antibodies (1:200), reacted using the avidin–biotin complex elite kit (Vector Laboratories, Burlingame, CA), and counterstained with 0.1% thionin (Iba-1 and GFAP only) as previously described (13). Prior to incubation with anti-Olig2, -MBP, -Iba-1, -GFAP, and -NeuN, antigen retrieval in sodium citrate buffer (pH 6.0) was performed using a microwave oven. For SMI-312, the sections were pretreated with 0.02% proteinase K (30 min, 37 °C). Sections from each lobe were stained with DeadEnd Colorimetric TUNEL system (Promega, Madison, WI) to identify apoptotic and necrotic cell death (38). Identical staining parameters were used for each antibody, and sections from control and caffeine-treated animals were simultaneously reacted to reduce

staining variability. There was no staining when the primary antibodies were omitted.

Quantitative Analysis

Analyses were performed on coded slides (observer blinded to group) from the right cerebral hemisphere using an image analysis system (Image-Pro Plus v6.2; Media Cybernetics, Rockville, MD). Immunohistochemical analyses were performed on one section from each of the frontal, parietal, temporal, and occipital lobe from each fetus at equivalent areas within the WM and GM (TUNEL and NeuN only; **Figure 7**). All areal densities are expressed as cells per square millimeter, and OD is expressed in arbitrary units.

OD of MBP- and SMI-312. The intensity of MBP- and SMI-312-IR in the cerebral WM was determined using an OD analysis as validated by us (30). Prior to measuring, the image analysis system was calibrated using an image of a blank section of the glass slide (incident light) and obscured section of the slide (infinite OD). From each section, OD was assessed in three fields from equivalent regions of each of the subcortical WM, periventricular WM, and corpus callosum, with a total of 6–9 fields from each section (field 0.093 mm^2). A correction was applied to each of these images by subtracting the OD measurement from a region of background staining. The mean OD was then calculated within each region, for each animal, and a mean of means determined for control and caffeine-treated animals. Imaging and analysis of each of the immunostains were performed in a single day using identical parameters to maintain consistency and eliminate error.

Percentage WM occupied by Iba-1-immunoreactive microglia. The proportion of WM area occupied by Iba-1-immunoreactive microglia was assessed in every 625th section throughout the entire right cerebral hemisphere (n = 8 sections/animal) using a digitizer interfaced to image analysis software. The area of each aggregation within a section was totaled and divided by the area of the WM of that section; data were expressed as a percentage (%). The mean was then calculated for each animal, and a mean of means for control and caffeine-treated groups was determined.

Areal density of Iba-1-, GFAP-, Olig2-, TUNEL-, and NeuN-positive cells. Resting (ramified) and activated (amoeboid) Iba-1-positive microglia (distinguished by morphology), GFAP-positive astrocytes, and Olig2-positive oligodendrocytes were counted in 2–3 fields from both the subcortical and periventricular WM, with a total of 4–6 fields/section (field: 0.093 mm^2). TUNEL-positive cells were counted throughout the subcortical WM, periventricular WM, cortical GM, and striatum (including the internal capsule); the area of each region was also measured to determine the areal cell density. NeuN-positive neurons in the cortical GM were counted in one field (field: 0.56 mm^2) in three gyri; each field was then divided into four bins (bin 1: cortical layer I; bin 2: layers II and III; bin 3: layer IV; bin 4: layers V and VI). For each immunostain, the mean cell density was calculated for each animal and for each region or bin, and a mean of means for control and caffeine-treated groups was determined.

Qualitative Analysis

All SMI-312-immunoreactive sections were qualitatively assessed for the presence of disrupted axons and axonal spheroids.

Statistics

Power analysis was performed using the software package G*Power3. Using data (microglial cell density) from a previous study (13), we determined that 7 animals/group would enable us to detect 1.8 SD difference between group means (80% power, 5% type 1 error rate). Differences between treatment groups in body morphometry, organ weights, and histological data were analyzed by the Student's t-test for parametric data or a Mann–Whitney U-test for nonparametric data. For physiological data (days 1–3), separate one-way repeated measures ANOVA (factors: treatment and time of day (repeated factor)) was used for each day. For physiological data (days 4–15) and cardiovascular data (days 1–2), a single two-way repeated measures ANOVA (factors: day, treatment, and time of day (repeated factor)) was used. Data are presented as mean of means ± SEM (histology) or mean ± SEM (all other data) with $P < 0.05$ considered significant.

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