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REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

ON..... 6 April 2004.....

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Sec. Research Graduate School Committee

ERRATA

Page iv - replace title 1.5.3 "Regulators of sleep" with "Neural regulation of sleep"
Page x - replace title of Table 2.3 with "Recovery of steroids following extraction", Table 2.4 with "Recovery of steroids from brain tissue using different solvents" and Table 2.5 with "Recovery of steroids from brain tissue after different incubation times"
Page 3, line 23 - following "This thesis, along with previous work from our laboratory,..." insert "(Nicol *et al.*, 1997; Nicol *et al.*, 1999; Crossley *et al.*, 2000; Nicol *et al.*, 2001; Nguyen *et al.*, 2003)."
Page 7, line 12 - delete "ρ"
Page 18, line 2 - insert "...as well as apoptosis and white *and grey* matter damage (Penning *et al.*, 1994)"
Page 20, line 13 - replace "a human model" with "the human"
Page 20, line 14 - replace the title "Regulators of sleep" with "Neural regulation of sleep"
Page 20, line 22 - replace "1.5.3.1" with "1.5.3.3"
Page 23, line 28 - delete "...increased retention of brown fat around adrenal glands,..."
Page 24, line 3 - insert "(compared to the fetus)" following "...lung volume..."
Page 25, line 9 - delete the extra "cross"
Page 25, line 12 - replace "Lambs were allowed to deliver spontaneously..." with "Lambs were born spontaneously..."
Page 25, line 15 - insert "respective" before mother
Page 31, line 12 - insert "(Nicol et al., 1997; Nicol et al., 1999)" at the end of the sentence
Page 34, line 16 - replace "Letharbarb" with "Lethabarb"
Page 34, line 17 - delete "at the conclusion of experiments"
Page 37, line 24 - insert "(Ultra-Trarax T25; Labtecknik, Germany)"
Page 38, line 8-9 - replace "brought to dryness" with "dried"
Page 38 - replace title of Table 2.3 with "Recovery of steroids following extraction"
Page 38 - include a legend below Table 2.3 stating, "Data are expressed as mean ± S.E.M."
Page 39 - replace title of Table 2.4 with "Recovery of steroids from brain tissue using different solvents"
Page 40 - replace title of Table 2.5 with "Recovery of steroids from brain tissue after different incubation times"
Page 40, line 3 - delete sentence and replace with "Four incubation periods were assessed; 1, 24, 48 and 72 hours."
Page 40 - within Table 2.5 replace "48 hours" with "48-72 hours"
Page 47 - within Table 2.7 replace "Concentration (nmol/L)" with "Allopregnanolone concentration (nmol/L)"
Page 48, line 25 & Page 50, line 25 - replace "vortexing" with "vortex mixing"
Page 57, line 7 - delete this sentence
Page's 59, 62, 152 - replace "E-Coli" with "E.coli."
Page 61, line 32 - delete "minor convulsions" and replace with "shivering (a consequence of fever)".
Page 66, Figure 3.5 - Replace key for the closed circle on graphs with "SAL 12d".
Page 78 - replace title of Table 3.4 with "Effect of LPS treatment on plasma ACTH, cortisol and allopregnanolone at 20 days of age".
Page 88, line 11 - insert "...intrapleural balloons for monitoring respiratory rate..." following "...vascular catheters for blood sampling".
Page 90, line 22 - replace "different" with "separate".
Page 110 - within Table 4.4, replace the 3 hour HYP allopregnanolone data "46.45" with "40.45" and remove the asterisk and dagger.
Page 113, line 5 - replace "stimuli" with "stimulus"
Page 113, line 3 - delete "but not in plasma."
Page 113, line 20 - insert "Although an additional control group that had the hood fitted would have been desirable, to ensure that this process..."
Page 114, line 16 - delete this sentence as well as the word "moderate" from the following.
Page 115, line 19 - replace "...remain unclear" with "...are likely to be due to the decreased $PaCO_2$ (Figure 4.3 C&G)".
Page 117, line 4 - replace "reversed" with "prevented"
Page 118, line 5 - delete "Overall, the incidence...two bouts of hypoxia."
Page 123, line 2 - insert "(pre-mortem)" following the sentence
Page 127, line 8 - replace "...except for 1 and 1.5 hours..." with "...except for 1 hour..."
Page 139, line 14 - delete "This is beneficial in the adult..." and replace with "The increase in sleep often observed following infection, is beneficial in the adult..."
Page 139, line 19 - insert "(measurement of IL-1β was not available)" at the conclusion of the sentence
Page 151 – delete the final two sentences of the introductory paragraph to the General Discussion
Page 151, line 11 - delete "The clinical focus...aetiology of SIDS" and replace with "One such sleep-related phenomenon, SIDS, was a focus for this thesis."
Page 152, line 10 - replace "while" with "whilst"
Page 156, line 7 - replace "This thesis hypothesised..." with "A hypothesis addressed in this thesis was that..."
Page 163, line 15 - replace "Respiratory effort..." with "Respiratory rate..."
Page 173, line 4 - delete "that contributed to the reduced...no evidence of an increased respiratory rate."
Page 173, line 12 - insert "...but may be a reflection of the changes in $PaCO_2$ observed in these lambs"

Neurosteroid and somnogenic responses to endotoxin and hypoxia treatments in lambs

by

Saraid Sheelagh Billiards

B.Sc. (Hons)

A thesis submitted in fulfillment of the requirements
for the degree of Doctor of Philosophy

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This thesis is dedicated to

parents who have lost precious children

and still have no answers.

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List of abbreviations

ABE	Acid-base excess
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
AP	Allopregnanolone
APP	Acute phase proteins
APR	Acute phase response
AS	Active sleep
AW	Wakefulness
BBB	Blood brain barrier
bpm	Beats per minute
BSA	Bovine serum albumin
BZD	Benzodiazepine
Cl^-	Chloride ion
cm	Centimetre
CNS	Central nervous system
CO_2	Carbon dioxide
CONT	Control
cpm	Counts per minute
CRP	C-reactive protein
CSP	Charcoal stripped plasma
CVO	Circumventricular organs
d	Days

Da	Dalton
dH_2O	Deionised water
DHEA	Dehydroepiandrosterone
dL	Decilitre
DOC	Desoxycorticosterone
dpm	Disintegrations per minute
ECoG	Electrocorticographic
E-Coli	*Escherichia Coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
EMG	Electromyographic
EOG	Electrooculargraphic
Fe^{2+}	Iron ion
g	Gram
GABA	Gamma-aminobutyric acid
GPI	Glycosylphosphatidylinositol
H_2SO_4	Hydrogen sulfate
Hb	Haemoglobin
HCl	Hydrogen chloride
HCO_3^-	Bicarbonate ion
hLPS	LPS followed by hypoxia
HPA	Hypothalamo-pituitary-adrenal
HRP	Horse radish peroxidase
hSAL	Saline followed by hypoxia
HSD	Hydroxysteroid dehydrogenase
HV	High voltage
HYP	Hypoxia
Hz	Hertz
ID	Inner diameter
IgG	Immunoglobulin G
IL	Interleukin
i.p.	Intraperitoneal
IS	Indeterminate sleep
i.v.	Intravenous
kg	Kilogram
L	Litre
LBP	Lipopolysaccharide-binding protein
LC	Locus coeruleus

LPS	Lipopolysaccharide
LSD	Least significant difference
LV	Low voltage
m	Metre
M	Molar
mA	Milliamp
mCi	Millicurie
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mmol	Millimole
mmHg	Millimetres of mercury
mol	Mole
mRNA	Messenger ribonucleic acid
mV	Millivolt
MW	Molecular weight
n	Sample size
N_2	Nitrogen
ND	Not detected
ng	Nanogram
nm	Nanometre
nmol	Nanomole
NREM	Non-rapid eye movement
NS	Not significant
NSB	Non-specific binding
O_2	Oxygen
OD	Outer diameter
OVX	Ovariectomised
$PaCO_2$	Partial pressure of carbon dioxide in arterial blood
PaO_2	Partial pressure of oxygen in arterial blood
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PG	Prostaglandin
PGE_2	Prostaglandin E_2
pmol	Picomole
PNS	Peripheral nervous system

POA	Preoptic area
QS	Quiet sleep
REM	Rapid eye movement
RIA	Radioimmunoassay
rpm	Rotations per minute
S.E.M.	Standard error of the mean
SAA	Serum amyloid A
SAL	Saline
SaO_2	Oxygen saturation
SAP	Serum amyloid P
SIDS	Sudden Infant Death Syndrome
TBPS	Tert-butylbicyclophosphorothionate site
TH-DOC	Tetrahydrodeoxycorticosterone
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
µCi	Microcurie
µg	Microgram
µl	Microlitre
µmol	Micromole
w/v	Weight by volume
°C	Degrees celsius

Summary

Infection and hypoxia are common stressors faced by the neonate and have been implicated in Sudden Infant Death Syndrome (SIDS). These stressors elicit 'stress-like' responses, such as increased activation of the hypothalamo-pituitary-adrenal (HPA) axis and clinical symptoms such as lethargy. However, little more is known about the changes that occur in the brain and particularly how these changes affect somnogenic behaviour. Neurosteroids can be produced *de novo* in the brain and can alter the excitability of the CNS in response to stress. Since neurosteroids such as allopregnanolone have sedative and anaesthetic properties, their elevation in response to stress may contribute to the somnolence that is typically associated with infection and/or hypoxia. The primary aim of this thesis is to examine the effects of infection and/or hypoxia on somnogenesis and neurosteroid concentrations in lambs.

The first experimental chapter of this thesis was designed to examine the effects of a mild infection on somnogenic and neurosteroid profiles. Lipopolysaccharide (LPS) *Escherichia Coli* has been used widely to examine the effects of infection and inflammation and was deemed to be a suitable model to examine neurosteroid responses in this study. LPS treatment (0.7 μg/kg) produced the expected biphasic increase in rectal temperature and reduction in plasma iron concentration, both indicators of fever. LPS treatment also increased the concentration of allopregnanolone, in both plasma and brain tissue. The magnitude of allopregnanolone increase was different between plasma and brain tissue,

suggesting independent regulation of central and peripheral neurosteroidogenesis in the lamb. The effect of LPS treatment on brain allopregnanolone concentration was also region specific, with a 4-fold increase observed in the midbrain and parietal cortex, but no change in the thalamus/hypothalamus or cerebellum. These findings suggest that regionally distinct differences occur in the *in situ* production of this neurosteroid. The increase in allopregnanolone concentration following LPS treatment coincided with a decrease in the incidence of wakefulness and a concomitant increase in the incidence of sleep. Thus, it was proposed that the increased allopregnanolone concentration in response to LPS treatment may contribute to the increased somnolence in these lambs.

Moderate hypoxia was induced by delivering a N_2/air (8 % O_2 in N_2) gas mixture through a custom made bag. Lambs were exposed to two bouts of hypoxia, defined as a drop in arterial oxygen saturation to ~50 %. Hypoxia exposure elicited marked increases in allopregnanolone concentration in the brain. In contrast, there was no change in plasma allopregnanolone, despite large changes in plasma cortisol concentration, indicating activation of the HPA axis. The regional effect of hypoxia treatment on the brain was similar to LPS treatment, with significant ($P<0.05$) increases in all regions examined except for the thalamus/hypothalamus, hippocampus and cerebellum. Increases in the incidence of sleep were also found following hypoxia exposure. This effect was restricted largely to quiet sleep, a sleep state that has been reported to have higher arousal thresholds in infants and during which infants at risk for SIDS have a greater susceptibility to hypoxic challenges.

The effect of a combined LPS and hypoxia treatment on somnogenic and neurosteroid profiles was also examined. This combined treatment resulted in greater increases in brain allopregnanolone concentration compared to either treatment alone. Importantly, regions that remained unaffected by LPS or hypoxia treatments alone, the thalamus/hypothalamus and cerebellum, showed an increase of this neurosteroid following the combined treatments, suggesting a higher level of resistance for these regions in response to stress. Surprisingly, there was no additional effect on somnogenesis when compared to LPS-treated lambs. It is possible that the low incidence of wakefulness that was produced by LPS treatment may not have been able to be depressed any further with the addition of hypoxia exposure.

Pro-inflammatory cytokines, the first line of defence in response to infectious challenge, have been reported to have somnogenic properties. The final experimental chapter of this thesis examines the effects of LPS and/or hypoxia treatment on the pro-inflammatory cytokines, TNF-α and IL-6. It was found that LPS treatment increased both plasma TNF-α and IL-6 concentrations, whilst hypoxia had no effect. However, when these treatments were combined, there was an additive effect on increased IL-6 concentrations, but not on TNF-α. Allopregnanolone has been reported to inhibit the production of TNF-α, a potent somnogenic cytokine. The marked increase in allopregnanolone concentration found in Chapter 5, in response to the combined LPS and hypoxia treatment, may inhibit the additive effects of the combined treatment on plasma TNF-α concentrations and therefore contribute to the absent additive effect on somnogenesis that was expected in these lambs.

In summary, the results presented in this thesis provide the first evidence of a potential somnogenic role of neurosteroids in response to either LPS or hypoxic challenge. The increased allopregnanolone concentration could be argued as having neuroprotective effects by increasing the incidence of sleep and thereby minimising energy costs associated with infection. However, substantial increases in allopregnanolone concentrations may also have a detrimental role by altering the degree of sleep to a level where arousal mechanisms are depressed. If this is the case, increased neurosteroid concentrations may have a causative role in the aetiology of SIDS.

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university, or other institution, and to the best of my knowledge, it contains no material published previously or written by another person, except where due reference is made in the text. All experiments reported in this thesis complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and had approval from the Standing Committee on Ethics and Animal Experimentation.

[redacted]

Saraid Sheelagh Billiards

List of publications

Papers arising from this thesis

BILLIARDS, S.S., SCOTT, K.M., NGUYEN, P.N., CANNY, B.J., WALKER, D.W. & HIRST, J.J. (2003). The effect of combined endotoxin and hypoxia treatments on brain allopregnanolone concentration in lambs. *Experimental Physiology* (Submitted).

BILLIARDS, S.S., WALKER, D.W., CANNY, B.J. & HIRST, J.J. (2002). Endotoxin increases sleep and brain allopregnanolone concentrations in newborn lambs. *Pediatric Research* **52,** 892-899.

Papers arising from techniques established for this thesis

NGUYEN P.N., **BILLIARDS S.S.,** WALKER D.W. & HIRST J.J. (2003). Allopregnanolone in the perinatal sheep. *Pediatric Research* (*In Press*).

NGYUEN P.N., **BILLIARDS S.S.,** WALKER D.W. & HIRST J.J. (2003). Changes in 5α-pregnanc steroids and neurosteroidogenic enzyme expression in fetal sheep with umbilico-placental embolization. *Pediatric Research* (*In Press*).

Invited Article

BILLIARDS, S.S., WALKER D.W. & HIRST J.J. (2003). Sudden Infant Death: New role for neurosteroids. *Australasian Science*, Jan/Feb Issue, p17-18.

Abstracts

BILLIARDS S.S., WILKINSON V.E., SCOTT K.M., WALKER D.W., CANNY B.J. & HIRST J.J. (2003). Deoxycorticosterone: Its role in the stress response in the newborn lamb. *The Perinatal Society of Australia and New Zealand, 7th Annual Congress*, Hobart, Australia.

SCOTT K.M., WILKINSON V.E., **BILLIARDS S.S.,** WALKER D.W., CANNY B.J. & HIRST J.J. (2003). Deoxycorticosterone in the ovine fetus and neonate and its role in the stress response. *The 17th National Workshop on Fetal and Neonatal Physiology*, Hobart, Australia.

BILLIARDS S.S., WALKER D.W., CANNY B.J., PHILLIPS DJ, SCHEERLINCK J.P. & HIRST J.J. (2002). Contribution of endotoxin and hypoxia to neurosteroid concentrations in newborn lambs. In *Ceske Gynekologie*, vol. 16, *Fetal and Neonatal Physiological Society*, Prague, Czech Republic.

BILLIARDS S.S., WALKER D.W., CANNY B.J. & HIRST J.J. (2002). The effect of combined endotoxin and hypoxic treatment on brain allopregnanolone levels in newborn lambs. In *Ceske Gynekologie*, vol. 16, *Fetal and Neonatal Physiological Society*, Prague, Czech Republic.

BILLIARDS S.S., WALKER D.W., CANNY B.J. & HIRST J.J. (2002). Additive effects of the combined treatment of hypoxia and endotoxin on brain allopregnanolone levels. *Monash University Postgraduate Research Symposium*, Melbourne, Australia.

NGUYEN P.N., **BILLIARDS S.S.,** WALKER D.W. & HIRST J.J. (2002). Allopregnanolone and P450scc/5α-reductase type II expression in the brain and adrenal glands of the sheep fetus and neonate. In *Ceske Gynekologie*, vol. 16, *Fetal and Neonatal Physiological Society*, Prague, Czech Republic.

NGUYEN P.N., **BILLIARDS S.S.,** WALKER D.W. & HIRST J.J. (2002). Changes in allopregnanolone concentrations and neurosteroidogenic enzyme expression in the IUGR fetus. In *Ceske Gynekologie*, vol. 16, *Fetal and Neonatal Physiological Society*, Prague, Czech Republic.

NGUYEN P.N., **BILLIARDS S.S.,** HIRST J.J. & WALKER D.W. (2002). Ontogeny of allopregnanolone synthesis in the perinatal brain. *Australian Neuroscience*, Sydney, Australia.

BILLIARDS S.S., WALKER D.W., CANNY B.J. & HIRST J.J. (2001). Neuroactive steroid and behavioural responses to endotoxin treatment in the newborn lamb. *Fetal and Neonatal Physiological Society*, Christchurch, New Zealand.

BILLIARDS S.S., SCOTT K.M., WALKER D.W., CANNY B.J. & HIRST J.J. (2001). Hypoxia in the newborn lamb causes an increase in the concentration of the sedative neuroactive steroid allopregnanolone in the brain. *Perinatal Development and Early Origins of Adult Diseases*, Melbourne, Australia.

BILLIARDS S.S., WALKER D.W., CANNY B.J. & HIRST J.J. (2001). Behaviour, cortisol and cytokine responses to lipopolysaccharide (LPS) in the newborn lamb: Relation to sudden infant death. *The Perinatal Society of Australia and New Zealand, 5th Annual Congress*, Canberra, Australia.

BILLIARDS S.S., WALKER D.W., CANNY B.J. & HIRST J.J. (2001). Pathophysiological effects of hypoxia and infection in newborns. *The 15th National Workshop on Fetal and Neonatal Physiology*, Canberra, Australia.

BILLIARDS S.S., WALKER D.W., CANNY B.J. & HIRST J.J. (2000). Endocrine and behavioural effects of lipopolysaccharide (LPS) in newborn lambs: Implications for SIDS. *The Perinatal Society of Australia and New Zealand, 4th Annual Congress*, Brisbane, Australia.

BILLIARDS S.S., WALKER D.W., CANNY B.J. & HIRST J.J. (2000). Infection and neurosteroids: Their interaction and role in the brain and on behaviour in the newborn. *The 14th National Workshop on Fetal and Neonatal Physiology*, North Stradbroke Island, Brisbane, Australia.

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Chapter 1: Literature review

Neurosteroids are a unique group of steroids that influence the excitability of the central nervous system (CNS). They can be produced in the brain or in other steroidogenic tissues such as the adrenal glands and gonads. Allopregnanolone, a potent neurosteroid with sedative and anaesthetic properties, has been shown to increase during periods of stress, and to induce sleep in adults and even anaesthesia at higher doses. Although there has been considerable interest in the role of neurosteroids during stress, nearly all experimental studies have been largely restricted to the adult. The role of these important endogenous compounds in the neonate and infant is less well understood.

Endotoxin treatment and hypoxia have been shown to elicit 'stress-like' responses, such as alterations in the hypothalamo-pituitary-adrenal (HPA) axis, to stimulate glucocorticoid secretion. Since some glucocorticoids may be precursors for neurosteroid production, endotoxin and hypoxia exposure could result in increased neurosteroid concentrations. These, in turn, may increase the concentration of allopregnanolone (or other potent neurosteroids) in the brain, which may explain the increased lassitude and drowsiness experienced during infection and the altered sleep-wake profiles observed during hypoxia.

The general aim of the studies presented in this thesis is to explore the effects of bacterial endotoxin treatment and induced hypoxaemia on neurosteroid concentrations and

behaviour in the early postnatal period in lambs. At this time, sleep patterns, arousal mechanisms, respiratory and thermal regulation are still being established and the newborn is still dependent on the mother for nutrition. The endocrinology of the stress response, including the HPA axis and sympatho-adrenal systems, have been reasonably well studied but the involvement of neurosteroids is not understood. Most studies investigating the role of neurosteroids during stress have used the rat as the experimental model but since neurosteroids can alter CNS excitability, the use of a more precocial species such as the sheep may give better insight into the possible effects that these stressors may have on the postnatal human infant. The main hypothesis of this thesis is that endotoxin treatment and hypoxia cause increased neurosteroid concentration, in the brains of lambs, that is associated with increased somnogenesis. The increased concentration of neurosteroids could be a potential factor in 'sleep' disease states, including Sudden Infant Death Syndrome (SIDS) and, therefore, may have clinical significance.

1.1 Neurosteroids

Research conducted over the past twenty years has revealed that the brain, like the adrenal gland and gonads, is a steroidogenic organ (review Compagnone & Mellon, 2000). The steroids synthesised by the CNS, termed *neurosteroids*, have unique properties in that they have a wide variety of functions affecting CNS excitability and behaviour principally through actions at the gamma-aminobutyric acid (GABA) receptor (Paul & Purdy, 1992; review Mellon & Griffin, 2002). This thesis examines the alteration of neurosteroid concentrations in response to stressful stimuli including endotoxin treatment and hypoxia in the lamb. The following is a brief summary of the literature on neurosteroids, including their mode of action and the physiological relevance that these steroids may have in certain human diseases.

1.1.1 Neuromodulation by steroids

Neurosteroids are a group of steroids that can be produced in the brain *de novo* from cholesterol or steroid hormone precursors and/or from peripheral sources such as the adrenal glands and gonads (Baulieu, 1998). Studies by Corpechot *et al.* (Corpechot *et al.*, 1981; Corpechot *et al.*, 1983) reported that, in the rat, concentrations of pregnenolone, dehydroepiandrosterone (DHEA) and their sulphated and lipoidal esters were higher in brain tissue than in plasma and, in addition, the concentrations in the brain underwent a

circadian variation that did not correlate with the concentrations of circulating steroids. Further, studies using models of gonadectomy and adrenalectomy revealed that these steroids remained in the nervous system, even after removal of the steroid-synthesising organs (Corpechot *et al.*, 1981; Corpechot *et al.*, 1983). This confirmed that these steroids were not present in the brain as a result of peripheral synthesis and transportation into the brain (as they can readily cross the blood brain barrier; BBB). Thus, the term *neurosteroid* was assigned to steroids that modulate CNS function to differentiate them from other classical steroids that are produced solely by the adrenals, gonads and placenta (Baulieu, 1991; review Mellon & Griffin, 2002).

1.1.2 Biosynthesis and metabolism of neurosteroids

At the time, the finding that steroids were synthesised in the brain was remarkable and led to an investigation of whether enzymes that were known to be involved in the synthesis of steroids from 'classical' organs were also involved in the synthesis of neurosteroids. These studies reported that the enzymes involved in the process of steroidogenesis of classical hormones were indeed present in the CNS and were involved in neurosteroid synthesis (review Compagnone & Mellon, 2000; review Mellon & Griffin, 2002). These studies supported the hypothesis that the brain is capable of steroid synthesis independently of adrenal and gonadal input.

The biosynthesis of neurosteroids is complex and has been reviewed extensively (Mellon, 1994; Compagnone & Mellon, 2000; Mellon & Griffin, 2002), however, reference to the early postnatal period is limited. This thesis, along with previous work from our laboratory, has focused primarily on the production of allopregnanolone in the sheep fetus and newborn. A brief summary of the production and metabolism of allopregnanolone via the enzymes P450scc (side chain cleavage) and 5α-reductase is given below.

1.1.2.1 Allopregnanolone production

Most neurosteroids are derived from a common precursor, cholesterol, through a series of enzymatic reactions that include both P450 and non-P450 enzymes (see Figure 1.1). The first step is the production of pregnenolone from cholesterol via the rate-limiting and hormonally-regulated enzyme P450scc. P450scc is expressed in a variety of cell types in both the CNS and the peripheral nervous system (PNS). For example, it has been detected in the white matter throughout the rat brain (Le Goascogne *et al.*, 1987) and in

oligodendrocytes and Schwann cells in the spinal cord (Baulieu, 1998). In addition, high concentrations of P450scc mRNA have been detected in the rat cerebral cortex and to a lesser extent in the amygdala, hippocampus and midbrain (Mellon & Deschepper, 1993). Expression is similar in male and female rat brains and has also been detected in the nervous system of developing rodent embryos (review Mensah-Nyagan *et al.*, 1999). Thus, the expression of this enzyme does not appear to be restricted to any stage of development.

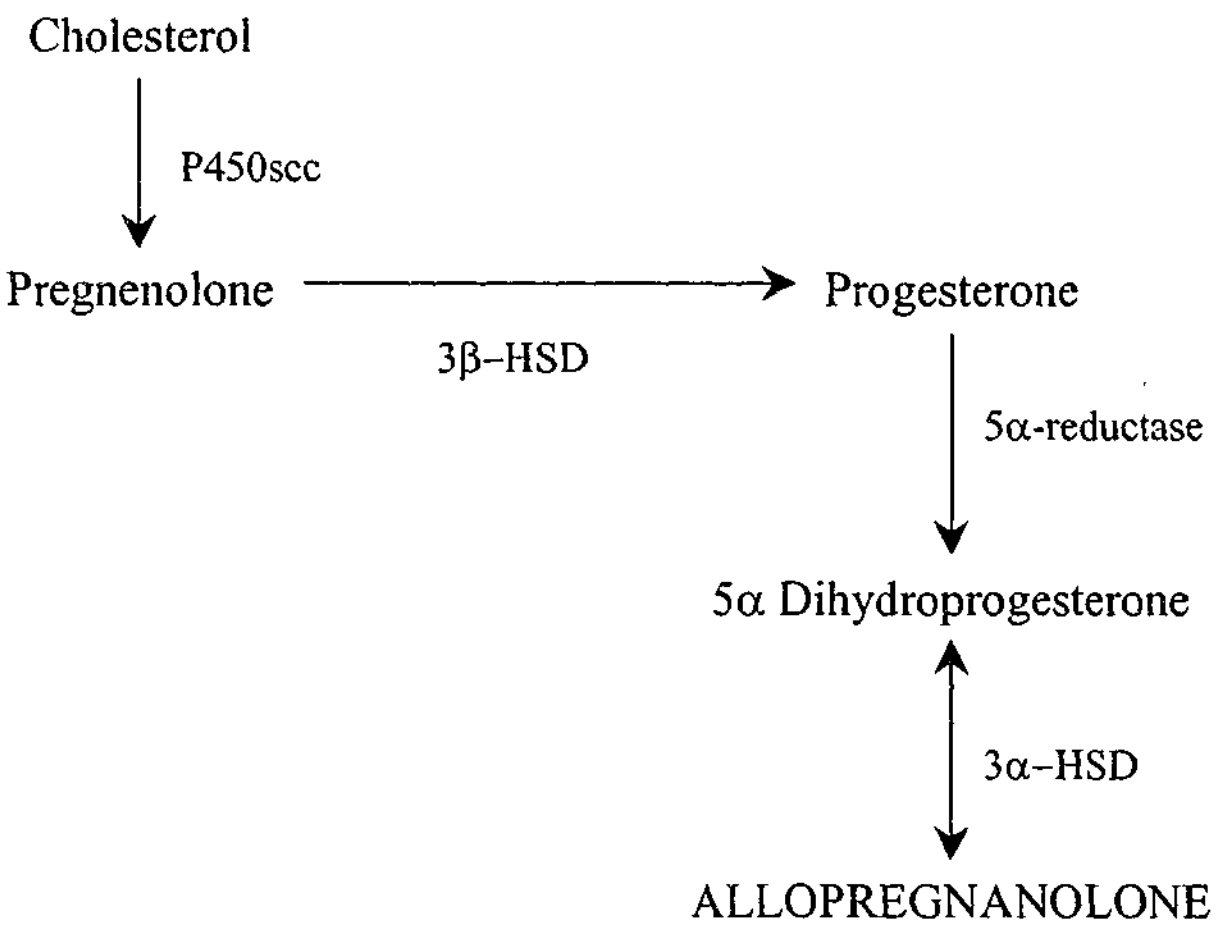


Figure 1.1: Production of allopregnanolone

A simplified schematic diagram of the production of allopregnanolone. The enzymes P450scc and 5α-reductase catalyse key rate-limiting steps in the production of allopregnanolone and the activity of these enzymes may regulate allopregnanolone concentration in the brain.

Pregnenolone, via the enzyme 3β-hydroxysteroid dehydrogenase 3β-(HSD) forms progesterone, the obligate precursor for allopregnanolone synthesis. Progesterone is converted to allopregnanolone via the consecutive catalytic actions of 5α-reductase and

3α-hydroxysteroid dehydrogenase (3α-HSD). 5α-reductase is present in two isoforms, type I and type II, both of which are regulated differentially during development (review Mensah-Nyagan *et al.*, 1999; review Compagnone & Mellon, 2000). In the human, the type I isoform is not expressed during embryogenesis, is transiently expressed during infancy and is expressed more readily from puberty onwards. In contrast, 5α-reductase type II is expressed throughout development. The expression of 5α-reductase, like P450scc, has been reported in several cell types, such as glia, throughout the CNS and neurons in the PNS (review Compagnone & Mellon, 2000). Further, *in vitro* studies have reported that 5α-reductase is expressed in astrocytes and oligodendrocytes of the adult rat brain (Melcangi *et al.*, 1990; Celotti *et al.*, 1992; Melcangi *et al.*, 1994). However, the data suggest that 5α-reductase is more readily expressed in glial cells compared to neurones, in contrast to P450scc, which is found primarily in neuronal cells (Celotti *et al.*, 1992; Melcangi *et al.*, 1994). Cellular localisation of enzyme expression may explain some of the regional differences found throughout the brain of rats (Barbaccia *et al.*, 1994; Barbaccia *et al.*, 1996; Bernardi *et al.*, 1998) and sheep (Nguyen *et al.*, 2003).

1.1.2.2 Developmental changes of allopregnanolone production

Little is known about the production and concentration of allopregnanolone postnatally. P450scc and 5α-reductase, two key enzymes involved in the synthesis of allopregnanolone have been detected in the brain, spinal cord and PNS of the adult rat and the nervous system of the embryonic rat (review Mellon & Griffin, 2002). After birth, however, it has been reported that these enzymes have differential developmental profiles (Lauber & Lichtensteiger, 1996; Jacobson *et al.*, 1997). Whilst P450scc and 5α-reductase are both expressed in the amygdala and frontal cortex of neonatal rat pups (Jacobson *et al.*, 1997), 5α-reductase mRNA expression has also been found in the developing lens of the eye and in differentiating regions of the brain, suggesting a role for this enzyme during brain development in the rat pup (Lauber & Lichtensteiger, 1996).

Fadalti *et al.* (1999) were the first group to document circulating levels of allopregnanolone in humans from infancy through to adulthood. In these studies, they found that between birth and two years of age, allopregnanolone levels did not change, however, at puberty, allopregnanolone levels increased significantly. These investigators suggested that this neurosteroid may have a role in adaptive neuroendocrine mechanisms associated with puberty. Other studies in the neonatal rat have also shown that

allopregnanolone is present in brain tissue with concentrations averaging about 8 ng/g (Bernardi *et al.*, 1998; Kehoe *et al.*, 2000).

1.1.3 Neurosteroid receptor interactions

Unique features of neurosteroids are their central depressant properties. First discovered by Cashin and Moravek in 1927 (see Holzbauer, 1976) and then further supported by studies performed by Seyle in the 1940s (Selye, 1941, 1942), the sedative-anaesthetic properties of neurosteroids were described as rapid and reversible (Majewska *et al.*, 1986). The mechanisms by which neurosteroids, including progesterone and allopregnanolone, exert these sedative effects on the CNS suggested that both genomic and non-genomic actions were involved (Paul & Purdy, 1992; review Compagnone & Mellon, 2000).

Jung-Testas and colleagues reported the idea of a genomic action of neurosteroids initially in 1991 (Jung-Testas *et al.*, 1991). They found that, when administering progesterone and estradiol to cultured rat glial cells, cell growth was inhibited by progesterone and stimulated by estradiol. However, both treatments elicited morphological changes in oligodendrocytes and astrocytes. Rupprecht *et al.* (1996) also reported a genomic action of neurosteroids. In human neuroblastoma cells treated with allopregnanolone or tetrahydrodeoxycorticosterone (TH-DOC), an increase in the gene expression for the intracellular progesterone receptor was found. These, and other studies (e.g. Koenig *et al.*, 1995), indicate that neurosteroids are capable of both genomic and non-genomic actions but the mechanisms are dependent on the target tissue and the presence of receptors and enzymes required for neurosteroid synthesis.

The non-genomic interactions of neurosteroids were implied by the rapid onset and disappearance of some effects of neurosteroids, and these actions were therefore likely to be mediated through neurotransmitter receptors (Majewska *et al.*, 1986; review Mellon & Griffin, 2002). The discovery that the derivatives of progesterone metabolism, such as allopregnanolone, exert their effects via the $GABA_A$ receptor ion complex (Majewska *et al.*, 1986; review Mellon & Griffin, 2002) gave a firm basis for this assumption. Actions of steroids at a number of neurostransmitters have been described, including glutamate and GABA receptors (review Mellon & Griffin, 2002), but the latter is the most important and has received the most attention.

1.1.4 GABA receptors

GABA is a major inhibitory neurotransmitter in the mammalian CNS. It is produced in GABAergic neurons and interacts with two types of GABA receptors, $GABA_A$ and $GABA_B$. Allopregnanolone acts on the $GABA_A$ receptor, therefore, for the purposes of this thesis, the following discussion is restricted to this receptor.

1.1.4.1 $GABA_A$ receptor

The $GABA_A$ receptor is a member of the ligand-gated ion channel family of receptors. It consists of a central chloride ion channel surrounded by five transmembrane proteins (Sieghart, 1992), which provide a hydrophilic environment that is essential for the movement of ions (Luddens & Korpi, 1995). The receptor exists in multiple forms in the mammalian brain (Luddens & Korpi, 1995) due to its several classes of subunits (α, β, γ, δ, ε, π and ρ). Various combinations of these subunits in the $GABA_A$ receptor are critical for specific ligand binding affinities and receptor activation (Puia *et al.*, 1990). For example, the efficacy of allopregnanolone is doubled in the presence of an α1 subunit, whereas other combinations of subunits are less sensitive to this steroid (review Compagnone & Mellon, 2000).

Several pharmacological agents and neurosteroids bind to the $GABA_A$ receptor to modulate the functions of the chloride ion channel. However, specific structural and stereochemical properties are required. There are five independent binding sites for the $GABA_A$ receptor, including the GABA agonist/antagonist site, the barbiturate site, the benzodiazepine (BZD) site, the picrotoxin/tert-butylbicyclophosphorothionate site (TBPS) and the steroid-binding site (Majewska *et al.*, 1986). GABA, barbiturates, BZDs and progesterone-derived metabolites potentiate the function of the $GABA_A$ receptor by increasing the influx of chloride ions and hyperpolarizing the nerve cell, whereas convulsants such as picrotoxin and TBPS inhibit receptor activity by blocking this influx (Majewska, 1992). Allopregnanolone binds to the $GABA_A$ receptor at physiological concentrations (10-30 nM) to induce hyperpolarisation of the cell membrane and, at higher doses (100 μM), allopregnanolone can directly open the gated chloride channel in the absence of the neurotransmitter GABA (Majewska *et al.*, 1986; review Smith, 2002). The binding of appropriate agents and metabolites of progesterone to each site therefore influences ion-gated channel responses of the receptor complex which, in turn, produce inhibitory or excitatory physiological effects on the CNS.

The expression of the $GABA_A$ receptor is regulated developmentally. Ontogeny studies in guinea pigs (Bailey *et al.*, 1999) and sheep (Crossley *et al.*, 2000), investigating the expression of $GABA_A$ receptors in the developing brain, have reported that the density of this receptor decreases dramatically after birth. These results suggest that this receptor may play a functional role in brain growth and development during fetal life. In addition, Crossley *et al.* (2000) reported regional variation in the binding characteristics of the $GABA_A$ receptor in the newborn lamb brain. Using TBPS, a convulsant that is modulated by many neurosteroids, these investigators found that there were higher binding densities of TBPS to the receptor in the frontal cortex and lower densities in the hypothalamus and brainstem.

1.1.5 Neurosteroids and behaviour

The roles of neurosteroids in the mammalian species appear to have evolved to aid in the complex behavioural responses to stressful stimuli and psychohormonal events such as anxiety, somnogenesis, depression and aggression. In the following sections, the homeostatic role of neurosteroids will be examined, particularly those associated with stressful conditions.

1.1.5.1 Stress and anxiety

Stress elicits a range of physiological responses including activation of the HPA axis (Kehoe *et al.*, 2000), regulation of the mesolimbocortical dopamine pathway (McCormick *et al.*, 2002), increased neurosteroid production and, subsequently, changes in behaviour (Rupprecht & Holsboer, 1999; review Mellon & Griffin, 2002). Until recently, it was believed that progesterone mediated the majority of the behavioural effects induced by stress, however, it has now been suggested that the production of allopregnanolone, the metabolite of progesterone, is responsible (Mellon, 1994; Stonestreet *et al.*, 1999).

The endogenous neurosteroid response to stressful stimuli has been studied extensively in the rat using several different stress-inducing paradigms including swim stress (Purdy *et al.*, 1991; Khisti *et al.*, 2000), foot shock (Barbaccia *et al.*, 1994), CO_2 inhalation (Barbaccia *et al.*, 1994; Barbaccia *et al.*, 1996) and social isolation (Kehoe *et al.*, 2000; Serra *et al.*, 2000). In each of these studies, marked and selective increases in neurosteroids, including allopregnanolone, to levels that altered GABA function were observed (Kehoe *et al.*, 2000). For example, CO_2 inhalation elicited increases in

allopregnanolone concentration in the cortex and hippocampus, regions that are considered to have functional roles in the coordination of behavioural responses to stress. In addition, the increases observed were at a time when the anxious behaviour and [^{35}S]TBPS binding had begun to decrease, reflecting the role of neurosteroids in restoring GABAergic tone after stress (Barbaccia *et al.*, 1996). Although increases in neurosteroid concentrations were observed in both plasma and brain tissue, it was found that the increase in neurosteroid concentration, regardless of the stressful condition, was greater in brain tissue than in blood, further supporting the role of *in situ* synthesis of steroids in the brain in response to stress (Barbaccia *et al.*, 1994; Barbaccia *et al.*, 1996; Serra *et al.*, 2000).

Administration of neurosteroids has also been shown to elicit anxiolytic effects. When infusing allopregnanolone directly into the central nucleus of the amygdala, a region that is involved in the regulation of fear and anxiety, anxiolytic behaviour was observed after stressful stimuli were presented (review Mellon & Griffin, 2002). Similarly, administration of TH-DOC to rat pups, which like allopregnanolone is also a potent neurosteroid agonist of the $GABA_A$ receptor, reduced anxiety levels induced by separation (Patchev *et al.*, 1997). Overall, these data show that 5α-reduced derivatives of progesterone are effective in reducing the physiological responses to stressful stimuli and that their actions are mediated through their interaction with the $GABA_A$ receptor.

1.1.5.2 Somnogenesis

Sleep is an integral behavioural function for the maintenance of homeostasis. Neurosteroids such as progesterone and its metabolites allopregnanolone and TH-DOC have been shown to have somnogenic (Lancel, 1997; review Muller-Preuss *et al.*, 2002) and anaesthetic effects (Mok *et al.*, 1993; Korneyev & Costa, 1996) through actions at the $GABA_A$ receptor. Initial studies showed that administration of progesterone to rats altered sleep profiles in a dose-dependent manner (Gandolfo *et al.*, 1994; Lancel *et al.*, 1996). However, further studies showed that, as reported previously with anxiolytic responses, the somnogenic effects of neurosteroids were not a result of the administration of progesterone *per se* but rather a result of its conversion to allopregnanolone (Lancel *et al.*, 1997) or TH-DOC (review Muller-Preuss *et al.*, 2002). The administration of either allopregnanolone or TH-DOC altered the sleep profiles of rats by reducing the latency of non-rapid eye movement (NREM) sleep and dose-dependently influenced brain activity

during rapid eye movement (REM) and NREM sleep. The rapid hypnotic actions of these steroids were reported to be similar to those found with progesterone and BZD's, *albeit* at much smaller doses, suggesting that the somnogenic actions of these steroids are accomplished through the modulation of the $GABA_A$ receptor (Lancel *et al.*, 1997; review Muller-Preuss *et al.*, 2002). Although the somnogenic properties of neurosteroids are well described, the role of endogenous neurosteroids in inducing sleep is less clear. This thesis examines the effects of stressful stimuli that are known to affect sleep architecture such as endotoxin treatment and induced hypoxaemia, on neurosteroid concentrations and somnogenesis.

1.1.5.3 Neuroprotection

Several observations suggest that neurosteroids may have a neuroprotective role (Barbaccia *et al.*, 1994; Kehoe *et al.*, 2000; Khisti *et al.*, 2000). It is well known that women are less prone to stroke and that oestrogen is neuroprotective (review Mellon & Griffin, 2002). Several studies using models of ischaemia have reported that administration of neurosteroids decreased infarct size (Jiang *et al.*, 1996; Gonzalez-Vidal *et al.*, 1998; Kumon *et al.*, 2000) and neuronal and glial cell injury (Lapchak *et al.*, 2000). The administration of allopregnanolone in neonatal rat pups subjected to intermittent maternal deprivation also abolished changes in central mechanisms that control the HPA axis, thus protecting the developing brain from the stressful insult (review Compagnone & Mellon, 2000). These studies indicate that neurosteroids may act as endogenous neuroprotectants in situations where neural damage might occur otherwise (review Mellon & Griffin, 2002).

1.1.5.4 Depression, cognition and other clinical effects

Alterations in neurosteroid concentrations have also been associated with the aetiology of several disorders in humans and animals (predominately rodents) including depression (Uzunova *et al.*, 1998), seizures (Mellon, 1994) and impaired cognition (Flood *et al.*, 1992). Several studies have supported a relationship between altered neurosteroid concentrations and depressive states including pre-menstrual syndrome (Genazzani *et al.*, 1998; Monteleone *et al.*, 2000), post-partum depression (Concas *et al.*, 1998) and unipolar depression (Uzunova *et al.*, 1998). In each of these studies, low neurosteroid concentrations, leading to decreased GABAergic neurotransmission, were found and suggested to be the cause of the depression. The possibility of clinical administration of

neurosteroids to help control the endogenous fluctuations are currently under investigation (review Mellon & Griffin, 2002).

Seizure disorders such as epilepsy are also associated with alterations in GABAergic neurotransmission and neurosteroid levels (Mellon, 1994; review Mellon & Griffin, 2002). Factors such as stress, puberty and pregnancy affect the frequency of seizures, suggesting a hormonal involvement. For example, in woman with partial focal epilepsy, it has been reported that the frequency of seizures is lower during the luteal phase, when progesterone levels are high (Mellon, 1994), suggesting that progesterone or neurosteroids derived from progesterone may play a role in raising seizure thresholds.

Administration of allopregnanolone into the basal magnocellular nucleus of rodents subjected to foot shock aversion tests was found to affect cognition by decreasing memory performance, whereas administration of the antagonist, pregnenolone sulphate, enhanced memory performance (Ladurelle *et al.*, 2000). Similarly, pregnenolone, DHEA and their sulphated esters all enhanced memory processes in mice when administered into the cerebral ventricles (Mellon, 1994). The exact mechanisms by which neurosteroids affect cognition and memory are still relatively unknown but altered neurosteroidogenesis that is associated with aging may contribute to memory loss (review Mellon & Griffin, 2002).

1.2 Infection

Dorland's Medical Dictionary (edition 28, 1994) defines infection as the 'invasion and multiplication of micro-organisms in body tissues, which may be clinically inapparent or result in local cellular injury due to competitive metabolism, toxins, intracellular replication or antigen-antibody response. It may remain localised, subclinical and temporary depending on the body's defence mechanisms'. Infectious challenge is usually accompanied by profound physiological and behavioural changes including immune responses (Elmquist *et al.*, 1997), fever (Kluger, 1980), acute phase responses (Sagar, 1994) and sickness behaviour (Dantzer, 2001). However, the specific response patterns vary depending upon both the invading organism and the host (Elmquist *et al.*, 1997). Many, if not most, of these responses are thought to be due to the release of cytokines from infected tissue and this, in turn, induces the release of bacterial products that induce expression of pro-inflammatory genes. Lipopolysaccharide (LPS or endotoxin), a component of the cell wall of gram-negative bacteria, is the most well characterised

bacterial endotoxin and has been used widely as an immune stimulus. Its use has given insight into the mechanisms and physiological processes involved in infection. The following sections provide a brief description of some of the processes applicable to this thesis.

1.2.1 Pro-inflammatory cytokines and infection

Part of the immune response to an infectious challenge includes increased synthesis and release of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α. These cytokines are released from activated circulating monocytes in response to micro-organism invasion and can elicit a host of CNS-mediated responses that have been well documented including fever, activation of the HPA axis, somnolence and anorexia (Elmquist *et al.*, 1997; Dantzer, 2001; Konsman *et al.*, 2002; Reyes & Sawchenko, 2002) through their potent effects on the brain. Circulating cytokines are large molecules (15,000-30,000 Da MW) that cannot penetrate the BBB readily. Recent studies have revealed that their effects on the brain are via two communication pathways: 1) a faster neural route affecting primary afferent neurons localised to the place of infection and 2) a slower humoral route involving the diffusion of cytokines from phagocytic cells in the circumventricular organs (CVOs) and choroid plexus to targeted brain areas (Dantzer, 2001; Konsman *et al.*, 2002). The integrity of the BBB can be compromised in some clinical conditions, such as bacterial infection, leading to increased entry of immune cells and cytokines from the periphery into the brain. This results in an enhanced cytokine-induced neuroimmune response (Turrin & Plata-Salaman, 2000).

LPS is responsible for most of the physiological and behavioural responses observed with gram-negative infections (Turrin *et al.*, 2001). Several studies have reported that administration of this endotoxin elicits an ordered series of effects that begin with the increased production of cytokines (McCann *et al.*, 1994; Schlafer *et al.*, 1994; Fantuzzi *et al.*, 1995). LPS is able to induce the synthesis of IL-1 by binding to receptors (see Section 1.3 for further detail) present on circulating phagocytic cells (Vitkovic *et al.*, 2000). IL-1, in turn, can induce its own synthesis and the synthesis of other cytokines such as TNF-α and IL-6 (Konsman *et al.*, 2002).

1.2.2 Fever and infection

Fever is an adaptive defence response of the host to infectious challenge, as higher body temperatures are unfavourable for the growth of many bacterial and viral pathogens (Dantzer, 2001). In contrast to hyperthermia, fever is a physiological adjustment of the thermoregulatory set point that is common in both humans and mammals (Kluger, 1980). Fever is associated with increases in metabolic heat production through shivering and non-shivering thermogenesis, and decreases in heat loss (Blatteis & Sehic, 1997). The development of fever is believed to be a consequence of the actions of exogenous pyrogens, such as LPS, the production of pro-inflammatory cytokines and the production of prostaglandins (PG; Romanovsky & Blatteis, 1995). That is, the invasion of micro-organisms or exogenous pyrogens into the host elicits increases in pro-inflammatory cytokines, such as TNF-α and IL-6 (Blatteis & Sehic, 1997; review Blatteis, 2000). These cytokines are then able to stimulate the biosynthesis of PGE_2, a BBB permeable lipid produced by activated Kupffer cells which then act at the preoptic area (POA) of the hypothalamus to increase the thermoregulatory set-point so that body temperature rises (Blatteis & Sehic, 1997; review Blatteis, 2000).

Many studies have shown that the systemic administration of LPS induces increases in both pro-inflammatory cytokines (Dinarello, 1984; Zuckerman *et al.*, 1991) and prostaglandins (Rotondo *et al.*, 1988; Coceani *et al.*, 1995) and, in turn, fever. In addition, administration of cytokines or PGs also elicits fever (McCann *et al.*, 1994; Noah *et al.*, 1995). Inhibition of IL-6 production in knockout mice (Chai *et al.*, 1996; Leon *et al.*, 1998), or the use of anti-inflammatory cytokines such as IL-10 (Leon *et al.*, 1999), results in reduction or inhibition of LPS-induced fever. Similarly, a study by Davidson *et al.* (2001) reports that administration of the anti-steroidal, anti-inflammatory drug, ketoprofen, decreases PGE_2 levels and, subsequently, abolishes the febrile response to LPS challenge. Therefore, because pro-inflammatory cytokines are able to stimulate the biosynthesis of PGE_2, a review by Blatteis (2000) suggests that PGE_2 is the 'most proximal' putative mediator of fever, supporting the idea that this lipid may function as the final mechanism involved in the induction of fever responses to infectious challenge.

1.2.3 Acute phase response

The acute phase response (APR) is a cytokine-driven, coordinated reaction of the host to infectious and traumatic challenges that include immunological, endocrinological,

haematological, neurological, metabolic and cardiorespiratory components to help maintain homeostasis (Dinarello, 1984; Bopst *et al.*, 1998; Black, 2002). Alterations in circulating plasma proteins, collectively termed acute phase proteins (APP), are required to induce these homeostatic physiological changes (Bopst *et al.*, 1998). These liver-derived proteins, including protease inhibitors, haptoglobin, serum amyloid A (SAA), serum amyloid P (SAP), C-reactive protein (CRP) and LPS-binding protein (LBP), increase rapidly after infection (Baumann & Gauldie, 1994). They also provide increased protection against micro-organisms and modify the inflammatory processes that are part of the APR through effects on cell trafficking and mediator release (Baumann & Gauldie, 1994; Bopst *et al.*, 1998; Suffredini *et al.*, 1999). APP production is mediated primarily by pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α but it is also modulated by catecholamines, growth factors and glucocorticoids (Baumann & Gauldie, 1994; Gabay & Kushner, 1999).

Clinically, APPs are useful predictive markers of inflammation, although species differ in their APP response to infection. For example, in humans, the APR is often associated with increases in CRP, SAA and α-1 antiproteinase inhibitor (Hilliquin, 1995), whereas in mice, SAP and SAA predominate (Steel & Whitehead, 1994), while SAA and haptoglobin are identified as key markers of inflammation in cattle (Schroedl *et al.*, 2001). To date, there is no study that has investigated the APP changes in sheep. The APR generally runs its course within 24 to 48 hours, after which acute inflammatory responses subside (Baumann & Gauldie, 1994; Black, 2002). Although the host can usually maintain a controlled APR, in some cases, an overwhelming APR resulting from an uncontrolled cytokine cascade can lead to septicaemia (Sagar, 1994). Thus, an understanding of the host's response to infectious and inflammatory challenges is important to reduce the severity of the APR and subsequent symptoms.

1.2.4 Sickness behaviour

Sickness behaviour is the term given to a collection of non-specific symptoms, including somnolence, muscle weakness, depression and anorexia, that result from increased pro-inflammatory cytokine production (Johnson, 2002). The physiological benefit of sickness behaviour is that it aids in the maintenance of homeostasis and promotes recovery from the infectious challenge by reducing energy consumption and expenditure (Dantzer, 2001; Reyes & Sawchenko, 2002). Pro-inflammatory cytokines, such as IL-1β and

TNF-α, when administered directly into the brain or into the periphery of rodents, can induce somnolence (Krueger *et al.*, 1998; Krueger *et al.*, 2001), depressed behaviour (see Bluthe *et al.*, 2000) and anorexia (Plata-Salaman *et al.*, 1988; Reyes & Sawchenko, 2002). These symptoms have also been reported in humans (Vedder *et al.*, 1999), birds (Johnson *et al.*, 1993), rats (Schiffelholz & Lancel, 2001) and cattle (Steiger *et al.*, 1999) following LPS administration, which also increases endogenous levels of these cytokines. Moreover, the exogenous administration of inhibitors of cytokine action, such as IL-1 receptor antagonist, inhibits or reduces the degree of sickness behaviour induced by pro-inflammatory cytokines or LPS administration (Turrin *et al.*, 2001). The regulation of the expression and action of these cytokines is controlled tightly (Konsman *et al.*, 2002). The changes and reorganisation of behaviour in the host response to infectious challenge is dependent on the synthesis and release of pro-inflammatory cytokines, and is beneficial in the short term. If, however, repeated or sustained, the sickness behaviour that helps to maintain homeostasis may become injurious.

1.2.5 Infection in neonates

In utero, fetal protection from the adverse effects of invading micro-organisms is accomplished through the transport of maternal antibodies, in the form of immunoglobulin G (IgG), across the placenta into the fetal circulation. This transfer of maternal immunity continues early into the neonatal period via IgG contained in the colostrum and maternal milk (Kasting *et al.*, 1979). However, this protection is afforded only for the first few weeks of life, after which susceptibility to infectious challenge increases due to reduced maternal antibody supply and immaturity in the host defence response of the newborn (Fantazia, 1983). This immaturity results from an inability to localise the infectious challenge (Fantazia, 1983), a naïve immune system that includes delayed antibody production compared to the adult (Haeney, 1994) and poor thermoregulatory responses (Kluger, 1980). Taken together, they predispose the infant to a higher risk of mortality (Gessler *et al.*, 1996). The precise age at which immunological functions involved in infection become similar to the adult is unknown, although it appears that the risk of severe infection does decrease by 2 to 4 months of age in the human (Wilson, 1986).

Based on the data presented thus far, it is clear that pro-inflammatory cytokines are the first line of defence of the host in response to most infectious challenges. The initiation of

cytokine release results in a cascade of effects on a wide variety of behavioural and physiological parameters. The precise response, however is complex, and depends on the specific insult and tissue affected. To further complicate matters, because cytokines exhibit multiple functions, the specific roles of individual cytokines can shift from being beneficial to deleterious. For example, immunocompromised hosts, such as the newborn, can have amplified physiological responses that render them susceptible to sepsis and even death during the advanced stages of infection.

1.3 Lipopolysaccharide administration as a model of infection

Bacterial endotoxins, such as LPS, have been used widely to study the effects of host response defence mechanisms in numerous animal and human models. LPS elicits immune responses in the host similar to those of a natural infection by activating monocytes and macrophages to produce pro-inflammatory cytokines. These cytokines then serve as endogenous mediators for the broad spectrum of physiological and behavioural responses to infectious challenges as discussed previously (Section 1.2.1).

LPS-mediated activation of inflammatory responses in mammalian cells is a complex pathway, many details of which still require elucidation (Medzhitov *et al.*, 1997; Triantafilou & Triantafilou, 2002). Current research opinion is that initiation of LPS-induced inflammation occurs through the binding of LPS with LBP (Heumann & Roger, 2002; Triantafilou & Triantafilou, 2002), a protein that is also a component of the APR (Vreugdenhil *et al.*, 1999). The LPS/LBP complex then binds to the glycoprotein receptor CD14, of which there are two forms: membrane-bound or soluble (Heumann & Roger, 2002). CD14 expression is found peripherally on monocytes (Wright *et al.*, 1990) and myeloid cells (Laflamme & Rivest, 2001) and expression of CD14 mRNA has been found in CVOs, leptomeninges and microvasculature of the brain (Lacroix *et al.*, 1998). CD14 is a glycosylphosphatidylinositol (GPI)-anchored protein and thus lacks a transmembrane domain, rendering it unable to participate directly in the intracellular signalling processes involved in LPS-induced inflammation (Dobrovolskaia & Vogel, 2002; Erridge *et al.*, 2002). A member of the Toll-like receptor (TLR) family, TLR-4, together with MD-2, a secreted protein, has been suggested as the receptor complex involved in this signalling process (Medzhitov *et al.*, 1997). The cytoplasmic domain of the TLR-4/MD-2 receptor complex transmits the LPS-induced signal and is homologous

to the cytoplasmic domain of the pro-inflammatory cytokine IL-1 receptor family (Kopp & Medzhitov, 1999), hence, the release of cytokines in models treated with LPS.

In the adult, systemic or central administration of LPS causes a wide spectrum of effects within the body including alterations in immune (Vedder *et al.*, 1999), thermoregulatory (Blatteis *et al.*, 1988; Parkes *et al.*, 1995), cardiorespiratory (Takeuchi *et al.*, 1997), neuroendocrine (Dent *et al.*, 1999; Vedder *et al.*, 1999), metabolic (Takeuchi *et al.*, 1997) (Steiger *et al.*, 1999) and somnogenic (Schiffelholz & Lancel, 2001) properties. It also results in BBB breakdown (Xaio *et al.*, 2001), thus allowing for the potential influx of cytokines into the brain. In addition, several studies have shown that repeated or continuous exposure to LPS can have attenuating effects on various physiological processes including cytokine release (Roth *et al.*, 1994), fever (Kanoh *et al.*, 1977), feed intake (O'Reilly *et al.*, 1988) and HPA axis responsiveness (Hadid *et al.*, 1996). Little is known about the physiological and behavioural effects of LPS treatment in the early postnatal period, however, studies have suggested that early exposure to LPS may disrupt or alter the development of neural mechanisms involved in endocrine responses to stress in later life (Shanks *et al.*, 1995; Shanks *et al.*, 2000). Considering that the early postnatal period is also a time of increased susceptibility to infectious challenges, the need for a greater understanding of the mechanisms involved in the infants response to infection is required.

1.4 Hypoxia

Hypoxia can result from a number of clinical conditions that involve compromised cardiorespiratory function. For example, in the fetus, reduced perfusion and gas exchange at the placenta can lead to hypoxia (Parer, 1998), whilst postnatally, hypoxia may result from infection (Steinschneider, 1975), chronic lung disease (see Fewell & Konduri, 1989), obstructed breathing and intermittent apnoea (see Fewell & Baker, 1987). Hypoxia elicits a number of compensatory mechanisms including increased ventilatory response (Cohen *et al.*, 1997), redistribution of blood flow to vital organs (see Stonestreet *et al.*, 1998), increased HPA activity (Krugers *et al.*, 1995), altered metabolic status (Richardson & Bocking, 1998), decreased body temperature (Almeida *et al.*, 1999) and arousal from sleep (Lewis & Bosque, 1995). These compensatory mechanisms are crucial as they aid in protecting and returning homeostasis to the body. However, if prolonged, the effects of hypoxia are severe with reports of altered CNS activity such as reduced brain activity

(Moss, 2000) and increased seizure susceptibility (Rodriguez Gil *et al.*, 2000) as well as apoptosis and white matter damage (Penning *et al.*, 1994). A reduced oxygen supply to vital organs is one of the most common contributors to neonatal morbidity and mortality due to the neonates low pulmonary oxygen stores and high metabolic demands (Scholz, 2003). Although the short and long-term adaptations to hypoxia have been well documented in fetal and adult models, less is known about the effects of this stressor in the newborn, particularly in relation to neurosteroid synthesis as a response to conditions of low tissue oxygenation.

It has been reported that that the incidence of single or repeated bouts of hypoxia are as common as each other clinically (Moss, 2000), however, little work has been done to investigate the different physiological and behavioural effects of single and repeated hypoxic exposures. Most of the work to date has focussed on the differential effects of hypoxia on ventilatory mechanisms, critical in the processes of arousal from sleep. The tolerance that develops in ventilatory responses (relative hypoventilation) during the neonatal period is reviewed by Moss (2000). This apparent adaptation appears to involve the reduction of oxygen to superoxide, to generate reactive oxygen species that act as chemical signals for the activation of transcription factors including hypoxia inducible factor-1 (HIF-1). Activation of these transcription factors has been reported to up-regulate oxygen responsive genes, which encode a variety of substances and functions involved in the response to hypoxia, such as cytokine release (Moss, 2000). It is of interest to determine if this adaptation applies to other physiological and behavioural parameters when confronted with repeated hypoxic challenges.

1.5 Fundamentals of sleep

From the discussion above, it is apparent that one of the prime physiological responses to infection and/or hypoxia is alteration of behavioural state and, in particular, induction of sleep or drowsiness. Our idea of the essential nature of sleep has changed dramatically over the last forty years from the simple, yet naïve, concept that sleep was merely a state of physical inactivity to the current idea that sleep is a complex behavioural state requiring the interaction of several brain regions and neuronal processes. The organisation of sleep-wake behaviour can be disrupted by the presence of infection (Kadlecova *et al.*, 1972; Mullington *et al.*, 2000) or in cases of hypoxic challenge (Lewis & Bosque, 1995),

however, although much progress has been made to understand the regulation of sleep and wakefulness, the neurophysiological functions remain unknown.

1.5.1 Definitions of sleep and wakefulness

Sleep can be defined as being one of two states, REM sleep or NREM sleep. Electrophysiological techniques, including recordings of nuchal muscle electromyographic (EMG) activity, electrooculargraphic (EOG) activity from eye movements and electrocorticographic (ECoG) activity of the cortex, along with other physiological parameters including heart rate, blood pressure and respiration, have allowed standardised criteria for sleep-wake characterisation to be developed (Szeto & Hinman, 1985).

NREM sleep is unique in that it is the only state that presents high voltage (HV), slow wave cortical activity. It is also identified as having reduced or little EOG activity and nuchal muscle tone, reduced variability in respiration and blood pressure, and few or no body movements. Studies have reported that during NREM sleep, which usually occurs just before and after bouts of REM sleep (Fagioli, 2002), there is a general decrease in brain activity and energy consumption (Steriade & McCarley, 1990), suggesting that NREM sleep is important in restoring energy homeostasis to the body. REM sleep, as the name suggests, is characterised by the presence of REM, and absent or reduced nuchal muscle activity during low voltage (LV), fast wave ECoG activity. Variability in respiration and blood pressure can be observed along with occasional twitches of the face and limbs. REM sleep has long been associated with the processes of dreaming (Hobson *et al.*, 1998), and has also been implicated in maturational processes of the brain (Marks *et al.*, 1995; see Section 1.5.2 for further information) and cognitive function (Karni *et al.*, 1994). Finally, wakefulness is characterised as having low voltage, fast wave ECoG activity, along with eye movements and tonic, but highly variable, EMG activity in the neck muscles. These criteria have enabled consistent analysis between experiments and laboratories, thereby allowing meaningful comparisons to be made.

1.5.2 Development of sleep

The organisation of sleep-wake patterns during development in different species appears to be a reflection of the maturation of the CNS (Szeto & Hinman, 1985). For example, animals born with immature CNS processes, such as rabbits and rats, undergo a long

postnatal developmental period to achieve sleep-wake patterns similar to that of an adult. In contrast, species such as the sheep, which has a more developed CNS at birth, show adult-like sleep patterns from birth (Szeto & Hinman, 1985). The most obvious difference in sleep architecture between the newborn and adult is that the newborn spends more time asleep and also spends a greater percentage of time in REM sleep than do adults, suggesting that this sleep state contributes, in part, to the facilitation of plastic processes in the early development of the CNS (Hobson & Pace-Schott, 2002). The sleep cycle (between NREM and REM sleep) is also of shorter duration in the young and they may pass from wakefulness directly into REM sleep, unlike adults, where a NREM episode always precedes a REM one. Sheep and humans have a developed CNS at birth and the neural processes governing sleep are similar in both species. This makes the sheep an ideal model for the study of somnogenic behaviour, particularly if parallels are to be made with a human model.

1.5.3 Regulators of sleep

Numerous studies have tried to identify a CNS-derived substance that may hold the key to inducing somnolence. For example, cytokines, prostaglandins and neurosteroids have all been identified as having somnogenic properties, but whether sleep is regulated by one factor or a combination of two or more factors remains to be elucidated. The following sections describe the somnogenic capabilities of the neurotransmitter GABA and neurosteroids, with particular attention to the role of allopregnanolone and its precursor progesterone.

1.5.3.1 GABA, $GABA_A$ receptors and sleep

Microdialysis experiments in the cat have revealed the presence of GABA in areas that have been associated with the regulation and/or maintenance of sleep, such as the locus coeruleus (LC; Nitz & Siegel, 1997). This study reported that noradrenergic neurons in the LC fire at their highest rate during wakefulness, are slow during NREM sleep and stop completely during REM sleep. Analysis of GABA concentrations during the sleep periods revealed that levels were higher during REM sleep compared to NREM sleep and wakefulness, suggesting that GABAergic inhibition may be selective in the LC depending on the behavioural state. GABAergic neurons have also been found in the pedunculopontine tegmental nucleus (PPT) of the cat (Torterolo *et al.*, 2002), a region implicated in the process of wakefulness and critical for the generation and/or

maintenance of REM sleep. When muscimol, a $GABA_A$ agonist, was microinjected into the PPT, it increased the amount of time spent in REM sleep, had no effect on NREM sleep and subsequently decreased the total amount of wakefulness. The same authors found that bicuculline, a $GABA_A$ antagonist, had the opposite effect to muscimol, by increasing wakefulness and decreasing the percentage of time spent in either REM or NREM sleep (Torterolo *et al.*, 2002). This study concluded that GABA, through actions at the $GABA_A$ receptor, is involved in the processes of REM sleep generation and in the promotion of wakefulness.

Intraperitoneal (i.p.) administration of the selective $GABA_A$ receptor agonists, muscimol and 4,5,6,7-tetrahydroisoxazolo-pyridin-3-ol (THIP), into the adult rat has also been reported to alter sleep-wake behaviour in a dose-dependent manner (Lancel, 1997). The effect was restricted to NREM sleep as was the case in studies on humans where oral administration of THIP increased the duration of NREM sleep (review Lancel, 1999). To the best of my knowledge, there is only one study that has investigated the role of the $GABA_A$ receptor on behaviour in the early postnatal period. In this study, muscimol was dialysed into the rostral ventral medulla (RVM; a region involved in cardiorespiratory regulation) of newborn piglets (Darnall *et al.*, 2001). This study found that muscimol altered the sleep-wake architecture of these animals inasmuch as that the usual sleep cycling that is common in newborns was abolished and the 'depth' of sleep was increased. This study highlights the somnogenic capabilities of the $GABA_A$ receptor agonists and lends support to the idea that neurosteroids such as allopregnanolone (as described in Section 1.1.5.2) may play a role in the regulation of sleep-wake behaviour in the newborn.

1.5.4 Clinical factors affecting sleep

Given that sleep is a highly organised state, it should not be surprising that there are many situations and/or diseases that can disrupt the architecture of sleep. The following sections will describe the effects of two stressors applicable to this thesis, infection and hypoxia, and their effects on sleep-wake behaviour.

1.5.4.1 Infection

Increased lassitude or drowsiness, common symptoms of systemic infection, is regarded as a homeostatic mechanism that is part of the APR to facilitate the healing process. Several studies have reported that administration of endotoxin alters sleep-wake behaviour by

increasing the incidence of NREM sleep. In rats (Kadlecova *et al.*, 1972), rabbits (Toth & Krueger, 1989) and at low doses in humans (Mullington *et al.*, 2000), administration of endotoxin disturbs sleep cycling by increasing the incidence of NREM sleep whilst having little or no effect on REM sleep, suggesting that increased NREM sleep is critical for the host defence response. The exact mechanism(s) and/or substance(s) involved in somnogenesis remain unclear, however, it is likely that the release of pro-inflammatory cytokines, particularly those with somnogenic capabilities (Krueger *et al.*, 1990), begins the process during infection.

1.5.4.2 Hypoxia

To date, most studies that have investigated the relationship between somnogenesis and hypoxia have focused on arousal responses, which are critical for survival. Hypoxic challenges act on mechanoreceptors and/or oxygen sensitive chemoreceptors to trigger ventilatory and cardiovascular responses to induce arousal (Berry & Gleeson, 1997). However, during REM sleep, it appears that these arousal mechanisms are depressed. In studies performed on lambs, arousal from NREM sleep occurred more quickly than from REM sleep and also occurred at a higher arterial oxygen level (Fewell & Baker, 1987). In addition, repeated hypoxic exposures were also found to become ineffective as an arousing stimulus during REM sleep (Johnston *et al.*, 1998) due to a depression of ventilatory and blood pressure responses (Johnston *et al.*, 1999). Given that NREM sleep increases during infectious challenge (Mullington *et al.*, 2000), one would assume that not only is this part of the host defence response but also a mechanism to increase survival of the host (through arousal) to further challenges such as hypoxia. Considering that the newborn spends a greater percentage of time in REM sleep, and that it is at an increased risk of infectious challenges that can result in hypoxia, a failure to increase the incidence of NREM sleep and, hence, arousal responses could have detrimental consequences.

1.6 Sudden Infant Death Syndrome

Sudden Infant Death Syndrome, a phenomenon that occurs during sleep, remains one of the leading causes of death in infants under one year of age (review Sullivan & Barlow, 2001). It is defined as the 'sudden and unexpected death of an apparently healthy infant whose death cannot be explained even after a thorough case investigation and autopsy' (Goldwater, 1992). The possible contributing factors to SIDS are numerous. A triple-risk hypothesis proposed by Filiano and Kinney (1994) suggests that SIDS is the result of the

interaction of three overlying factors: 1) a vulnerable infant; 2) a critical period in development of homeostatic control; and 3) an exogenous stressor. However, a recent review by Guntheroth and Spiers (2002) questioned the reliability of this model, suggesting that it brings us no closer to understanding the pathophysiology involved in a SIDS death. Due to the contentious issues associated with SIDS, this brief summary of the literature will examine the epidemiological evidence of two possible risk factors proposed to be associated with SIDS, infection and hypoxia.

1.6.1 Infection and SIDS

A number of general observations have suggested a role for infection in the aetiology of SIDS. Most SIDS deaths occur between the ages of 2 and 6 months (Goldwater, 1992), a period where susceptibility to infection can be high due to a decline in the availability of maternally-transmitted antibodies (Morris, 1999). During this time, infants are also establishing sleep patterns and arousal mechanisms, and any disturbances resulting from infection may lead to a life-threatening event (Toth & Chaudhary, 1998). Several papers have reported that the prevalence of SIDS is reduced in breast-fed babies (Raza & Blackwell, 1999) and is distributed seasonally with higher death rates occurring in winter months (Raza & Blackwell, 1999; review Sullivan & Barlow, 2001). Both respiratory and bacterial infections have been identified in babies who have died of SIDS (Yolken & Murphy, 1982; Jakeman *et al.*, 1991; Lindgren *et al.*, 1996; Kerr *et al.*, 2000). Although these infections are not considered to be the direct cause of death, it is suggested that they may be triggers for an uncontrolled or abnormal inflammatory response, eliciting symptoms similar to septic shock (Raza & Blackwell, 1999; review Sullivan & Barlow, 2001).

1.6.2 Hypoxia and SIDS

Hypoxia can result from a number of factors including infection, smoking, chronic lung disease and intermittent apnoea (Steinschneider, 1975; Fewell & Baker, 1987; Fewell & Konduri, 1989). It has been hypothesised that hypoxia is one of the final factors in the aetiology of SIDS, based on pathological studies reporting increased retention of brown fat around adrenal glands, increased levels of cortisol in the plasma (Naeye, 1988), and increased gliosis (Kinney *et al.*, 1983) and substance P (Ozawa & Takashima, 2002) in the brainstem of the victims. Recently, Jones *et al.* (2003) reported that levels of vascular endothelial growth factor (VEGF), a sensitive marker to changes in partial oxygen tension

in tissues, was significantly higher in the cerebrospinal fluid of SIDS victims than in infants that died of known causes, thereby lending further support to a role for hypoxia in the aetiology of SIDS. Since the newborn has a reduced lung volume that is associated with irregular breathing and diminished intercostal muscle function (Henderson-Smart & Read, 1979), arousal from progressive hypoxia is critical for survival. Reasons as to why some babies appear to be more susceptible to hypoxic challenges than others remain to be elucidated.

1.7 Summary

Neurosteroids act on the $GABA_A$ receptor to produce alterations in CNS excitability in response to stressful stimuli. Allopregnanolone, a potent modulator of the $GABA_A$ receptor, increases in response to stress, suggesting a possible neuroprotective role for this steroid. Studies focusing on the effects of neurosteroids in response to stressful stimuli have been restricted largely to the adult rat model, therefore, little information is available for other species and the different stages of development. Therefore, the first aim of this thesis is to examine the effects of stressful stimuli on neurosteroid production during the early postnatal period in the sheep.

It has been reported that susceptibility to infection and hypoxia is heightened during the early postnatal period. This could be due to a number of factors including an immature immune system or decreased lung function. However, it appears that a common link between these two stress-inducing paradigms is their effects on sleep architecture. Infection has been reported to increase the incidence of sleep whilst hypoxia has been shown to 'deepen' the level of sleep by raising arousal thresholds. Since allopregnanolone and other potent neurosteroids have somnogenic properties, a hypothesis of this thesis is that allopregnanolone concentration will increase in response to infection and hypoxic challenge, resulting in increased sleep. This thesis will examine the effects of endotoxin and/or hypoxia treatments on neurosteroid concentrations and behaviour in lambs.

Chapter 2: Materials and methods

This chapter presents the general methodologies followed for the experiments used in this thesis. However, where there are methods that are unique to a particular study, the details are presented in the relevant chapter. The Monash University Standing Committee on Ethics and Animal Experimentation (SCEAE) granted prior approval for the use of the animals and all procedures performed.

2.1 Animals

Pregnant Merino cross Border Leicester cross ewes of known gestational age were brought into the Department of Physiology's animal house at least 2 weeks prior to their delivery date. Each ewe was held in a lambing pen, monitored daily for well being and fed lucerne chaff once a day. Water was available *ad libitum*. Lambs were allowed to deliver spontaneously and were weighed, sexed and the number of siblings recorded within 12 hours. Only lambs delivered from singleton or twin pregnancies were used for the experiments presented in this thesis. All lambs remained with their mother except for during the experimental periods described in Chapters 3, 4, 5 and 6.

2.2 Preparation of electrodes

Electrodes for recordings of sleep-wake states were prepared using the following method. One length (1.5 m) of maternal vascular catheter tubing (inner diameter, ID, 1.5 mm, outer

diameter, OD, 2.5 mm, Critchley Electrical Products, NSW, Australia) was cut and threaded with either two (for EOG and EMG recordings) or three (for ECoG recordings) strands of insulated stainless steel wire (1.7 m, Cooner Wire Co., Chatsworth, CA, USA). Approximately 50 mm of each length of wire was left extending from one end of the catheter and 150 mm from the other. The lengths of wire were secured in the tubing by silastic sealant that was injected using a small gauge needle and syringe. After the silastic had set (usually within 24 hours), approximately 10 mm of insulation was stripped from the end of the short wires using a soldering iron. These ends were then soldered into gold pins (1 mm diameter, Radio Spares, VIC, Australia). The wires at the other end of the tubing were prepared for measurement of the behavioural parameters as follows: (1) ECoG was recorded via a 3-wire electrode. A rubber disc (1 mm thick, 5 mm in diameter) was threaded over two wires and the ends of these wires (~5 mm) were exposed. The third wire served as a ground electrode that had a portion of the wire (~5 mm) exposed mid-way; (2) EOG and EMG were recorded via 2-wire electrodes. These wires were prepared similarly to the ground wire of the ECoG. All electrodes were sterilised prior to surgery by ethylene oxide.

2.3 Surgery

2.3.1 Care of lambs

Ewes rely heavily on their sense of smell to recognise their young. Unfortunately, the smells associated with surgery would often result in the ewe rejecting the lamb post-operatively. This created a major problem for the lambs, as they would have difficulty in feeding from the ewe. Following initial pilot studies, it was found that preparing lambs for surgery shortly after birth minimised the risk of post-operative maternal rejection. A protective jacket (Dialex, VIC, Australia), that was used to cover the lamb post-surgery and thereby reduce the risk of catheters being chewed by the mother, was placed on the floor of the lambing pen on the day of the birth to allow association to occur. This coat was then placed on the lamb within 48 hours. An antibacterial spray (oxytetracycline hydrochloride, 2 mg/g, Terramycin Pinkeye Aerosol, Pfizer, Australia), later used to treat the surgical incision sites, was sprayed in the lambing pen, on the ewe's nose and on the jacket at this time. This reduced the sudden influx of foreign smells on the lamb associated with surgery. No ewe rejected her lamb post-operatively once these procedures were in place.

2.3.2 Pre-operative preparation

Lambs were anaesthetised by inhalation of 1-2 % Flurothane (BOC Gases, Australia) in O_2 (Midget anaesthetic machine, BOC Gases, Australia). Anaesthesia was maintained throughout surgery following tracheal intubation with an inflatable-cuffed endotracheal tube (size 5-6 mm, Portex, England). Lambs breathed spontaneously whilst anaesthetised, removing the need for mechanical ventilation.

Surgical sites were shorn following anaesthesia. These included the top of the lamb's head, extending over the left eye and down to the nose, the dorsal and ventral sides of the neck, along the midline of the back, the left flank and the thorax. Once shorn, these areas were scrubbed using Hibitane antiseptic solution (Cetamide 150 mg/ml 15 % w/v, ICI, NSW, Australia) and Betadine antibacterial scrub (7.5 % w/v povidone-iodine, Faulding & Co., SA, Australia). The skin was wiped down with warm water and the process repeated. Antiseptic solution (10 % w/v povidone-iodine, Faulding, SA, Australia) was then applied liberally to the area. Lambs were placed prone on heating pads on the operating table and their necks supported with a rolled drape. Antibiotics (Procaine penicillin, 200 mg; Dihydrostreptomycin, 250 mg) were given intramuscularly just prior to surgery. All aseptic surgery was performed on lambs aged between 3 and 6 days.

2.3.3 Electrodes

Incisions were made between the scapulae of the lamb (5-7 cm), at the neck (3-5 cm) and medially over the skull (5-7 cm long) followed by blunt dissection between these sites. A 20 cm length of tubing was inserted subcutaneously between the scapulae and the neck. The electrodes (ECoG, EOG and EMG) were passed through this tubing, which was then removed. This process was repeated again between the neck and skull.

2.3.3.1 Electro-oculogram (EOG)

A subcutaneous path to the left eye was formed from the medial incision over the skull. Two small incisions were made (1-2 cm) at the inner and outer canthi of the left eye and the two-wire EOG electrode fed through. Each wire was sewn under the skin, so that the bared portion of the wire lay completely within the muscle, and the incisions were then sutured closed. This allowed EOG activity to be recorded.

2.3.3.2 Electrocorticogram (ECoG)

Skin was freed from the underlying skull by blunt dissection on either side of the medial incision (2-3 cm each side). The skull was then scraped clean with a scalpel blade. Two holes (approximately 1 mm in diameter) were drilled through the parietal bones, located sagitally to the midline. Care was taken to not penetrate the dura mater. The bared portion of the wire was inserted into the hole far enough so that it lay on the dura mater. The wires were then secured in place using a rubber disc (5 mm diameter) attached to the surface of the skull with adhesive (Vetbond, 3M Animal Care Products, MN, USA). A third wire was sewn into the subcutaneous tissue of the skull to act as a common earth for all three electrodes. Both the EOG and ECoG electrodes were sewn securely under the skin at the base of the skull and the incision sutured closed.

2.3.3.3 Nuchal muscle electromyogram (EMG)

The nuchal muscles located bilaterally of the midline of the neck were exposed. A single wire of the 2-wire EMG electrode was then sewn into each muscle so that the bared portion lay completely within the muscle. At this point, all three electrodes were secured by attaching them to the underlying skin. The incision was not closed until after catheterisation of the carotid artery and jugular vein (see Section 2.3.4).

2.3.4 Arterial and venous catheterisation

Arterial and venous catheters (~1.0 m; ID 0.86 mm, OD 1.52 mm, Dural Plastics and Engineering, NSW, Australia) were inserted through the incision between the scapulae and fed through tubing to the incision site at the neck. Blunt dissection was performed to create a subcutaneous pathway from the dorsal to the lateral side of the neck. A small incision (~5 cm) was made on the lateral side of the neck and the catheters fed through. The carotid artery was located using blunt dissection and separated from the vagus nerve. Two silk ties were placed approximately 3 cm apart and the artery occluded by securing the silk thread on the cranial side. A bulldog clip was placed distally to the caudal tie and a small incision was made in the arterial wall and the appropriate catheter inserted. The caudal tie was then secured around the vessel and the clip removed. Patency of the heparinised, saline-filled catheter was tested and, once confirmed, the catheter was advanced 75 mm towards the heart and secured with the second tie. The catheter for the jugular vein was inserted 75 mm into the vessel using a similar method as for the carotid

artery. Catheters were secured subcutaneously at both neck sites, and each site sutured closed.

2.3.5 Intrapleural balloon

One length (1.5 m) of maternal vascular catheter tubing (ID 1.5 mm, OD 2.5 mm) was cut and a custom made latex balloon (2-3 cm long, constructed from a condom tip, non-lubricated, Ansell, Australia) secured onto one end. Each balloon-tipped catheter was tested for leaks with saline prior to surgery and then sterilised. This catheter was used as an arbitrary indicator of respiratory rate.

Blunt dissection was performed to create a subcutaneous pathway from the incision site between the scapulae to the left flank between the 5^{th} and 6^{th} ribs. A 3-4 cm incision was made at this site (over the 5^{th} intercostal space, so as to avoid the diaphragm) and the balloon catheter fed through tubing from the back of the lamb. The intercostal muscle layer was separated using blunt dissection until the pleural membrane was located. A purse-string suture (size 3-0 silk) was placed into the muscle layer (15 mm diameter) and tied loosely. A small incision in the pleural membrane, in the middle of the purse-string suture, allowed the balloon-tipped catheter to be introduced and placed 3-4 cm into the pleural cavity. The lungs were then over-inflated manually to expel air from the intrapleural space and the purse-string suture was tightened to draw the intercostal muscles together around the catheter. The catheter was secured under the skin and the incision site was sutured closed.

2.3.6 Exteriorising the catheters

At the completion of surgery, all electrodes and catheters were exteriorised through the same incision site (between the scapulae) to reduce the need for a second incision site. Each catheter was anchored under the skin and the incision sutured closed using synthetic thread (Vetafil, WDT, Germany). All catheters were then secured externally by anchoring them to the outer surface of the skin. Sterile three-way stopcocks (Discofix-3, Braun, Germany) were attached to the exposed end of each of the three catheters and antibacterial spray (2 mg/g oxytetracycline hydrochloride, Terramycin, Pfizer, Australia) was applied liberally. Electrodes and catheters were placed into plastic bags secured to elastic netting (Setonet, Seton Healthcare Group, UK) that was placed around the torso of the lamb.

2.3.7 Post-operative care

Each lamb was kept under a heating lamp following surgery and observed until it appeared to have recovered fully. A protective jacket was placed on the lamb (over the netting) before it was returned to its mother. Lambs were able to feed within 2-3 hours following surgery. Antibiotics were given for two days post-surgery and all catheters were flushed with heparinised saline daily for 3-4 days, at which time experiments commenced.

2.4 Behavioural parameters: recording and analysis

2.4.1 Recordings

Within 2 days of surgery, all animals were connected to a Grass 8-Channel Polygraph machine (Model 7D Polygraph, Grass Instrument Co., Quincy, MA, USA) via the electrodes and catheters. This allowed calibration of the recordings of ECoG, EOG, nuchal muscle EMG, heart rate, blood pressure and respiratory rate before experimental protocols commenced. All channels on the Grass Polygraph used 50 Hz low-pass filters to eliminate interference from other pieces of electrical apparatus within the room. A video camera was focused on the upper torso and head of the lamb to further aid the identification of sleep-wake behaviour.

2.4.1.1 ECoG, EOG and nuchal muscle EMG

ECoG electrical activity from the lamb was amplified by passing the signal through a Wide Band AC EEG Pre-Amplifier (Model 7P5B, Grass Instrument Co., MA, USA) with high-input impedance. The signal was then passed through a DC amplifier that was calibrated with the ½ amplitude high frequency set at 35 Hz, enabling the signal to be passed through and recorded on the polygraph. EOG activity and nuchal muscle activity were recorded on the polygraph similarly to ECoG, except that the EOG signal was recorded with the ½ amplitude high frequency set at 15 Hz. All pen driver amplifiers were calibrated before each experiment so that a 1 cm vertical deflection on the trace recording corresponded to a 1 mV change. All recordings were displayed on the 8-Channel Polygraph machine at a chart speed of 5 mm/min.

2.4.1.2 Cardiorespiratory parameters

Blood pressure and an arbitrary measurement of respiratory rate of lambs were measured directly from the arterial and intrapleural balloon catheters, respectively. Both catheters

were connected to fluid filled pressure transducers (Pa23, Gould-Statham, USA) that were sterilised and calibrated prior to use. Calibration to 100 mmHg was performed using a water filled manometer connected to a mercury column. Pressure output from the catheters was passed directly into DC amplifiers and displayed at a paper speed of 5 mm/min. Rate meters triggered by the arterial and intrathoracic pressure waveforms were used to determine heart rate and breathing rate, respectively. Calibration of driver pens with the transducers open to air, ensured baseline positions remained constant within and between experiments. Sensitivity of the driver pens was set so that 100 mmHg was the equivalent of a 1 V signal.

2.4.2 Analysis of recordings

For each experiment performed, all parameters (ECoG, EOG, nuchal EMG, blood pressure, heart rate and respiratory rate) were measured minute-by-minute. Consistent determination of behavioural state was achieved using the following scoring system. ECoG was scored as having either a high voltage (HV; 0) or low voltage (LV; 1) signal, with the threshold established during basal recordings. The presence or absence of EOG and nuchal EMG signals were scored as 0 or 1, respectively, using the amplitude of the signal. These individual scores and a sleep-wake definition were determined from the recording (see Figure 2.1 for example), for each minute, and entered into a spreadsheet (Table 2.1). If signals for any parameter measured lasted less than one minute, they were deemed to be random movements and were excluded.

2.4.3 Definition of sleep states

Sleep-wake states determined from the chart records, following the method described previously, were confirmed by videotape recordings. Briefly, active sleep (AS), otherwise known as REM sleep, was characterised by the presence of rapid eye movements and absent or reduced nuchal muscle EMG activity during low voltage, fast wave ECoG activity. Lambs in AS also showed increased variability in respiration and blood pressure and exhibited occasional twitches of the face and limbs. Quiet sleep (QS), otherwise known as NREM sleep, was identified as episodes with reduced or no EOG activity and tonic EMG discharge of the nuchal muscles during high voltage, slow wave ECoG. During QS, there was also reduced variation in respiration, blood pressure and body movements. Stages of sleep that could not be identified clearly as AS or QS, but were nonetheless a 'drowsy-like' state, were defined as indeterminate sleep (IS). Wakefulness

was characterised by low voltage, fast wave ECoG accompanied by eye movements and tonic and highly variable EMG activity in the neck muscles.

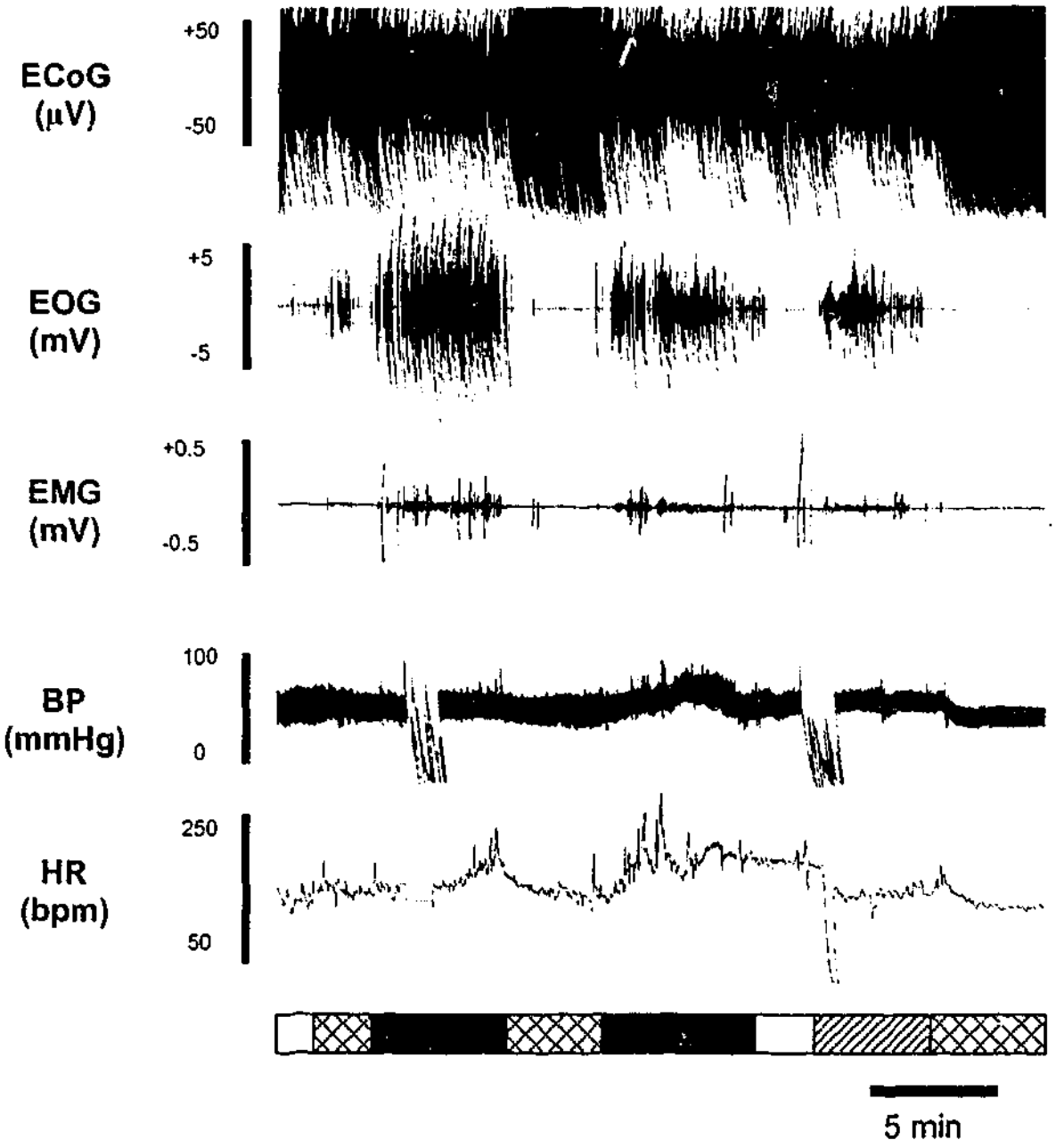


Figure 2.1: Trace recording from a lamb in a control experiment

A one hour polygraph recording showing behavioural states in a 21 day old control lamb: wakefulness (*black bars*), quiet sleep (*white bars*), active sleep (*hatched bar*) and indeterminate sleep (*checked bars*). Breaks in blood pressure and heart rate recordings are indicators of when sampling took place.

Table 2.1: Spreadsheet for behavioural analysis

Hour	*Minute*	*ECoG*	*EOG*	*EMG*	*Sleep State*
1	16	1	1	1	AW
1	17	1	1	1	AW
1	18	1	1	1	AW
1	19	1	1	1	AW
1	20	0	0	1	QS
1	21	0	0	1	QS
1	22	0	0	1	QS
1	23	0	0	0	IS
1	24	0	0	0	IS
1	25	1	1	1	AW
1	26	1	1	1	AW
1	27	1	1	0	AS
1	28	1	1	0	AS
1	29	1	1	0	AS
1	30	1	1	1	AW

An example of a spreadsheet used to record the sleep-wake state of lambs: AW (wakefulness), QS (quiet sleep), AS (active sleep) and IS (indeterminate sleep). Analysis was performed on a minute-by-minute basis using the criteria described in Section 2.4.2.

2.5 Blood and tissue collection

2.5.1 Blood collection

Blood samples were collected at various time points during each experiment using sterile syringes and transferred into chilled sterile ethylenediaminetetraacetic acid (EDTA; for measurement of ACTH only) or lithium heparin (for all other parameters) primed tubes. Blood samples were then centrifuged at 3000 rpm for 10 minutes at 4 °C. Aliquots of plasma for iron, glucose, lactate, ACTH, cortisol, pregnenolone, progesterone, allopregnanolone and cytokines were then stored at -20 °C until required for analysis. Sterility was maintained at all times.

2.5.2 Blood gases and plasma glucose and lactate

Arterial blood gases (O_2 saturation, SaO_2; partial pressure of O_2, PaO_2; and CO_2, $PaCO_2$), pH and haemoglobin (Hb) were determined using a haemoximeter and an ABL-30 Acid-Base analyser (Radiometer, Copenhagen, Denmark) with values adjusted for the prevailing rectal temperature of the lamb. Plasma glucose and lactate concentrations were measured using a 2300 STAT analyser (YSI, Yellow Springs, Ohio, USA).

2.5.3 Euthanasia

At the conclusion of all studies, all lambs used in these studies were killed with an injection of a lethal dose (5-10 mL) of 325 mg/mL sodium pentobarbitone (Letharbarb, Peakhurst, NSW, Australia) at the conclusion of experiments.

2.5.4 Tissue collection

Immediately after the lamb was killed, the spinal cord was exposed and cut caudal to the medulla. The entire brain and upper spinal cord was then removed carefully. Viewing the brain with the ventral side up, a coronal cut was made at the optic chiasm to separate the cerebrum from the other structures. The cerebrum was divided into two hemispheres, the hippocampi removed and each hemisphere divided coronally into the frontal, primary and occipital cortices. The remaining cortex was further separated into the parietal and the less defined temporal cortex with a ventral cut, 5 mm from the dorsal surface. A coronal cut was made caudal to the mammillary bodies and the thalamus/hypothalamus blocked. The cerebellum was then removed from the remaining hindbrain by carefully stripping away

any remaining dura and arachnoid from the medulla. Finally, the midbrain, pons and medulla were separated with coronal cuts made at the pontine-midbrain junction (to separate the midbrain from the pons), the trapezoid body (rostral end of the medulla) and the medulla-spinal cord junction. Each brain region was then snap-frozen in liquid N_2 and stored at -70 ^{0}C until required for analysis of steroid concentrations.

2.6 Plasma iron

Iron concentrations in plasma were determined manually using an Avanta Atomic Absorbance Spectrophotometer (AAS, GBC Scientific Electronics, Australia). Standard solution (1.035 mg/mL in 1 % HCl) was obtained from Sigma Chemical Company (NSW, Australia). An initial stock solution (1:100) of 185 µM was made in 2.5 % TCA (10.35 mg/L). Final concentrations of 10-160 µM were prepared with a final volume of 10 mL per standard. A hollow cathode iron lamp was purchased from GBC Scientific (NSW, Australia). An air-acetylene flame with a lamp current of 7 mA, wavelength of 248.3 nm and a slit width of 0.2 nm were used for greatest sensitivity.

2.6.1 Measurement of iron

After calibration of the AAS with standards and deionised water (dH_2O), a manual process was used to feed plasma samples (~100 µl; in duplicate) directly into the nebuliser. Manual administration of samples allowed smaller volumes to be used. The nebuliser distributed the sample as an aerosol. This generated smaller particles that were easier to atomise. The minimal detectable concentration of iron using this method was 1.68 ± 0.23 µmol/L (n=4).

2.7 Plasma ACTH

Plasma ACTH concentrations were measured in unextracted samples using a kit available commercially (ICN Biomedical Australasia Pty Ltd, NSW, Australia). Standards were used at concentrations ranging from 6.8-170 pmol/L, reconstituted in dH_2O. Cross reactivities of the antiserum are presented in Table 2.2. The minimal detectable level of ACTH was 2.89 ± 0.03 pmol/L (n=4). The intra and interassay coefficients of variance were 10 % (n=6) and 7 % (n=10), respectively.

2.7.1 Measurement of ACTH

Duplicate plasma samples (10-100 µl) or ACTH standards (100 µl) were incubated overnight at 4 °C with dH_2O (100 µl), ACTH antiserum (100 µl) and ^{125}I-ACTH (100 µl). The next day, a second antibody/polyethylene glycol (PEG) (500 µl; BDH Chemicals, USA) solution was added and all tubes were centrifuged at 3500 rpm for 15 minutes at 4 °C to separate bound from free fractions. The supernatant was aspirated and the precipitate counted for radioactivity (γ) using a Multidetector Radioimmunoassay (RIA) system (United Technologies Packard, USA).

Table 2.2: ACTH antiserum cross reactivity with closely related steroids

Peptide	*Cross Reaction (%)*
Human ACTH 1-24	100.00
Porcine ACTH 1-39	100.00
α-MSH	<0.01
β-Endorphin	<0.01
β -Lipotropin	<0.01
Leucine Enkephalin	<0.01
Methionine Enkephalin	<0.01
Bombesin	<0.01
Calcitonin	<0.01
Parathyroid Hormone	<0.01
FSH	<0.01
Human Growth Hormone	<0.01
Vasopressin	<0.01
Oxytocin	<0.01
Substance P	<0.01

2.8 Tissue and plasma allopregnanolone

Methods for allopregnanolone extraction and quantification in plasma were established by Purdy *et al.* (1990). Subsequent studies by Purdy *et al.* (1991) progressed the allopregnanolone extraction process to include brain tissue. There were several disadvantages to the methods established by Purdy and colleagues, including a requirement for large quantities of plasma (0.3 mL), the use of heavy solvents such as hexane and ether for plasma extraction, and the additional purification step of high-performance liquid chromatography (HPLC). These extraction procedures were also established specifically for the quantification of allopregnanolone only. Several improvements were made due to: (1) a limitation on the amount of plasma available and (2) the need to find an extraction procedure that would allow for the simultaneous extraction of several steroids in both brain tissue and plasma. A later study by Barbaccia *et al.* (1992) modified the extraction method to allow for the simultaneous extraction of pregnenolone, progesterone and desoxycorticosterone (DOC) in brain tissue. However, these methods did not include allopregnanolone. Thus, one of the aims of this study was to establish a method of extraction that allowed for the simultaneous measurements of steroids in both brain tissue and plasma. The following is a description of the steps taken to achieve optimal results.

2.8.1 Brain tissue and plasma extraction

2.8.1.1 Homogenate preparation

Frozen brain samples were crushed with a mortar and pestle on dry ice and approximately 100 mg of sample was added to 1mL (1:10 dilution) of ice-cold 50 % methanol containing 1 % acetic acid. This mixture was then homogenised on the maximum setting for 3 x 30 second bursts using a hand-held homogeniser. Samples remained on ice for approximately one minute between each burst. The homogenate was then centrifuged at 3000 rpm for 15 minutes at 4 °C and the supernatant collected. The pellet was resuspended twice in the above solution, centrifuged and the supernatants collected, combined and stored at -70 °C until required. Plasma samples (110 µl) were combined with ice-cold 50 % methanol containing 1 % acetic acid (990 µl; 1:10 dilution) and mixed well.

2.8.1.2 Extraction by Sep-Pak cartridges

Steroids were extracted from plasma and the supernatants from brain homogenates by passing 1 mL of acidified sample through a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA), equilibrated previously by washing first with 2.5 mL 100 % methanol, followed by 2.5 mL 50 % methanol and finally with 2.5 mL 50 % methanol containing 1 % acetic acid. After loading the samples, the cartridge was washed with 2.5 mL 50 % methanol containing 1 % acetic acid followed by 50 % methanol, and the steroids eluted with 2 mL 100 % methanol. All eluent washes were discarded and the final eluent was then brought to dryness under nitrogen. Brain tissue was reconstituted in 2 mL 0.1 M phosphate buffer (pH 7.4), whilst plasma was reconstituted in 0.5 mL of the same assay buffer. Additional samples of brain and plasma containing tritium-labeled steroid (n=3) were homogenised and extracted to estimate recovery. The recovery of each steroid through the extraction procedure (75-90 %) was determined for each assay run by adding 100 µl of sample to 1 mL of EcoScint (National Diagnostics, USA) for progesterone and 100 µl of sample to 2.5 mL of EcoScint for pregnenolone and allopregnanolone. All samples were counted using a Beckman LS 3801 liquid scintillation counter (Beckman Instruments Inc., Irvine, CA, USA) to assess β-activity and the data obtained at the end the assay was corrected for this procedural loss. Table 2.3 presents the average recovery for each steroid.

Table 2.3: Recoveries for steroid extraction

Steroid Compound	*Recovery (%)*
Pregnenolone	89.48 ± 1.56
Progesterone	76.32 ± 1.53
Allopregnanolone (brain)	78.88 ± 1.29
Allopregnanolone (plasma)	88.31 ± 1.72

2.8.1.3 Optimisation of tissue preparation and extraction

Several steps were taken to ensure optimal conditions for tissue preparation and steroid extraction. The following are a series of parameters investigated to achieve high recoveries.

2.8.1.3.1 Homogenising tissue in buffer or solvent?

In initial experiments, tissue samples were homogenised in either 0.1 M phosphate buffer (pH 7.4) or 50 % methanol containing 1 % acetic acid at *room temperature* and the supernatants extracted with solvent. These methods showed recoveries in the range of 50-75 %, which was not deemed sufficient to continue with the assay procedure. Subsequent studies using *ice-cold* 50 % methanol containing 1 % acetic acid resulted in recovery rates of between 75 and 90 %. Recoveries were also more consistent between experiments using this ice-cold homogenate solution and, hence, it was used for all samples assayed in this thesis. Plasma samples were also combined with different buffers/solvents. Recovery rates were as follows: *ice-cold* 50 % methanol containing 1 % acetic acid had higher and more consistent recovery rates (~98 %) than 0.1 M phosphate buffer (~85 %) or diethyl ether (~75 %).

2.8.1.3.2 Solvent for elution of steroids

The final step of the extraction process was the elution of the steroids from the Sep-Pak cartridge. A number of solvents were tested to achieve optimal recovery rates. Table 2.4 presents the recovery rates for the different solvents used. Methanol (100 %) provided the highest and most consistent recovery rate. Dichloromethane proved to be difficult to handle with separation of the more aqueous phase from the dichloromethane, and hexane required two elution steps to achieve satisfactory recovery.

Table 2.4: Recoveries from the Sep-Pak cartridge using different solvents

Solvent	*Recovery (%)*
100% Methanol	100
Dichloromethane	98
Hexane	71 + 24
Acetonitrile	68

Note: two numbers for hexane indicates the need for two elution steps to achieve satisfactory recovery.

2.8.1.3.3 Incubation times

Preliminary experiments also assessed the reconstitution/incubation times of samples in buffer solution following extraction, but prior to assaying. Three incubation periods were assessed; 1, 24 and 48 hours. Table 2.5 presents the recovery rates for the different incubation times. This assessment determined that 48 hours was optimal for all samples to achieve equilibrium prior to being assayed.

Table 2.5: Recoveries for different incubation times

Incubation time	*Recovery (%)*
1 hour	30-70
24 hours	50-80
48 hours	75-90

2.8.2 Measurement of allopregnanolone

2.8.2.1 General reagents

2.8.2.1.1 Standards

Allopregnanolone standard (provided generously by Dr RH Purdy, San Diego, CA, USA) was prepared as a stock solution in absolute ethanol (314 µmol/L) and stored at -20 °C. The stock standard was further diluted with ethanol, to working dilutions of 31.4 µmol/L, 3.14 µmol/L, and 314 nmol/L, on the day of the assay. Final concentrations of 0.34-25.12 nmol/L in 0.1 M phosphate buffer (pH 7.4) were used in the assay procedure.

2.8.2.1.2 Tracer

Allopregnanolone tracer (25 µCi of Pregnan-3α-ol-20-one,5α-[9,11,12-^{3}H(N)]-; NEN Life Science Products, Inc., MA, USA) was made up to 1 mL in ethanol and stored at -20 °C. On the day of the assay, a small amount of tracer was added to 0.1 M phosphate buffer (pH 7.4) to give ~25000 dpm/0.25mL/tube.

2.8.2.1.3 Antiserum

Allopregnanolone antiserum, raised in sheep against allopregnanolone carboxymethyl ether coupled to bovine serum albumin (BSA), was purchased from Dr RH Purdy (San Diego, CA, USA). Neat serum was diluted (1:100) with 0.1 M phosphate buffer (2 mL) and aliquoted into 200 µl lots. The cross reactivities of the allopregnanolone antiserum are presented in Table 2.6. The limit of detection for allopregnanolone was 0.19 ± 0.03 pmol/tube (n=11). The intra and interassay coefficients of variance were 5 % (n=4) and 9 % (n=17), respectively.

2.8.2.1.4 Charcoal stripped plasma

Sheep plasma was used for the production of charcoal stripped plasma (CSP), a plasma sample free of steroids. Charcoal was added to plasma in a ratio of 10 mg charcoal to 1 mL plasma. This mixture was incubated at 35 °C for 30 minutes and vortexed every 10 minutes for 30 seconds. After incubation, the charcoal/plasma mix was centrifuged at 3000 rpm for 20 minutes to layer the charcoal. The supernatant was decanted carefully, stored at -20 °C and used for validation of the assay.

2.8.2.2 Allopregnanolone RIA methods

Following extraction, 100 µl of each sample was assayed in duplicate using the following procedure. Brain and plasma samples were incubated overnight at 4 °C with 0.1 M phosphate buffer (150 µl), antiserum (250 µl) and ^{3}H-allopregnanolone (250 µl). Allopregnanolone standards (100 µl) were also incubated overnight with antiserum (250 µl) and ^{3}H-allopregnanolone (250 µl). The bound and free fractions were separated by the addition of 200 µl of a cold suspension of dextran-coated charcoal (0.625 % charcoal activated, Sigma Chemical Co., NSW, Australia; 0.0625 % Dextran T70, Pharmica Fine Chemicals, Uppsala, Sweden). After centrifuging at 2500 rpm for 10 minutes at 4 °C, 500 µl of the supernatant was aliquoted into 6 mL polyethylene scintillation vials (Pony Vials, Packard-Camberra Company, Meriden, CT, USA). Scintillation fluid (4 mL; EcoScint) was added to all tubes, vortexed and β-radioactivity was determined for each sample.

Table 2.6: Allopregnanolone antiserum cross reactivity with closely related steroids

Steroid Compound	*Cross Reaction (%)*
3α-hydroxy-5α-pregnan-20-one	100.00
3α-hydroxy-5β-pregnan-20-one	6.50
Pregn-4-ene-3,20-dione	0.70
5α-pregnane-3,20-dione	0.10
5β-pregnane-3,20-dione	0.10
20β-hydroxy-5α-pregnan-3-one	0.10
5α-pregnane-3α,20α-diol	0.10
3β-hydroxy-5α-pregnan-20-one	0.05
5β-pregnane-3α,20α-diol	0.03
3β-hydroxy-5β-pregnan-20-one	0.01
5α-pregnane-3β,20α-diol	0.01

2.8.3 Validation of allopregnanolone RIA procedures

Due to modifications of the extraction procedure, validation of the RIA was required to ensure that sensitivity of the assay remained high whilst not affecting the specificity. The following sections detail the validation methods.

2.8.3.1 Validation of allopregnanolone antiserum dilution

Serial dilutions of the allopregnanolone antiserum stock (1:10 to 1:80) were incubated with a fixed amount of ^{3}H-Allopregnanolone (~25000 dpm). To assess the optimum antiserum dilution, a ~50 % binding capacity of the tracer ligand (Bmax) was required. This was achieved at a final antiserum dilution of 1:5000 (initial 1:100; Figure 2.2).

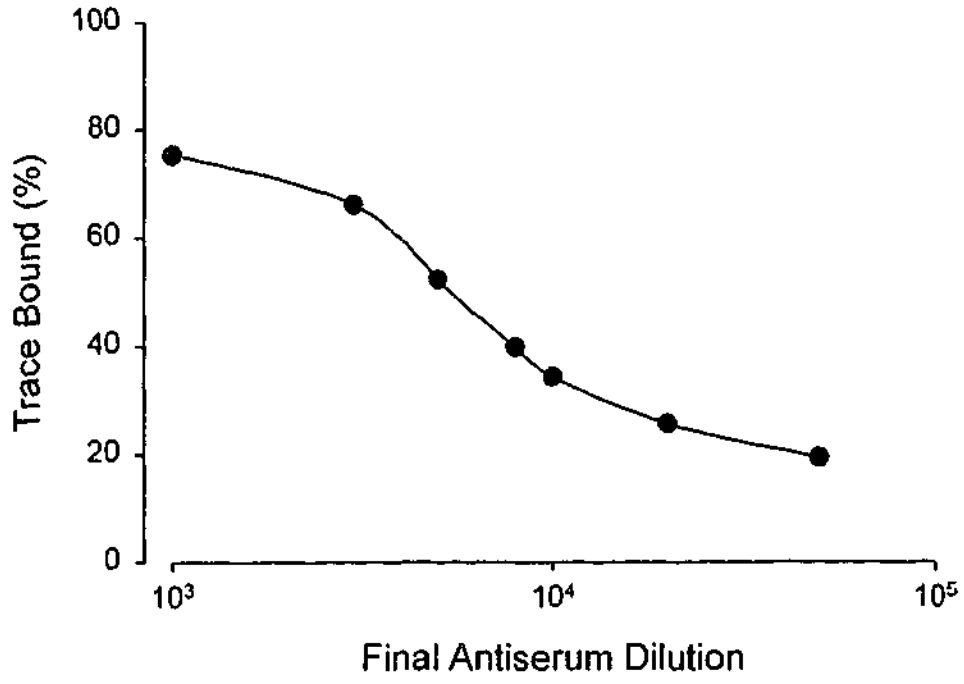


Figure 2.2: Allopregnanolone antiserum titration curve

Antiserum titration curve showing serial dilutions of the antiserum with a fixed amount of ^{3}H-Allopregnanolone (~25000 dpm). The percentage of tracer ligand bound to the antiserum decreases with increasing antiserum dilution. The optimal final dilution of the allopregnanolone antiserum used for the assays (1:5000) corresponds to the concentration that bound 52.3 % of the tracer.

A standard curve using different concentrations of unlabelled ligand was then established using the fixed amount of tracer (~25000 dpm) and the optimised dilution of antiserum (1:5000). Figure 2.3 presents the standard curve for allopregnanolone. The sigmoidal shape of the curve highlights that samples reading in the upper and lower ends of the curve have a greater risk of imbalance between bound and free hormones, rendering results less reliable, whereas the steepest part of the slope represents the most effective range with more consistent and reliable results. All samples measured for allopregnanolone concentration were assayed in a volume that ensured that they would fall between the standards located on the steepest part of the slope.

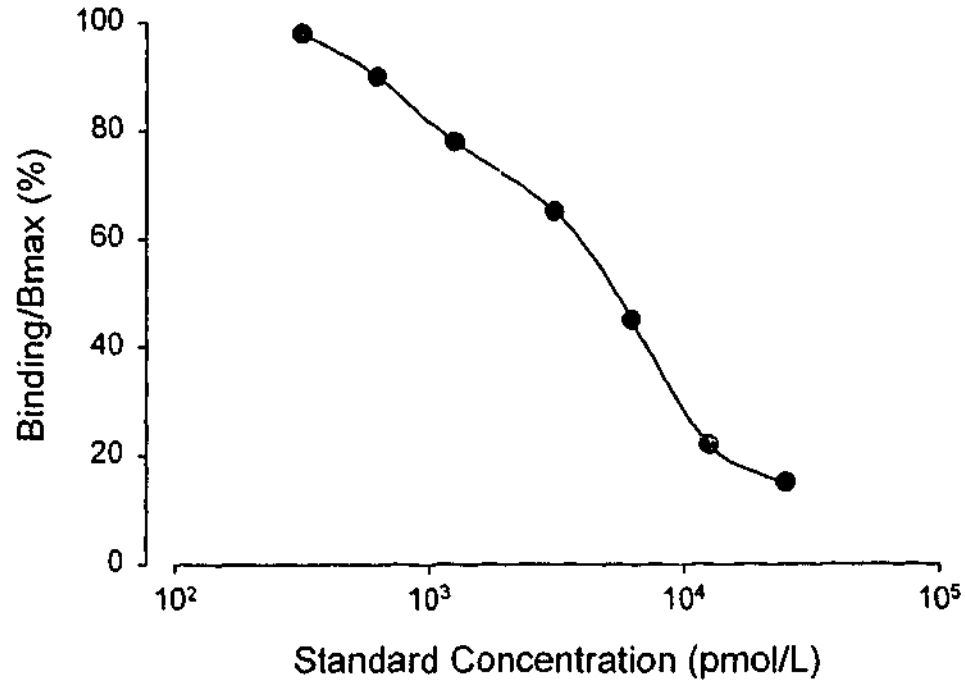


Figure 2.3: Allopregnanolone standard curve

Fixed amounts of ^{3}H-Allopregnanolone and antiserum were incubated with varying concentrations of standard unlabelled ligand. Note the sigmoidal shape and logarithmic scale of the X-axis.

2.8.3.2 Parallelism of allopregnanolone antiserum

Parallelism tests were performed to validate the accuracy of binding properties of the antiserum to allopregnanolone and to exclude potential non-specific interference. Brain and plasma samples treated with a known concentration of standard were diluted serially. These samples were then assayed along with the standards. Validation of the accuracy of the antiserum to bind to allopregnanolone was confirmed as samples lay close to parallel to the allopregnanolone standard curve (Figure 2.4).

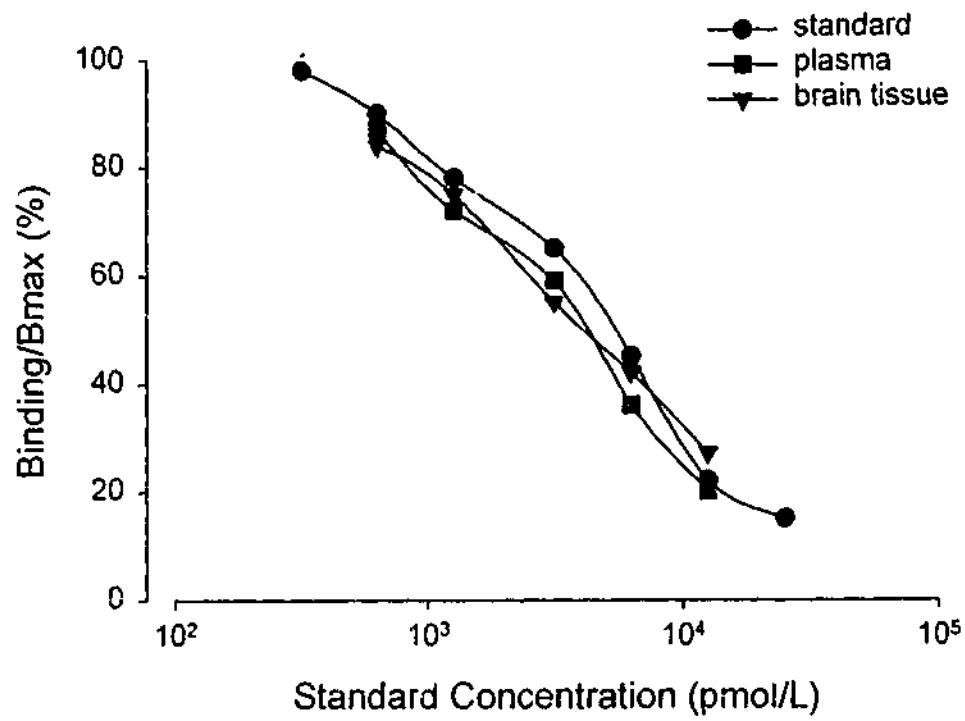


Figure 2.4: Allopregnanolone parallelism validation

To ensure accuracy of allopregnanolone detection in brain samples and plasma samples, samples were spiked with a known standard and assayed with allopregnanolone standards. Validation was confirmed as the samples diluted serially lay close to parallel with the standard curve. Logarithmic scale as per previous figure.

2.8.3.3 Optimisation of buffers, charcoal and incubation times for allopregnanolone

In addition to validation of the parameters described earlier, different buffers, charcoals and incubation times were examined and optimised. The following is a brief description of these parameters. Different **buffers** used for the reconstitution of steroids after extraction and for the assay were tested. Phosphate buffer (0.1 M, pH 7.4) was found to be optimal for both extraction and assay conditions and was also the buffer that was used for the other neurosteroid assays. The type of **charcoal** used also influenced the outcome of the assay by affecting the amount of non-specific binding (NSB). The finer grade (<100 mesh) charcoal produced a lower NSB (8 %) than the coarser grade (100-400 mesh, NSB = 15 %). Finally, the **incubation times** of ^{3}H-Allopregnanolone in sample tubes during the assay procedure was assessed. Of the different times tested (1, 12 and 24 hours), a 24 hour incubation produced more consistent and sensitive results.

2.8.3.4 Antiserum cross reactivity validation

Although the cross reactivity of the allopregnanolone antiserum had been characterised previously, further validation was performed on the cross reactivity of the antiserum with cortisol. Results obtained from the studies presented in Chapters 3 and 5 demonstrate that lambs treated with LPS exhibit large increases in both allopregnanolone and cortisol in the plasma. To ensure that the increase in allopregnanolone was a true increase, and not a by-product of the cortisol increase, CMP samples and samples obtained from ovariectomised ewes (OVX) were treated with a range of cortisol standards (6.25-50 ng/mL) alone, or in combination with allopregnanolone standard (2 ng/mL), and assayed for allopregnanolone. These values are reported in Table 2.7. The results demonstrate that there is no detectable cross reactivity between cortisol and allopregnanolone, consistent with results published by Purdy *et al.* (1990). All values reported in this thesis are therefore considered to be true changes of allopregnanolone concentration in response to treatment.

Table 2.7: Allopregnanolone antiserum validation

Plasma Sample	*Concentration (nmol/L)*
CSP	ND
CSP + cortisol (50 ng/mL)	ND
CSP + cortisol (25 ng/mL)	ND
CSP + cortisol (12.5 ng/mL)	ND
CSP + cortisol (6.25 ng/mL)	ND
CSP + cortisol (50 ng/mL) + AP	8.20
OVX	18.62
OVX + cortisol (50 ng/mL)	18.31
OVX + cortisol (25 ng/mL)	19.44
OVX + cortisol (12.5 ng/mL)	19.69
OVX + cortisol (6.25 ng/mL)	19.19
OVX + cortisol (50 ng/mL) + AP	32.53

ND indicates that allopregnanolone was not detected in these samples. Note that CSP samples alone and with cortisol had no detectable allopregnanolone, whereas OVX samples had detectable concentrations.

2.9 Plasma cortisol

Plasma cortisol concentrations were measured in extracted samples according to the methods of Bocking *et al.* (1986).

2.9.1 Measurement of cortisol

2.9.1.1 General reagents

2.9.1.1.1 Standards

Cortisol standard (Hydrocortisone, H-4001, Sigma Chemical Co., NSW, Australia) was prepared as a stock solution of 5 µg/mL concentration in ethanol and stored at -20 °C. Final concentrations of 0.195-50 ng/mL in absolute ethanol, evaporated with air at 37 °C, were used in the assay procedure.

2.9.1.1.2 Tracer

Cortisol tracer (250 mCi of [1,2,6,7-^{3}H]; Amersham Australia Pty. Ltd.) was made up to 5 mL in toluene/ethanol (9:1) and stored at -20 °C. On the day of the assay, a small amount of tracer was dried down under air and reconstituted in 0.05 M phosphate buffer (pH 7.4) to give ~10000 cpm/0.1mL/tube.

2.9.1.1.3 Antiserum

Cortisol antiserum (#3368(090878)) that was raised in sheep was supplied by Dr RI Cox (CSIRO, Divison of Animal Production, NSW, Australia). Cross reactivities of the antiserum are presented in Table 2.8 (Dr RI Cox, *personal communication*). The limit of detection for cortisol was 2.15 ± 0.22 nmol/L (n=10). The intra and interassay coefficients of variance were 4 % (n=6) and 15 % (n=10), respectively. The average recovery for the cortisol extraction procedure was 92.37 ± 1.56 (n=14).

2.9.1.2 Cortisol RIA methods

Duplicate plasma samples (25-100 µl) and standards were extracted with dichloromethane (2 mL; Merck, Darmstadt, Germany) and dH_2O (100 µl) in glass tubes. After vortexing thoroughly twice and allowing the layers to settle, the upper aqueous phase was removed and discarded. One mL of the remaining solvent layer was then transferred into a plastic

polypropylene tube and evaporated under air at 37 °C. Bovine γ-globulin (0.8 mg/mL; Commonwealth Serum Laboratories, VIC, Australia), cortisol antiserum (100 µl; initial dilution 1:8000, final dilution 1:24000) and ^{3}H-cortisol (10000 cpm/100µl) were added to both standard and sample tubes. After an overnight incubation at 4 °C, 1 mL 22 % (PEG) was added to separate bound from free hormones. After centrifuging at 3000 rpm for 15 minutes at 4 °C, the supernatant was aspirated, discarded and the precipitate reconstituted in 200 µl 0.05 M phosphate buffer. Scintillation fluid (1 mL; Aqueous Counting Solution, Amersham Australia Pty Ltd) was added to each tube and mixed well. Radioactivity (β) was determined for each sample.

Table 2.8: Cortisol antiserum cross reactivity with closely related steroids

Steroid Compound	*Cross Reaction (%)*
Cortisol	100.00
Cortisone	20.50
Corticosterone	1.00
Progesterone	0.57
17α-hydroxy-progesterone	3.90
Betamethasone	0.50
4-Androstene-3,17-dione	0.03
Testosterone	0.02
Dexamethasone	<0.01

2.10 Tissue and plasma progesterone

2.10.1 Brain tissue extraction

Brain tissue from lambs was extracted in acidified homogenates (1:10) through Sep Pak cartridges as described previously (Section 2.8.1).

2.10.2 Measurement of progesterone

2.10.2.1 General

2.10.2.1.1 Standards

Progesterone standard (Sigma Chemical Co., NSW, Australia) was prepared as a stock solution (4 mM) in absolute ethanol and further diluted with ethanol to working dilutions of 10 μM, 200 nM and 10 nM. All standards were stored at -20 °C. Final concentrations of 0.01-10.0 pmol/L in absolute ethanol, evaporated with air at 37 °C and reconstituted in 50 μl CMP, were used in the assay procedure.

2.10.2.1.2 Tracer

Progesterone tracer (250 μCi of [1,2,6,7-^{3}H(N)]-Progesterone; Amersham/NEN Australia Pty. Ltd.) was made up to 2 mL in absolute ethanol and stored at -20 °C. On the day of the assay, a small amount of tracer was dried down under air at 37 °C and reconstituted in 0.1 M phosphate buffer (pH 7.4) to give ~5000 cpm/0.1mL/tube.

2.10.2.2 Antiserum

Progesterone antiserum (S23), raised in sheep against progesterone-11-α-BSA, was supplied by Dr J Malecki (Regional Veterinary Centre, Department of Agriculture and Rural Affairs, VIC, Australia). Cross reactivities of the antiserum are presented in Table 2.9 (Rice *et al.*, 1986). The minimum detectable level of progesterone was 0.12 ± 0.02 pmol/tube (n=10). The intra and interassay coefficients of variance were 8 % (n=4) and 19 % (n=29), respectively.

2.10.2.3 Progesterone RIA methods

Brain and plasma progesterone were measured in extracted samples (in duplicate) according to the methods of Rice *et al.* (1986). Distilled H_2O (100 μl) was added to all samples (50-200 μl) and standards. Progesterone was then extracted from both brain and plasma with n-hexane (2 mL; Merck, Darmstadt, Germany) in glass tubes. After vortexing thoroughly, the aqueous phase was frozen using an ethanol-freezing bath (Hetofrig, Birkerood, Denmark) and the solvent layer containing the extracted progesterone was decanted into plastic polypropylene tubes. This solvent layer was then evaporated under

air at 37 °C and the samples reconstituted in 50 µl 0.1 M phosphate buffer (pH 7.4). Antiserum (100 µl; 1:4000 initial dilution, 1:10000 final dilution) and ^{3}H-progesterone (100 µl) were added to the tubes, mixed gently and incubated overnight at 4 °C. The bound and free fractions were separated by the addition of 50 µl bovine γ-globulin (0.8 mg/mL) and 22 % PEG (1 mL). After centrifuging at 3200 rpm for 15 minutes at 4 °C, the supernatant was aspirated, discarded and the pellet reconstituted in 50 µl of absolute ethanol. Scintillation fluid (1.5 mL; Ecoscint) was added to all tubes, vortexed and then sonicated for 30 minutes. Radioactivity (β) was determined for each sample.

Table 2.9: Progesterone antiserum cross reactivity with closely related steroids

Steroid Compound	*Cross Reaction (%)*
Progesterone	100.00
11α-hydroxy-progesterone	43.80
5α-pregnane,3α-ol,20-one	15.90
5β-pregnane,3α-ol,20-one	10.00
Corticosterone	1.05
17α-hydroxy-progesterone	0.70
20α-hydroxy-pregnane,3-one	0.30
11-deoxycortisol	<1.00
5α-pregnane,3β-ol-2-one	<1.00
5α-pregnane,3α,17α-diol-20-one	<1.00
5β-pregnane,3α,17α,20α-triol-20-one	<1.00
5β-pregnane,3α,17α,20α-triol	<1.00
Dehydroepiandrosterone	<0.40
Cortisol	<0.20

2.11 Tissue and plasma pregnenolone

2.11.1 Brain tissue extraction

Extraction of pregnenolone from acidified brain homogenates (1:10) using Sep Pak cartridges is as described previously (Section 2.8.1).

2.11.2 Measurement of pregnenolone

2.11.2.1 General

2.11.2.1.1 Standards

Pregnenolone standard (Sigma Chemical Co., NSW, Australia) was prepared similarly to that for progesterone. Pregnenolone standard stock solution (4 mM) was diluted serially with ethanol to working dilutions of 10 µM, 200 nM and 10 nM. Final concentrations of 0.01-10.0 pmol/L diluted in absolute ethanol, evaporated with air at 37 °C and reconstituted in 50 µl CMP were used in the assay procedure.

2.11.2.1.2 Tracer

Pregnenolone tracer (250 µCi of [7-^{3}H(N)]-Pregnenolone; NEN Australia Pty. Ltd.) was made up to 2 mL in absolute ethanol and stored at -20 °C. On the day of the assay, a small amount of tracer was added to 0.1 M phosphate buffer (pH 7.4) to give ~5000 cpm/0.1mL/tube.

2.11.2.1.3 Antiserum

A commercial antiserum (R3-8BO; ICN Biomedicals) was used for this assay. Cross reactivities of the antiserum are presented in Table 2.10. The minimum detectable level of pregnenolone was 0.09 ± 0.02 pmol/tube (n=6). The intra and interassay coefficients of variance were 5 % (n=4) and 16 % (n=14), respectively.

Table 2.10: Pregnenolone antiserum cross reactivity with closely related steroids

Steroid Compound	*Cross Reaction (%)*
Pregnenolone	100.00
Pregnenolone-sulfate	100.00
Progesterone	3.10
5α-dihydroprogesterone	0.85
Desoxycorticosterone	0.03
17α-hydroxypregnenolone	0.02
Cholesterol	<0.025
17α-hydroxyprogesterone	<0.025
20α-dihydroprogesterone	<0.025
Cortisol	<0.025
Dihydroepiandrosterone	<0.025
11-desoxycortisol	<0.025
Corticosterone	<0.025
Androsterone	<0.025
5α-dihydrotestosterone	<0.025
Ethiocholanolone	<0.025
Estradiol-17β	<0.025
Estradiol-17α	<0.025
Estriol	<0.025
Estrone	<0.025
20β-dihydroprogesterone	<0.025
Testosterone	<0.025
Aldosterone	<0.025
Androstenedione	<0.025

2.11.2.2 Pregnenolone RIA methods

Brain and plasma pregnenolone was measured in a similar assay system as progesterone but with minor modifications. Following extraction with n-hexane, brain and plasma samples (50-200 µl) along with standards (both in duplicate) were vortexed thoroughly and the aqueous phase frozen using an ethanol-freezing bath. The solvent layer containing the extracted pregnenolone was decanted into plastic polypropylene tubes and evaporated under air at 37 °C. All samples were then reconstituted in 50 µl 0.1 M phosphate buffer (pH 7.4). Antiserum (100 µl; 1:23 initial dilution, 1:2300 final dilution) and ^{3}H-pregnenolone (100 µl) were added to the tubes, mixed gently and incubated overnight at 4°C. The bound and free fractions were separated by the addition of 400 µl of a cold suspension of dextran-coated charcoal (0.625 % charcoal, 0.0625 % Dextran T70). The supernatant was centrifuged at 2000 rpm for 20 minutes at 4 °C and, subsequently, decanted into scintillation vial inserts. Scintillation fluid (2.5 mL; EcoScint) was added to all tubes and vortexed. β radioactivity was determined for each sample.

2.11.3 Validation of pregnenolone RIA procedures

Due to modifications of the original pregnenolone assay, and because this assay had not been used previously for brain tissue, validation of the RIA was required to ensure that sensitivity of the assay remained high without affecting the specificity. The following sections detail the validation methods performed, which are similar to those performed for allopregnanolone.

2.11.3.1 Validation of pregnenolone antiserum dilution

Serial dilutions of the pregnenolone antiserum stock (1:10 to 1:400) were incubated with a fixed amount of ^{3}H-Pregnenolone (~5000 cpm). The final dilution of 1:2300 (initial 1:23) was found to have a ~50 % binding capacity, indicating that this was the preferred dilution required for the assay. Figure 2.5 shows the antiserum titration curve for pregnenolone.

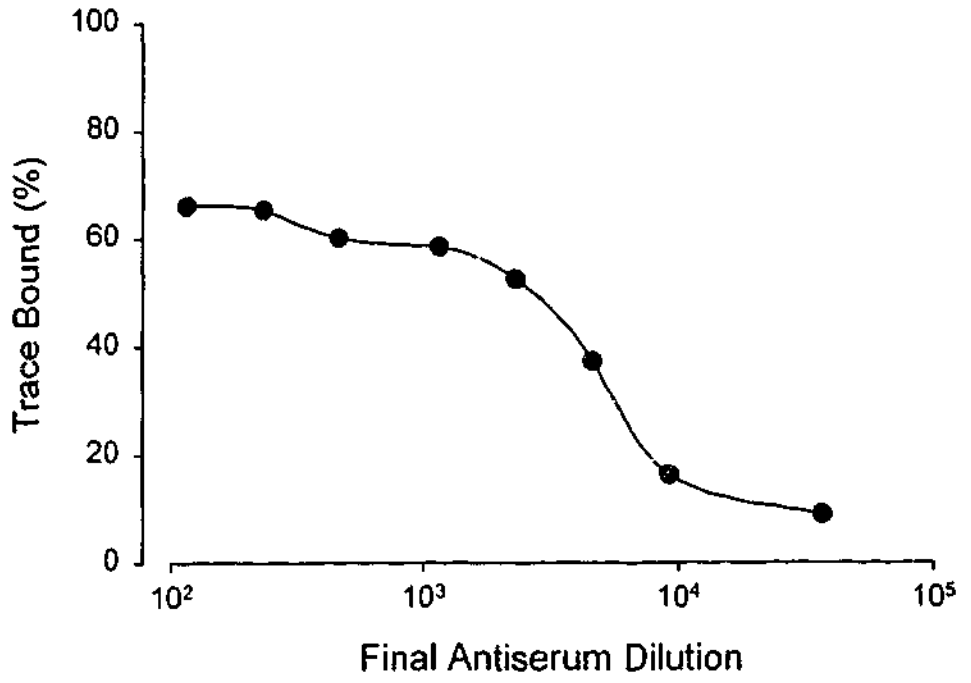


Figure 2.5: Pregnenolone antiserum titration curve

Antiserum titration curve showing serial dilutions of the antiserum with a fixed amount of ^{3}H-Pregnenolone (~5000 cpm). The optimal final dilution of the pregnenolone antiserum used for the assays (1:2300) corresponds to the concentration that bound 52 % of the tracer. Note the logarithmic scale of the X-axis.

A standard curve, using different concentrations of unlabelled ligand, was then established using a fixed amount of tracer (~5000 cpm) and the optimised dilution of antiserum (1:2300). Figure 2.6 presents the standard curve established for pregnenolone. All samples measured for pregnenolone concentration were assayed in a volume that ensured they would fall between the standards located on the steepest part of the slope.

2.11.3.2 Parallelism of pregnenolone antiserum

Parallelism tests were performed to validate the accuracy of binding properties of the antiserum to pregnenolone in brain samples. This was not performed in plasma as it had been performed previously in our laboratories (Dr Sue McKay, *personal communication*). Brain samples treated with a known concentration of standard were diluted serially and assayed along with the standards. Validation of the specificity of the antiserum was confirmed since samples lay close to parallel to the pregnenolone standard curve (Figure 2.7).

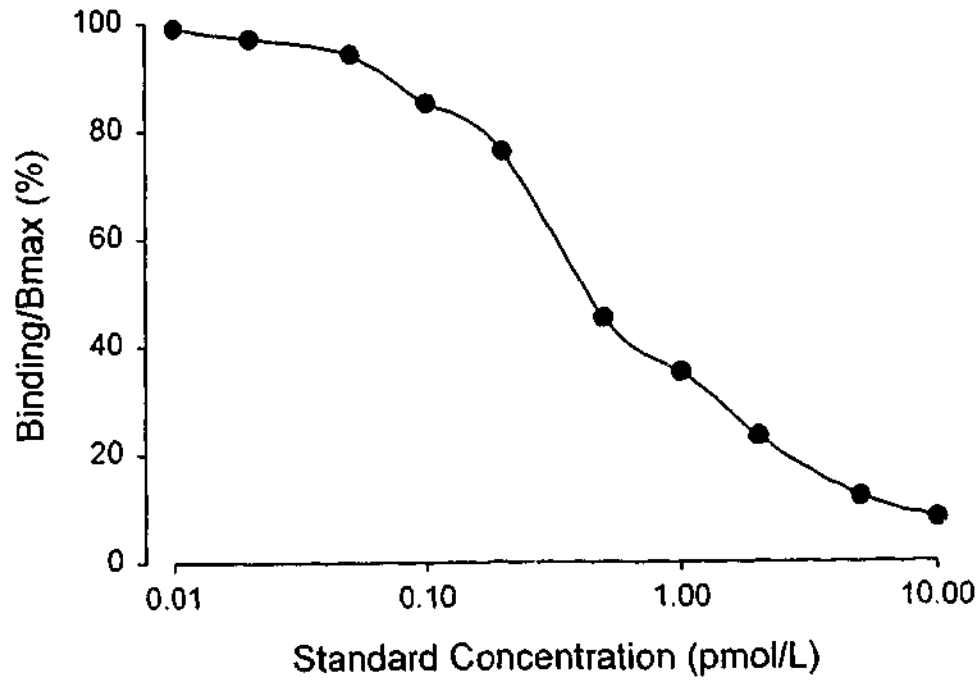


Figure 2.6: Pregnenolone standard curve

Fixed amounts of ^{3}H-Pregnenolone and antiserum were incubated with varying concentrations of standard unlabelled ligand. Note the logarithmic scale of the X-axis.

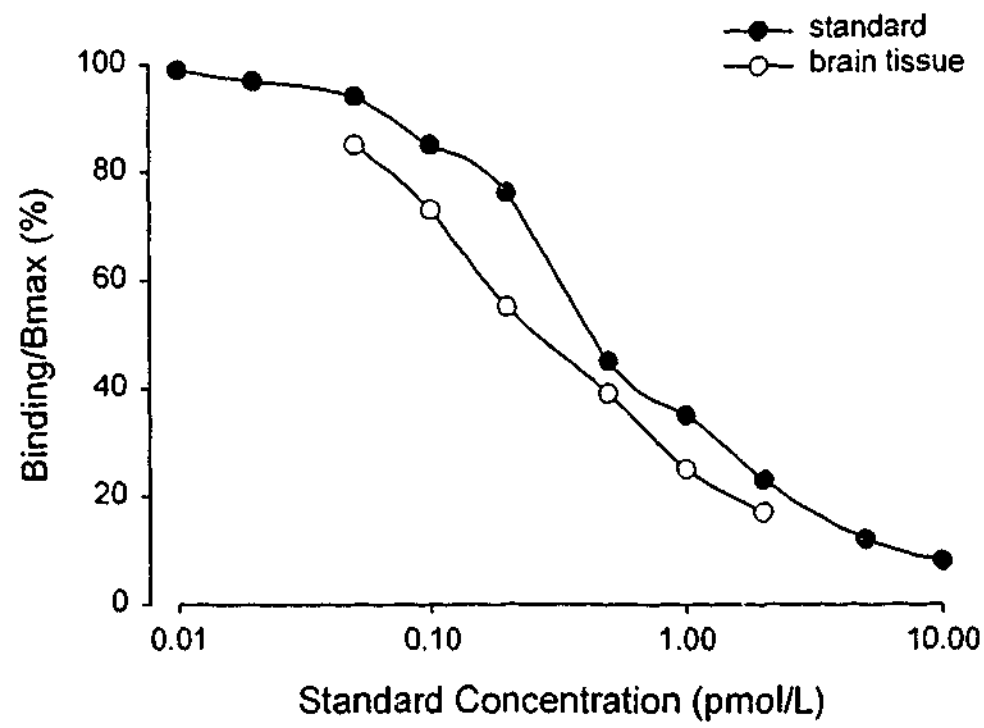


Figure 2.7: Pregnenolone parallelism validation

To ensure specificity of the antibody, brain samples were spiked with a known standard and assayed along with pregnenolone standards. Validation was confirmed since the sample curve lay close to parallel with the standard curve. Note the logarithmic scale of the X-axis.

Chapter 3: Endotoxin increases sleep and allopregnanolone concentrations in lambs

Infection is a stress often faced by neonates and has been proposed as a risk factor in SIDS. The potential mechanisms by which infection could increase susceptibility to SIDS remain unclear. It is likely, however, that a SIDS death results from the coincidence of a number of events that together create a period of increased risk (Filiano & Kinney, 1994). An hypothesis of this chapter is that one of the mechanisms involved in a SIDS death is the increased production of sedative neurosteroids in response to infection, and that these steroids contribute to the lethargy and increased sleep associated with infection in neonates.

The pathology of infection and its effects on sleep-wake states has been studied extensively (Kadlecova *et al.*, 1972; Toth & Krueger, 1989; Mullington *et al.*, 2000). Together with fever, reduced plasma iron concentration and other 'acute phase' changes (such as increased cytokine production and cortisol levels) increased somnogenesis are part of the cardinal responses to infection in adults. Several components of the body's response to infection, including increased production of cytokines and prostaglandins, have been shown to induce sleep. Muramyl peptides and lipopolysaccharides (LPS) associated with the cell wall of bacteria have also been shown to be somnogenic (Krueger *et al.*, 1982; Krueger & Karnovsky, 1987). While sleepiness may have an adaptive role that promotes rest and conserves energy, the combination of infection and somnolence in

the newborn, with its high metabolic rate and low pulmonary oxygen stores, may increase the susceptibility of the infant to episodes of periodic breathing, sleep-associated apnoea and SIDS by ablating essential cardiorespiratory responses to hypoxia and asphyxia.

Neurosteroids are a group of compounds that influence CNS function. In the adult, neurosteroids can be produced in the brain from cholesterol, or alternatively in peripheral organs, such as the adrenal cortex and gonads, from precursors (review Compagnone & Mellon, 2000). Progesterone, a key precursor for the synthesis of neurosteroids, has been shown to have a number of suppressive effects on brain function. These effects are mediated primarily by 5α-reduced metabolites, including allopregnanolone (Lancel *et al.*, 1997). Allopregnanolone is a positive modulator of the ligand-gated ionotrophic $GABA_A$ receptor, enhancing $GABA_A$ receptor function by prolonging the channel 'open' time and increasing the inward Cl^- current, thereby producing sedative, anaesthetic, anticonvulsant and anxiolytic effects (Barbaccia *et al.*, 1996; Brot *et al.*, 1997; see Chapter 1). Studies in the adult rat have shown that the administration of allopregnanolone induces anaesthesia (Mok *et al.*, 1993; Korneyev & Costa, 1996) and alters sleep patterns (Lancel *et al.*, 1997). In addition, it has been suggested that these responses to allopregnanolone may be neuroprotective, particularly in the newborn (Kehoe *et al.*, 2000; see Chapter 1).

Previous studies in fetal sheep have shown that neurosteroids have potent suppressive effects on CNS activity (Nicol *et al.*, 1999). Further, inhibition of neurosteroid production increase arousal-like behaviour in the late gestation fetus and reduce the incidence of spontaneous breathing movements (Nicol *et al.*, 2001). These findings suggest that neurosteroids increase $GABA_A$ activity to tonically inhibit the fetal CNS and suppress fetal breathing episodes. The source of steroid precursors in pregnancy is likely to be the placenta (Nicol *et al.*, 1997). However, it is not clear if neurosteroidogenesis in the newborn is responsive to stressful stimuli or if 5α-reduced steroids are produced in sufficient amounts to have physiological effects on behaviour, in particular, on sleep-wake activity.

Therefore, the general aim of this study was to investigate the effects of infection on sleep-wake behaviour and neurosteroid concentrations in the lamb. LPS was used to mimic the effects of bacterial infection in the lamb. Previous studies have reported that mammalian neonates do not develop fever during infection (Blatteis *et al.*, 1981; Goelst & Laburn, 1991). However, these neonates do display a reduction in plasma iron

concentration, a peripheral indicator of infection (Goelst & Laburn, 1991). Therefore, measurements of both body temperature and plasma iron concentration were performed for this study. Experiments were also repeated three times between 12 and 20 days of age to determine if the responses were attenuated by repeated exposure to LPS, as has been observed in adult animals (Whyte *et al.*, 1989; Roth *et al.*, 1994; Hadid *et al.*, 1996).

3.1 Materials and methods

3.1.1 Animals

Twelve lambs of Merino-Border Leicester cross ewes were delivered spontaneously in lambing pens in the Physiology Department Animal House. All lambs were allocated randomly to a treatment group and remained with their mothers except for during the experimental periods described below.

3.1.2 Surgical preparation

Aseptic surgery was performed on all lambs between 5 and 10 days of age to insert indwelling vascular catheters to obtain blood samples and to implant electrodes for recording sleep states as described in Section 2.3. After surgery, lambs recovered in a sling under a heating lamp before being returned to their mothers. Experiments did not commence until at least four days after surgery.

3.1.3 Experimental design

All experiments were conducted in a dark, sound-proofed sleep chamber with the lambs resting in a canvas sling. Temperature (22-23 °C) and humidity (~40 %) were kept constant throughout each study. ECoG, EOG and nuchal muscle activities together with arterial pressure and intrathoracic pressure were recorded on a polygraph. Rectal temperature was monitored using a temperature probe inserted at the beginning of each experiment. All signals were displayed on a polygraph (Model 7D, Grass Instrument, Co., Quincy, MA, USA) using a chart speed of 5 mm/minute. Rate meters triggered by the arterial and intrathoracic pressure waveforms determined heart rate and breathing rate, respectively.

To avoid possible effects of diurnal rhythms, all experiments began at 0800 hours. Recordings of sleep-wake states, rectal temperature and other physiological variables commenced one hour prior to injection of endotoxin or vehicle. *E-Coli* LPS (0127:B8,

Sigma Chemical Co. St Louis, USA) was administered intravenously as a bolus at a dose of 0.7 μg/kg body weight dissolved in 5 mL of saline (n=7 lambs). Vehicle-treated lambs received an intravenous bolus of 5 mL saline (n=5 lambs). Sleep states and physiological parameters were recorded continuously for 6 hours after LPS treatment. Lambs that were treated with saline became restless after approximately 4 hours in the sleep chamber (i.e. at 3 hours post-treatment) and had to be returned to the ewe for feeding. This interruption was usually for 15-20 minutes hence the recording of temperature, heart rate, blood pressure, respiratory rate and sleep was interrupted at this time.

3.1.3.1 Blood sampling and tissue collection

Arterial blood samples (4 mL) were collected at –1, -0.5, 0, 0.5, 1, 2, 3, 4, 5 and 6 hours with respect to the saline or LPS injection (time = 0) to determine changes in blood gases, pH and Hb, and the plasma concentrations of glucose, lactate, iron, ACTH, cortisol, pregnenolone, progesterone and allopregnanolone (see Chapter 2 for methodologies). This 7 hour experimental protocol was carried out on two separate occasions on each lamb that was LPS or vehicle treated, at approximately 12 (range, 7-15) and 15 (range, 13-18) days of postnatal age. At least 3 days elapsed between these two experiments. A third and final experiment was conducted at approximately 20 (range, 17-21) days of age during which each lamb received LPS or vehicle and was killed after 3 hours using intravenous (i.v.) pentobarbitone. The brain was removed immediately (n=4 per group) and divided into blocks incorporating the cerebellum, midbrain, pons, medulla, diencephalon (thalamus and hypothalamus), hippocampus, and the frontal, primary, parietal, temporal and occipital cortices. Each block was frozen immediately in liquid N_2 and stored at -70 °C until required.

3.1.4 Data analysis

Identification of sleep-wake states based on the chart records was as described previously (Section 2.4.2). For each experiment, the sleep-wake states of the entire recording were scored at 1 minute intervals and this data combined to obtain an average value for each 30 minute or 1 hour epoch. The proportion (%) of the total time spent in a particular state (wakefulness, AW; quiet sleep, QS; active sleep, AS; or indeterminate sleep, IS) after saline or LPS treatment was then calculated.

3.1.5 Statistical analyses

Data were analysed using SPSS software (SPSS for Windows, Version 10.0, Chicago, IL, USA). Data were tested for homogeneity using Levene's test before analysis and, when necessary, the data were transformed using square root or logarithmic transformations. A two-way repeated measures analysis of variance (ANOVA) was used to compare treatment, age and the interaction between treatment and age over time. Where an ANOVA revealed a significant difference between treatment and/or age over time, the Fisher's Least Significant Difference (LSD) *post hoc* test was used. Data (original, untransformed) are presented as mean ± S.E.M. $P<0.05$ was considered statistically significant. The absence of error bars on some graphs presented in this chapter indicates that they are within the symbol.

3.2 Results

3.2.1 Preliminary experiments

All experiments reported in this thesis used LPS to mimic the effects of a mild endotoxin exposure. LPS has been used widely to examine the effects of inflammation and other parameters, however, it has been found that there are inter-species differences to this treatment (Kanoh *et al.*, 1977; Blatteis *et al.*, 1988; Takeuchi *et al.*, 1997). Therefore, preliminary studies were performed on three lambs to ascertain the optimal concentration of LPS to be used. The concentrations of LPS tested were 0.6, 0.7 and 0.8 μg/kg body weight, which were pre-determined based on previous work in sheep (Blatteis *et al.*, 1987; Blatteis *et al.*, 1988; Singh & Atwal, 1997). Plasma cortisol and general behavioural parameters were monitored as indicators for optimal LPS concentration.

LPS, given as a bolus dose of 0.6 μg/kg body weight, produced an increase in plasma cortisol concentration (Figure 3.1) but had very little effect on any other parameter measured. These lambs were also restless and, hence, could not be maintained in the experimental conditions for the 6 hour duration. A dosage of 0.7 μg/kg of LPS produced results that were mild enough to not cause great discomfort to the lambs but strong enough to elicit physiological and behavioural responses comparable to those observed for a mild bacterial challenge. LPS given at 0.8 μg/kg produced dramatic results. These lambs exhibited minor convulsions and were still drowsy and/or sedated 6 hours post-LPS administration. However, the rapid and sustained changes in plasma cortisol

concentrations were not different between this dose of LPS used and the 0.7 µg/kg dose (Figure 3.1). Therefore, a dosage of 0.7 µg/kg body weight of LPS was used for all experiments.

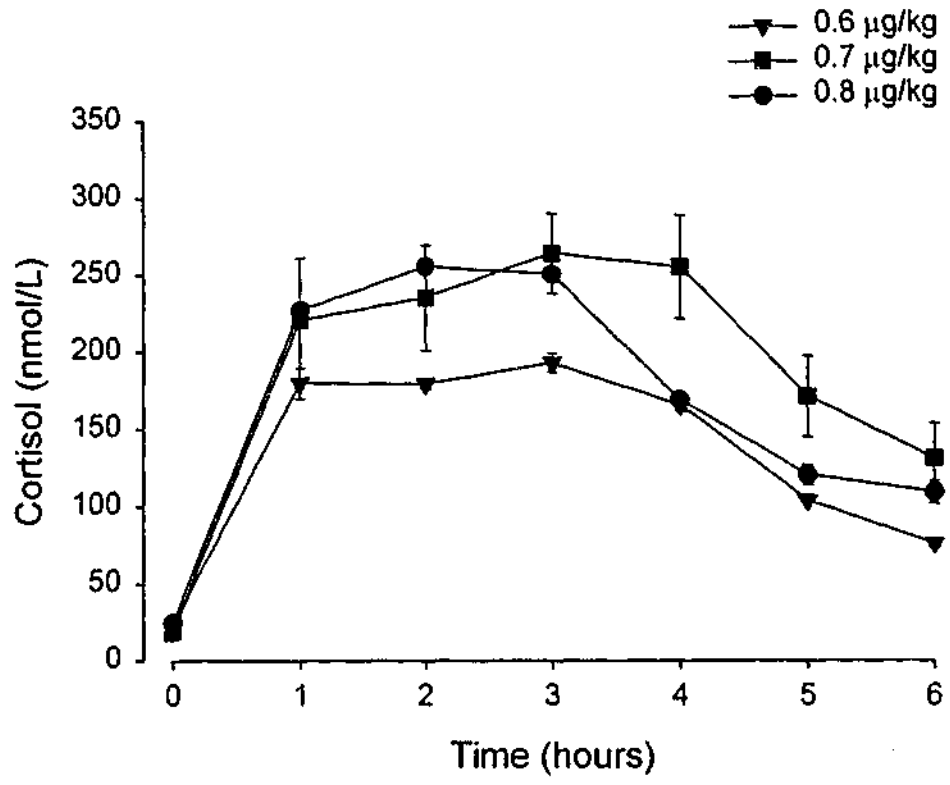


Figure 3.1: Effect of different doses of LPS *E-Coli* on plasma cortisol concentrations

Effect of LPS treatment on plasma cortisol concentrations for the doses; 0.6 µg/kg (n=2), 0.7 µg/kg (n=7) and 0.8 µg/kg (n=3). Data are expressed as mean ± S.E.M.

3.2.2 LPS treatment at 12 and 15 days of age

3.2.2.1 Effect of LPS treatment on temperature and plasma iron concentrations

Injection of saline had no effect on rectal temperature whereas LPS resulted in an increase of rectal temperature (P<0.001) that was biphasic (Figure 3.2). The peak of the first phase (40.04 ± 0.24 °C) occurred approximately 1 hour post-LPS treatment and the peak of the second phase (40.46 ± 0.32 °C) occurred approximately 3 hours post-LPS treatment. Thereafter, rectal temperature decreased until, at 6 hours post-LPS treatment, it was slightly, but significantly, greater than in the control period (Figure 3.2). The febrile response differed between the two ages: at 12 days of age, the increase in temperature was significantly greater at 3 to 4.5 hours post-LPS compared to the response at 15 days of

age. At 15 days of age, the increase in temperature at 1 hour post-LPS was greater than at 12 days of age. However, in all other respects, the biphasic change of temperature was remarkably similar at the two postnatal ages.

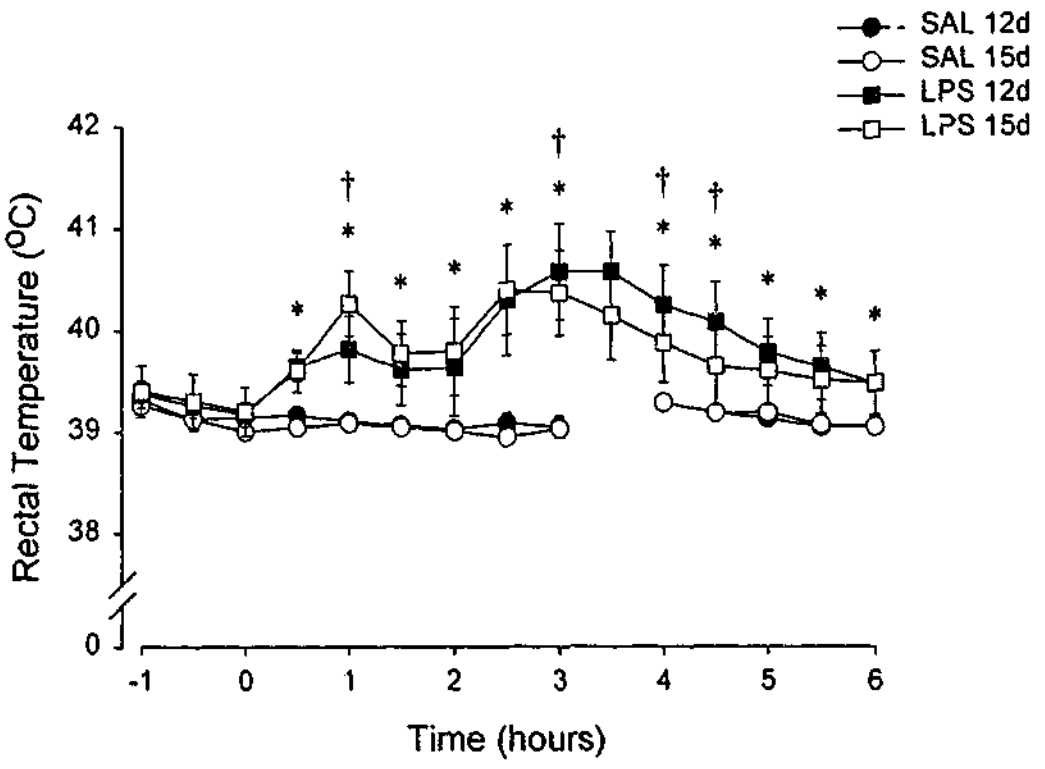


Figure 3.2: Effect of LPS treatment on rectal temperature

Effect of saline (n=5) or LPS (n=7) treatment on rectal temperature. * P<0.05 between saline and LPS-treated lambs at 12 and 15 days, † P<0.05 between LPS groups at 12 and 15 days of age. The break in the saline data represents the time when lambs had to be returned to their mothers for feeding. Data are expressed as mean ± S.E.M.

Plasma iron, an indicator of fever (Goelst & Laburn, 1991), was found to decrease in the LPS-treated lambs, at both ages, 6 hours post-treatment (Figure 3.3). The effect, however, was different between the ages (P<0.05). This may be due to a reduction in plasma iron concentrations with increasing age, as a reduction in plasma iron concentration was found in the control and LPS groups. However, when analysing the data based on the percentage decrease of iron concentrations at 6 hours compared to its age-matched pre-treatment values, the significant difference between the age groups was lost; iron concentrations were reduced by 27.03 ± 7.04 % for 12 day old lambs and by 29.44 ± 6.26 % for 15 day old lambs.

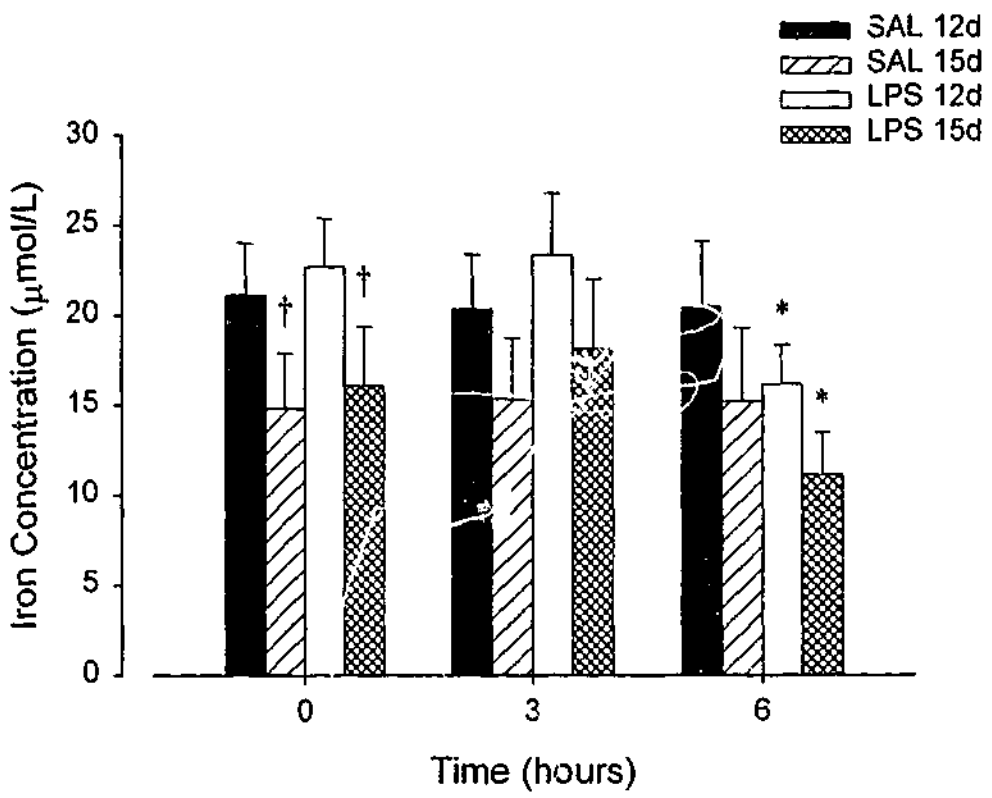


Figure 3.3: Effect of LPS treatment on plasma iron concentrations

Effect of saline (n=5) or LPS (n=7) treatment on plasma iron concentrations. * P<0.05 for LPS-treated lambs compared to time (0). † P<0.05 between 12 day and 15 day old lambs within each treatment group. Data are expressed as mean ± S.E.M.

3.2.2.2 Effect of LPS treatment on blood gases, pHa and Hb

LPS treatment resulted in significant decreases in arterial O_2 saturation (from 96.81 ± 1.10 to 92.94 ± 2.10 %, Figure 3.4A) and PaO_2 (from 117.29 ± 3.48 to 95.36 ± 6.87 mmHg, Figure 3.4B) at 0.5 hours post-LPS compared to time (0). $PaCO_2$ decreased 1 hour after LPS treatment (from 36.36 ± 0.41 to 31.57 ± 1.11 mmHg, Figure 3.4C). LPS had no effect on pHa (Figure 3.4D) or Hb (data not shown). Saline treatment had no effect on blood gas parameters (Figure 3.4 A-C), pHa (Figure 3.4D) or Hb (data not shown).

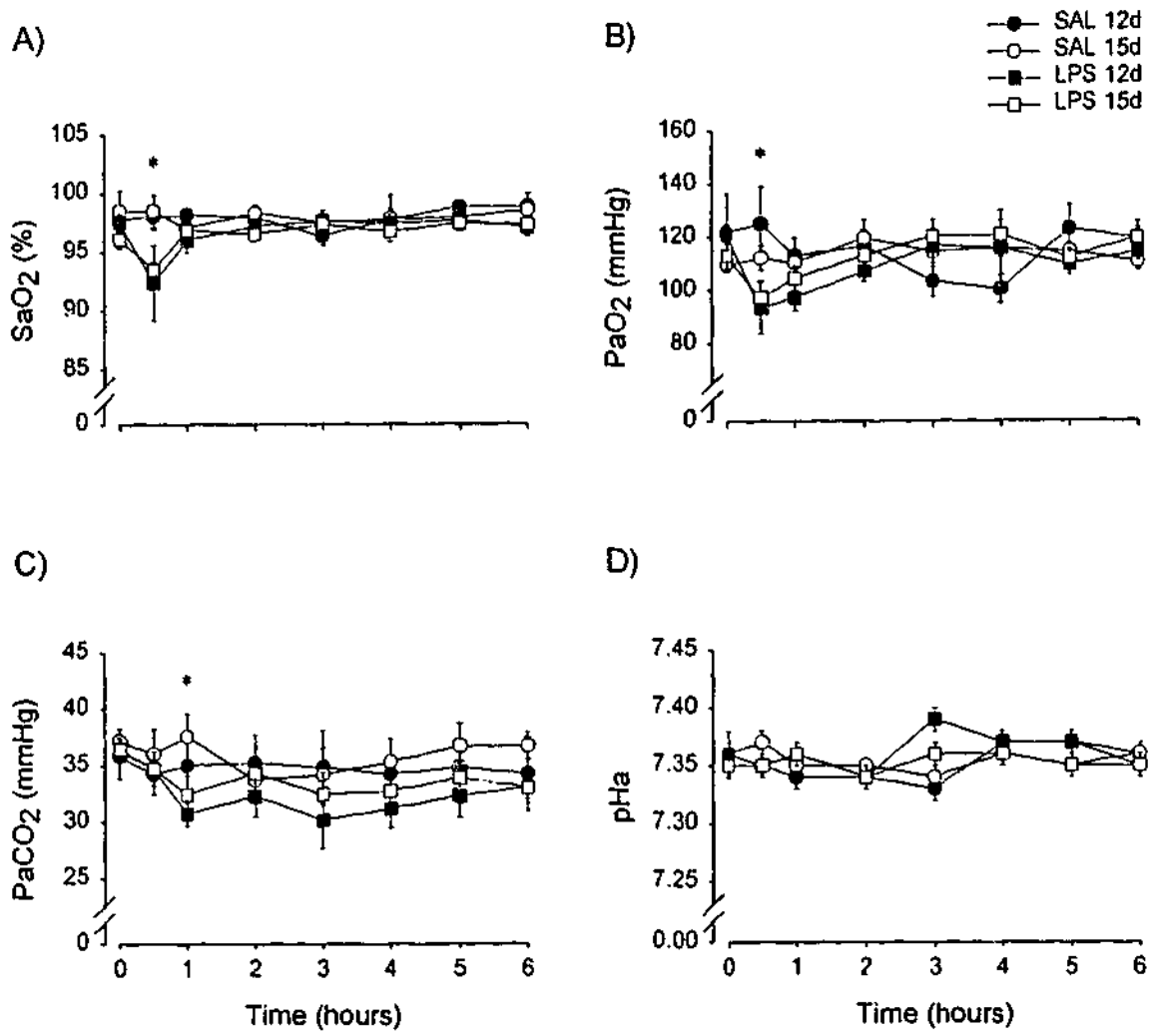


Figure 3.4: Effect of LPS treatment on blood gases and pHa

Effect of saline (n=5) or LPS (n=7) treatment on SaO_2 (A), PaO_2 (B), $PaCO_2$ (C) and pHa (D). * P<0.05 between saline and LPS-treated lambs at 12 and 15 days. Data are expressed as mean ± S.E.M.

3.2.2.3 Effect of LPS treatment on plasma glucose and lactate concentrations

In the 12 day old lambs, plasma glucose concentration was increased significantly at 1 and 2 hours after LPS treatment but then decreased markedly (P<0.05) to below control levels by 3 hours post-treatment (Figure 3.5A). In the 15 day old lambs, plasma glucose did not increase at 1-2 hours post-LPS treatment but a significant (P<0.05) reduction was observed by 3 hours after treatment. The magnitude of reduction in glucose concentration was similar to that observed at 12 days of age. Plasma lactate concentrations had increased significantly by 1 hour post-LPS treatment in both experiments at 12 and 15 days of age (P<0.001) and remained elevated significantly for the duration of the experiment (Figure 3.5B). Plasma glucose and lactate concentrations were not affected by saline treatment.

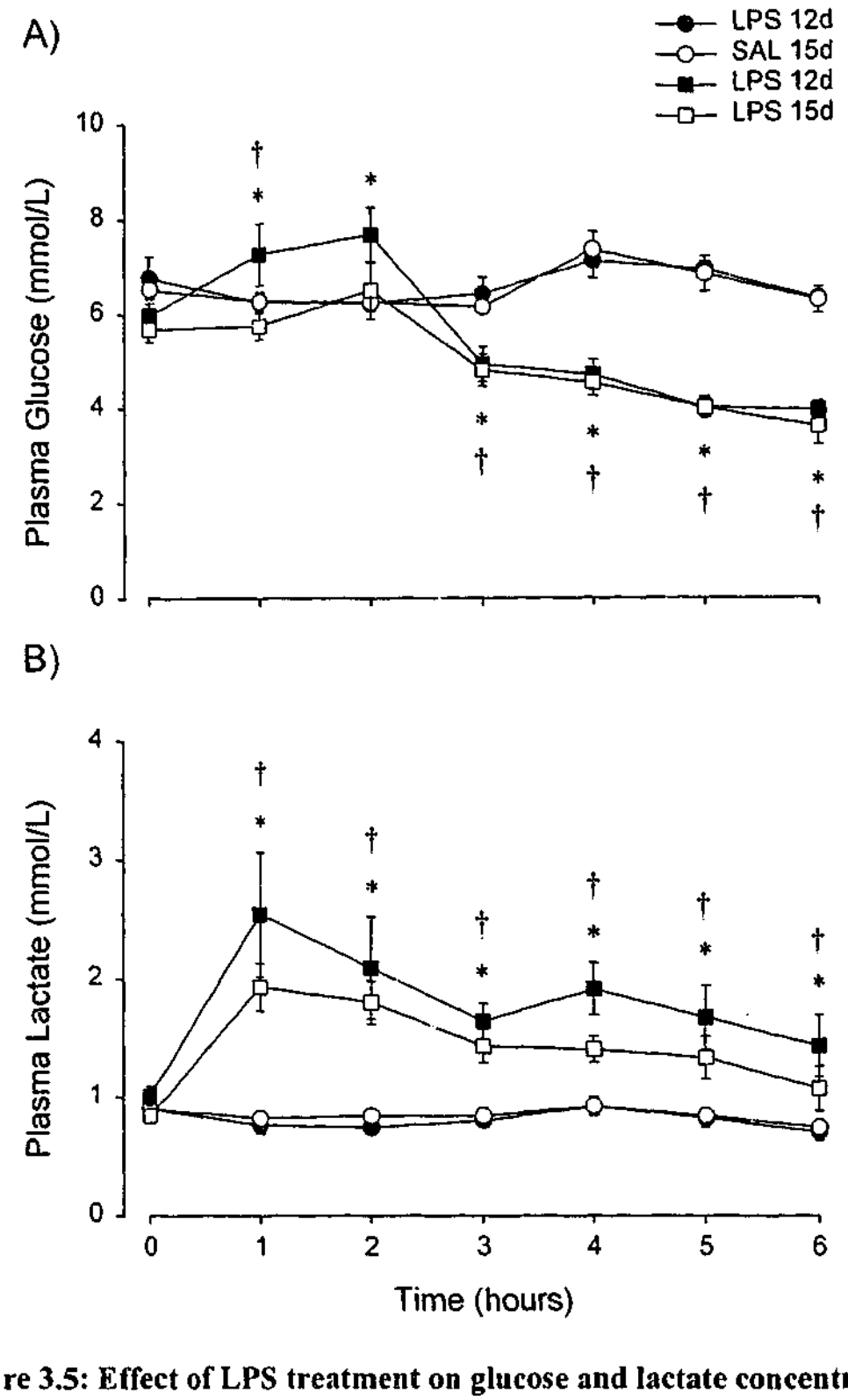


Figure 3.5: Effect of LPS treatment on glucose and lactate concentrations

Effect of saline (n=5) or LPS (n=7) treatment on plasma glucose (A) and lactate concentrations (B). * P<0.05 between saline and LPS-treated lambs at 12 days; † P<0.05 between saline and LPS-treated lambs at 15 days. Data are expressed as mean ± S.E.M.

these lambs at the time of tissue collection, blood samples were taken immediately prior to tissue collection.

5.1.3 Data analysis

Identification and scoring of sleep-wake states based on the chart records was conducted using the methods described in Section 2.4.2. For each experiment, sleep-wake states of the entire recording were scored at 1 minute intervals and these data were combined to obtain an average value for a combination of 30, 45 and 60 minute epochs. The variation in epoch length was used to assess the effects of the hypoxia challenge by incorporating the entire 45 minute period(s). The proportion (%) of the total time spent in a particular state (AW, QS, AS, IS) after saline or LPS treatment, with or without hypoxia, was then calculated.

5.1.4 Statistical analyses

Data were analysed using SPSS software (SPSS for Windows, Version 10.0, Chicago, IL, USA). The data presented include values for the saline and LPS groups (Chapter 3) and for the hypoxia alone group (Chapter 4), however, a completely new analysis was performed to compare treatment groups. Data were assessed for homogeneity using Levene's test and, where necessary, the data were transformed into square root or logarithmic data before analysis. All analyses were performed using a two-way factorial ANOVA with LPS and hypoxia treatments as the main factors. Where a significant interaction was found between the treatments, individual mean values were compared using Fisher's LSD test. Data (original, untransformed) are presented as mean ± S.E.M. $P<0.05$ was considered statistically significant. The absence of error bars on some graphs presented in this chapter indicates that they are within the symbol.

5.2 Results

This chapter presents results for four treatment groups: saline controls (SAL), saline with hypoxia (hSAL), LPS and LPS with hypoxia (hLPS). For the purposes of this chapter, the data presented include the effects of these treatments on somnogenic behaviour, plasma cortisol and neurosteroid concentrations only. Measurements of rectal temperature, blood gases, pHa, Hb and plasma iron, glucose and lactate were performed, however, the results were similar to those reported in previous chapters. That is, there was no difference between the two groups of hypoxic lambs in the measurements of blood gases, pHa and

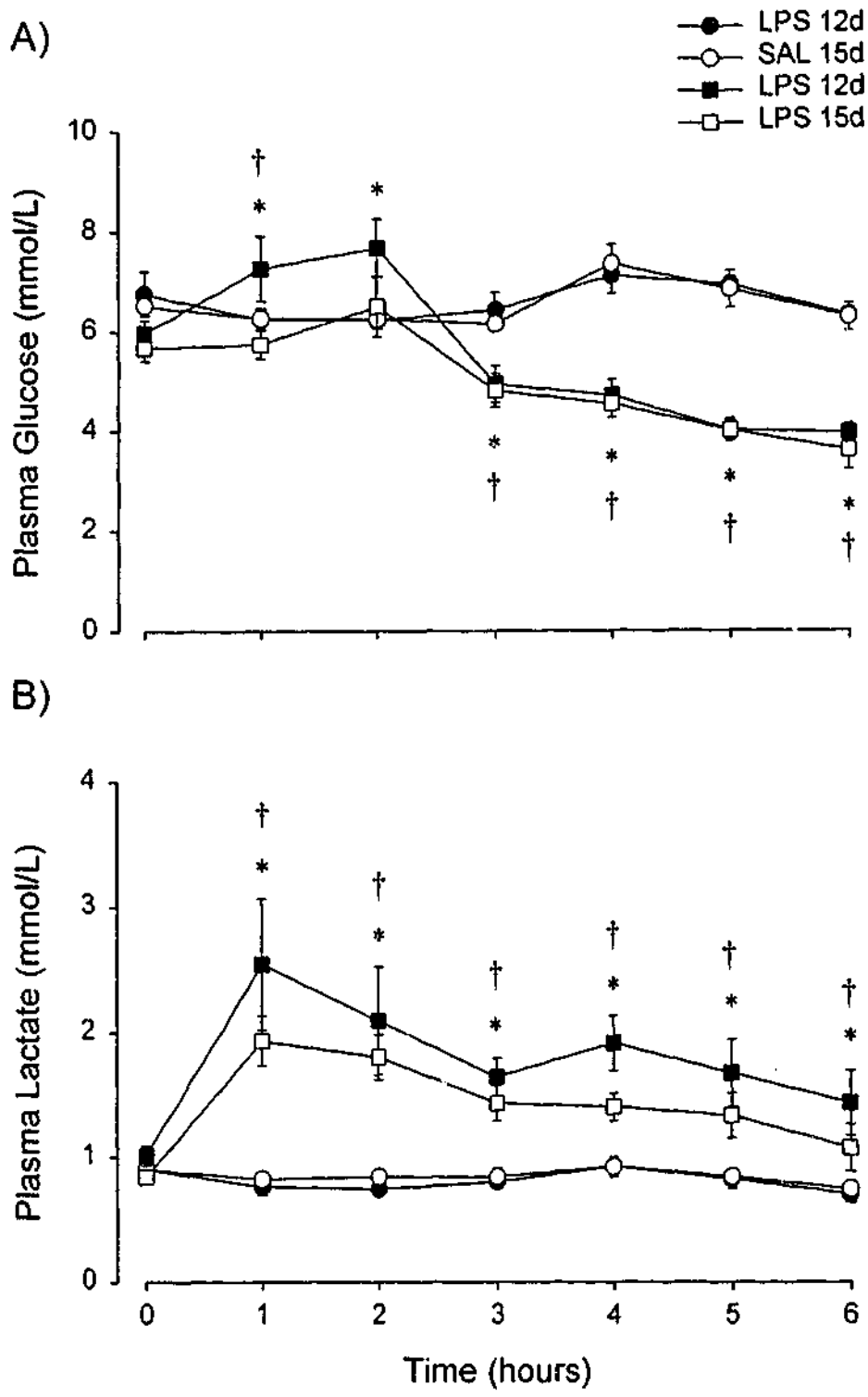


Figure 3.5: Effect of LPS treatment on glucose and lactate concentrations

Effect of saline (n=5) or LPS (n=7) treatment on plasma glucose (A) and lactate concentrations (B). * P<0.05 between saline and LPS-treated lambs at 12 days; † P<0.05 between saline and LPS-treated lambs at 15 days. Data are expressed as mean ± S.E.M.

3.2.2.4 Effect of LPS treatment on cardiorespiratory parameters

Heart rate increased in a biphasic manner in response to LPS treatment between 2 and 3 hours and from 4.5 hours post-treatment (Figure 3.6A). Blood pressure was not affected by LPS treatment (Figure 3.6B). An initial increase in respiratory rate was observed at 0.5 hour post-LPS administration, returning to basal levels thereafter (Figure 3.6C). There was no effect of age on LPS-induced changes of heart rate and respiratory rate, and there was no effect of saline over time for any of the parameters examined.

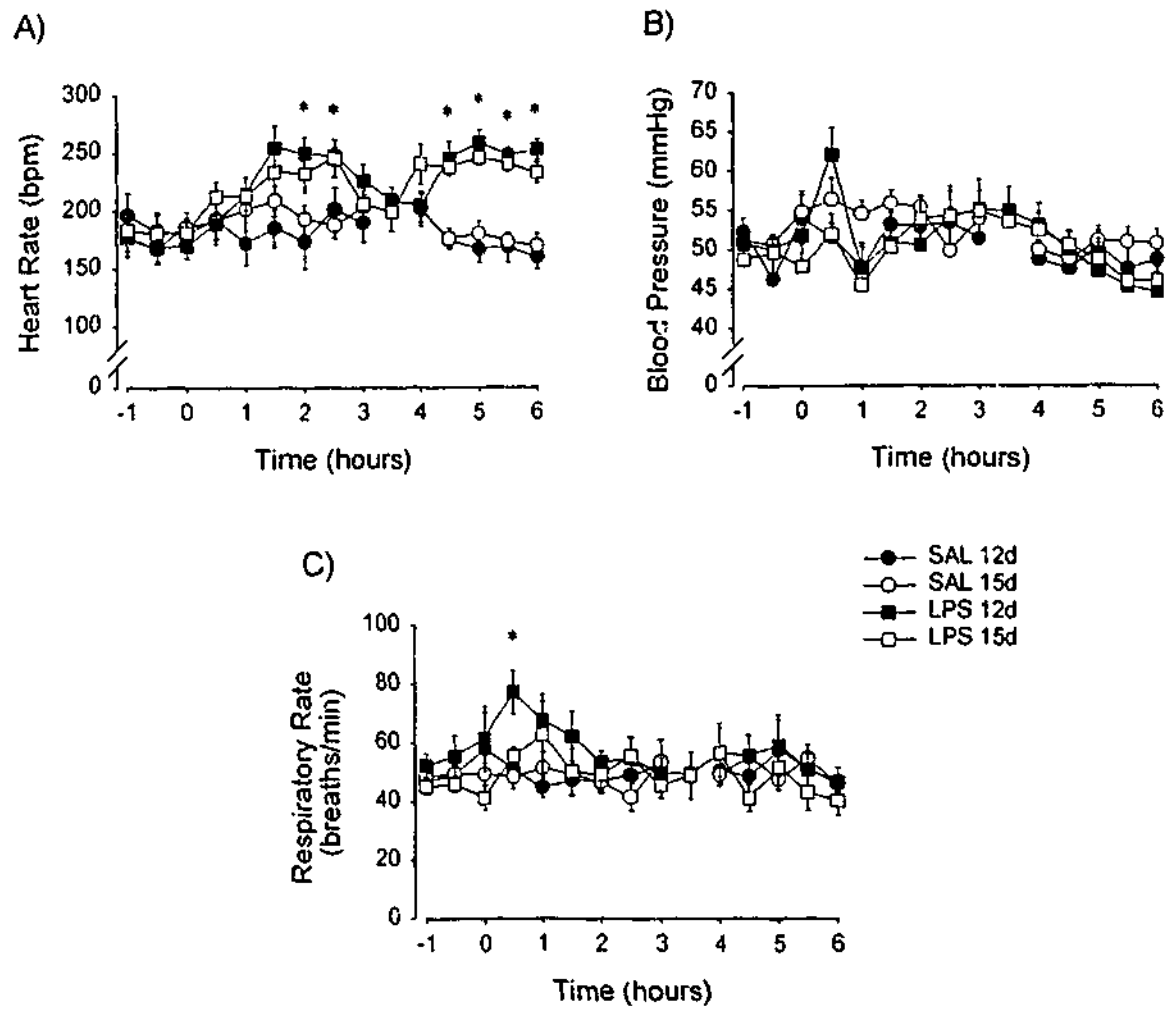


Figure 3.6: Effect of LPS treatment on cardiorespiratory parameters

Effect of saline (n=5) or LPS (n=7) treatment on heart rate (A), blood pressure (B) and respiratory rate (C). * P<0.05 between saline and LPS-treated lambs at 12 and 15 days. The break in the saline data represents the time when lambs had to be returned to their mothers for feeding. Data are expressed as mean ± S.E.M.

3.2.2.5 Effect of LPS treatment on somnogenic behaviour

The duration of the experiments at 12 and 15 days of age, including the pre-treatment period, was 7 hours. When treated with saline, lambs became restless after approximately 4 hours in the sleep chamber (i.e. at 3 hours post-treatment) and had to be returned to their mothers for feeding. This interruption was usually for 15-20 minutes, hence, the recordings of sleep and other physiological parameters measured were interrupted at this time. In contrast, LPS-treated lambs remained asleep or were drowsy for the entire 6 hour period following endotoxin administration; hence the records of rectal temperature (Figure 3.2), heart rate (Figure 3.6A), blood pressure (Figure 3.6B), respiratory rate (Figure 3.6C) and sleep states for these lambs were continuous.

The recording of sleep-wake parameters in lambs during the pre-treatment period presented normal behavioural, cardiovascular and respiratory patterns (Figure 3.7A). In contrast, following LPS treatment, lambs displayed variability in all parameters measured (Figure 3.7B). This variability usually occurred 2 hours following LPS administration and lasted for approximately 2 hours. Figure 3.8 presents the effect of LPS treatment on sleep-wake behaviour in one hour epochs. LPS administration reduced the incidence of wakefulness significantly 2 hours after treatment ($P<0.05$; Figure 3.8A). This reduction continued until the end of the experiment ($P<0.05$). The reduced wakefulness was accompanied by concomitant increases in the incidences of QS (Figure 3.8B) and IS (Figure 3.8D) at 2 and/or 4 hours post-treatment. The incidence of AS did not change over time with LPS treatment (Figure 3.8C). Saline treatment had no effect on the incidences of wakefulness or sleep for the duration of the experiment. Age was also not a factor in variability of sleep-wake states with both age groups displaying similar patterns of behaviour.

The main effect of LPS treatment on behaviour occurred 2-3 hours post-treatment. Incidentally, saline-treated lambs were returned to their mothers after this time. Therefore, Figure 3.9A shows the effect of treatment on sleep-wake behaviour for the hour beginning 2 hours after treatment. LPS treatment resulted in a significant decrease ($P<0.05$) in the incidence of wakefulness (~68 %) and a significant increase ($P<0.05$) in the incidence of indeterminate sleep (~93 %) compared to control animals. When sleep-wake states were calculated as a percentage over the entire 6 hour post-treatment period, LPS treatment reduced the incidence of wakefulness significantly (~44 %) and increased the incidence of IS significantly (~87 %) compared to saline treatment (Figure 3.9B; $P<0.05$). The

incidence of QS tended to be greater following LPS treatment in both analyses, but this did not reach statistical significance (Figure 3.9A: P=0.071; Figure 3.9B: P=0.095). LPS treatment had no effect on the incidence of AS. There were no differences in the effects of LPS on the incidences of sleep and wakefulness between the experiments at 12 and 15 days of age for either the hour preceding the return of the saline-treated lambs or for the entire duration of the experiment.

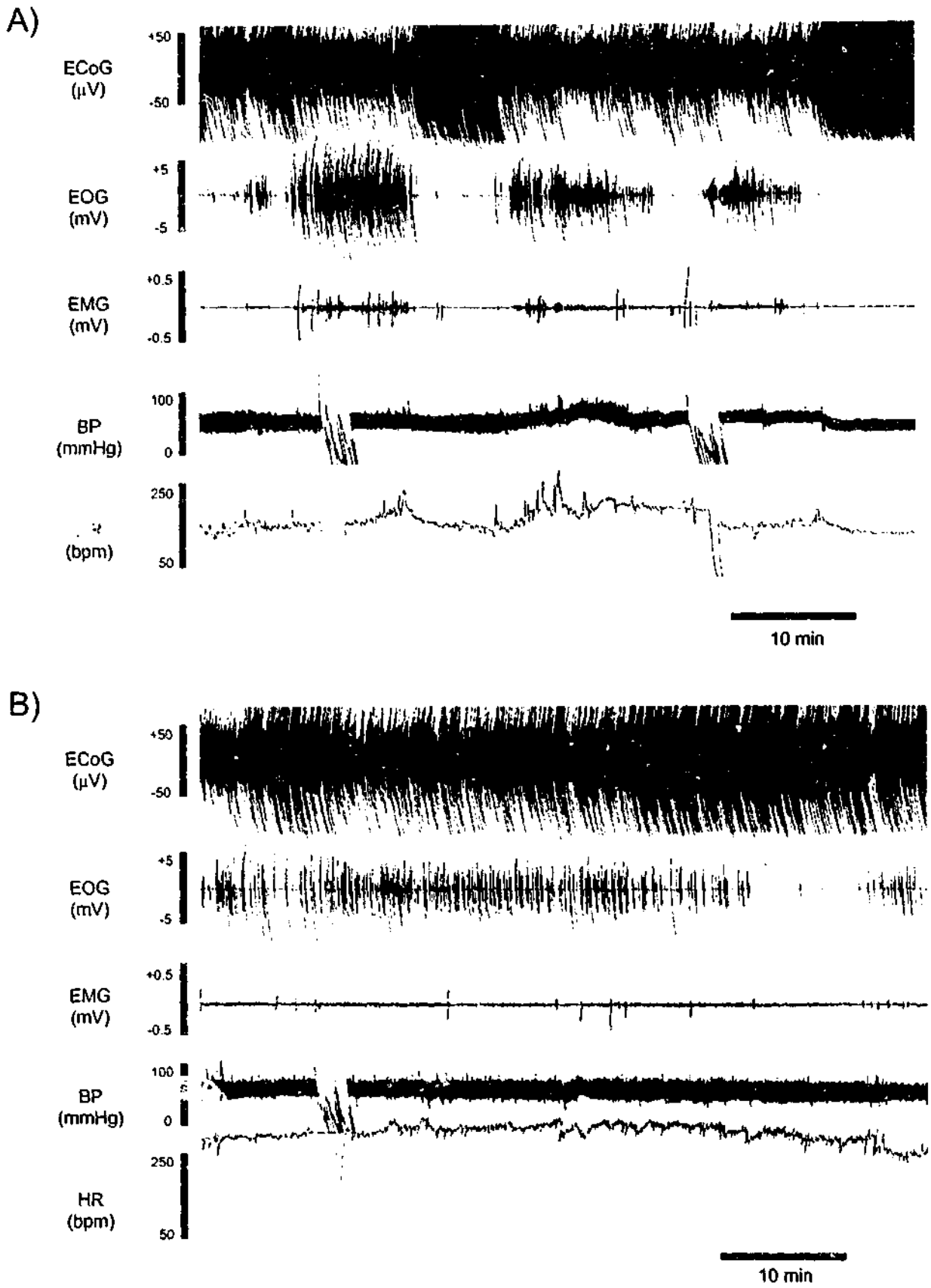


Figure 3.7: Polygraph recording of behavioural parameters

Polygraph recording showing ECoG, EOG, EMG, blood pressure (BP) and heart rate (HR) from a 21 day old lamb. The records show (A) 1 hour of basal activity and (B) 1 hour of recording 2 hours after LPS treatment (0.7 μg/kg, i.v.). Following LPS treatment, there was an increased incidence of high amplitude ECoG and EOG activity. Tonic EMG discharge was also observed after LPS. There was a large increase in heart rate following LPS treatment, however, there was little change in blood pressure post-LPS treatment. The break in the blood pressure and heart rate recordings is when blood sampling occurred.

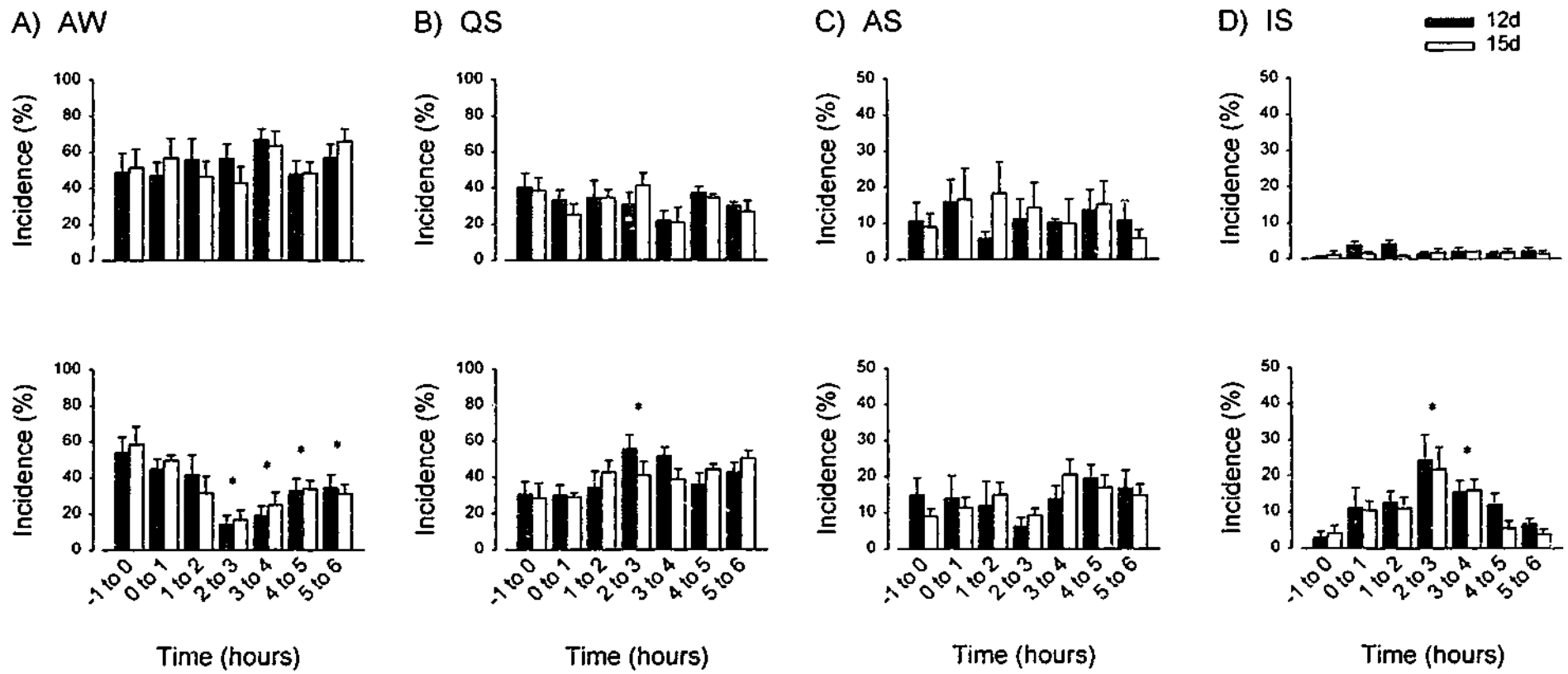


Figure 3.8: Effect of LPS treatment on somnogenic behaviour over time

Effect of saline (top row; *n*=5) or LPS (bottom row; *n*=7) treatment on wakefulness (A), quiet sleep (B), active sleep (C) and indeterminate sleep (D) over the 7 hour experimental period. Note the reduced scale of the y-axis in Figures C & D. * $P<0.05$ as compared to pre-treatment values. Data are expressed as mean ± S.E.M.

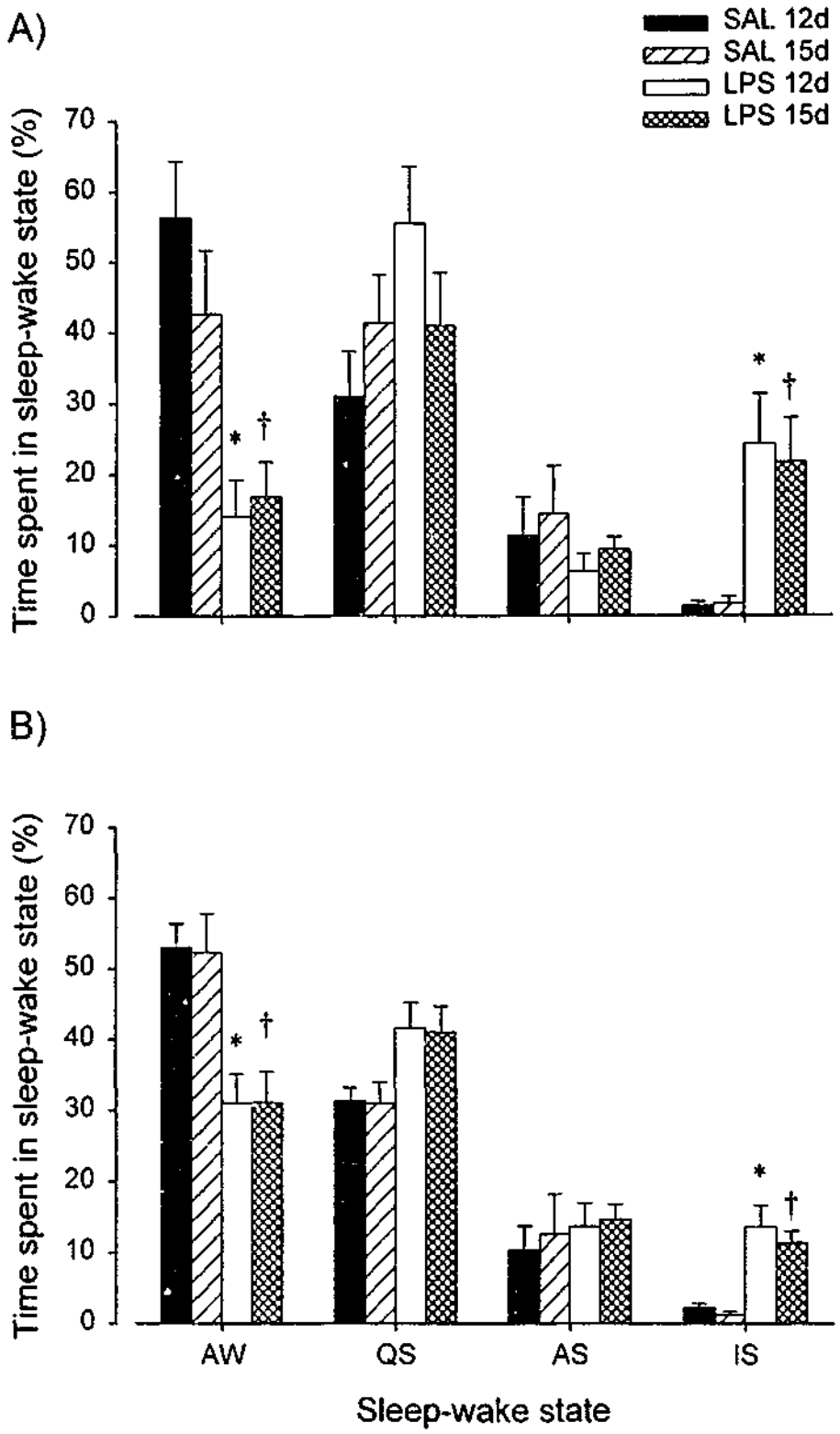


Figure 3.9: Effect of LPS treatment on somnogenesis

Effect of saline (n=5) or LPS (n=7) treatment on sleep-wake behaviour in lambs at 2-3 hours post-treatment (A), and calculated over the duration of the 6 hour experiment (B). * P<0.05 between saline and LPS-treated lambs at 12 days; † P<0.05 between saline and LPS-treated lambs at 15 days. Data are expressed as mean ± S.E.M.

3.2.2.6 Effect of LPS treatment on plasma ACTH concentrations

Plasma ACTH concentrations increased significantly ($P<0.05$) 0.5 hour after LPS administration for both age groups examined. There was no difference between the age groups studied (Figure 3.10). The increase in plasma ACTH concentration following LPS treatment was maximal at 2 hours post-treatment with concentrations increasing from 23.41 ± 0.86 pmol/L at time (0) to 173.09 ± 20.08 pmol/L at 2 hours post-treatment. Saline treatment had no effect on ACTH concentrations over time. At 6 hours post-treatment, concentrations of ACTH for LPS animals were still significantly higher than pre-treatment values and those obtained for saline-treated control lambs (Figure 3.10).

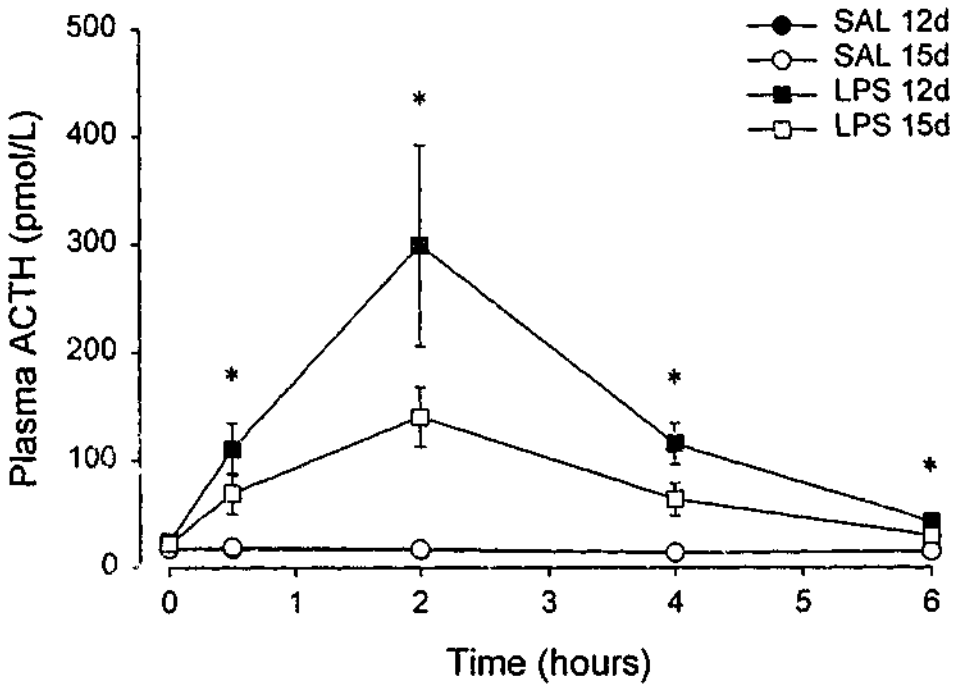


Figure 3.10: Effect of LPS treatment on plasma ACTH concentrations

Effect of saline (n=5) or LPS (n=7) treatment on plasma ACTH concentrations. * $P<0.05$ between saline and LPS-treated lambs at 12 and 15 days. Data are expressed as mean ± S.E.M.

3.2.2.7 Effect of LPS treatment on plasma cortisol concentrations

Plasma cortisol concentrations increased approximately 9-fold after LPS treatment ($P<0.05$; Figure 3.11). The increase was significant by 1 hour post-treatment ($P<0.05$) and peaked at approximately 3 hours, after which it declined but was still significantly greater than for the saline treatment at 6 hours ($P<0.05$). In the saline-treated lambs, there was a small but significant change in plasma cortisol levels ($P<0.05$), but the mean concentration observed at the end of the 6-hour period was not different from the pre-treatment value (Figure 3.11).

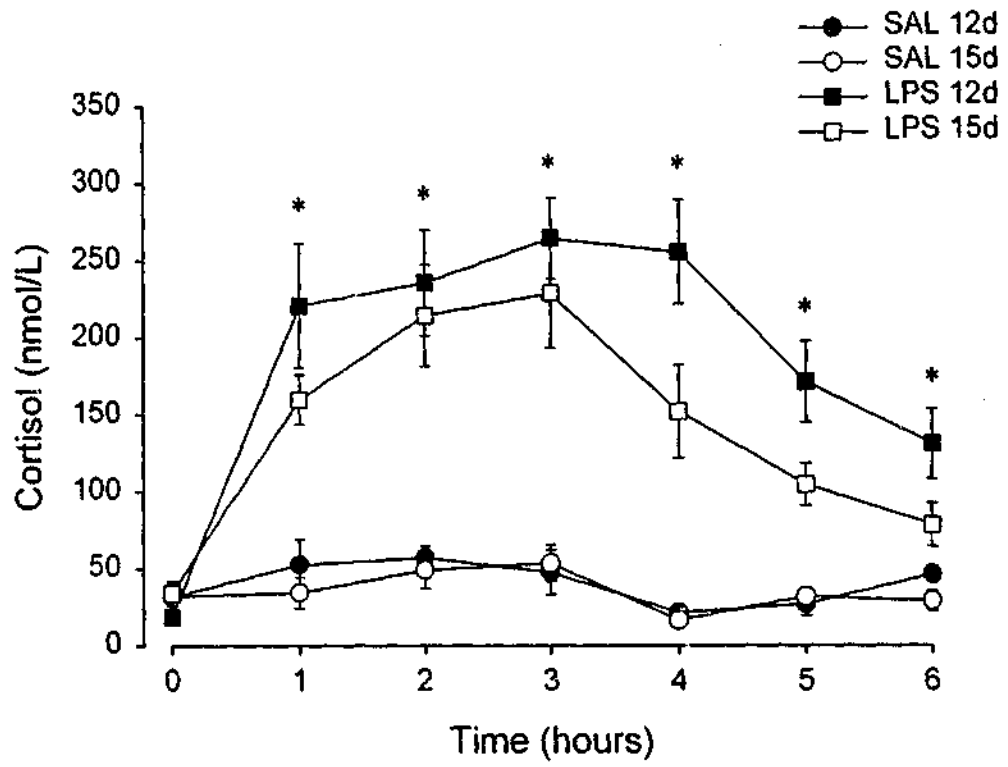


Figure 3.11: Effect of LPS treatment on plasma cortisol concentrations

Effect of saline (n=5) or LPS (n=7) treatment on plasma plasma cortisol concentrations. * $P<0.05$ between saline and LPS-treated lambs at 12 and 15 days. Data are expressed as mean ± S.E.M.

3.2.2.8 Effect of LPS treatment on plasma neurosteroid concentrations

Plasma pregnenolone concentrations were below the level of detection of the assay for the duration of all experiments. Plasma progesterone was undetectable throughout the experiments in the saline-treated lambs, but low plasma concentrations (~0.03 nmol/L) were detected after LPS treatment.

For experiments at both postnatal ages, plasma allopregnanolone concentrations increased significantly ($P<0.05$) following LPS treatment from 1 hour after treatment until the end of the experiment (Figure 3.12). The magnitude of increase was not different between the ages with maximal increases of allopregnanolone concentration of 51.86 ± 8.71 % for 12 day old lambs and 55.62 ± 7.10 % for 15 day old lambs. This similar increase in response to the LPS challenge was in spite of changing basal concentrations of allopregnanolone with age. Resting concentrations of circulating allopregnanolone decreased with increasing age (Figure 3.12). No significant changes in plasma allopregnanolone concentrations were observed following saline treatment.

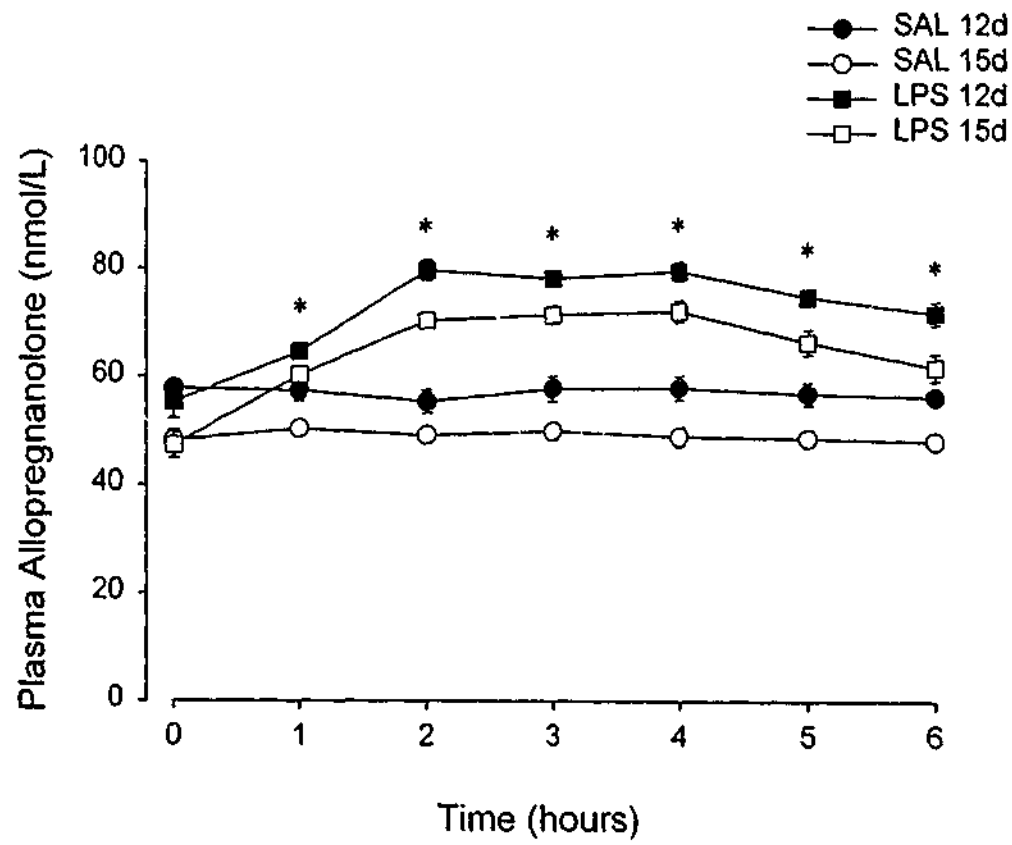


Figure 3.12: Effect of LPS treatment on plasma allopregnanolone concentrations

Effect of saline (n=5) or LPS (n=7) treatment on plasma allopregnanolone concentrations. * $P<0.05$ between saline and LPS-treated lambs at 12 and 15 days. Data are expressed as mean ± S.E.M.

3.2.3 LPS treatment at 20 days of age, prior to tissue collection

3.2.3.1 Effects of age on physiological outcomes

In these experiments, lambs were killed 3 hours following LPS or saline treatment. Tables 3.3-3.6 present data for time (0) and for 3 hours post-saline or LPS treatment, the time prior to euthanasia and subsequent tissue collection. The effects of LPS on rectal temperature (Table 3.1), blood gases, acid-base status, plasma glucose and lactate (Table 3.2), cardiovascular and respiratory parameters (Table 3.3), plasma ACTH and cortisol (Table 3.4) and sleep-wake states (Figure 3.13) for the time period of 0-3 hours were not different to those observed at 12 and 15 days of age. Basal plasma iron concentrations were significantly lower at 20 days of age compared to 12 and 15 days of age, however, there was no significant effect of treatment at 3 hours post-treatment regardless of basal concentrations (Table 3.1). Resting plasma allopregnanolone concentrations decreased significantly between 12 (57.04 ± 0.71 nmol/L) and 15 days (49.05 ± 0.63 nmol/L; $P<0.01$), and between 15 and 20 days (40.12 ± 1.36 nmol/L; $P<0.05$). The relative increase in plasma allopregnanolone following LPS treatment was greater at 20 days of age (92.80 ± 12.06 %; $P<0.05$) than at either 12 (51.86 ± 8.71 %) or 15 days of age (55.62 ± 7.10 %; Table 3.4).

Table 3.1: Effect of LPS treatment on temperature and plasma iron concentrations at 20 days of age

		Time (hours)	
Parameter	***Treatment***	***0***	***3***
Temperature (°C)	SAL	39.00 ± 0.11	39.06 ± 0.07
	LPS	39.36 ± 0.29	40.84 ± 0.42*†
Iron (μmol/L)	SAL	11.65 ± 2.99	10.51 ± 2.79
	LPS	11.30 ± 2.22	13.54 ± 3.20

Effect of saline (n=5) or LPS (n=7) treatment on rectal temperature and plasma iron concentrations at time (0) and at 3 hours post-treatment, prior to tissue collection. * $P<0.05$ between saline and LPS-treated lambs; † $P<0.05$ effect of LPS treatment at 3 hours as compared to time (0). Data are expressed as mean ± S.E.M.

Table 3.2: Effect of LPS treatment on blood gases, pHa, Hb, and plasma glucose and lactate concentrations at 20 days of age

		Time (hours)	
Parameter	***Treatment***	***0***	***3***
SaO_2 (%)	SAL	98.38 ± 2.41	97.80 ± 1.86
	LPS	97.37 ± 1.08	97.39 ± 0.83
PaO_2 (mmHg)	SAL	108.80 ± 11.73	108.40 ± 11.57
	LPS	122.57 ± 2.88	117.14 ± 3.08
$PaCO_2$ (mmHg)	SAL	37.86 ± 0.91	36.29 ± 2.11
	LPS	35.57 ± 1.00	30.57 ± 1.81*†
pHa	SAL	7.36 ± 0.01	7.35 ± 0.01
	LPS	7.38 ± 0.01	7.39 ± 0.01
Hb (g/dL)	SAL	7.34 ± 1.04	7.56 ± 0.93
	LPS	6.31 ± 0.28*	6.69 ± 0.30*
Glucose (mmol/L)	SAL	6.46 ± 0.30	6.30 ± 0.18
	LPS	6.27 ± 0.17	4.67 ± 0.35*†
Lactate (mmol/L)	SAL	0.92 ± 0.09	0.80 ± 0.08
	LPS	0.90 ± 0.07	2.46 ± 0.82*†

Effect of saline (n=5) or LPS (n=7) treatment on blood gases, pHa and plasma glucose and lactate concentrations at time (0) and at 3 hours post-treatment, prior to tissue collection. * $P<0.05$ between saline and LPS-treated lambs; † $P<0.05$ effect of LPS treatment at 3 hours as compared to time (0). Data are expressed as mean ± S.E.M.

Table 3.3: Effect of LPS treatment on cardiorespiratory parameters at 20 days of age

		Time (hours)	
Parameter	***Treatment***	***0***	***3***
Heart rate (bpm)	SAL	173.80 ± 14.33	184.00 ± 8.35
	LPS	168.43 ± 10.73	216.57 ± 14.80
Blood pressure (mmHg)	SAL	54.98 ± 1.56	54.53 ± 1.28
	LPS	49.49 ± 1.45	54.67 ± 1.87
Respiratory rate (breaths/min)	SAL	45.00 ± 3.05	44.20 ± 5.26
	LPS	40.14 ± 3.22	58.00 ± 5.15

Effect of saline (n=5) or LPS (n=7) treatment on cardiovascular and respiratory parameters at time (0) and at 3 hours post-treatment, prior to tissue collection. Data are expressed as mean ± S.E.M.

Table 3.4: Effect of LPS treatment on plasma steroids at 20 days of age

		Time (hours)	
Parameter	***Treatment***	***0***	***3***
ACTH (pmol/L)	SAL	18.02 ± 2.39	19.00 ± 2.51
	LPS	24.29 ± 1.12*	221.02 ± 31.23*†
Cortisol (nmol/L)	SAL	18.09 ± 5.67	26.93 ± 6.28
	LPS	25.48 ± 9.25	248.90 ± 29.00*†
Allopregnanolone (nmol/L)	SAL	39.32 ± 2.32	40.37 ± 3.49
	LPS	42.16 ± 1.81	74.37 ± 3.24*†

Effect of saline (n=5) or LPS (n=7) treatment on plasma ACTH, cortisol and allopregnanolone concentrations at time (0) and at 3 hours post-treatment, prior to tissue collection. * P<0.05 between saline and LPS-treated lambs; † P<0.05 effect of LPS treatment at 3 hours as compared to time (0). Data are expressed as mean ± S.E.M.

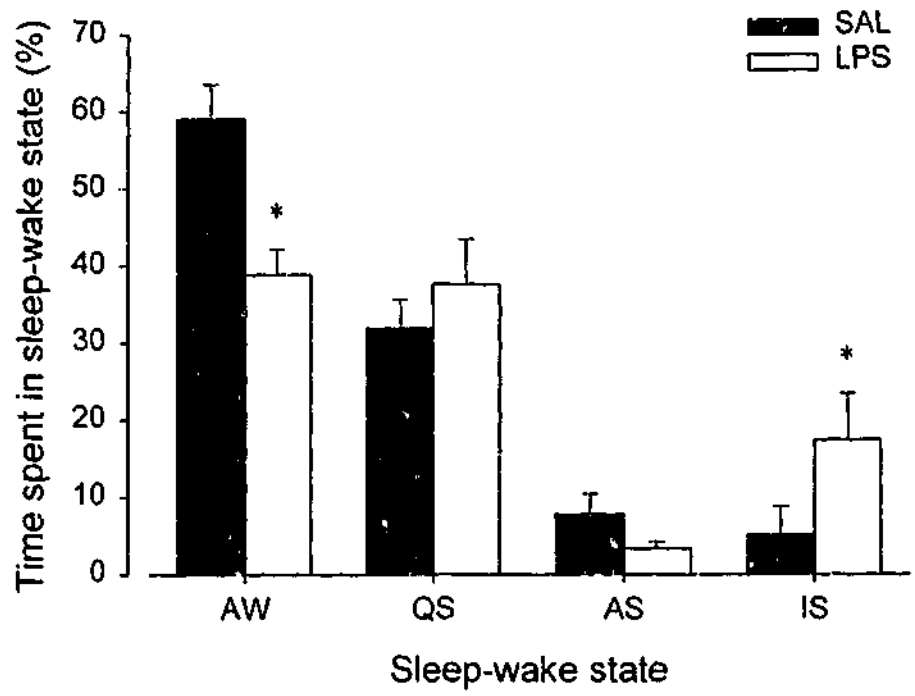


Figure 3.13: Effect of LPS treatment on somnogenesis at 20 days of age

Effect of saline (n=5) or LPS (n=7) treatment on sleep-wake behaviour in lambs between 0 and 3 hours post-treatment. * P<0.05 between saline and LPS-treated lambs. Data are expressed as mean ± S.E.M.

3.2.3.2 Effect of LPS treatment on brain allopregnanolone concentrations

Allopregnanolone concentrations measured in the different regions of the brain that had been obtained 3 hours after saline or LPS treatment are shown in Figure 3.14. LPS treatment increased allopregnanolone concentration significantly in the medulla, pons, midbrain and hippocampus, compared to the saline-treated controls (Figure 3.14A; P<0.05). Allopregnanolone concentration was also increased markedly in all regions of the cortex in response to LPS treatment (Figure 3.14B; P<0.05). There was no effect of LPS treatment on allopregnanolone concentration in the cerebellum or diencephalon. Pregnenolone concentration was <0.08 pmol/g in all brain regions and there was no significant difference between the LPS and saline-treated groups. Progesterone concentrations in all brain regions were below the level of detection of the assay for both treatment groups.

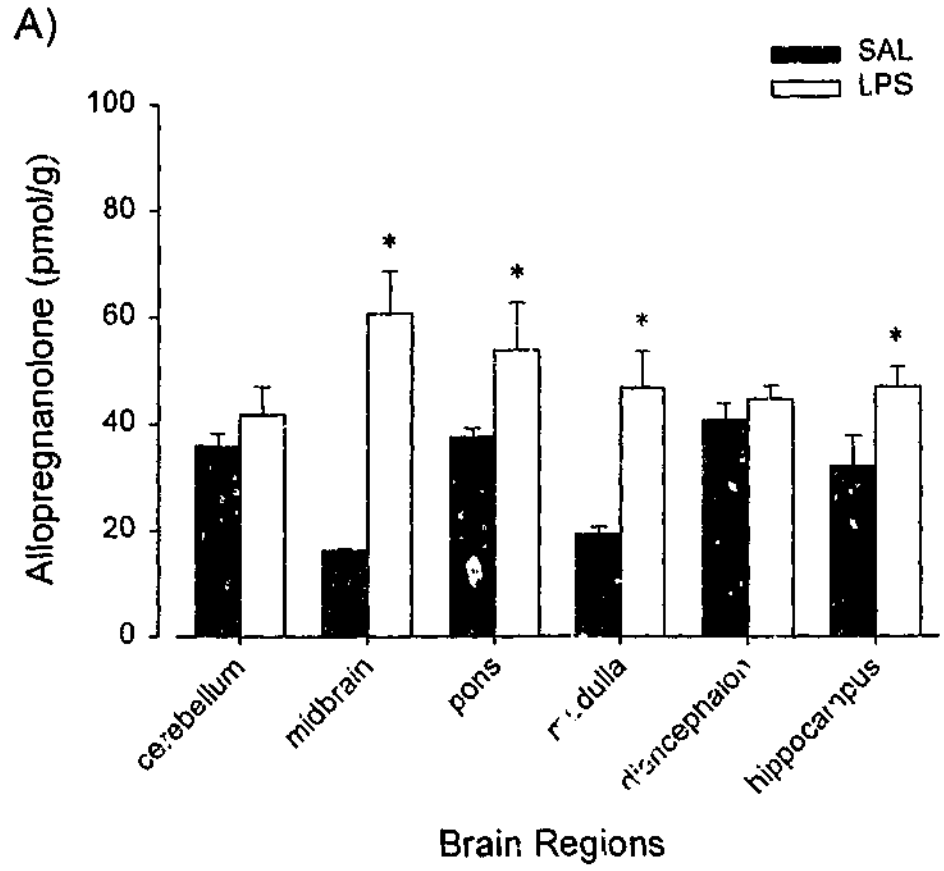


Figure 3.14: Effect of LPS treatment on brain allopregnanolone concentrations

Allopregnanolone concentrations in the major regions of the brain (A) and in specific regions of the cerebral cortex (B) of saline (n=4) or LPS-treated (n=4) lambs. * $P<0.05$ between saline and LPS treatment at 20 days of age. Data are expressed as mean ± S.E.M.

3.3 Discussion

The principal finding of this study is that allopregnanolone concentrations are increased significantly in the brain and plasma of lambs 3 hours after LPS administration. This coincided with a marked decline in wakefulness and a concomitant increase in sleep and drowsiness, suggesting a link between stimulated neurosteroid production and somnolence in these lambs.

3.3.1 Temperature and plasma iron responses to LPS treatment

This study used a model of infection that has been used extensively to examine the acute phase response (APR) to infection in a number of species. Following injection of LPS, body temperature increased in a well-established biphasic manner (Goelst *et al.*, 1992) in all lambs. This rise has been proposed to result from an initial release of prostaglandins and inflammatory mediators such as TNF-α followed by a more sustained release of cytokines such as IL-1β and IL-6 (Romanovsky & Blatteis, 1995; Miller *et al.*, 1997). In addition to fever, a decrease in plasma iron concentrations was evident 6 hours after LPS administration. Measurements of body temperature and plasma iron together represent a coordinated host defence mechanism as part of the APR which, as shown by these results, is present in the lamb at 12 days of age. The decrease in iron in response to an endotoxin challenge is believed to result from the increased synthesis or release of endogenous pyrogens that cause an increased uptake of iron from the liver (Goelst & Laburn, 1991). The increased iron uptake is beneficial as it reduces or prevents the growth of bacteria both *in vitro* and *in vivo* (Kochan *et al.*, 1977). Kluger and Rothenburg (1979) reported that rabbits, challenged with *Pasteurella multocida*, exhibit an increase in rectal temperature that is associated with a decrease in plasma iron concentration. Further, *in vitro* studies (Kluger & Rothenburg, 1979) reported that, at higher temperatures, this bacterium grew poorly in the presence of low iron concentrations, thus supporting a role for the coordinated response between temperature and iron levels in protecting the host against infection.

The observed reduction in plasma iron concentrations with age is most likely to result from the increased size of the liver and a greater demand for haemoglobin synthesis early in neonatal life (Linder & Munro, 1973). After birth, the levels of stored iron in the liver are reduced and these levels are further depleted during suckling as the mother's milk provides inadequate quantities of iron (Linder & Munro, 1973). Thus, the reduction of

circulating iron concentrations observed in lambs with increasing age reflects the greater utilisation of available iron.

3.3.2 Effects of LPS treatment on blood gases, pHa, Hb, glucose and lactate

LPS treatment resulted in an initial arterial O_2 desaturation accompanied by a mild and relatively brief hypocapnia and hypoxaemia. Plasma glucose levels were significantly lower from 3 hours after LPS treatment compared to the basal period, while lactate concentrations increased in response to LPS administration. Although both groups of lambs were separated from their mothers and, consequently, did not feed for a minimum of 4 hours, saline treatment had no effect on plasma glucose or lactate levels during this time. A moderate biphasic increase in heart rate was observed, with no consistent change in systemic blood pressure or respiratory rate, supporting previous studies using low doses of pyrogen administration in young lambs (Fewell *et al.*, 1991). There are considerable interspecies differences in responses and sensitivities to LPS treatment (McKuskey *et al.*, 1984). No animals died during or after LPS treatment in the current study, indicating that the dose of LPS used was adequate to induce a response similar to a relatively mild acute infection.

3.3.3 Plasma steroid responses to LPS treatment

Acute stressors such as CO_2 inhalation and swim stress in adult rats have been reported to lead to increases in neurosteroid concentrations in plasma and brain (Purdy *et al.*, 1991; Barbaccia *et al.*, 1994). The current finding that allopregnanolone was elevated in plasma and brain following LPS treatment is consistent with these studies and confirms that neurosteroid production is stimulated during stressful events, including infection. The marked rise in plasma ACTH and cortisol concentrations after LPS treatment is consistent with the activation of the HPA axis (Faggioni *et al.*, 1995; Takeuchi *et al.*, 1997). Allopregnanolone concentrations have been reported to rise after adrenal stimulation in adult subjects where gonadal production of steroids is prevented, suggesting that the adrenal gland is the source of plasma allopregnanolone (Genazzani *et al.*, 1998; Genazzani *et al.*, 2000). The increase in plasma allopregnanolone concentration observed following LPS treatment in the current study most likely results from the stimulation of adrenal secretion, since gonadal neurosteroidogenesis would be minimal in these sexually immature lambs.

3.3.4 Brain allopregnanolone response to LPS treatment

The increase in allopregnanolone concentrations in the brain was different between regions; from insignificant changes in the cerebellum and diencephalon to a 2-3 fold increase in other regions of the cerebral cortex and an approximate 4-fold increase in the midbrain and parietal cortex. This suggests that there were regionally distinct changes in the *in situ* production of this steroid in the brain. Some of the increases in brain allopregnanolone were greater, relatively, than the increase in plasma allopregnanolone, suggesting that the steroid changes observed in the brain are distinct from the peripheral changes in allopregnanolone. The very low concentrations of pregnenolone and progesterone in plasma suggest that delivery of these precursors from the periphery would be unlikely to support the increased cerebral production of allopregnanolone. A previous study from our laboratory reported that two of the key enzymes involved in the synthesis of allopregnanolone, $P450_{scc}$ and 5α-reductase, are strongly expressed in the neonatal brain (Petratos *et al.*, 2000). Although these findings provide support for the role of local production and regulation of allopregnanolone in the newborn brain, it has not yet been established that LPS treatment actually up-regulates either gene expression or enzyme activity.

3.3.5 Somnogenic effects of LPS treatment

In the current study, LPS-treated lambs were able to remain separated from the ewe for up to 7 hours. These lambs displayed significantly greater sleep and drowsiness compared to the saline-treated lambs. The saline-treated lambs became restless after 3 hours and were evidently hungry and had to be returned to their mothers. This suggests that one of the effects of LPS is to suppress appetite, as these lambs displayed no sucking reflex or desire to feed from a bottle. Furthermore, the observation that the LPS-treated lambs became hypoglycaemic and yet did not display behaviours consistent with hunger is consistent with a possible suppressive effect of LPS on appetite and the desire to feed. Saline-treated lambs were not hypoglycaemic when they were returned to the ewe suggesting that the behavioural response observed was a non-hypoglycaemic hunger response or a result of stress at being separated from the ewe. The short-term anorectic properties of LPS administration appeared to have long lasting effects with reductions in body and tissue weights at post-mortem (see Appendix 8.1). These results support previous findings that LPS administration reduced feed intake in heifers (Steiger *et al.*, 1999) and altered body

and tissue mass in rats (Raina *et al.*, 2000), and is consistent with the actions of some cytokines (e.g. IL-1β) in the hypothalamus to induce anorexia (Sonti *et al.*, 1996).

The LPS-treated lambs showed decreased wakefulness and increased drowsiness regardless of whether this was calculated over the entire recording period in one hour epochs, from one hour of data immediately before the saline-treated lambs were returned to their mother, or from all of the data obtained post-treatment. Several mechanisms may contribute to the increased sleep that occurs after LPS treatment, such as the induction of sleep-inducing prostaglandin D_2 (Hayaishi, 2000), as well as a contribution by increased levels of sedative neurosteroids in the brain. Allopregnanolone is a potent sedative steroid with positive modulatory actions with GABA at the $GABA_A$ receptor, thereby increasing GABAergic inhibition (Barbaccia *et al.*, 1996). In newborn piglets, dialysis of the $GABA_A$ agonist, muscimol, into the brainstem disturbs normal sleep patterns and produces a state of drowsiness (Darnall *et al.*, 2001). It is possible that the 2-3 fold increase in allopregnanolone in the brain observed in this study was sufficient to increase sleep, however, no study has reported increased sleepiness following the direct infusion of the brain with similar concentrations of allopregnanolone.

3.3.6 Potential tolerance to LPS treatment

In order to test the hypothesis that the lamb becomes tolerant to infection-induced stimuli, the effect of injecting LPS on 3 occasions between 10 and 21 days of age was examined. Previous studies have shown that tolerance of a number of physiological parameters develops with repeated doses of endotoxin over a period of days (Whyte *et al.*, 1989; Roth *et al.*, 1994; Hadid *et al.*, 1996). However, no marked differences between the three LPS treatments in febrile measurements, blood gases, glucose, lactate, cardiovascular and respiratory parameters, ACTH, cortisol or behavioural responses were found in this study. Differences were observed in the response of plasma allopregnanolone to LPS treatment by the third experiment. However, because the responses to LPS were not diminished at this time, these data suggest that little tolerance to LPS develops with the dose used and with the relatively short-term response generated in these animals.

Basal plasma allopregnanolone concentrations decreased with age in lambs. The cause of this, and its relevance, remains to be elucidated. A recent study found that allopregnanolone concentrations in the brain decrease sharply at birth (Nguyen *et al.*, 2003), whereas plasma allopregnanolone concentrations decrease slowly with postnatal

age, as shown in the present study. This again suggests a separation of peripheral and CNS production of 5α-reduced steroids. It has been proposed that neurosteroids such as allopregnanolone may have a neuroprotective function (Kehoe *et al.*, 2000) but increases in the newborn brain could be detrimental by creating a higher arousal threshold and suppression or ablation of responses to hypoxia and apnoea, which are problems often faced by the newborn infant.

3.3.7 Possible role for neurosteroids in the aetiology of SIDS

The contribution of bacterial or viral infection in the aetiology of SIDS remains controversial but is supported by both autopsy and epidemiological findings (Filiano & Kinney, 1994). The potential link between SIDS and infection is supported by the findings that the prevalence of SIDS is distributed seasonally with higher death rates occurring in winter months and that several respiratory and bacterial infections have been isolated in SIDS victims (Blackwell *et al.*, 1992). The pathway by which these infectious challenges contribute to SIDS or apparent life-threatening events may involve several mechanisms. However, it is established that even minor infections lead to marked stimulation of the HPA axis (Dunn, 1993) such that a relatively mild infection may cause a marked increase in glucocorticoid concentrations as observed in the present study. Increased glucocorticoid secretion may stimulate neurosteroid synthesis, since glucocorticoids are potential precursors for neurosteroid production, and may also stimulate the expression of enzymes in the neurosteroidogenic pathway (Genazzani *et al.*, 1998; Genazzani *et al.*, 2000).

3.3.8 Summary

In summary, this study found that concentrations of the sedative steroid, allopregnanolone, were increased significantly in several brain regions following the administration of a small dose of LPS to mimic infection. This coincided with markedly increased plasma ACTH and cortisol concentrations, an increase in drowsiness and a decrease in wakefulness. These observations suggest that this steroid may contribute to a reduction in arousal responses that may increase the risk of SIDS following an infectious challenge.

Chapter 4: Hypoxia increases sleep and brain allopregnanolone concentrations in lambs

Hypoxia induces a range of physiological responses to restore homeostasis to the body including arousal (Johnston *et al.*, 1998), hypertension (Fletcher *et al.*, 1992), tachycardia (Sidi *et al.*, 1983), ventilatory stimulation (Moss *et al.*, 1995), increased glucocorticoid production (Lee *et al.*, 2002) and increased brain GABA concentration (Xiao *et al.*, 2000). Studies that have investigated the relationship between hypoxia and sleep-wake patterns have focused primarily on arousal mechanisms. From these studies, it has been reported that increased ventilation, mediated by peripheral chemoreceptors, in response to hypoxia is depressed significantly during sleep in the newborn (Phillipson & Sullivan, 1978; Bissonnette, 2000), suggesting an increased susceptibility to hypoxic challenges.

Stressful stimuli such as endotoxin challenge in lambs (see Chapter 3) and CO_2 inhalation in rats (Purdy *et al.*, 1991) have the effect of increasing neurosteroid synthesis in the brain. It has been suggested that increased allopregnanolone synthesis in response to stress has neuroprotective effects by counteracting the neurotoxic responses produced by the stressor (Patchev *et al.*, 1997). However, studies performed for this thesis (see Chapter 3) have suggested that large increases in allopregnanolone concentration in the brain could be detrimental, by increasing the degree of sedation to a level where innate arousal

mechanisms are depressed. As discussed at length in Chapter 1, allopregnanolone is a potent sedative steroid because of its positive modulatory actions with GABA at the $GABA_A$ receptor (Barbaccia *et al.*, 1996). It is synthesised in the brain from cholesterol and in peripheral organs such as the adrenal glands or gonads (Lancel *et al.*, 1997). These peripherally-produced neurosteroids are also able to cross the BBB, further contributing to neurosteroid concentrations in the brain (Zwain & Yen, 1999). The administration of allopregnanolone has been shown to alter sleep patterns (Lancel *et al.*, 1997; Darnaudery *et al.*, 1999) and induce anaesthesia at high doses (Korneyev & Costa, 1996).

SIDS continues to account for the largest number of deaths during the first year of life in Australia (SIDS for KIDS foundation, *personal communication*). Although many causes have been postulated, such as prematurity (review Sullivan & Barlow, 2001), low birth weight (review Sullivan & Barlow, 2001), maternal smoking (Kahn *et al.*, 1994) and infection (Bettelheim *et al.*, 1990; Crawley *et al.*, 1999; Harrison *et al.*, 1999), the exact mechanisms involved in the SIDS phenomenon are still relatively unknown. Pathological evidence suggests that hypoxia is one of the final triggers involved in SIDS (Kinney *et al.*, 1983; Jones *et al.*, 2003). Therefore, because SIDS infants have been reported to die in their sleep (review Sullivan & Barlow, 2001), an hypothesis addressed by the studies performed in this chapter is that hypoxia induces the production of sedative neurosteroids in the brain, leading to increased drowsiness and sleep.

Although extensive research has focused on the effects of hypoxia on arousal thresholds, there is little data available commenting on the effects of this stressor on the incidence of somnogenesis. Therefore, as allopregnanolone concentrations have been shown to increase in response to stressful stimuli, the primary aims of this study were (1) to investigate the effects of induced hypoxaemia on sleep-wake profiles and neurosteroid concentrations in lambs and (2) to assess the effects of single and repeated bouts of induced hypoxaemia on these parameters. It has been reported that repeated episodes of hypoxia, as seen in obstructive sleep apnoea, can lead to a tolerance in ventilatory responses (Moss, 2000). Therefore, experiments performed as part of this chapter examined and compared the effects of exposure to a single or two episodes of induced hypoxaemia, to determine if physiological responses in the lamb adapted to the second challenge.

4.1 Materials and methods

4.1.1 Animals and surgical preparation

Eleven lambs obtained from Merino-Border Leicester crossbred ewes were delivered spontaneously in lambing pens. To conserve animal numbers, results from lambs that received saline treatment alone (n=5) are those reported in Chapter 3. In addition, single (n=4) and repeated hypoxia (n=6) experiments were performed on the same lamb. The reduced number of animals for the single hypoxia experiments was due to experimental difficulties. In each case, lambs were allocated randomly to a treatment group.

Aseptic surgery was performed between 5 and 10 days of age, using halothane inhalation, to insert indwelling vascular catheters for blood sampling and to implant electrodes for recording sleep states as described in Section 2.3. Experiments did not commence until at least four days after surgery.

4.1.2 Experimental design

All experiments began at 0800 hours, to avoid possible effects of diurnal rhythms. The lamb was then placed in a sling and recording of the physiological variables commenced. ECoG, EOG and nuchal muscle EMG activity, together with arterial pressure and intrathoracic pressure, were recorded on a polygraph. Rectal temperature was monitored using a temperature probe. Saline (5 mL; n=15) was administered intravenously as a bolus to all lambs, one hour after recording began. This enabled the use of saline-treated lambs from Chapter 3 to be used as controls for the studies reported in this chapter. Note: saline treatment had no effect on the majority of parameters measured (see Chapter 3). Recordings continued for 6 hours, thus, the lambs remained in the sleep chamber for 7 hours in total.

Preliminary studies were performed to assess the timing of hypoxia administration. To investigate the effects of a single episode of hypoxia, a one hour period was used. This length of time was long enough to induce endocrine changes that were maintained for a minimum of 30 minutes and to permit recovery following the challenge. In addition, one hour of hypoxia was sufficient to induce changes in the architecture of sleep, either within the challenge or immediately after. To assess the possible pre-conditioning effects of an earlier exposure to hypoxia, lambs were also treated with two episodes of hypoxia. Given

that lambs could not be removed from their mothers for any longer than 7 hours (see Chapter 3), the length of the episodes was reduced to 45 minutes each, with 75 minutes in-between. Although 15 minutes shorter than the single episode, preliminary observations found that this length of time was still sufficient to induce endocrine and somnogenic changes. In addition, a 75 minute break between exposures was sufficient to allow blood gases and cardiorespiratory parameters to return to normal.

Lambs that were to be treated with a hypoxic challenge(s) had a custom-made plastic hood (100 x 120 cm) placed over the head and upper torso, thirty minutes following the commencement of the basal recording period (t = -60 minutes). Air was passed through the plastic hood continuously, at a flow of 30 L/min, except during a single one hour episode (n=4) or two 45 minute episodes of hypoxia (n=6) when lambs breathed a mixture of 8 % O_2 in N_2. This level of gas mixture was sufficient to reduce the arterial oxygen saturation to ~50 %, which was monitored from arterial blood samples drawn every 15 minutes. The single period of hypoxia commenced 2.5 hours after saline administration and the repeated hypoxia challenge commenced at 0.5 and 2.5 hours after the saline had been given. At least 3-4 days elapsed before studies were performed on the same lamb to ensure that no tolerance occurred (as assessed by blood gases, pHa and plasma cortisol concentration; see results). Saline-treated controls also breathed air throughout the experiment, but this was not within the custom-made bag. All apparatus used for the administration of air or hypoxia, sampling or recording remained outside the sleep chamber to ensure minimal disruption during the experiment.

The 7 hour experimental protocol (1 hour pre-treatment and 6 hours post-saline treatment) was carried out at approximately 12 days (range, 7-15) of age for the first of the two repeated hypoxia experiments and at 15 days (range, 13-18) of age, for the single hypoxia challenge. During a third experiment at approximately 20 days (range, 17-21) of age, each lamb was given another repeated hypoxia challenge. These lambs were then euthanased using an overdose of i.v. pentobarbitone, 3 hours after the administration of saline (or 30 minutes after the onset of the second hypoxia challenge). The brain was removed immediately, divided into blocks incorporating the cerebellum, midbrain, pons, medulla, thalamus/hypothalamus (diencephalon), hippocampus, and frontal, primary, parietal, temporal and occipital cortices, and each block was snap-frozen in liquid N_2 and stored at -70 °C until required for analysis of steroid concentrations. Blood samples were taken

immediately prior to tissue collection to ensure that hypoxia had been induced in these lambs.

4.1.2.1 Blood sampling

Arterial blood samples (4 mL) were collected as reported in Section 3.1.3.1 during non-hypoxic periods and every 15 minutes during hypoxia. Blood gases, pHa and Hb were measured immediately in 0.2 mL of the blood sample. Plasma was recovered from the remaining blood and kept frozen at -20 °C until required for assays of glucose, lactate, iron, cortisol, pregnenolone, progesterone and allopregnanolone (see Chapter 2 for methodologies).

4.1.3 Data analysis

Identification of sleep-wake states based on the chart records was conducted using the methods described (Section 2.4.2). For each experiment, the sleep-wake states of the entire recording were scored at 1 minute intervals and these data were combined to obtain an average value for either 60 minute epochs or a combination of 30, 45 and 60 minute epochs. This variation in epoch length was to assess the effects of the hypoxic challenges by incorporating the entire 1 hour or 45 minute challenge(s). The proportion (%) of the total time spent in a particular state (AW, QS, AS, IS) after saline treatment, with or without hypoxia, was then calculated.

4.1.4 Statistical analyses

Data were analysed using SPSS software (SPSS for Windows, Version 10.0, Chicago, IL, USA). The data presented include values reported previously for the saline control group (Chapter 3). However, a different analysis was conducted for the purpose of this study. Data were assessed for homogeneity using Levene's test and, when necessary, transformed into square root or logarithmic data. A two-way repeated measures ANOVA was used to compare treatment over time between age-matched groups. Where a significant interaction was found between the treatments, *post hoc* analysis was conducted using Fisher's LSD test. Data (original, untransformed) are presented as mean ± S.E.M. $P<0.05$ was considered statistically significant. The absence of error bars on some graphs presented in this chapter indicates that they are within the symbol.

4.2 Results

This chapter presents results of both single (sHYP) and repeated (rHYP) episodes of hypoxia. For both groups, comparisons are made with saline-treated controls (CONT). Saline-treated controls were unable to remain in the sleep chamber for the full duration of the experiment and were returned to the ewe for feeding (as discussed in Chapter 3). Therefore, a break in the data for rectal temperature and cardiorespiratory parameters represents this time.

4.2.1 Effect of hypoxia on temperature and plasma iron concentrations

Resting rectal temperatures were higher in hypoxic lambs than in normoxic lambs (Figure 4.1), for reasons that are not clear. Rectal temperature tended to decrease after each hypoxia challenge, however, this was not significant ($P>0.05$). Saline treatment had no effect on rectal temperature over time (Figure 4.1).

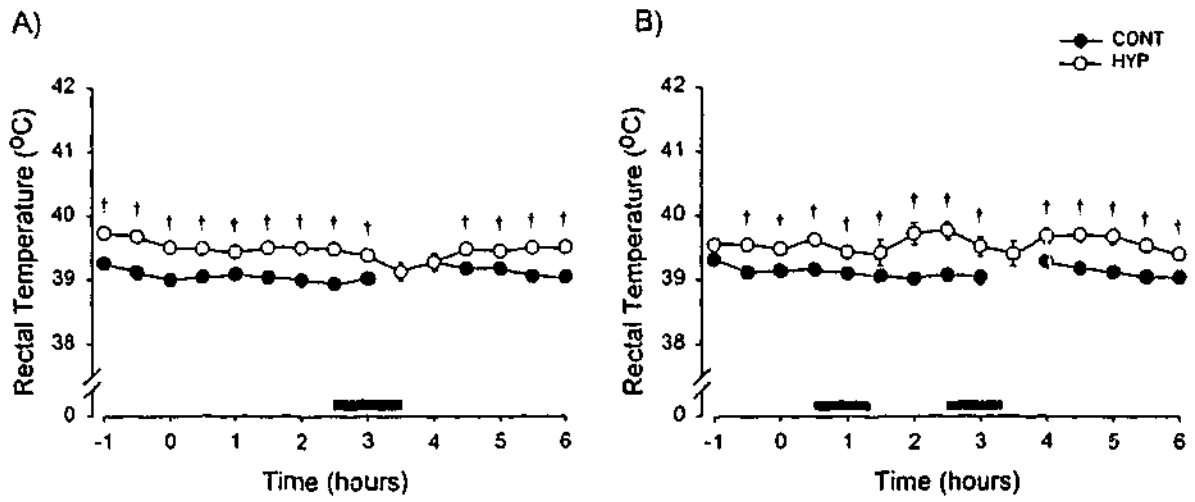


Figure 4.1: Effect of hypoxia on rectal temperature

Effect of control (n=5) or single hypoxia treatment (n=4) on rectal temperature (A). Effect of repeated hypoxia treatment (n=5) compared to control lambs (n=6) on rectal temperature (B). † $P<0.05$ between groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

Plasma iron concentrations were lower in lambs that received a single hypoxia challenge than those that received repeated hypoxia challenges (Figure 4.2). The lambs in the single hypoxia group were older than those in the repeated hypoxia group. The lower plasma iron concentration in the former group supports the findings of the previous chapter where plasma iron concentrations were found to decrease with age. Plasma iron concentrations were not affected by hypoxia treatment (Figure 4.2).

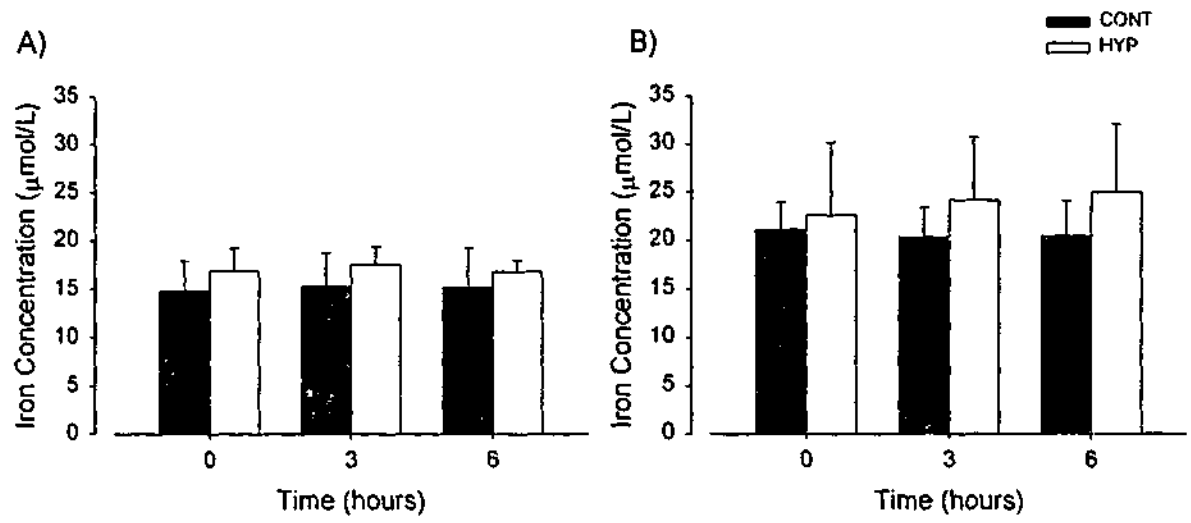


Figure 4.2: Effect of hypoxia on plasma iron concentrations

Effect of control (n=5) or single hypoxia treatment (n=4) on plasma iron concentrations (A). Effect of control (n=5) or repeated hypoxia treatment (n=6) on plasma iron concentrations (B). Data are expressed as mean ± S.E.M.

4.2.2 Effect of hypoxia on blood gases, pHa and Hb

Hypoxia administered as a single episode resulted in significant changes in blood gas parameters and pHa (Figure 4.3 A-D). Arterial O_2 saturation (SaO_2), PaO_2 and $PaCO_2$ decreased within 15 minutes of commencement of the hypoxia challenge by ~50 %, ~66 % and ~30 %, respectively. These parameters returned to control levels within 30 minutes of conclusion of the challenge (Figure 4.3 A-C). Arterial pH, although initially higher than controls, increased in response to hypoxia with levels increasing from 7.41 ± 0.02 at time (0) to 7.48 ± 0.02 at 3 hours post-saline administration (Figure 4.3D). Arterial pH returned to pre-treatment values in hypoxic lambs within 30 minutes of the challenge, but was still significantly higher compared to saline controls for the remainder of the experiment (Figure 4.3D). Haemoglobin was not affected by hypoxia (data not shown). Blood gases and pHa did not change over time in control lambs (Figure 4.3 A-D).

Repeated hypoxia challenges also elicited changes in blood gas parameters and pHa compared to pre-treatment and control values (Figure 4.3 E-H). The changes observed were not different between each episode and the degrees of change were not different to those observed for single hypoxia experiments. Arterial pH levels were also higher in hypoxic lambs compared to controls during the pre-treatment period, but the difference did not persist for the duration of the experiment as observed in the single hypoxia experiments (Figure 4.3H). Again, there was no observed change in blood gas parameters, pHa (Figure 4.3 E-H) or Hb (data not shown) in control lambs.

4.2.3 Effect of hypoxia on plasma glucose and lactate concentrations

Control and hypoxia treatments had no effect on plasma glucose concentrations over time (data not shown). There was no change in plasma lactate concentrations in control lambs, whereas hypoxia treatment induced large changes in this parameter (Figure 4.4). A single bout of hypoxia elicited increases in lactate concentrations within 30 minutes of the challenge and levels remained elevated until 6 hours post-saline administration (Figure 4.4A). Peak responses were observed 30 minutes following the completion of the hypoxia challenge with concentrations increasing from 0.90 ± 0.13 mmol/L at time (0) to 5.33 ± 0.27 mmol/L (an increase of ~83 %).

Repeated hypoxia challenges increased lactate concentrations within 30 minutes of the onset of each episode. The degree of change was not different between the two episodes, despite the apparently smaller change during the second episode (Figure 4.4B). Basal concentrations were not significantly different between single and repeated hypoxia-treated groups, however the relative increase was lower for repeated hypoxia treated-lambs (~67 %) than observed for the single hypoxia-treated lambs (~83 %). Plasma lactate concentrations were unchanged in control lambs (Figure 4.4).

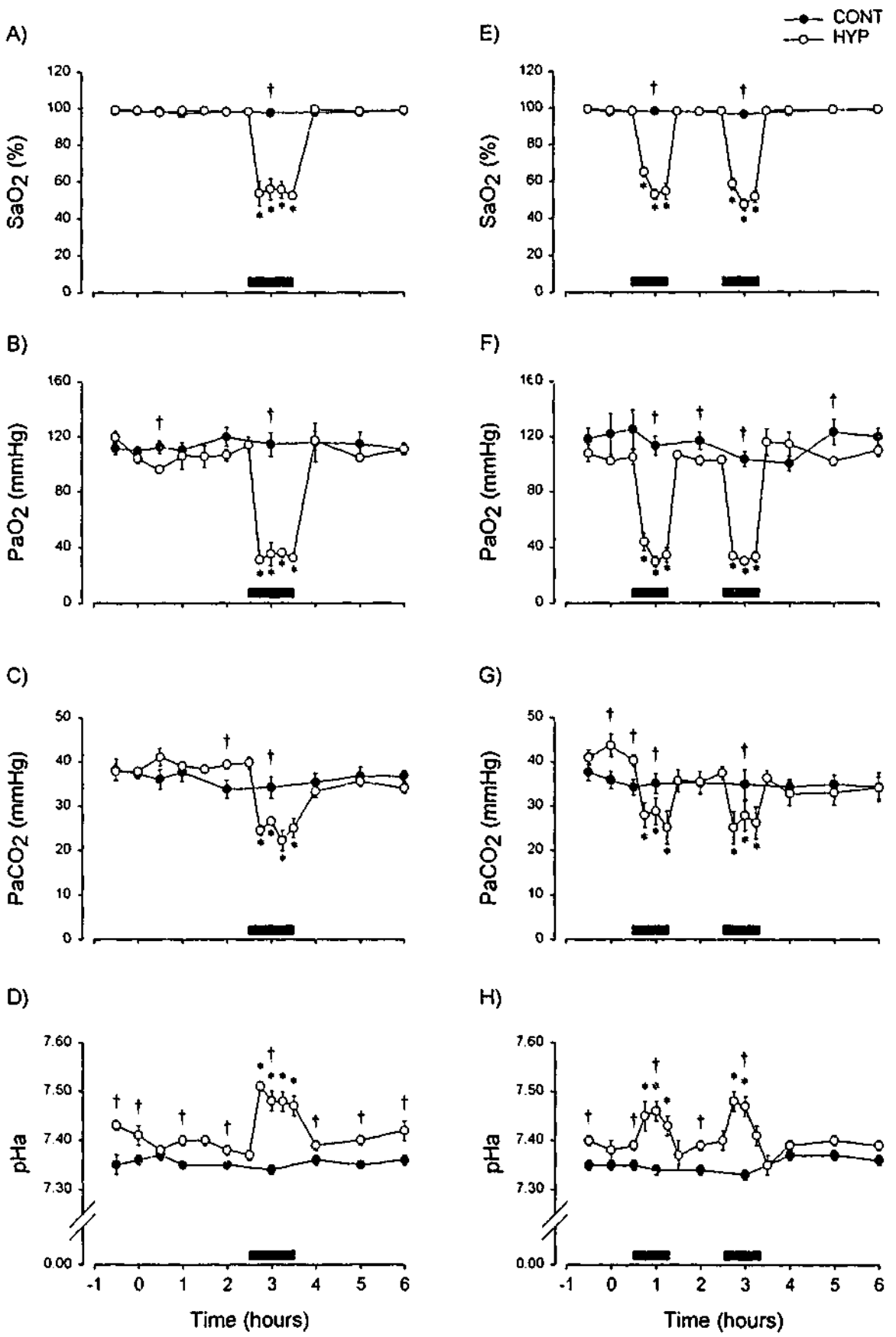


Figure 4.3: Effect of hypoxia on blood gases and pHa

Effect of control (n=5) or single hypoxia treatment (n=4) on SaO_2 (A), PaO_2 (B), $PaCO_2$ (C) and pHa (D). Effect of control (n=5) or repeated hypoxia treatment (n=6) on on SaO_2 (E), PaO_2 (F), $PaCO_2$ (G) and pHa (H). * P<0.05 for hypoxic lambs compared to time (0). † P<0.05 between groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

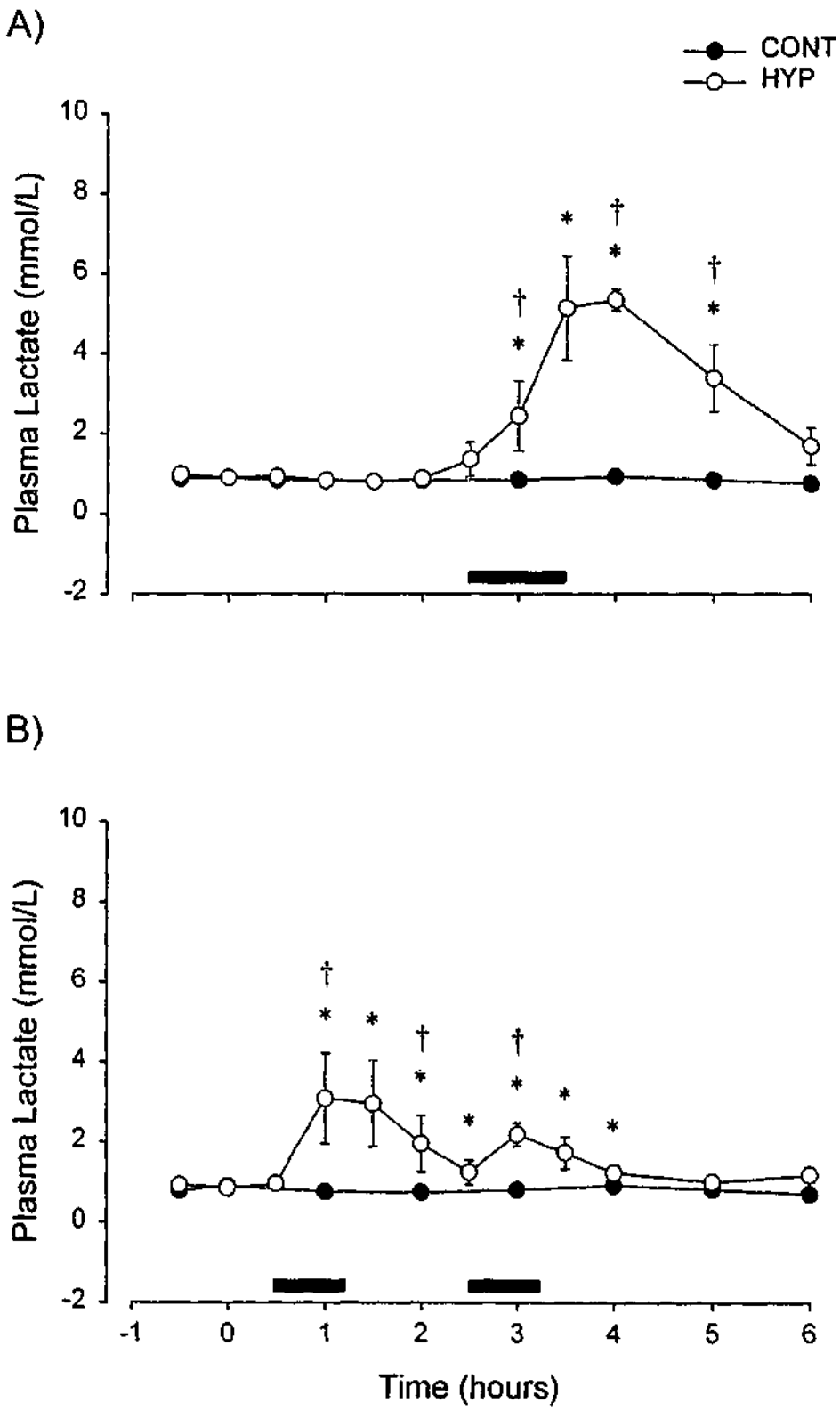


Figure 4.4: Effect of hypoxia on plasma lactate concentrations

Effect of control (n=5) or single hypoxia treatment (n=4) on lactate concentrations (A). Effect of control (n=5) or repeated hypoxia treatment (n=6) on lactate concentrations (B). * $P<0.05$ for hypoxic lambs compared to time (0). † $P<0.05$ between groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

4.2.4 Effect of hypoxia on cardiorespiratory parameters

Heart rate increased significantly after hypoxia challenge (Figure 4.5 A&D). Lambs challenged with a single episode of hypoxia experienced increases in heart rate that were observed within 30 minutes of the challenge (Figure 4.5A). This acute increase was also followed by a sustained elevation in heart rate that persisted after the hypoxia challenge was concluded. Repeated episodes of hypoxia also elicited increases in heart rate that were not different to the single challenge (Figure 4.5D). However, these increases were not observed until after the challenge had been removed. The degree of change in heart rate was similar between the hypoxia episodes and, by 6 hours post-saline administration, heart rate levels were back to control levels in these lambs. Blood pressure was not affected by hypoxia treatment over the course of the experiment. Note that normoxic lambs had significantly lower blood pressures than lambs subjected to hypoxia treatment (Figure 4.5 B&E). Reasons for this are unknown. Respiratory rates remained unchanged in control lambs for the duration of the experiment (Figure 4.5 C&F). In hypoxia-treated lambs, there was a tendency for respiration to increase following the hypoxia challenge, however, this was not found to be significant (Figure 4.5 C&F).

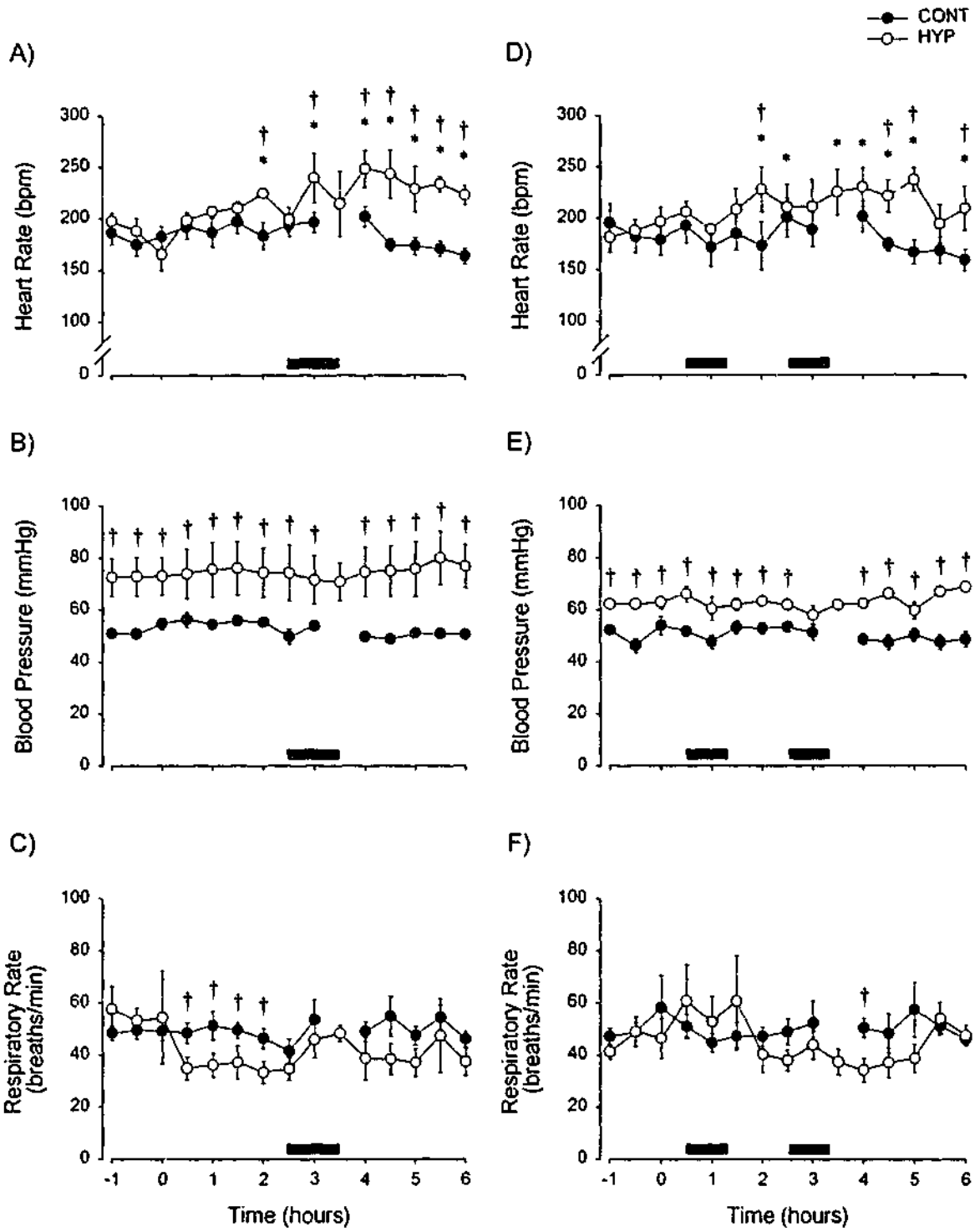


Figure 4.5: Effect of hypoxia on cardiorespiratory parameters

Effect of control (n=5) or single hypoxia treatment (n=4) on heart rate (A), blood pressure (B) and respiratory rate (C). Effect of control (n=5) or repeated hypoxia treatment (n=6) on heart rate (D), blood pressure (E) and respiratory rate (F). * P<0.05 for hypoxic lambs compared to time (0). † P<0.05 between groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

4.2.5 Effect of hypoxia on somnogenic behaviour

Physiological parameters were monitored over 7 hours, including the pre-treatment period. However, control lambs were unable to remain in the chamber for this length of time, as explained previously in Chapter 3. These lambs were returned to their mothers for approximately 15-20 minutes. Lambs treated with hypoxia, whether as a single episode or repeated episodes, remained asleep or were drowsy for the entire period of recording, allowing them to remain in the chamber for the duration of the experiment.

4.2.5.1 Effect of hypoxia on somnogenic behaviour using intermittent epochs

To gain an understanding of the immediate short-term effects of hypoxia, analysis of data using intermittent epoch lengths was used. These results are presented in Figures 4.6 and 4.7. Control treatment had no effect on wakefulness and, no effect on any of the sleep states (Figure 4.6A; *black bars*). In contrast, a single challenge of hypoxia reduced the incidence of wakefulness (Figure 4.6A; *hatched bars*) and increased the incidence of quiet sleep significantly (Figure 4.6B; *hatched bars*). However, no further increase was observed for the incidence of wakefulness (Figure 4.6A; *hatched bars*) or quiet sleep (Figure 4.6B; *hatched bars*) during the actual hypoxia episode. A single episode of hypoxia had no effect on the incidence of either active sleep or indeterminate sleep during this period (Figure 4.6 C&D; *hatched bars*). The reduction in wakefulness was observed from the first hour post-saline administration, (i.e. before the hypoxia challenge) and, therefore, may be a consequence of the initial high levels of wakefulness in these hypoxic lambs (Figure 4.6A; *hatched bars*).

The incidence of wakefulness in lambs treated with repeated episodes of hypoxia was reduced significantly within the first hour post-saline administration (Figure 4.7A; *hatched bars*), as observed in single hypoxia experiments. However, this reduction was amplified during the second challenge. Similar results were also obtained for the incidences of quiet sleep (Figure 4.7B; *hatched bars*) and active sleep (Figure 4.7C; *hatched bars*), with levels increasing further during the second challenge. The incidence of indeterminate sleep also increased, however, this was significant only for the first hypoxia challenge and the following 45 minutes (Figure 4.7D; *hatched bars*). The initial effects on wakefulness, quiet sleep and active sleep also occurred within the first 30 minutes post-saline administration, the period before commencement of hypoxia treatment, hence, this effect

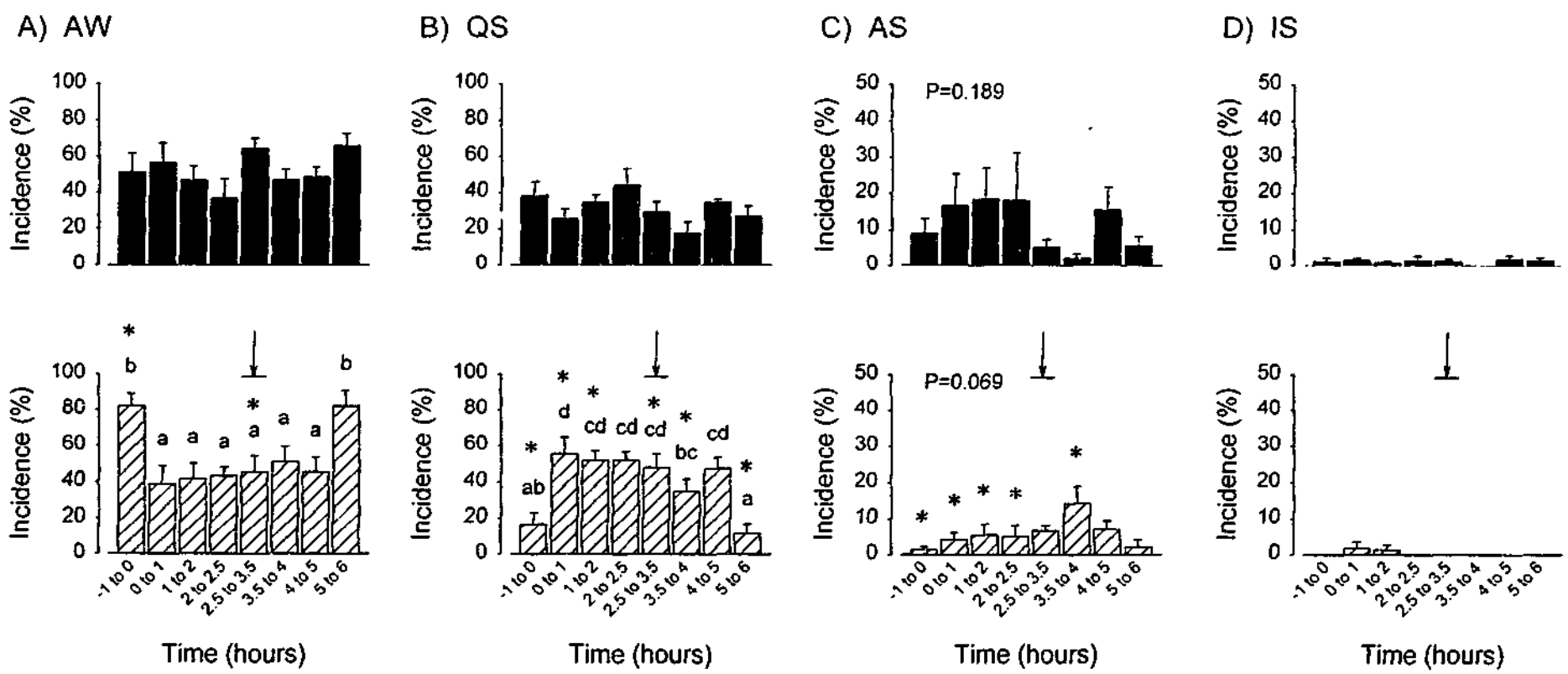
is most likely to be due to the very high pre-treatment levels of wakefulness compared to controls.

4.2.5.2 Overall effects of hypoxia on somnogenic behaviour

Additional analysis was conducted to determine the effects of hypoxia over different periods of time. Analysis of the first three hours post-saline treatment was conducted to incorporate the period of time when the saline-treated lambs were in the sleep chamber, before being returned to their mothers. Analysis of the period 0.5-3.5 hours post-saline administration was conducted to include the full duration of the single or repeated hypoxia episode(s) and to exclude the possible bias effect of the initially high incidence of wakefulness.

Figure 4.8 shows the effects of a single hypoxia challenge on behaviour. No significant effect of hypoxia was observed on the incidence of wakefulness. However, there was a significant ($P<0.05$) increase in the incidence of quiet sleep for all three analyses (Figure 4.8). The increase in quiet sleep, without a concomitant decrease in wakefulness, may be explained by a decreased incidence of active sleep, however, this reduction was not significant.

Repeated episodes of hypoxia decreased the incidence of wakefulness and increased the incidence of quiet sleep for all analyses (Figure 4.9). The degree of reduction in wakefulness was greatest during the period 0.5-3.5 hours post-saline administration (~41 %), followed by 0-3 hours (~32 %) and then 0-6 hours (~20 %). A similar pattern was found for quiet sleep. No effects of repeated hypoxia were observed on the incidences of active sleep or indeterminate sleep (Figure 4.9).



Amended Figure 4.6: Single hypoxia in intermittent epochs

Effect of control (*closed bars*; *n*=5) or single hypoxia treatment (*hatched bars*; *n*=4) on the incidence of wakefulness (A), quiet sleep (B), active sleep (C) and indeterminate sleep (D). Note the reduced scale of the y-axis in C and D. Letters indicate significant ($P<0.05$) differences across epochs within each treatment; * indicates significant ($P<0.05$) differences between groups. Data are expressed as mean ± S.E.M. *Arrows* represent periods of hypoxia.

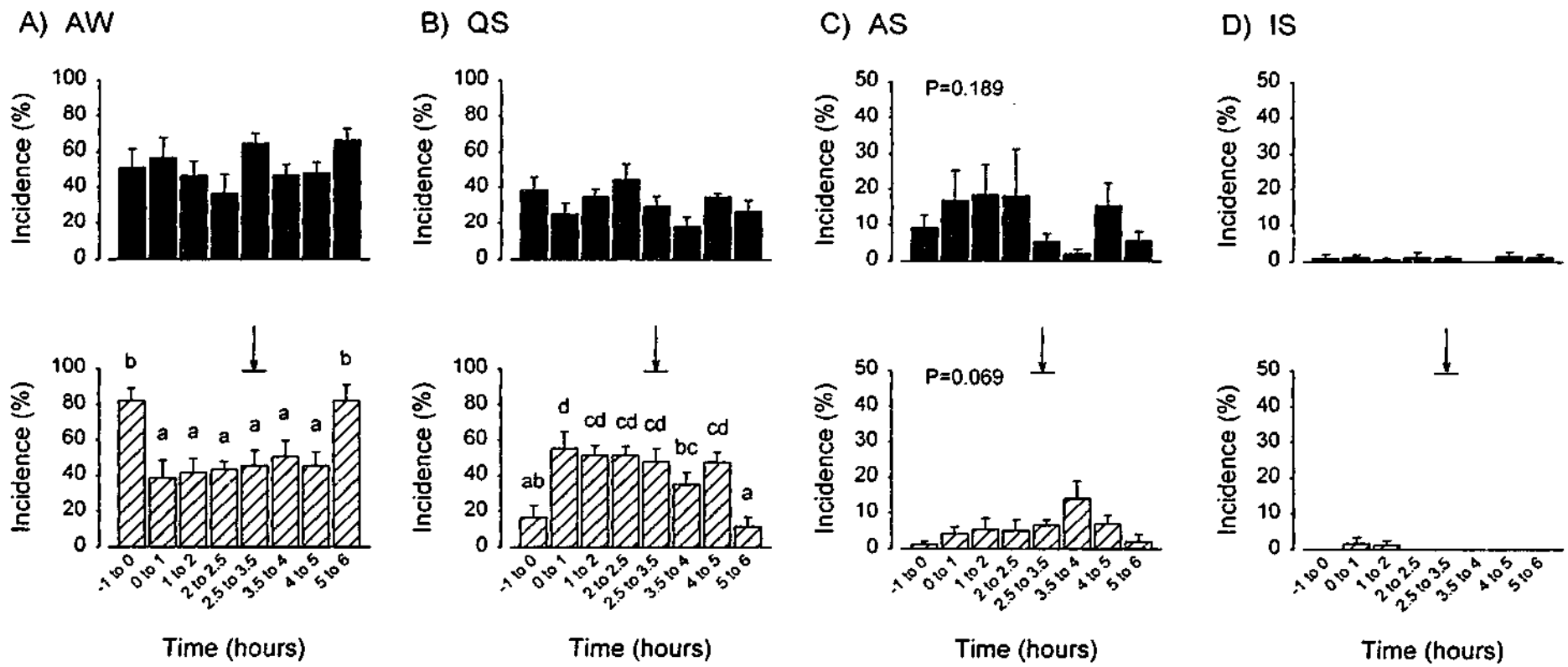
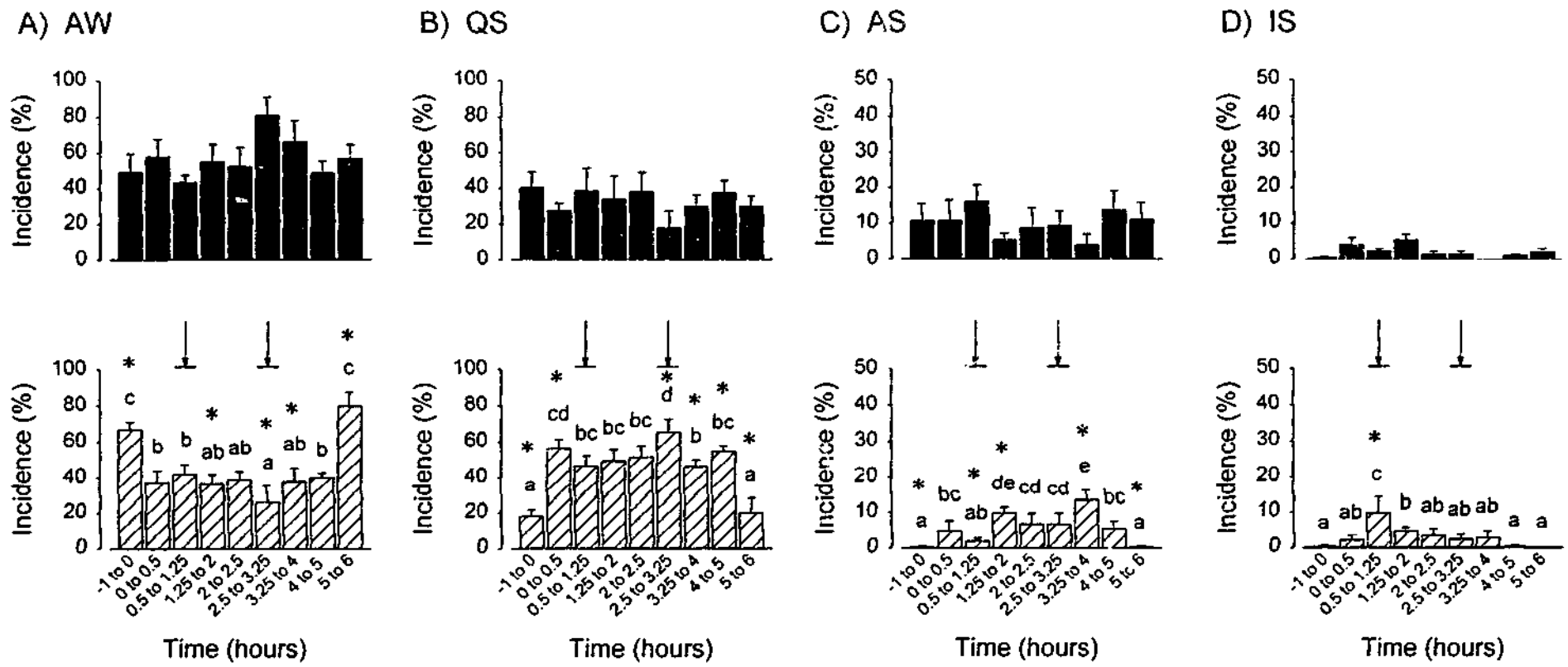


Figure 4.6: Single hypoxia in intermittent epochs

Effect of control (*closed bars*; *n*=5) or single hypoxia treatment (*hatched bars*; *n*=4) on the incidence of wakefulness (A), quiet sleep (B), active sleep (C) and indeterminate sleep (D). Note the reduced scale of the y-axis in C and D. Letters indicate significant ($P<0.05$) differences across epochs within each treatment. Data are expressed as mean ± S.E.M. *Arrows* represent periods of hypoxia.



Amended Figure 4.7: Repeated hypoxia in intermittent epochs

Effect of control (closed bars; n=5) or repeated hypoxia treatment (hatched bars; n=6) on the incidence of wakefulness (A), quiet sleep (B), active sleep (C) and indeterminate sleep (D). Note the reduced scale of the y-axis in C and D. Letters indicate significant ($P<0.05$) differences across epochs within each treatment; * indicates significant ($P<0.05$) differences between groups. Data are expressed as mean ± S.E.M. Arrows represent periods of hypoxia.

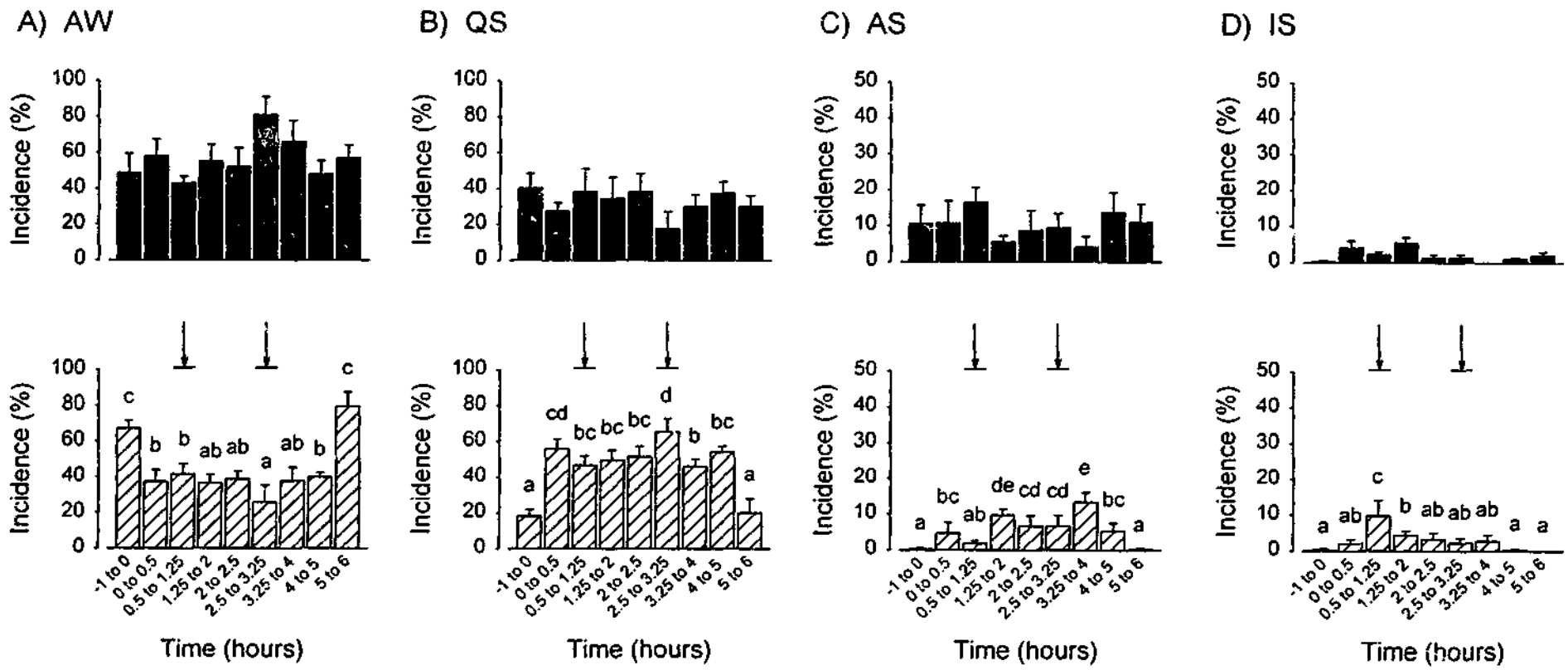


Figure 4.7: Repeated hypoxia in intermittent epochs

Effect of control (*closed bars*; *n*=5) or repeated hypoxia treatment (*hatched bars*; *n*=6) on the incidence of wakefulness (A), quiet sleep (B), active sleep (C) and indeterminate sleep (D). Note the reduced scale of the y-axis in C and D. Letters indicate significant ($P<0.05$) differences across epochs within each treatment. Data are expressed as mean ± S.E.M. *Arrows* represent periods of hypoxia.

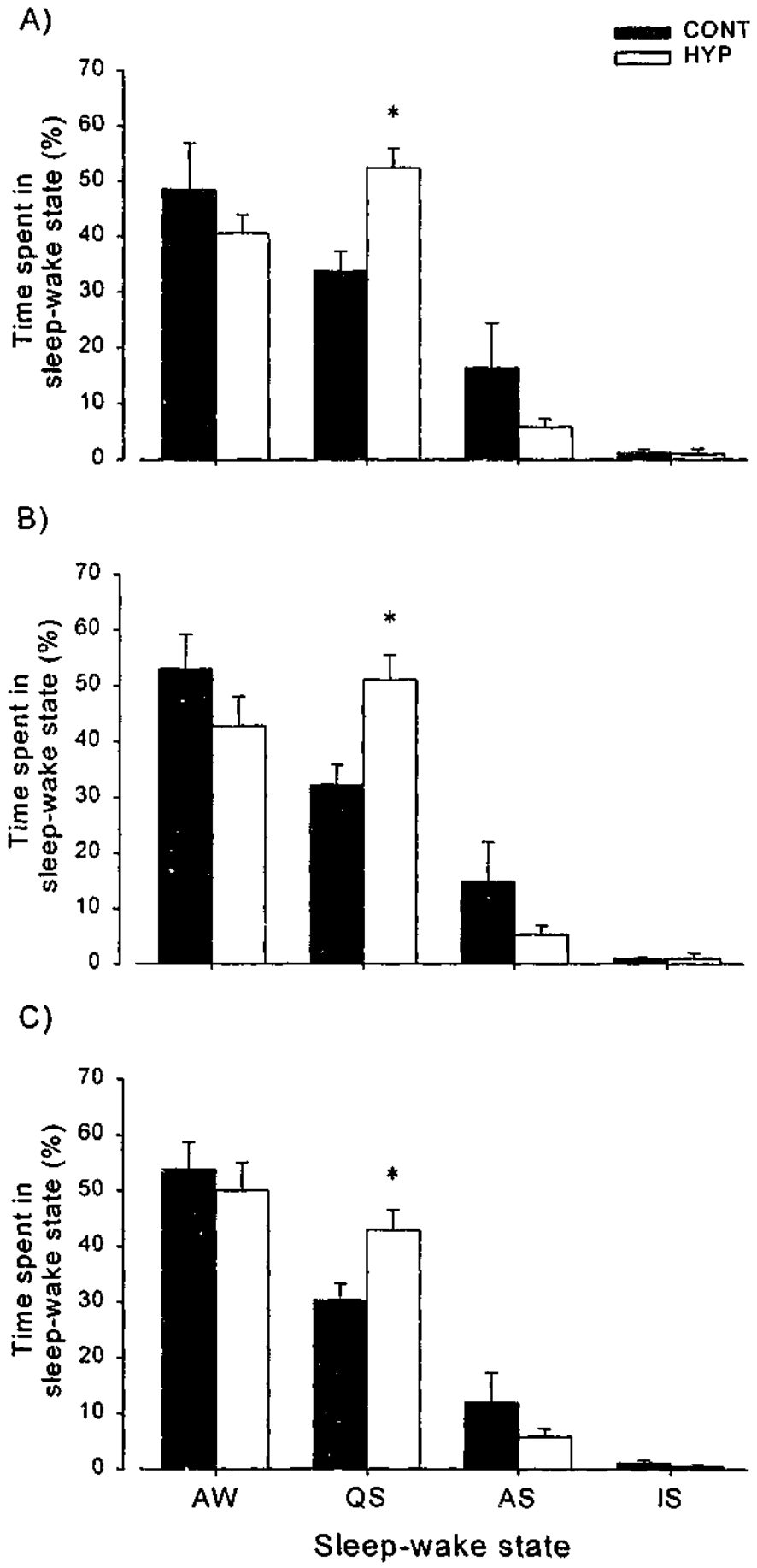


Figure 4.8: Effect of single hypoxia challenge on somnogenic behaviour

Effect of control (n=5) or single hypoxia treatment (n=4) on somnogenic behaviour for the periods 0-3 hours (A), 0.5-3.5 hours (B) and 0-6 hours post-saline administration. * P<0.05 between groups. Data are expressed as mean ± S.E.M.

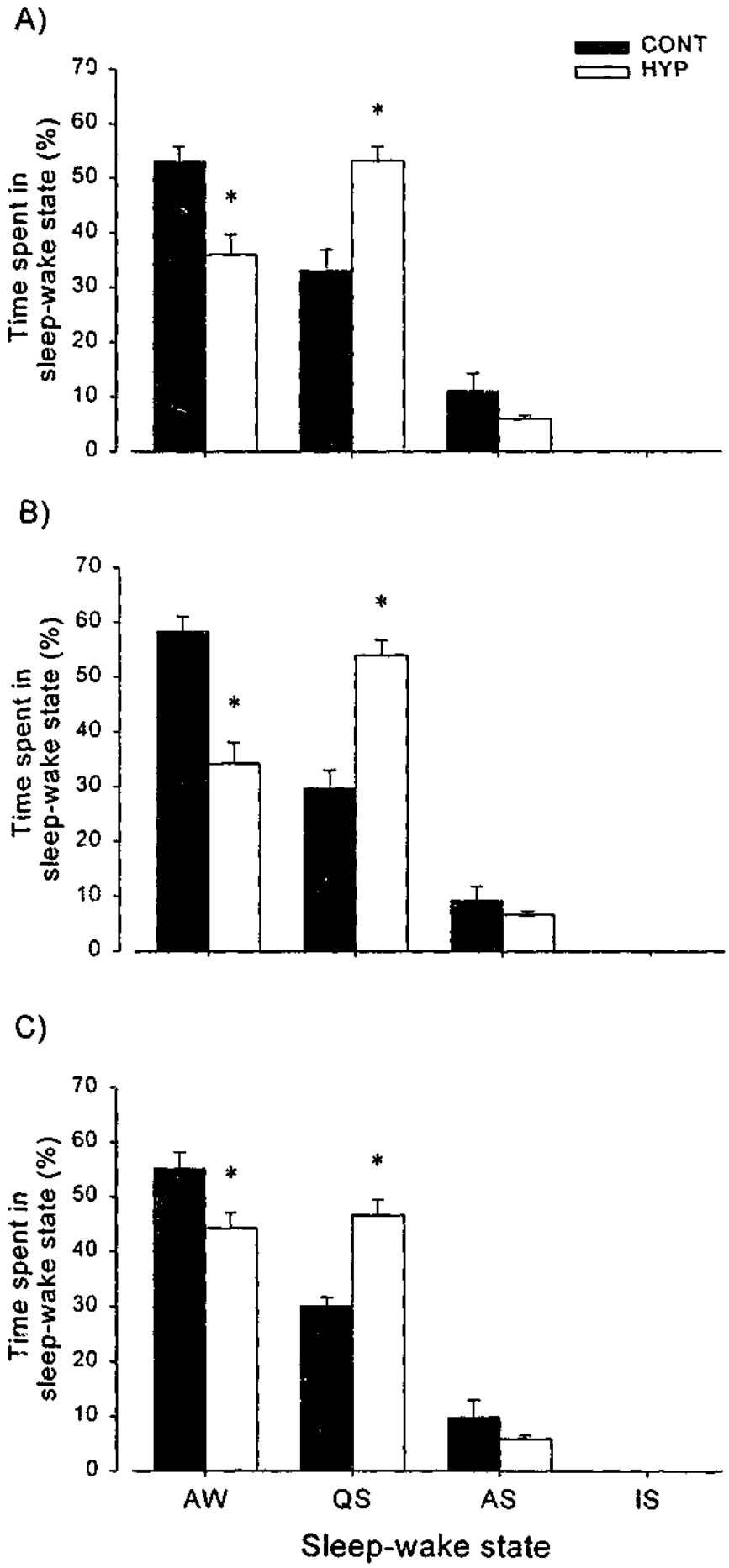


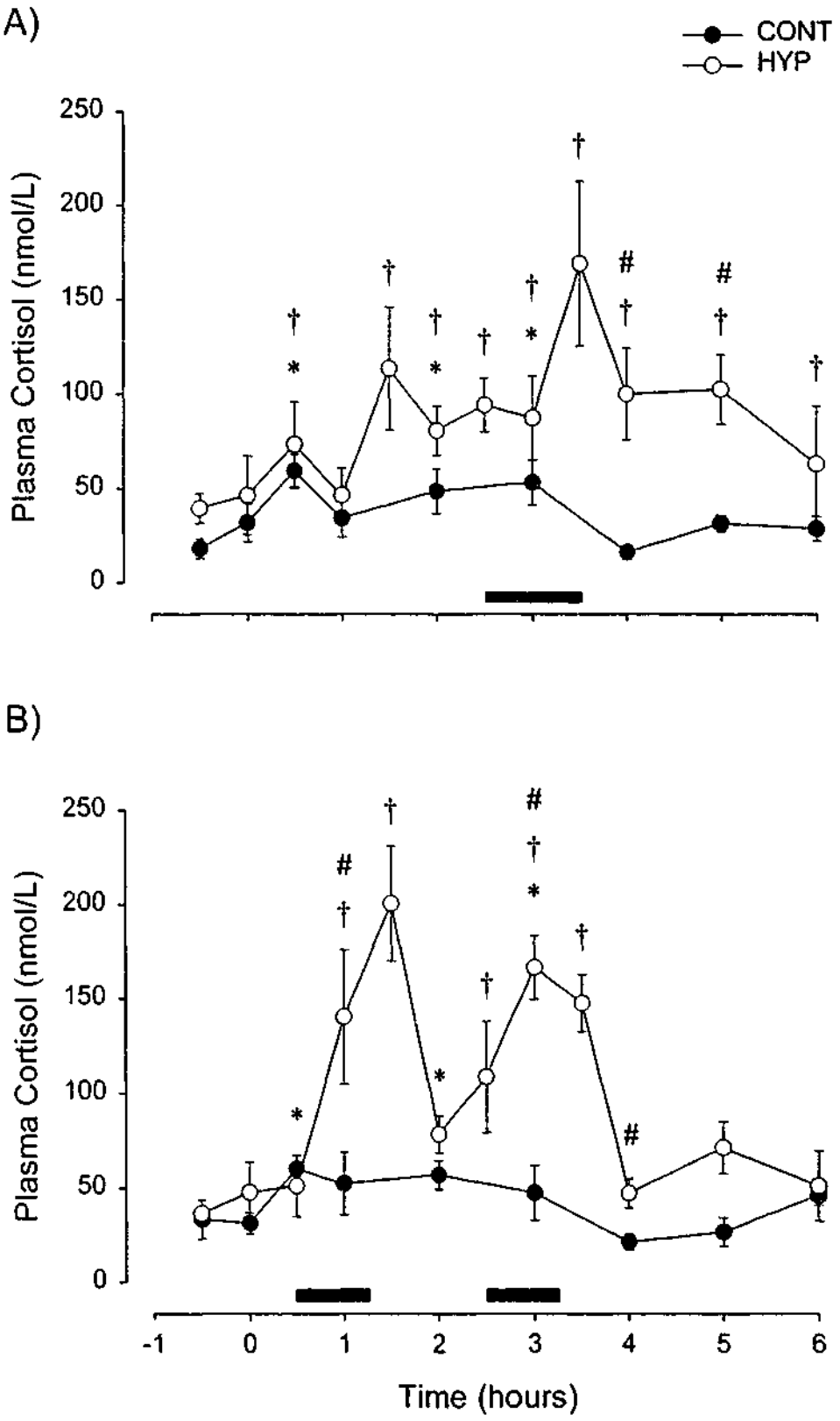
Figure 4.9: Effect of repeated hypoxia on somnogenic behaviour

Effect of control (n=5) or repeated hypoxia treatment (n=4) on somnogenic behaviour for the periods 0-3 hours (A), 0.5-3.5 hours (B) and 0-6 hours post-saline administration. * P<0.05 between groups. Data are expressed as mean ± S.E.M.

4.2.6 Effect of hypoxia on plasma cortisol concentrations

Plasma cortisol concentrations fluctuated over time in saline controls, but returned to pre-treatment values by the end of the experiment (Figure 4.10A). Lambs treated with a single episode of hypoxia experienced an increase in cortisol concentration, compared to pre-treatment levels, within 30 minutes of saline administration (Figure 4.10A). The increase in plasma cortisol that occurred before the hypoxia challenge may be due to diurnal variations as observed with saline controls. However, a further increase was also found towards the end of the hypoxia period in these lambs. The peak plasma cortisol concentration was approximately 4-fold greater compared to time (0) and concentrations remained elevated for the duration of the experiment.

Lambs treated with repeated hypoxia displayed elevated cortisol concentrations during each hypoxia episode ($P<0.05$; Figure 4.10B). During the first period of hypoxia, cortisol concentrations increased from 33.92 ± 5.12 nmol/L to 200.58 ± 30.41 nmol/L and, during the second hypoxia episode, concentrations increased to 166.68 ± 16.88 nmol/L. The degree of increase was similar between hypoxia episodes and was similar to that found with a single hypoxia challenge. Elevated cortisol concentrations during hypoxia returned to pre-treatment values between the two episodes and within 45 minutes of the second hypoxia challenge (Figure 4.10B). Fluctuations in plasma cortisol concentrations similar to those described previously were observed for the control lambs used in these experiments (Figure 4.10B).



Amended Figure 4.10: Effect of hypoxia on plasma cortisol concentrations

Effect of control (n=5) or single hypoxia treatment (n=4) on plasma cortisol concentrations (A). Effect of control (n=5) or repeated hypoxia treatment (n=6) on plasma cortisol concentrations (B). * P<0.05 for control lambs compared to time (0); † P<0.05 for hypoxic lambs compared to time (0); # P<0.05 between groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

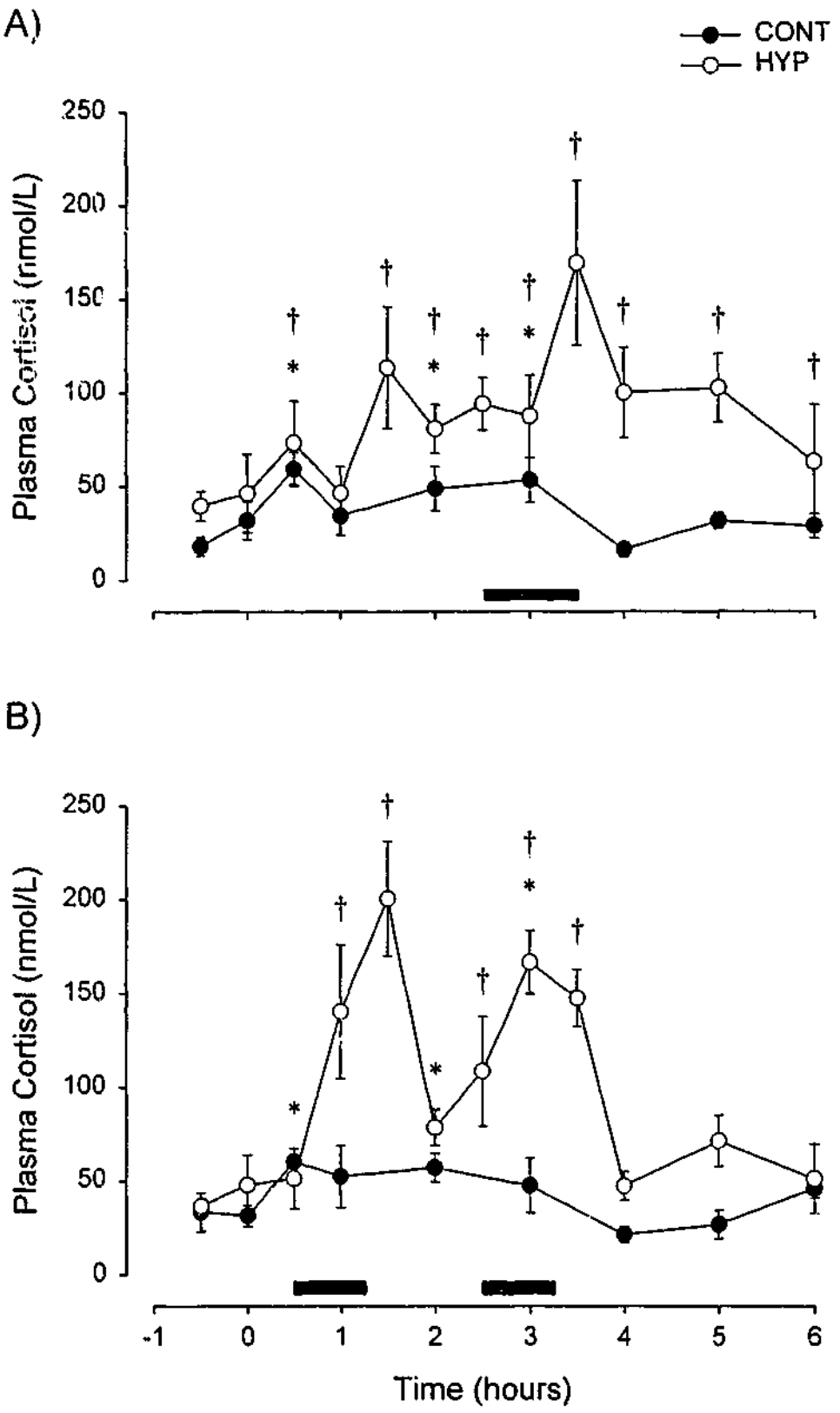


Figure 4.10: Effect of hypoxia on plasma cortisol concentrations

Effect of control (n=5) or single hypoxia treatment (n=4) on plasma cortisol concentrations (A). Effect of control (n=5) or repeated hypoxia treatment (n=6) on plasma cortisol concentrations (B). * P<0.05 for control lambs compared to time (0); † P<0.05 for hypoxic lambs compared to time (0). Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

4.2.7 Effect of hypoxia on plasma neurosteroid concentrations

Pregnenolone and progesterone concentrations in plasma samples from control lambs remained below the level of detection of the assay for all time points examined. Very low concentrations (~0.01 nmol/L) of plasma pregnenolone were detected occasionally in lambs that were treated with a single or repeated hypoxia challenge(s), however, the data were inconsistent. Plasma concentrations of progesterone, up to a maximum of 0.02 nmol/L, were also detected periodically in hypoxia-treated lambs.

Plasma allopregnanolone concentrations did not change in control lambs (Figure 4.11A). In contrast, lambs that were treated with a single episode of hypoxia exhibited a small but significant increase in plasma allopregnanolone concentration that was sustained for the first 4 hours post-saline administration (Figure 4.11A). This increase was observed within the first hour of the experimental period, before hypoxia had been introduced. The reason(s) for the increase in plasma allopregnanolone concentration prior to hypoxia exposure remain(s) unclear. However, increases in plasma cortisol that were also observed for this time (see Figure 4.10) suggest that increased adrenal activation may have occurred at this time.

No differences in pre-treatment concentrations of allopregnanolone were found between control and repeated hypoxia-treated lambs (Figure 4.11B). In addition, no changes in plasma allopregnanolone concentrations were observed as a result of the repeated hypoxia challenges. Although there were significant differences between the two groups at 3 and 6 hours post-saline administration, no overall effect on plasma allopregnanolone concentrations were observed for either group. Finally, basal concentrations of allopregnanolone were significantly higher for lambs used in the repeated hypoxia studies than those in the single hypoxia studies (Figure 4.11). Lambs used in repeated hypoxia studies were older (~15 days of age) than lambs used in the single hypoxia study (~12 days of age), supporting data reported in Chapter 3, which showed that plasma allopregnanolone concentrations decrease with increasing age.

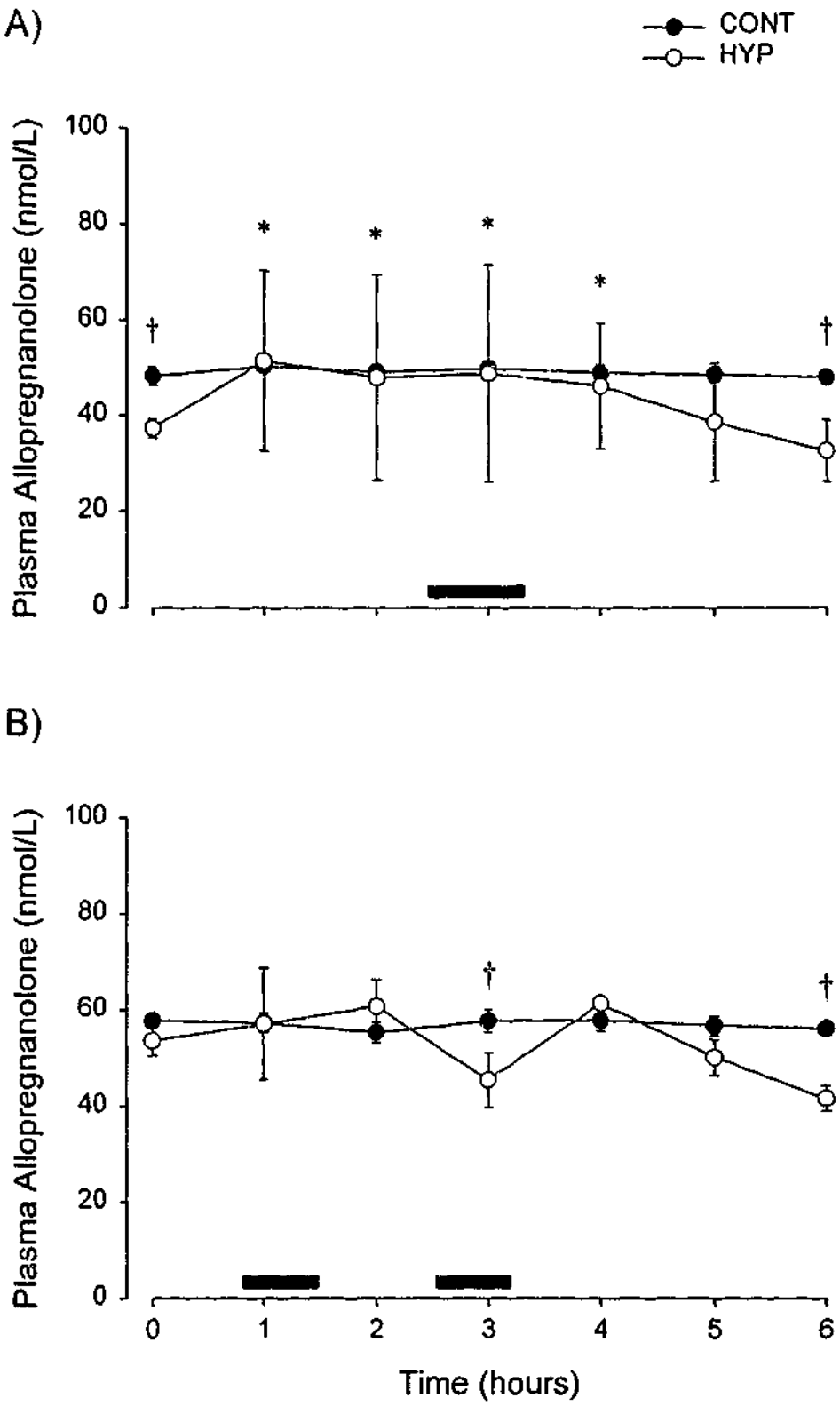


Figure 4.11: Effect of hypoxia on plasma allopregnanolone concentrations

Effect of control (n=5) or single hypoxia treatment (n=4) on plasma allopregnanolone concentrations (A). Effect of control (n=5) or repeated hypoxia treatment (n=4) on plasma allopregnanolone concentrations (B). * P<0.05 for hypoxic lambs compared to time (0); † P<0.05 between groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

4.2.8 Effect of hypoxia on physiological parameters, prior to tissue collection

In these experiments, where brain tissue was collected, a decision to use repeated hypoxia rather than a single challenge was made based on the data collected in the experiments performed at 12 and 15 days of age. From these data, it was apparent that most responses to repeated hypoxia were not attenuated, nor were they amplified, when compared to a single episode of hypoxia. In addition, since the analysis of somnogenic behaviour is important for this thesis, the variability and lack of clarity of results obtained from lambs treated with a single episode of hypoxia meant that, for this last experiment, data from the repeated hypoxia challenge was used. Tables Table 4.1 to Table 4.4 contain data obtained from these lambs at time (0), before treatment had commenced, and at 3 hours post-saline treatment or during the second hypoxia episode for control and experimental lambs, respectively.

At the time immediately prior to tissue collection, lambs treated with hypoxia were hypocapnic, hypoxaemic, hypocarbic and hyperglycaemic. They also exhibited hyperlactaemia, tachycardia and elevated plasma cortisol and allopregnanolone concentrations. The magnitude of change in the parameters measured were not different to those at the equivalent time in the previous experiments, except for pHa, which did not increase in this experiment, and plasma allopregnanolone, which did not increase in response to hypoxia in the previous experiment (Figure 4.11B).

Table 4.1: Effect of hypoxia on temperature and plasma iron concentrations, prior to tissue collection

		Time (hours)	
Parameter	***Treatment***	***0***	***3***
Temperature (°C)	CONT	39.00 ± 0.11	39.06 ± 0.07
	HYP	39.55 ± 0.11	39.63 ± 0.10
Iron (μmol/L)	CONT	11.65 ± 2.99	10.51 ± 2.79
	HYP	9.38 ± 1.83	10.59 ± 1.30

Effect of control (n=5) or repeated hypoxia treatment (n=6) on rectal temperature and plasma iron concentrations. Data are expressed as mean ± S.E.M for time (0) and 3 hours post-saline administration, just prior to tissue collection. There was no change in rectal temperature or plasma iron concentrations for either treatment group.

Table 4.2: Effect of hypoxia on blood gases, pHa, Hb, and plasma glucose and lactate concentrations prior to tissue collection

		Time (hours)	
Parameter	*Treatment*	*0*	*3*
SaO_2 (%)	CONT	98.38 ± 2.41	97.80 ± 1.86
	HYP	99.80 ± 0.20	52.40 ± 4.06*†
PaO_2 (mmHg)	CONT	108.80 ± 11.73	108.40 ± 11.57
	HYP	116.20 ± 5.64	32.20 ± 3.88*†
$PaCO_2$ (mmHg)	CONT	37.86 ± 0.91	36.29 ± 2.11
	HYP	38.42 ± 2.13	26.86 ± 2.47*†
pHa	CONT	7.36 ± 0.01	7.35 ± 0.01
	HYP	7.43 ± 0.02	7.49 ± 0.02
Hb (g/dL)	CONT	7.34 ± 1.04	7.56 ± 0.93
	HYP	6.08 ± 0.67	6.90 ± 0.63*
Glucose (mmol/L)	CONT	6.46 ± 0.30	6.30 ± 0.18
	HYP	7.08 ± 0.27	8.73 ± 1.69*†
Lactate (mmol/L)	CONT	0.92 ± 0.09	0.80 ± 0.08
	HYP	0.58 ± 0.19*	1.75 ± 0.70*†

Effect of control (n=5) or repeated hypoxia treatment (n=6) on blood gases, pHa, Hb and plasma glucose and lactate concentrations. * P<0.05 between treatment groups; † P<0.05 within each treatment as compared to its own time (0). Data are expressed as mean ± S.E.M for time (0) and 3 hours post-saline administration, just prior to tissue collection. Control treatment had no effect on the parameters measured. Hypoxia resulted in hypocapnia, hypoxaemia, hyperglycaemia, and elevated Hb and lactate levels.

Table 4.3: Effect of hypoxia on cardiorespiratory parameters, prior to tissue collection

		Time (hours)	
Parameter	***Treatment***	***0***	***3***
Heart rate (bpm)	CONT	173.80 ± 14.33	184.00 ± 8.35
	HYP	164.50 ± 9.09	234.94 ± 16.60*†
Blood pressure (mmHg)	CONT	54.98 ± 1.56	54.53 ± 1.28
	HYP	63.85 ± 3.03*	63.27 ± 4.20*
Respiratory rate (breaths/min)	CONT	45.00 ± 3.05	44.20 ± 5.26
	HYP	46.89 ± 11.78	49.57 ± 5.17

Effect of control (n=5) or repeated hypoxia treatment (n=6) on cardiorespiratory parameters. * P<0.05 between treatment groups; † P<0.05 within each treatment as compared to its own time (0). Data are expressed as mean ± S.E.M for time (0) and 3 hours post-saline administration, just prior to tissue collection. Control treatment had no effect on the parameters measured. Hypoxia increased heart rate significantly. Basal blood pressure was elevated in hypoxic lambs compared to controls.

Table 4.4: Effect of hypoxia on plasma steroid concentrations, prior to tissue collection

		Time (hours)	
Parameter	***Treatment***	***0***	***3***
Cortisol (nmol/L)	CONT	18.09 ± 5.67	26.93 ± 6.28
	HYP	48.28 ± 6.30*	165.06 ± 12.06*†
Allopregnanolone (nmol/L)	CONT	39.32 ± 2.32	40.37 ± 3.49
	HYP	36.14 ± 4.78	46.45 ±3.47*†

Effect of control (n=5) or repeated hypoxia treatment (n=6) on plasma cortisol and allopregnanolone concentrations at time (0) and 3 hour post-administration, prior to tissue collection. * P<0.05 between treatment groups as compared to saline only; † P<0.05 within each treatment as compared to its own time (0). Data are expressed as mean ± S.E.M. Hypoxia treatment resulted in increases of plasma cortisol and allopregnanolone, whereas control treatment had no effect.

4.2.9 Effect of hypoxia on brain allopregnanolone concentrations

Allopregnanolone was detected in all brain regions of all lambs studied (Figure 4.12). In control lambs, concentrations ranged from 15 to 40 pmol/g, whereas pregnenolone and progesterone remained below the level of detection of the assay. Hypoxia treatment increased allopregnanolone concentration significantly in all brain regions except the thalamus/hypothalamus (diencephalon), hippocampus and cerebellum (Figure 4.12). The degree of increase ranged from 27 % in the primary motor cortex (from 34.40 ± 1.79 pmol/g to 47.20 ± 4.85 pmol/g) to 72 % in the midbrain (from 16.13 ± 0.45 pmol/g to 58.12 ± 3.14 pmol/g). Pregnenolone and progesterone concentrations were below the level of detection in the brain of lambs treated with hypoxia.

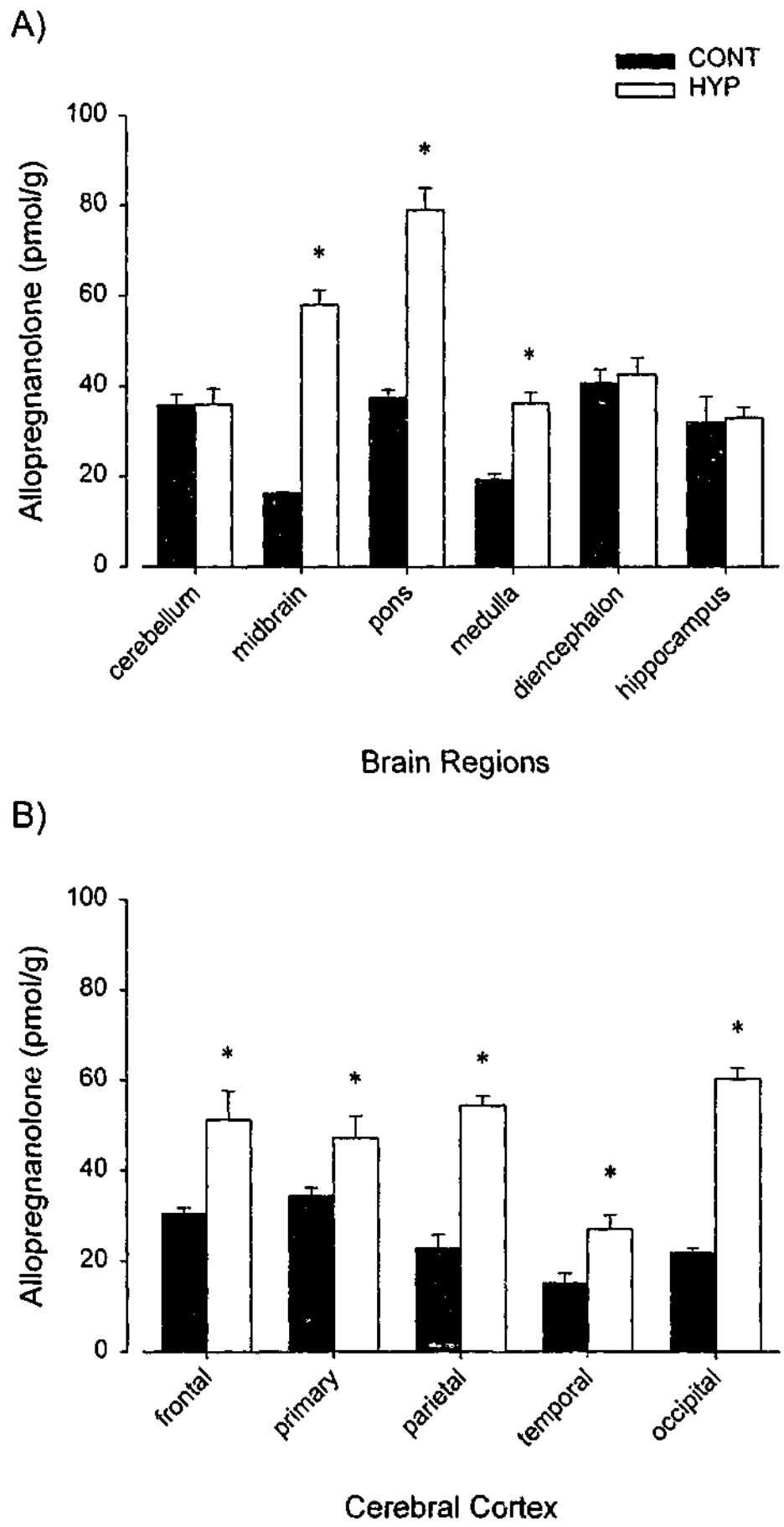


Figure 4.12: Effect of hypoxia on brain allopregnanolone concentrations

Allopregnanolone concentrations in the major regions of the brain (A) and in specific regions of the cerebral cortex (B) from control (n=4) or hypoxia-treated (n=4) lambs. * P<0.05 between groups. Data are expressed as mean ± S.E.M.

4.3 Discussion

The primary finding of this study was that hypoxia treatment increased the concentration of allopregnanolone significantly in the brain, but not in plasma. In addition, the increased brain allopregnanolone concentration was associated with an increased incidence of sleep and drowsiness and, subsequently, reduced wakefulness. These results are similar to those found in Chapter 3, where LPS was used as the stressful stimuli. They also support previous studies that have reported increases in neurosteroid concentrations following exposure to other types of stressful stimuli in the adult rat (Barbaccia *et al.*, 1994; Barbaccia *et al.*, 1996; Khisti *et al.*, 2000). The somnogenic properties of neurosteroids suggest that the increased incidence of sleep observed in lambs exposed to hypoxia challenges may be attributable to these unique compounds. Therefore, neurosteroids, such as allopregnanolone, may play a crucial role in the aetiology of 'sleep' disorders, including SIDS.

4.3.1 Model of hypoxia

Induction of arterial hypoxemia with the use of the custom-made plastic hood ensured consistency within and between experiments. Placement of the hood over the lamb's upper torso 30 minutes prior to saline administration enabled lambs to become acclimatised to the setting and their surroundings. To ensure that this process had no effect on stress responses in the lamb, cortisol concentrations were measured both prior to and after hood placement. Results confirmed that placing the hood on the lamb's head had no effect on plasma cortisol concentrations (before 36.46 ± 3.14 nmol/L and after 33.92 ± 5.12 nmol/L), indicating that the hood itself did not stress the lambs significantly (Figure 4.10).

The hypoxia experiments used in this study were designed to ascertain which paradigm, single or repeated challenges of hypoxia, was more disruptive to behavioural patterns in the lamb. To determine the timing of administration of the hypoxia challenges, and to minimise animal use, a series of preliminary experiments was conducted. To be consistent with the protocol used in Chapter 3, the episode of hypoxia had to be administered during the period when brain collection would normally take place. Preliminary experiments found that a ½ hour period of hypoxia was sufficient to elicit changes in a number of physiological parameters, including brain allopregnanolone concentration. In addition,

these studies also found that an ~1 hour period between hypoxia challenges provided sufficient time for the majority of parameters to return to control values.

4.3.2 Temperature and plasma iron responses to hypoxia

The challenge of hypoxia elicits a number of compensatory mechanisms in mammals, including a reduction of body temperature (Almeida *et al.*, 1999). This reduces O_2 demand by shifting the O_2 dissociation curve to the left which, in turn, minimises the energy costs associated with 'normal' physiological responses to hypoxia, such as increased ventilation and cardiac output (Almeida *et al.*, 1999; Bicego *et al.*, 2002). In the studies performed for this chapter, lambs challenged with a single or repeated episode(s) of hypoxia showed a slight trend towards reduced temperatures, however, this change was not significant. These lambs were found to have significantly higher baseline temperatures than saline-treated lambs, which may have amplified any reductions observed. The moderate hypoxia experienced by these animals may not have been sufficient to induce the expected anapyrexia, however, the reasons for a lack of effect remain unclear.

4.3.3 Effect of hypoxia on blood and plasma parameters

The severity of hypoxia is described in terms of its effects on blood gas parameters and pHa. In this study, moderate hypoxia was induced for both single and repeated challenges, eliciting the desired reductions in O_2 saturation, PaO_2, and $PaCO_2$ (Figure 4.3). These reductions were similar between hypoxia-treated lambs and consistent between episodes in the repeated hypoxia experiments. The reduction in PaO_2 as a consequence of hypoxia was expected to have resulted in increased ventilation. Measurement of respiratory rate was variable and, although there appeared to be a slight increase in respiration following hypoxia, this was not significant (Figure 4.5 C&F). However, as there was no evidence of increased respiration, this indicates that an increase in gas exchange took place to compensate for the hypoxia. This is supported by an increased heart rate in lambs treated with hypoxia challenge, suggesting that cardiac output increased in these lambs. The decreased $PaCO_2$ was therefore a physiological response to the hypoxia. A consequence of the reduced $PaCO_2$, increased pHa, is consistent with the induction of respiratory alkalosis.

Glucose concentrations remained unchanged in lambs treated with hypoxia challenge(s), despite the 7 hour separation from the ewe. This may be due to increased neurosteroid

action at the $GABA_A$ receptor, which has been shown to suppress appetite (Engel & Grant, 2001). The concentration of peripheral allopregnanolone in lambs treated with hypoxia exhibited little variation (see Section 4.3.4) in contrast to central allopregnanolone concentrations which increased following the hypoxia challenge (see Section 4.3.5). This suggests that central, rather than peripheral, neurosteroid concentration influences appetite suppression in lambs.

Hypoxia resulted in hyperlactaemia in all lambs studied (Figure 4.4). The relative increase was greater for a single hypoxia episode (~83 %) than it was for either of the repeated episodes (~67 %). The greater effect of a single hypoxia episode on plasma lactate levels in these lambs may be a result of the longer duration of the single episode (1 hour) compared to the repeated episodes (45 minutes each), however, it is unclear whether this length of time is sufficient to induce such dramatic changes. Lactic acidaemia resulting from hypoxia has been reported previously (Parer, 1998; Bicego *et al.*, 2002), however, the increased lactate concentration is usually associated with a decreased arterial pH. In the present study, increased arterial pH concentrations were observed following both single and repeated hypoxia episodes, and the degree of pH increase was not different between the two types of hypoxia administration. The reasons for an increased pHa associated with an increased lactate concentration in the current study remain unclear.

4.3.4 Plasma steroid responses to hypoxia

In the previous chapter, it was found that plasma allopregnanolone concentrations increased following a bolus dose of LPS administration in lambs. This finding supports previous studies suggesting that neurosteroid production is stimulated in response to stressful stimuli (Purdy *et al.*, 1991; Barbaccia *et al.*, 1994). The increased release of neurosteroids in response to stressful stimuli such as LPS (see Chapter 3) or CO_2 inhalation (Purdy *et al.*, 1991) has been suggested as playing a role in neuroprotection, through modulation of the excessive responses that are common between the stressors (Engel & Grant, 2001). Thus, it is surprising that the moderate hypoxia used in the present study elicited little or no response in plasma allopregnanolone, despite the large changes in plasma cortisol concentrations observed in these lambs. It is widely accepted that hypoxia activates the HPA axis, which stimulates adrenal steroidogenesis sufficiently to increase cortisol secretion (Krugers *et al.*, 1995; Carmichael *et al.*, 1997). The present

findings indicate that adrenal activation in this situation does not result in increased release of precursors or allopregnanolone into the circulation.

The minimal change in plasma allopregnanolone concentration may result from a greater activity of the glucocorticoid pathway and the increased utilisation of precursors for glucocorticoid production. Alternatively, greater metabolism or degradation of allopregnanolone by peripheral tissues under hypoxic conditions may have occurred. The increased allopregnanolone concentration induced by hypoxia in some parts of the brain (see discussion in Section 4.4.5), without an increase in neurosteroid concentration in plasma, further indicates that the CNS response to hypoxia is not dependent on peripheral steroid precursors.

4.3.5 Brain allopregnanolone response to hypoxia

The increase in allopregnanolone concentration following exposure to hypoxia was significant in all regions of the brain examined except for the diencephalon, hippocampus and cerebellum, and, as with LPS treatment, the effects were different between regions (Figure 4.12). Allopregnanolone was also detected in varying concentrations in all regions of the brain examined from control lambs. This differential expression of allopregnanolone may be the result of variability between brain regions in the lamb in the level of P450scc and 5α-reductase type II enzyme expression, two enzymes crucial for the synthesis of allopregnanolone (Nguyen *et al.*, 2003). P450scc is expressed strongly in the hippocampus, thalamus/hypothalamus and cerebellum of the neonatal lamb brain and, to a lesser degree, in the medulla and primary motor cortex (Nguyen *et al.*, 2003). 5α-reductase type II expression is also expressed strongly in the hippocampus and, cerebellum and to a lesser degree, in the primary motor cortex (Nguyen *et al.*, 2003). These differences in enzyme expression and, presumably, steroidogenic activity between brain regions may relate to the cell types primarily expressing each enzyme. P450scc expression is found in astrocytes, oligodendrocytes and neurons (Zwain & Yen, 1999), with glial expression being greater than that of neurons (Jung-Testas *et al.*, 1989; Akwa *et al.*, 1991; Baulieu, 1991). 5α-reductase type II expression is present in neurons, oligodendrocytes and astrocytes (Celotti *et al.*, 1992; Lauber & Lichtensteiger, 1996) but, in contrast to P450scc expression, 5α-reductase type II is more highly expressed in neurons than in glial cells (Melcangi *et al.*, 1994).

Allopregnanolone has been reported to have neuroprotective effects (review Compagnone & Mellon, 2000; Frank & Sagratella, 2000). A study investigating the 'irreversible' neurotoxic effects of hypoxia on rat hippocampal slices *in vitro* reported that the effects could be reversed by pre-treating the cells with allopregnanolone (Frank & Sagratella, 2000). It is possible, therefore, that regions of the lamb brain that did not exhibit increases in allopregnanolone concentration after hypoxia challenge (the thalamus/hypothalamus, hippocampus and cerebellum) may have already had a higher level of neuroprotection due to the higher basal levels of allopregnanolone in these regions.

The relative increase in allopregnanolone concentration was, overall, greater for LPS treatment (see Section 3.2.3.2) than for repeated hypoxia, suggesting that the level of hypoxia used in the present study had a reduced inhibitory effect on $GABA_A$ receptor function in the lamb brain compared to LPS. In addition, the minimal change in plasma allopregnanolone after repeated exposures to hypoxia, despite increases in brain allopregnanolone concentrations, also suggests that central and peripheral neurosteroidogenesis is regulated differentially. The studies reported in this thesis thus far support the notion of *de novo* production of allopregnanolone in the lamb brain after exposure to stressful stimuli. The mechanisms by which central neurosteroid concentration changes occur are unclear although it does appear that, after hypoxia treatment, increased central allopregnanolone concentration is not regulated by factors that control adrenal steroid production.

4.3.6 Somnogenic responses to hypoxia

Hypoxia can have major implications for CNS activity in infants and adults, depending on severity. It can alter brain activity (Moss, 2000) and arousal responses (Vertes & Perry, 1993), and has been suggested as a factor contributing to SIDS deaths (see Ozawa & Takashima, 2002). In the current study, when analysing data in variable epoch lengths over the course of the experiment (Figure 4.6), a single episode of hypoxia reduced the incidence of wakefulness with concomitant increases in the incidences of quiet and active sleep. However, when examining the somnogenic effects of this treatment in blocks of time (i.e. between 0 and 3 hours etc.), the effect on wakefulness was abolished, despite the significant increase in quiet sleep (Figure 4.8). The increased quiet sleep was most likely offset by a tendency for active sleep to decrease during the same time periods and therefore ultimately not affecting wakefulness. Although similar results were observed for

repeated hypoxia exposures over the course of the experiment (with the addition of an increase in the incidence of indeterminate sleep) compared to a single exposure, the general effect was a significant decrease in the incidence of wakefulness along with a significant increase in the incidence of quiet sleep (Figure 4.9).

Overall, the incidence of sleep increased significantly in lambs that had been exposed to two bouts of hypoxia. To the best of my knowledge, this is the first study that has highlighted extensively the somnogenic effects of hypoxia in young lambs. Interestingly, the increased incidence of sleep coincided with increases in the sedative neurosteroid allopregnanolone in the brain. As mentioned previously, allopregnanolone administration has been reported to have potent somnogenic properties (Damianisch *et al.*, 2001). Therefore, allopregnanolone and other neurosteroids may be responsible for the increased sleep observed in these lambs following hypoxia challenge. It has been reported that arousal thresholds in human infants are higher during periods of quiet sleep (Read *et al.*, 1998) and that infants at risk of SIDS are more susceptible to the stress of hypoxia during this sleep state (McCulloch *et al.*, 1982). It is possible that elevated neurosteroid concentrations in association with increased quiet sleep may increase the risk of SIDS following hypoxia challenge by altering sleep-wake behaviour and possibly arousal thresholds.

4.3.7 Single versus repeated exposures to hypoxia in the lamb

Previous studies have investigated the effects of single and repeated hypoxia challenge(s) on respiratory responses (review Moss, 2000), however, very little work has examined the effects of single and repeated bouts of hypoxia on other physiological parameters. The studies presented in this chapter investigated whether a range of physiological responses to a single hypoxia episode was the same or different to that of a repeated exposure and whether subsequent exposures to hypoxia within the same experimental protocol had additive or habitual effects.

Some differences were found between the results of the single and repeated hypoxia protocols. A single hypoxia challenge had a greater effect on plasma lactate concentration and resulted in slightly more variable behavioural results. Repeated hypoxia challenges resulted in more defined and conclusive behavioural responses without any evidence of habituation between exposures. Due to the importance of this parameter for this thesis, a

repetitive model of hypoxia was used to study neurosteroid concentrations in brain tissue. Therefore, this thesis has not investigated the effects of a single episode of hypoxia on central allopregnanolone concentration.

4.3.8 Summary

Exposure to two repeated episodes of hypoxia was found to elicit increases in allopregnanolone concentration in the lamb brain. In contrast, little or no increase in peripheral allopregnanolone concentration was found following hypoxia, suggesting that the role of neurosteroid production (both centrally and peripherally) in the young lamb is regulated differentially. The effects of hypoxia treatment on central neurosteroid response, although not as profound, were similar to those found following LPS administration (see Chapter 3). The results reported thus far are the first detailed description of the effects of stressful stimuli on neurosteroid concentrations in the early post-natal model. Given that allopregnanolone is well known for its sedative properties, the increase in allopregnanolone concentration along with increased sleep following *hypoxia* treatment suggests that this steroid may play a role in 'sleep' disorders such as sleep-associated apnoea and SIDS.

Chapter 5: Somnogenic and neurosteroid responses to a combined endotoxin-hypoxia regime in lambs

Infection and hypoxia have been identified as two of the most common factors contributing to neonatal morbidity and mortality (Gessler *et al.*, 1996; Scholz, 2003). Infection elicits a wide range of physiological responses, including increased sleep, that are consistent with the profile of sickness behaviour (Dantzer, 2001). These behavioural responses are mediated by the release of cytokines that act to induce protective immune mechanisms, but these processes can also blunt the CNS response to hypoxia (Dantzer, 2001). This suggests that in a newborn, where susceptibility to infection is heightened (see Section 1.2.5), subsequent challenges of hypoxia may impair basic CNS responses. The previous studies in this thesis reported increases in the incidence of sleep and in the concentration of the neurosteroid allopregnanolone when lambs were exposed to endotoxin (Chapter 3) or hypoxia (Chapter 4) challenges. Due to the sedative nature of allopregnanolone, it was proposed that this neurosteroid might contribute to the increased somnogenesis observed in these lambs. In the present study, an extension of the work reported in the previous chapters was performed, by combining the treatments of endotoxin and repeated hypoxia.

Although it is accepted that exogenous administration of neurosteroids, such as allopregnanolone, alters sleep-wake architecture (Korneyev & Costa, 1996; Lancel *et al.*,

1997), the endogenous production of neurosteroids and the effect these have on somnogenesis are still relatively unknown. The studies presented in this thesis (see Chapters 3 and 4) are the first to describe in detail the effects of stressful stimuli on somnogenic behaviour and neurosteroid concentration in the brain during the early developmental period. It has been reported previously that increases in endogenous concentrations of allopregnanolone may have a neuroprotective function in the adult brain (Majewska *et al.*, 1986; Frank & Sagratella, 2000). The increased synthesis of central neurosteroid concentration after acute (Barbaccia *et al.*, 1996) or chronic (Nguyen *et al.*, 2002) stress suggests that these substances have a homeostatic function in the response and adaptation to stress. The increased allopregnanolone concentration in the brain of lambs treated with endotoxin (Chapter 3) or hypoxia (Chapter 4) may represent an endogenous neuroprotective mechanism in the developing brain. Alternatively, increased neurosteroid production could have a harmful effect by depressing 'normal' arousal responses.

Since the incidence of sleep and allopregnanolone concentration in the brain were found to increase in response to endotoxin (Chapter 3) and hypoxia (Chapter 4) challenges individually, it was hypothesised that the combined treatment would have an additive effect on both parameters measured. Therefore, the primary aim of this study was to investigate the interaction and/or effects of a combined endotoxin-hypoxia regime on sleep-wake profiles and neurosteroid concentrations in the lamb. These results are compared to the data for the individual stressors reported in Chapters 3 and 4. Analysis of steroid responses in brain areas known to be associated with the regulation of sleep (e.g. thalamus/hypothalamus) were performed to assess the potential somnogenic effects of these stress-inducing paradigms.

5.1 Materials and methods

5.1.1 Animals

Twenty-five lambs obtained from Merino-Border Leicester crossbred ewes delivered spontaneously in lambing pens. All lambs remained with their mothers except for during the experimental periods described below. To conserve animal numbers, results from the groups of lambs that received saline (n=5) and LPS (n=7) treatments alone are the same as those reported in Chapter 3. In addition, saline-treated lambs used for repeated hypoxia

experiments in Chapter 4 (n=6) have been included. Therefore, a further 7 lambs treated with LPS and hypoxia combined were used for the studies performed in this chapter.

5.1.2 Experimental design

Each lamb was subjected to two experiments. The first experiment was conducted when the lambs were 12 days old (range, 7-15) and the protocol lasted for 7 hours (see Experiment 1 below). The lambs were allowed to recover from this experiment for no less than 3 days, following which they were subjected to a second experiment at 20 days of age (range, 17-21) which lasted 4 hours, at the end of which lambs were euthanased to obtain tissue samples (see Experiment 2 below).

Animal preparation and surgery protocols were as described previously in Section 2.3. Experiments did not commence until at least four days after surgery. Behavioural parameters were recorded in all experiments as described in Section 2.4. Blood sampling was as reported in Section 3.1.3.1 for normoxia lambs and 4.1.2.1 for hypoxia lambs.

Experiment 1

All experiments began at 0800 hours, when the lamb was placed in the sling and recording of the physiological variables commenced. One hour later, saline (5 mL; n=11) or LPS (0.7 μg/kg in 5 mL saline; n=14) was administered intravenously as a bolus and recordings were continued for 6 hours. The induction of repeated hypoxia has been described previously in Section 4.2.2. Briefly, a custom-made plastic hood was placed over the head and upper torso of the lamb and air or a N_2 rich gas mixture was passed continuously at a flow of 30 L/min. Thirteen lambs breathed air throughout the experiment except for two 45 minute episodes of hypoxia commencing at 0.5 and 2.5 hours after either the LPS or saline had been given. During each hypoxia episode, lambs breathed a mixture of 8 % O_2 in N_2, sufficient to reduce the arterial oxygen saturation to ~50 %, which was monitored from arterial blood samples drawn every 15 minutes (see Section 8.2.2 for data).

Experiment 2

During the second experiment, each lamb was killed using i.v. pentobarbitone, 3 hours after administration of either LPS or saline. The brain was removed immediately at the conclusion of the second experiment, divided into blocks (see Section 2.5.4) and stored at -70 °C until required for analysis of steroid content. To ensure hypoxaemia was induced in

these lambs at the time of tissue collection, blood samples were taken immediately prior to tissue collection.

5.1.3 Data analysis

Identification and scoring of sleep-wake states based on the chart records was conducted using the methods described in Section 2.4.2. For each experiment, sleep-wake states of the entire recording were scored at 1 minute intervals and these data were combined to obtain an average value for a combination of 30, 45 and 60 minute epochs. The variation in epoch length was used to assess the effects of the hypoxia challenge by incorporating the entire 45 minute period(s). The proportion (%) of the total time spent in a particular state (AW, QS, AS, IS) after saline or LPS treatment, with or without hypoxia, was then calculated.

5.1.4 Statistical analyses

Data were analysed using SPSS software (SPSS for Windows, Version 10.0, Chicago, IL, USA). The data presented include values for the saline and LPS groups (Chapter 3) and for the hypoxia alone group (Chapter 4), however, a completely new analysis was performed to compare treatment groups. Data were assessed for homogeneity using Levene's test and, where necessary, the data were transformed into square root or logarithmic data before analysis. All analyses were performed using a two-way factorial ANOVA with LPS and hypoxia treatments as the main factors. Where a significant interaction was found between the treatments, individual mean values were compared using Fisher's LSD test. Data (original, untransformed) are presented as mean ± S.E.M. $P<0.05$ was considered statistically significant. The absence of error bars on some graphs presented in this chapter indicates that they are within the symbol.

5.2 Results

This chapter presents results for four treatment groups: saline controls (SAL), saline with hypoxia (hSAL), LPS and LPS with hypoxia (hLPS). For the purposes of this chapter, the data presented include the effects of these treatments on somnogenic behaviour, plasma cortisol and neurosteroid concentrations only. Measurements of rectal temperature, blood gases, pHa, Hb and plasma iron, glucose and lactate were performed, however, the results were similar to those reported in previous chapters. That is, there was no difference between the two groups of hypoxic lambs in the measurements of blood gases, pHa and

Hb, and there was little difference in febrile responses between the two LPS-treated groups. Due to the large volume of data, these results have been included in Section 8.2.

5.2.1 Effect of treatment on somnogenic behaviour

The duration of the experiment, including the pre-treatment period, was 7 hours. As reported previously in Chapter 3, after at least 4 hours in the sleep chamber (i.e. at 3 hours post-treatment), control saline-treated lambs became restless and had to be returned to their mothers for feeding. In contrast, lambs that received either hypoxia (hSAL) or LPS alone, or were made hypoxic after LPS treatment (hLPS), remained asleep or were drowsy for the entire 6 hour period of recording. Analysis of sleep-wake profiles over the course of the experiment is presented in Section 8.2.5. The following is a summary of the effects of the different treatments on somnogenic behaviour over time.

5.2.1.1 Effect of treatment between 0.5 and 3.5 hours

Figure 5.1 shows the effect of the different treatments on sleep states for the period 0.5 to 3.5 hours after saline or LPS administration. Data for this 3 hour period was chosen as this includes both periods of hypoxia, which may highlight the more immediate effects of this stressor. It was also during this time period (3 hours post-saline or LPS administration) that lambs were euthanased in a subsequent experiment and brain samples collected for the analysis of neurosteroid content. Both hypoxia and LPS treatments reduced the incidence of wakefulness compared to saline controls, with LPS (with or without hypoxia) having a significantly greater effect than hypoxia alone ($P<0.01$; Figure 5.1A). The combined treatment of LPS and hypoxia reduced the incidence of wakefulness by ~50 %, but this was not different to LPS alone. The individual treatments of LPS or hypoxia increased the incidence of quiet sleep significantly compared to controls (Figure 5.1B). Lambs that were made hypoxic following LPS treatment also exhibited an increased incidence of quiet sleep compared to saline controls (Figure 5.1B). This increase was greater than that found with LPS treatment alone, but not with hypoxia alone (Figure 5.1B). There was no effect of LPS or hypoxia treatments on the incidence of active sleep when compared to controls (Figure 5.1C). However, when comparing the two groups that were treated with hypoxia, it was found that the combination of LPS and hypoxia increased the incidence of active sleep significantly over that observed with hypoxia alone (Figure 5.1C). The incidence of indeterminate sleep was increased significantly ($P<0.01$)

in lambs treated with LPS compared to control lambs (Figure 5.1D). Neither hypoxia nor hypoxia with LPS treatment had any effect on this sleep state (Figure 5.1D).

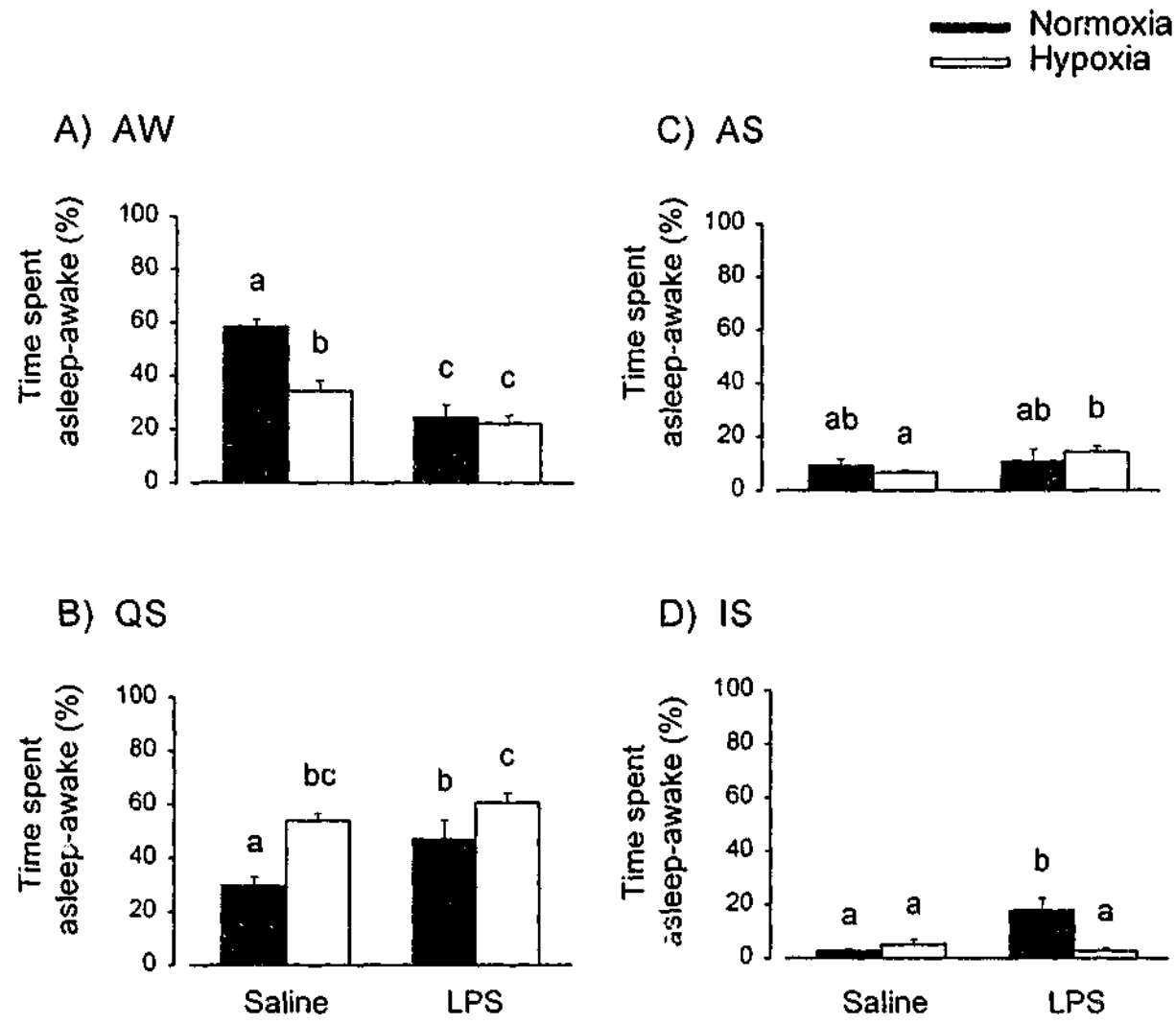


Figure 5.1: Effect of treatment on somnogenic behaviour (0.5-3.5 hours)

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) or hLPS (n=7) treatments on the incidence of wakefulness (A), quiet sleep (B), active sleep (C) and indeterminate sleep (D). Letters indicate significant (P<0.01) differences between treatment groups that showed an interaction during ANOVA analysis. Data are expressed as mean ± S.E.M.

5.2.1.2 Effect of treatment for the 6 hours post-saline or LPS administration

Figure 5.2 shows the effect of treatment on the incidence of sleep for the entire 6 hour post-treatment period. LPS treatment resulted in a significant decrease in the incidence of wakefulness compared to controls, while hypoxia had no effect (P<0.05; Figure 5.2A). The reduction in wakefulness following LPS treatment was also associated with an increase in the incidence of quiet sleep (Figure 5.2B). Although hypoxia did not affect

wakefulness, there was a significant increase in the incidence of quiet sleep in these lambs (Figure 5.2B). Active sleep was increased significantly following LPS, but not hypoxia, treatment (Figure 5.2C). A significant interaction ($P<0.01$) was found between the effects of LPS and hypoxia treatments on the incidence of indeterminate sleep (Figure 5.2D), with LPS-treated lambs displaying a higher level of this sleep state compared to any other treatment group (Figure 5.2D).

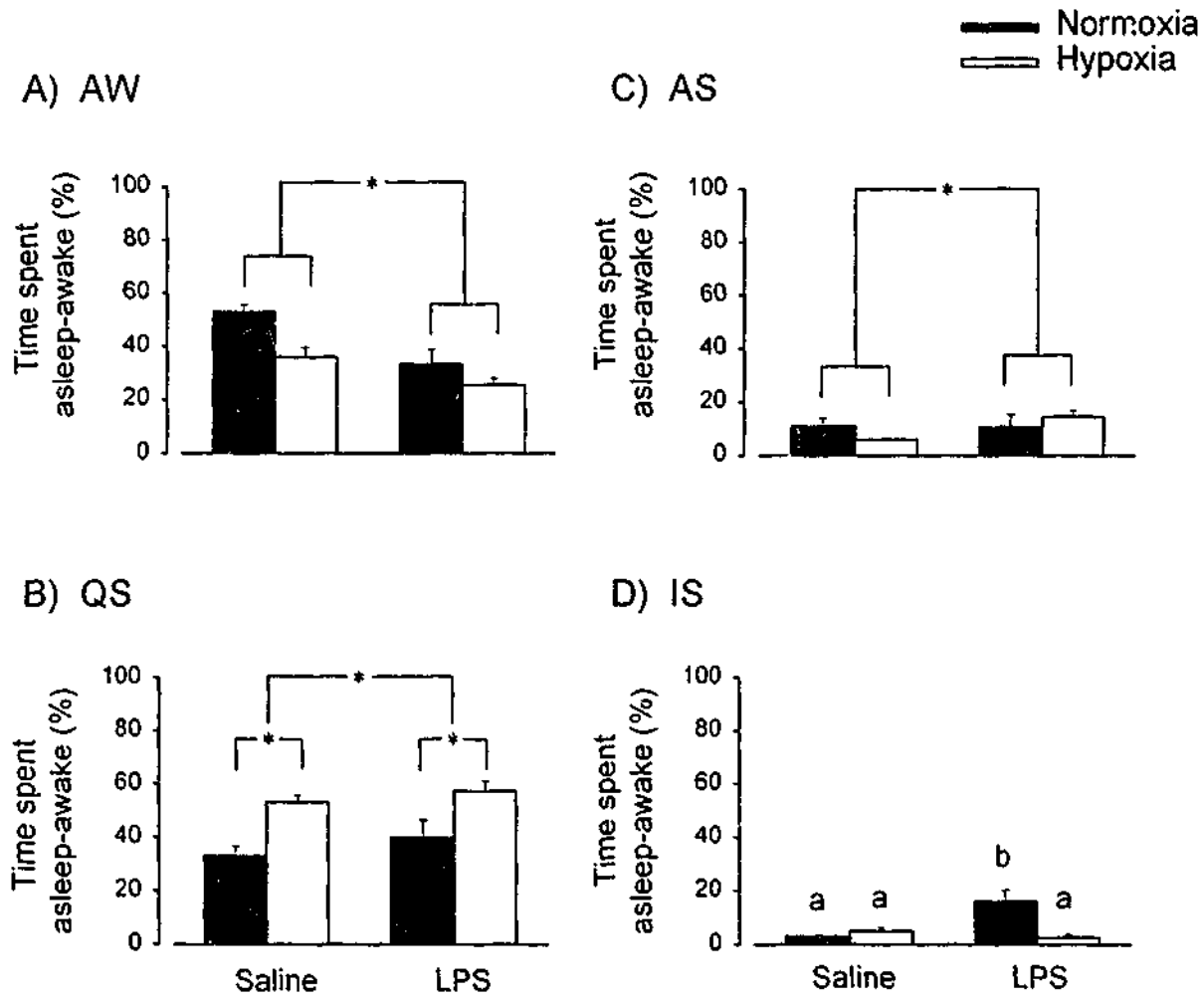


Figure 5.2: Effect of treatment on somnogenic behaviour (0-6 hours)

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) or hLPS (n=7) treatments on the incidence of wakefulness (A), quiet sleep (B), active sleep (C) and indeterminate sleep (D). * $P<0.05$ between treatments; letters indicate significant ($P<0.01$) differences between treatment groups that showed an interaction during ANOVA analysis. Data are expressed as mean ± S.E.M.

5.2.2 Effect of treatment on plasma cortisol concentrations

Cortisol concentrations in saline-treated hypoxic lambs were increased significantly ($P<0.05$) during each hypoxia episode when compared to pre-treatment control values (Figure 5.3). Cortisol concentrations returned to control levels between the two episodes and within 45 minutes following the second hypoxia episode. LPS-treated lambs, with or without hypoxia, showed an approximate 9-fold increase in cortisol concentrations, when compared to pre-treatment values, 3 hours following LPS administration. This increase was significantly greater than for hypoxia alone at all time points, except for 1 and 1.5 hours post-LPS treatment. Cortisol concentrations remained elevated in LPS-treated lambs for the duration of the experiment (Figure 5.3).

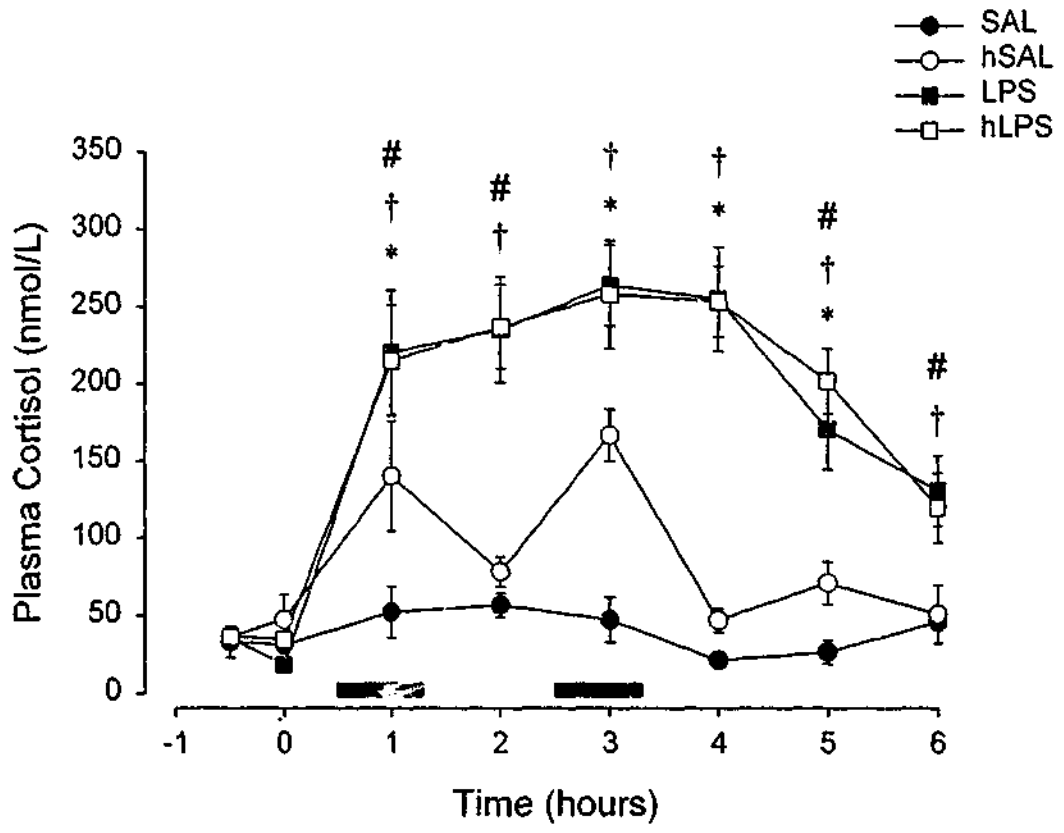


Figure 5.3: Effect of treatment on plasma cortisol concentrations

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) and hLPS (n=6) treatments on plasma cortisol concentrations. * $P<0.05$ between SAL and hSAL; † $P<0.05$ between SAL and both LPS groups; # $P<0.05$ between hSAL and both LPS-treated groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

5.2.3 Effect of treatment on plasma neurosteroid concentrations

5.2.3.1 Plasma pregnenolone and progesterone

Plasma pregnenolone concentrations were below the level of detection of the assay for the lambs that received either saline or LPS alone and remained normoxic. Pregnenolone was sometimes detected in plasma following the hypoxia treatments, but concentrations were always low (~0.01 nmol/L). Plasma progesterone was undetectable throughout the experiments in the saline-treated lambs, but low plasma concentrations were sometimes detected in lambs treated with hypoxia alone (range from undetectable to 0.02 ± 0.00 nmol/L), LPS alone (undetectable to 0.06 ± 0.02 nmol/L) and LPS with hypoxia (undetectable to 0.08 ± 0.01 nmol/L).

5.2.3.2 Plasma allopregnanolone

There was no significant change in plasma allopregnanolone concentration over the duration of the experiment following saline treatment in normoxic lambs (Figure 5.4). Further, hypoxia exposure in saline-treated lambs had no significant effect on plasma allopregnanolone concentrations over time (Figure 5.4). However, at 3 and 6 hours, allopregnanolone concentrations were significantly lower in lambs treated with hypoxia when compared to saline controls. LPS administration resulted in an increase in plasma allopregnanolone concentration 2 hours following treatment, which remained elevated for the duration of the experiment (Figure 5.4). This increase in plasma allopregnanolone was similar for lambs that received LPS treatment alone, and for lambs made hypoxic following LPS treatment (Figure 5.4).

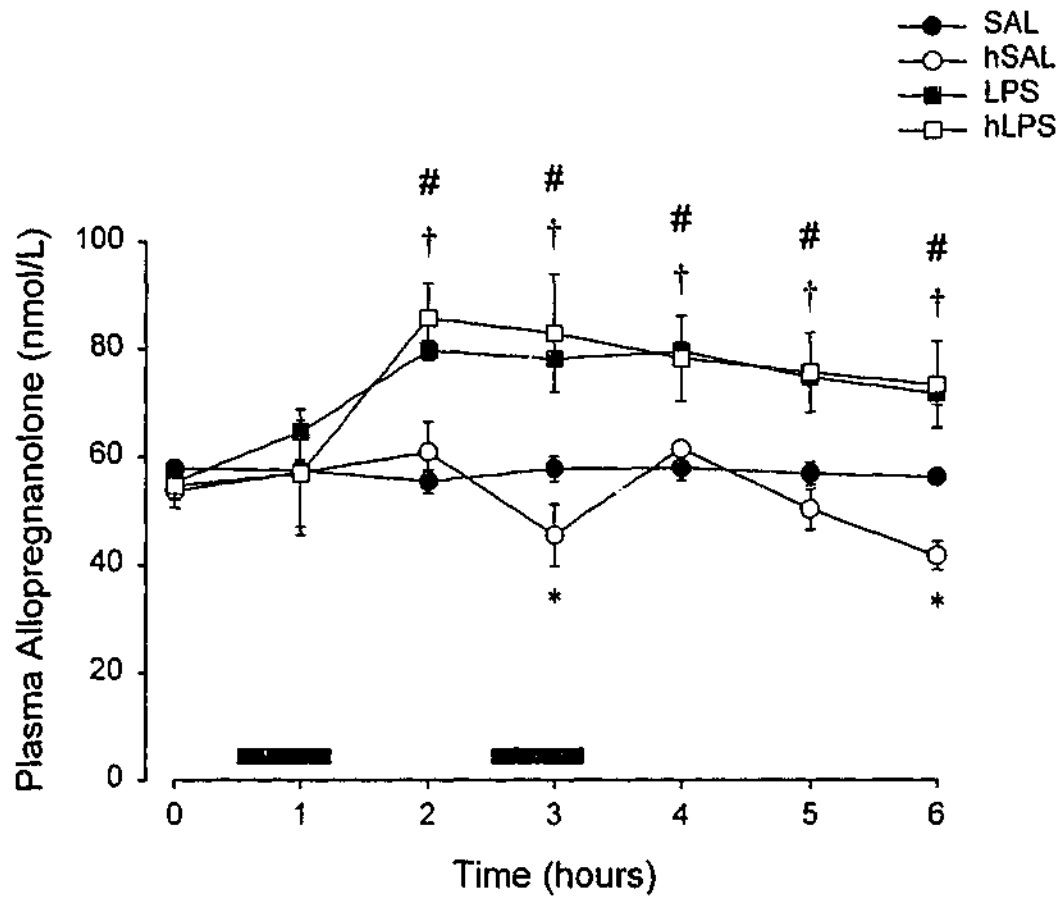


Figure 5.4: Effect of treatment on plasma allopregnanolone concentrations

Effect of SAL (n=5), hSAL (n=4), LPS (n=7) and hLPS (n=4) treatments on plasma allopregnanolone concentrations. * P<0.05 between SAL and hSAL; † P<0.05 between SAL and both LPS-treated groups; # P<0.05 between hSAL and both LPS-treated groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

5.2.4 Effect of treatment on physiological parameters at 20 days of age, prior to tissue collection

An additional experiment, using the same experimental conditions, was performed on lambs at 20 days of age for the collection of brain tissue. During this experiment, the effects of treatment on parameters measured were not different to those observed during the initial experiment (see Section 8.2). Table 5.1 presents the blood gas, pHa, Hb, plasma cortisol and allopregnanolone data obtained in these lambs, immediately prior to tissue collection. Saline treatment in normoxic lambs had no effect on any of the parameters measured. Hypoxia treatment resulted in hypoxaemia, hypocapnia, and increased pHa, cortisol and allopregnanolone levels. Normoxic, LPS-treated lambs exhibited a small but significant decrease in $PaCO_2$ that was associated with increases in plasma cortisol and

allopregnanolone. The combined treatment of LPS and hypoxia reduced SaO_2, PaO_2 and $PaCO_2$, as well as increasing pHa and plasma steroids. The degrees of change in SaO_2, PaO_2, $PaCO_2$ and pHa were not different between the two hypoxic lamb groups, i.e. LPS had no additional effect. The increase in plasma steroids was greater in lambs treated with LPS alone than in lambs treated with hypoxia alone. However, the increases in plasma steroids were not different between lambs treated with LPS alone or in lambs treated with LPS and hypoxia combined.

5.2.5 Effect of treatment on brain allopregnanolone concentrations

Allopregnanolone concentration was detected in all regions of the lamb brain examined, with concentrations ranging from 15 to 40 pmol/g in the saline-treated control lambs (Figure 5.5). In contrast, pregnenolone and progesterone concentrations were undetectable (<0.08 pmol/g and <0.17 pmol/g, respectively) in all regions examined from brains of saline-treated lambs.

Hypoxia alone increased allopregnanolone concentration in all brain regions examined except the thalamus/hypothalamus and cerebellum (Figure 5.5 D&J). The greatest relative increases occurred in the pons (~2 fold), occipital cortex (~3 fold) and midbrain (~3.5 fold). LPS alone also resulted in increased allopregnanolone concentration in most brain regions examined with, as for hypoxia alone, no changes observed for the thalamus/hypothalamus and the cerebellum. LPS alone also had no effect on allopregnanolone concentration in the pons (Figure 5.5B).

Significantly greater increases of allopregnanolone concentration occurred in several brain regions of lambs made hypoxic after receiving LPS. These regions were the medulla, midbrain, hippocampus and all regions of the cortex examined (Figure 5.5). Notably, allopregnanolone concentration was increased in the thalamus/hypothalamus and cerebellum after the combined hypoxia and LPS treatments, whilst the individual treatments had no effect (Figure 5.5 D&J). Neither pregnenolone nor progesterone was detected (<0.08 pmol/g and 0.17 pmol/g, respectively) in brains from hypoxia, LPS, or LPS with hypoxia-treated groups.

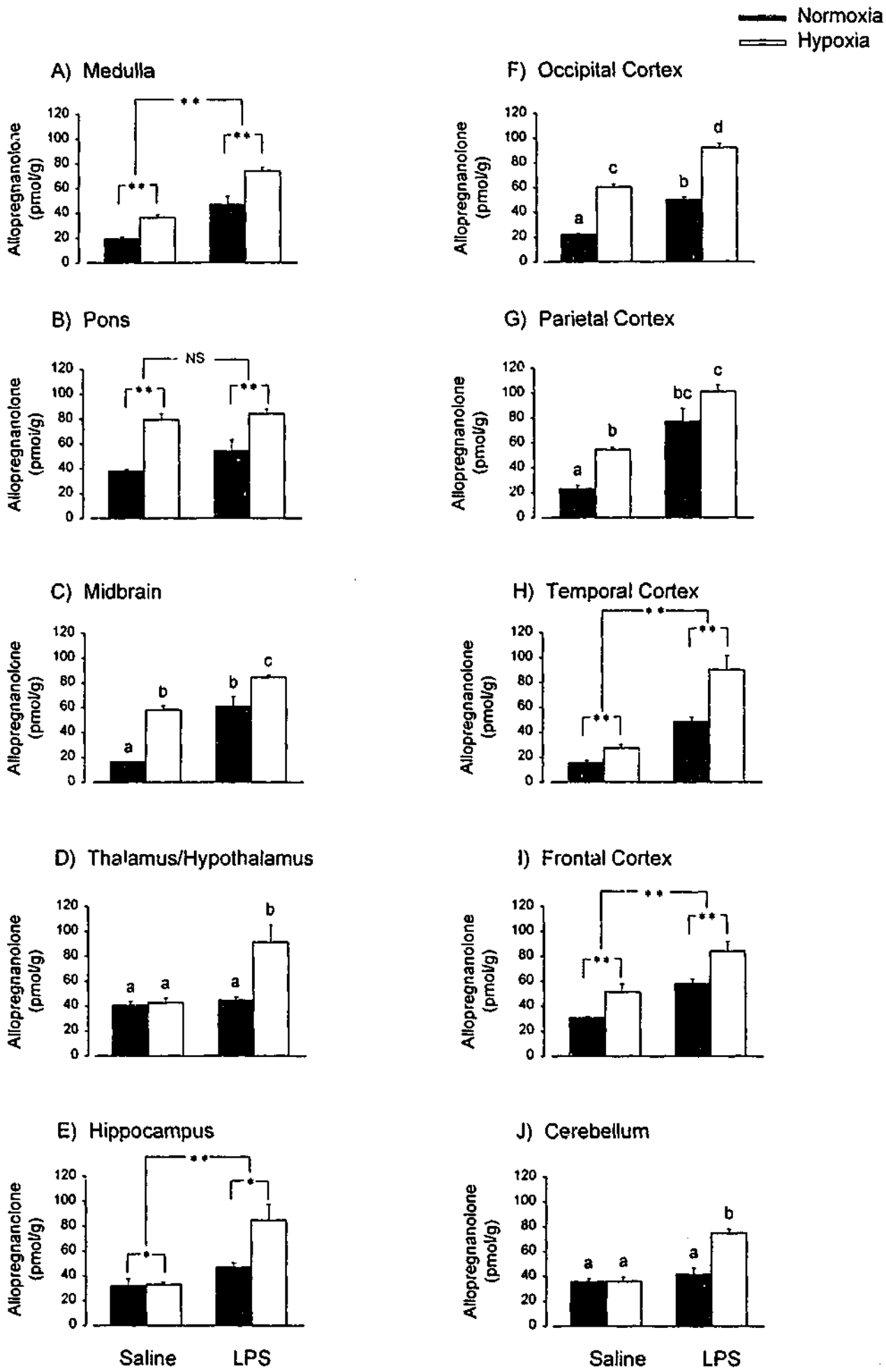
Table 5.1: Effect of treatment on blood gases, pHa, Hb, and plasma cortisol and allopregnanolone concentrations prior to tissue collection

	Treatment			
Parameter	***SAL (n=5)***	***hSAL (n=6)***	***LPS (n=7)***	***hLPS (n=7)***
SaO_2 (%)	97.80 ± 1.86	52.40 ± 4.06*†	97.39 ± 0.83	50.00 ± 1.79*†
PaO_2 (mmHg)	108.40 ± 11.57	32.20 ± 3.88*†	117.14 ± 3.08	36.80 ± 2.60*†
$PaCO_2$ (mmHg)	36.29 ± 2.11	26.86 ± 2.47*†	30.57 ± 1.81*†	28.64 ± 2.94*†
pHa	7.35 ± 0.01	7.49 ± 0.02*†	7.39 ± 0.01	7.42 ± 0.04*†
Hb (g/dL)	7.56 ± 0.93	6.90 ± 0.63	6.69 ± 0.30	6.46 ± 0.66
Cortisol (nmol/L)	26.93 ± 6.28	165.06 ± 12.06*†	248.90 ± 29.00*†	227.42 ± 47.17*†
Allopregnanolone (nmol/L)	40.37 ± 3.49	46.45 ± 3.47*† (4)	74.37 ± 3.24*†	97.60 ± 18.67*† (4)

Effect of SAL (n=5), hSAL (n=4), LPS (n=7) and hLPS (n=4) treatments on blood gases, pHa, Hb, and plasma cortisol and allopregnanolone concentration 3 hours post-saline or LPS administration, prior to tissue collection. * $P<0.05$ within each treatment group compared to its pre-treatment values (pre-treatment data not shown); † $P<0.05$ between treatment groups compared to saline controls. Data are expressed as mean ± S.E.M for the period 3 hours after saline or LPS administration, just prior to tissue collection.

Figure 5.5: Effect of treatment on brain allopregnanolone concentrations

Effect of treatment on allopregnanolone concentrations in specific brain regions examined (A-J). Lambs were treated with saline or LPS, in combination with normoxia or hypoxia. * $P<0.05$ between treatments; ** $P<0.01$ between treatments. Letters indicate significant ($P<0.01$) differences between treatment groups that showed an interaction during ANOVA analysis. Data are expressed as mean ± S.E.M., $n=4$ for all groups. NS=not significant.


Normoxia
Hypoxia
A) Medulla
B) Pons
C) Midbrain
D) Thalamus/Hypothalamus
E) Hippocampus
F) Occipital Cortex
G) Parietal Cortex
H) Temporal Cortex
I) Frontal Cortex
J) Cerebellum
Allopregnanolone (pmol/g)
NS
Saline
LPS

5.3 Discussion

A major finding of the studies described in this chapter is that LPS combined with hypoxia treatment had a greater effect on allopregnanolone concentration than either of the individual treatments in the majority of brain regions examined. Furthermore, only the combined hypoxia and LPS treatment was effective in eliciting increases in allopregnanolone concentration in the thalamus/hypothalamus and cerebellum. Thus, there were significant differences between brain regions in responses to hypoxia, LPS and the combined treatments, indicating regional differences in the cellular responses of the brain to these challenges. The changes in allopregnanolone concentration in the brain could not be accounted for by changes in the steroid or its precursors in the circulation, again suggesting *de novo* production is of primary importance in determining the concentration of this neurosteroid in the lamb brain. Finally, despite the large increases in allopregnanolone concentration in the brains of lambs treated with LPS and hypoxia, there was no additional effect on the incidence of somnogenesis in lambs when compared to LPS treatment alone.

5.3.1 Plasma steroid responses to combined hypoxia and LPS treatments

Previous studies have reported that neurosteroids increase in both plasma and brain in response to stressful stimuli (Purdy *et al.*, 1991; Barbaccia *et al.*, 1994; see Chapter 3). However, in Chapter 4, moderate hypoxia did not elicit an increase in circulating allopregnanolone concentrations despite the large increases observed centrally. This absent response in peripheral allopregnanolone concentration is in contrast with the increase in plasma cortisol concentration that occurred during this time. As discussed in the previous chapters, the source of peripheral neurosteroidogenesis in these sexually immature lambs is most likely to be the adrenal gland. The increase in cortisol secretion detected in this study indicates that an increased adrenal activation as a result of moderate hypoxia did occur but that it was not sufficient to induce an increased release of neurosteroids into the circulation. The absent effect of hypoxia on allopregnanolone concentrations, despite changes centrally (see Section 5.4.4), suggests that peripheral steroid precursors are not essential for the allopregnanolone response of the CNS to hypoxia.

LPS treatment (with or without hypoxia) increased plasma allopregnanolone concentrations approximately 2-fold, beginning 2 hours post-LPS treatment and lasting for

at least a further 4 hours. These findings are consistent with previous work in the adult rat where acute stressors, such as CO_2 inhalation, resulted in rapid increases in neurosteroid production (Barbaccia *et al.*, 1994; Barbaccia *et al.*, 1996). Large increases in plasma cortisol were also found, suggesting activation of the HPA axis. The rise in plasma allopregnanolone concentration is likely to be due to the stimulation of adrenal neurosteroidogenic enzymes by inflammatory cytokines. It is possible that stimulation of adrenal neurosteroidogenesis increases precursor availability, however, as there was little or no plasma pregnenolone detected in these lambs, this is unlikely. The adrenal gland is the most likely source of plasma allopregnanolone in these lambs, based on the large increase in plasma cortisol and the fact that these lambs were pre-pubertal and unlikely to have mature gonadal function. This proposed pathway of neurosteroid production is supported by previous studies that have reported increases in plasma allopregnanolone concentrations in adult subjects where gonadal production of steroids was inhibited (Genazzani *et al.*, 1998; Genazzani *et al.*, 2000).

5.3.2 Central allopregnanolone response to combined hypoxia and LPS treatments

Investigation of the central neurosteroidogenic response to stress in lambs revealed that moderate hypoxia increased allopregnanolone concentration significantly in all regions of the brain examined, except the thalamus/hypothalamus and cerebellum. LPS treatment also increased allopregnanolone concentration, with increases in all brain regions examined except the pons, thalamus/hypothalamus and cerebellum. These findings suggest that acute hypoxic stress and LPS treatment can up-regulate neurosteroidogenesis in the developing lamb brain, as reported previously for adult rats using other types of stress (Barbaccia *et al.*, 1996). Whether this up-regulation occurs through increased neurosteroidogenic enzyme gene expression or activity remains to be elucidated.

LPS treatment followed by hypoxia exposure resulted in further increases in allopregnanolone concentration in many brain regions examined including the medulla, midbrain, hippocampus, and the occipital, temporal and frontal cortices. The effects were additive in some of these regions including the medulla, hippocampus, and temporal and frontal cortices. In contrast, allopregnanolone concentration was increased only when both treatments were present in the thalamus/hypothalamus and cerebellum. The finding that the independent stressors, hypoxia and LPS, were not sufficient to alter allopregnanolone

concentration in the thalamus/hypothalamus and cerebellum of the lamb brain, suggests that these brain regions are relatively resistant to stress and that multiple challenges are required to have an affect.

5.3.3 Behavioural responses to combined hypoxia and LPS treatments

When examining the somnogenic effects of treatment between 0.5 and 3.5 hours post-saline and/or LPS administration (Figure 5.1), a time which included both hypoxic episodes, it was found that hypoxia treatment reduced the incidence of wakefulness significantly through a concomitant increase in the incidence of quiet sleep. LPS treatment was also found to reduce the incidence of wakefulness, however, this was associated with increases in the incidence of quiet sleep and indeterminate sleep. It was surprising to find that there was no additional effect on somnogenesis when hypoxia and LPS treatments were combined, despite there being a greater increase in brain allopregnanolone content, particularly in the thalamus/hypothalamus, a key region for the regulation of sleep. Considering that allopregnanolone has been reported as having somnogenic properties (Korneyev & Costa, 1996; Lancel *et al.*, 1997), it was expected that this treatment regime would result in further reductions in the incidence of wakefulness and, hence, increase the incidence of sleep compared to the individual stressors. It is possible that due to the already low levels of wakefulness produced as a result of the LPS treatment (see Section 8.2.5), the addition of hypoxia may not have been able to depress this behavioural state any further.

The increase in allopregnanolone concentration in the brain during hypoxia and/or LPS exposure raises a number of questions concerning the effect of this steroid on brain function. On the one hand, allopregnanolone may be neuroprotective due to its interaction with the $GABA_A$ receptor and the subsequent effect this has in counteracting excitotoxicity (review Compagnone & Mellon, 2000). The neuroprotective role of neurosteroids has been reported in studies where neurosteroid administration reduced or prevented chronic neurotoxicity (Jiang *et al.*, 1996; Kumon *et al.*, 2000) and reduced the incidence of neuronal cell death (review Mellon & Griffin, 2002). On the other hand, due to the sedative nature of allopregnanolone, large changes in brain allopregnanolone concentrations in response to stress may result in a degree of sedation that is sufficient to increase behavioural arousal thresholds and to blunt brainstem reflex mechanisms that regulate ventilatory and cardiovascular responses to challenges such as hypoxia and

apnoea. Further investigation of arousal responses is required to examine these possibilities.

The studies reported in this thesis have found that LPS administration to young lambs increases the incidence of quiet sleep in association with increases in the concentration of the sedative neurosteroid allopregnanolone in the brain. Since infection changes both the nature and type of sleep in animals and humans (Lindgren *et al.*, 1996), by increasing the incidence of quiet sleep following endotoxin exposure, it has been proposed that this increase is part of a defence mechanism of the host against infection, otherwise known as sickness behaviour (Toth & Krueger, 1989; Mullington *et al.*, 2000). Pro-inflammatory cytokines in the CNS have been suggested as contributing to the increased somnolence during infection, however, it could be that neurosteroids may also play a role though actions at the $GABA_A$ receptor.

5.3.4 Summary

The increase in brain allopregnanolone concentration that was observed following hypoxia, LPS or the combination of the two treatments, suggests that an increase in the production of neurosteroids is part of the response to stressful stimuli during the early developmental period, as well as in adulthood. The differential effects of LPS and hypoxia on plasma and brain levels of allopregnanolone indicate separate regulation of peripheral and CNS allopregnanolone production. The marked rise in the concentration of allopregnanolone in many regions of the brain and the increased incidence of quiet sleep following exposure to stress supports the sedative actions of this neurosteroid. The finding that the thalamus/hypothalamus and cerebellum did not produce any change in allopregnanolone concentration until LPS and hypoxia were combined suggests a level of resistance to stress in these regions. Whether the increased production of allopregnanolone in the thalamus/hypothalamus in response to LPS and hypoxia combined has any clinical implications for 'sleeping' disorders requires further investigation.

Chapter 6: Contribution of cytokines to endotoxin and hypoxia-induced increases in sleep in lambs

Cytokines are a diverse group of low molecular weight, potent, pleiotrophic glycoproteins that serve as chemical messengers within the immune system (Vitkovic *et al.*, 2000). They exhibit properties that are short-lived by binding to cells that express receptors for that cytokine (Vitkovic *et al.*, 2000) and act either in an autocrine, paracrine or endocrine fashion (Nash *et al.*, 1992). Cytokines are key regulators of the responses to infection and inflammation (McCann *et al.*, 1994; Vitkovic *et al.*, 2000; Aloisi, 2001). The interaction of multiple cytokines that are generated as part of a typical immune response are a part of the acute phase response (APR) profile (Krueger *et al.*, 1990), sickness behaviour (Dantzer, 2001; Reyes & Sawchenko, 2002) and fever (Kluger *et al.*, 1998). They have also been proposed as playing a role in the aetiology of SIDS (Sayers *et al.*, 1995; Thrane *et al.*, 1995; Blackwell *et al.*, 1999; Raza & Blackwell, 1999).

Cytokines may have pro-inflammatory and/or anti-inflammatory properties (Vitkovic *et al.*, 2000), depending upon the requirements of the immune system. The pro-inflammatory cytokines, IL-1, TNF-α and IL-6, initiate a wide spread of activity that aids in the coordination of the host's response to infectious challenges. These cytokines are produced by microglia and macrophages (Aloisi, 2001), have somnogenic properties (Krueger *et al.*,

1990; Krueger *et al.*, 1998) and are endogenous pyrogens as they can elicit fever (McCann *et al.*, 1994). Pro-inflammatory cytokines can act alone or in combination to induce or alter the expression and response of other cytokines; for example, TNF-α regulates the expression of IL-6, whereas IL-6 inhibits the production of TNF-α (McWaters *et al.*, 2000). Over-expression of pro-inflammatory cytokines has been shown to have harmful effects leading to chronic inflammation and toxic shock syndrome (McCann *et al.*, 1994). Anti-inflammatory cytokines produced by microglia, such as transforming growth factor (TGF)-β, IL-3 and IL-10, can reduce the severity of the inflammatory response by suppressing the expression of pro-inflammatory cytokines (Kronfol & Remick, 2000; Aloisi, 2001).

The endogenous production of cytokines during an infectious challenge is a primary component of the inflammatory response, which initiates a cascade effect including increased drowsiness or sleep (Krueger *et al.*, 1990). This is beneficial in an adult, however, in a newborn, where metabolic needs are high and pulmonary oxygen stores are low, the combination of infection and resulting somnolence may lead to increased susceptibility to the adverse effects of further challenges such as hypoxia. The current study investigates the effects of LPS, hypoxia and a combination of both treatments on the expression of the somnogenic cytokines TNF-α and IL-6. It has been reported previously that both LPS and hypoxia can elicit increases in mRNA expression of IL-6 (Ziesche *et al.*, 1996), as well as increases in the neurosteroid allopregnanolone, a potent somnogenic compound in the brain of young lambs (see Chapters 3, 4 and 5). However, a study by Ghezzi *et al.* (2000) reported that, when administered to cells pre-treated with LPS, allopregnanolone inhibits the production of TNF-α, suggesting that neurosteroids may be endogenous inhibitors of cytokine production (see Figure 6.1).

The individual stressors LPS and hypoxia were found to increase the production of allopregnanolone and increase the incidence of sleep (Chapters 3 and 4). Given this, it was hypothesised that both stressors together would result in an additive effect for both parameters (Chapter 5). Although there was a dramatic increase in allopregnanolone concentration in the brain above that observed for the individual stressors, the amount of time spent asleep was not different between the two LPS groups (Chapter 5). Since pro-inflammatory cytokines have somnogenic properties, their contribution to the somnogenesis observed in the present experimental paradigms used for this thesis

warrants investigation. LPS treatment has been reported to increase cytokine production (Zuckerman *et al.*, 1991; McCann *et al.*, 1994), whilst exposure to hypoxia has been reported to increase cytokine mRNA expression (Ziesche *et al.*, 1996). Therefore, it is possible that their combination may induce an uncontrolled cytokine cascade. Since it has been reported that allopregnanolone may be an endogenous inhibitor of cytokine production (Ghezzi *et al.*, 2000), the resulting increase in brain allopregnanolone concentration, beyond that observed for the individual stressors, may inhibit the production of cytokines and, as a consequence, partially reduce the somnogenic capacity of the stress as observed in Chapter 5 (see Figure 6.1). Therefore, the primary aim of the present study was to investigate the effects of LPS and/or hypoxia treatment on circulating TNF-α and IL-6 concentrations and, secondly, to compare these responses to the somnogenic and neurosteroid responses reported previously in this thesis.

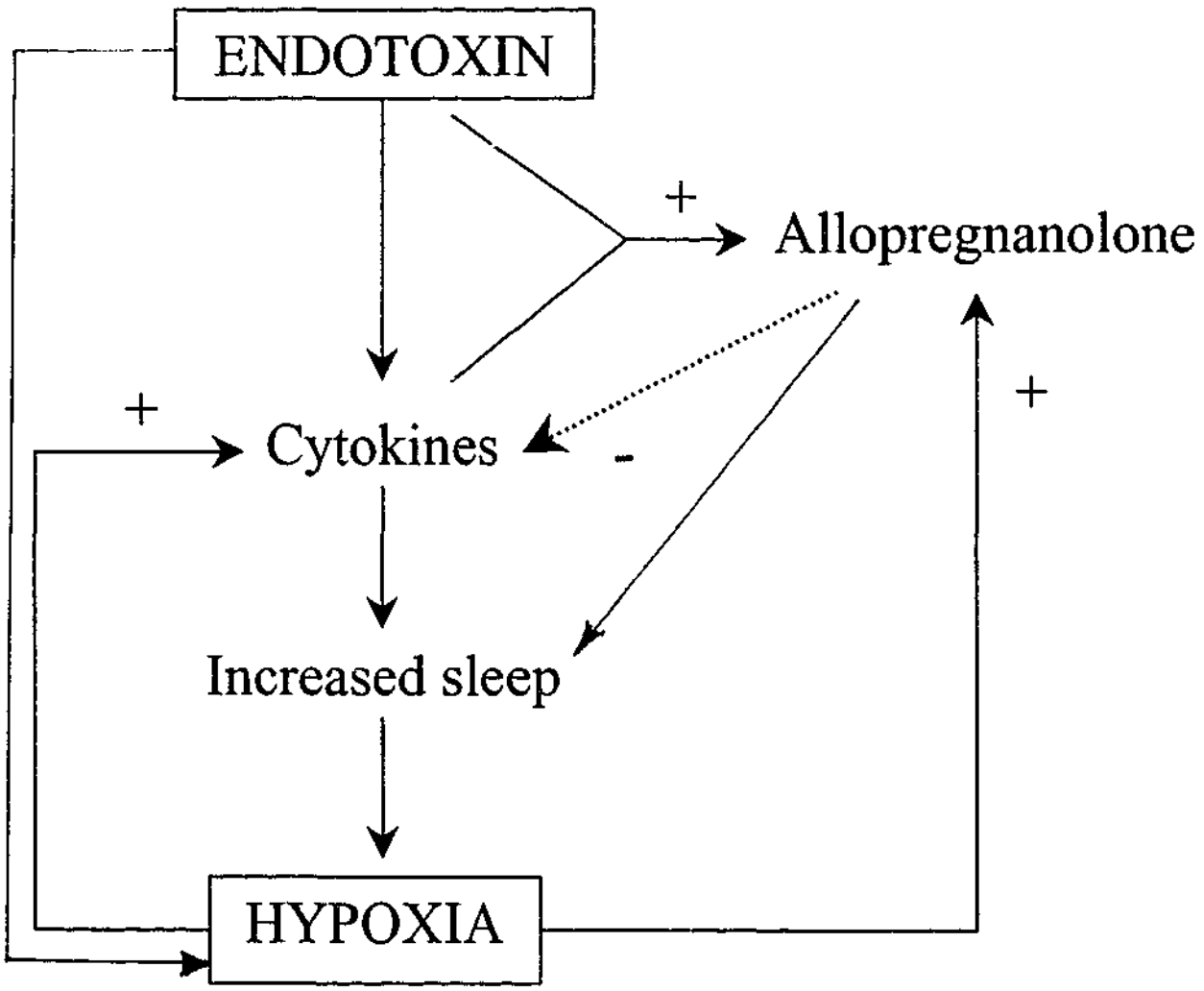


Figure 6.1: Effect of endotoxin and/or hypoxia treatment on somnogenesis

Diagrammatic representation of possible steps involved in the regulation of sleep as a result of endotoxin and/or hypoxia exposure. Dotted lines represent possible inhibitory actions of neurosteroids, such as allopregnanolone on cytokine production.

6.1 Materials and methods

Four groups of lambs were used for this study; lambs that received saline alone (n=4), LPS alone (n=4), saline treatment followed by two bouts of hypoxia (n=4) and LPS treatment followed by two bouts of hypoxia (n=4). The experimental protocols for normoxic lambs (Section 3.2.3) and hypoxic lambs (Section 4.2.2) are as described previously. Plasma samples collected as part of these previous studies (Chapter 3, 4 and 5) were analysed, using ELISA techniques (see below), for the presence of the pro-inflammatory cytokines TNF-α and IL-6.

6.1.1 Plasma tumour necrosis factor-α (TNF- α)

Plasma TNF-α concentrations were measured by enzyme-linked immunosorbent assays (ELISA) using a modification of the method described by Egan *et al.* (1994).

6.1.1.1 Measurement of TNF-α

6.1.1.1.1 General reagents

All reagents used in these assays were purchased from Sigma Chemical Company (NSW, Australia) unless stated otherwise. Phosphate-buffered saline (10 x PBS, pH 7.0) containing 0.4 M di-sodium hydrogen phosphate (Na_2HPO_4), 0.1 M potassium phosphate (K_2HPO_4) and 1.5 M sodium chloride (NaCl, pH 7.4) was used in all assays. The coating buffer (pH 9.6) contained 15 mM sodium carbonate (Na_2CO_3), 30 mM sodium hydrogen carbonate ($NaHCO_3$) and 3.1 mM sodium azide (NaN_3). The washing buffer (pH 8.0) contained 10 x PBS and 20 % Tween 20 (Polyoxyethelene sorbitan monolaurate) and the blocking buffer, which was filtered before use, contained 10 x PBS with 1 % skim milk powder (Diploma skim milk powder, Bonlac Foods Ltd., VIC, Australia). The diluent that was used in these assays was 5 % bovine serum albumin (BSA, w/v), made with 1 x PBS. Wells were washed with washing buffer four times unless stated otherwise and all incubations took place at room temperature in a humidified container unless stated otherwise.

TNF-α ovine recombinant standard was supplied by Dr JP Scheerlinck (Centre for Animal Biotechnology, University of Melbourne, VIC, Australia). Final concentrations of 0.47-120 ng/mL in 500 µl of diluent were used in the assay.

6.1.1.1.2 TNF-α ELISA

Each well of a 96-well ELISA plate (Microtiter 96 well plates, Thermo Labsystems, Finland) was coated with 50 µl of TNF-α mouse ascites monoclonal antibody (Centre of Animal Biotechnology, University of Melbourne, VIC, Australia) that had been diluted previously in coating buffer (1:250). Following incubation overnight, excess antibody was removed by washing each well with washing buffer using a 4 MK2 plate washer (Thermo Labsystems, Finland). Blocking buffer (250 µl) was added to each well and incubated for 1-2 hours before the plates were washed again.

Standard or plasma samples (50 µl) were added in duplicate and left to incubate overnight. Following this incubation, plates were washed and 50 µl of rabbit anti-TNFα polyclonal antibody (1:500; Centre of Animal Biotechnology, University of Melbourne, VIC, Australia) was added to each well and incubated for 2 hours. Sheep anti-rabbit Horse Radish Peroxidase (HRP) antibody (50 µl, 1:1000; Anti-rabbit Ig affinity isolated peroxidase conjugated; Silenus Labs Pty. Ltd., VIC, Australia) was added to each well that had been washed previously with washing buffer and incubated for 1 hour. Following a final wash, 100 µl of tetramethylbenzidine (TMB, Zymed, San Francisco, CA, USA) was added to each well and incubated for approximately 10-20 minutes in a darkened container. The reaction of TMB in the presence of TNF-α was stopped by adding 100 µl 0.5 M H_2SO_4. TNF-α concentrations in plasma samples were determined by the quantification of colour absorbency using a Multiskan RC plate reader (Thermo Labsystems, Finland), read at 450 nm, using 630 nm as a reference point. Concentrations were analysed using the Genesis (Version 3.04, Thermo Labsystems, Finland) program. The minimum detectable level of TNF-α was 0.68 ± 0.39 ng/mL (n=4). The intra and interassay coefficients of variance were 7 % (n=4) and 24 % (n=4), respectively.

6.1.2 Plasma Interleukin-6 (IL-6)

Plasma IL-6 concentrations were measured by the ELISA method according to a modification of the methods of McWaters *et al.* (2000).

6.1.2.1 Measurement of IL-6

6.1.2.1.1 General reagents

All reagents and buffers used in these assays are as described previously (Section 6.1.1.1.1). IL-6 ovine recombinant standard (1 μg/mL) was generated and supplied by Dr JP Scheerlinck (Centre for Animal Biotechnology, University of Melbourne, VIC, Australia). Final concentrations of 0.007-5.0 ng/mL in 400 μl of diluent were used in the assays.

6.1.2.1.2 IL-6 ELISA

The methods for the IL-6 ELISA were similar to the methods described for TNF-α with the following differences. The captured antibody (50 μl/well) was a mouse monoclonal anti-ovine IL-6 antibody (Epitope Technologies Pty. Ltd., VIC, Australia), diluted in coating buffer (1:200). The second antibody (50 μl/well) was a rabbit anti-ovine IL-6 polyclonal antibody (1:1000; Epitope Technologies Pty. Ltd., VIC, Australia). The minimum detectable level of IL-6 was 0.35 ± 0.15 ng/mL (n=6). The intra and interassay coefficients of variance were 8 % (n=4) and 19 % (n=4), respectively.

6.1.3 Statistical analyses

Data were analysed, as described in the previous chapter, using SPSS for Windows software (Version 10.0, Chicago, IL, USA). Two-way factorial ANOVAs were used to compare the hypoxia and LPS treatments following assessment of the data for homogeneity. Where a significant interaction was found between the treatments, individual means were compared using Fisher's LSD test. Data (original, untransformed) are presented as mean ± S.E.M. P<0.05 was considered statistically significant. The absence of error bars on some graphs presented in this chapter indicates that they are within the symbol.

6.2 Results

6.2.1 Effect of LPS treatment on cytokine concentrations

Plasma TNF-α concentrations increased significantly (P<0.05) 0.5 hour after LPS administration for lambs at 12 and 15 days of age (Figure 6.2A). This increase was

maximal at 1 hour post-LPS administration with concentrations increasing from 2.24 ± 1.45 ng/ml at time (0) to 76.66 ± 4.52 ng/ml. Thereafter, concentrations declined and returned to pre-treatment levels by 6 hours. Saline treatment had no effect on plasma TNF-α concentrations (Figure 6.2A).

Studies performed on lambs at 12 and 15 days of age revealed an increase in plasma IL-6 concentrations after LPS administration (Figure 6.2B). The increase was evident by 3 hours post-treatment and continued for the duration of the experiment. The effect of LPS treatment was greater in 12 day old lambs, with a higher mean increase observed from 3 hours post-treatment. At 6 hours post-LPS administration, IL-6 concentrations were 4.52 ± 0.58 ng/ml for 12 day old lambs and 1.71 ± 0.54 ng/ml for 15 day old lambs (Figure 6.2B).

6.2.2 Effect of hypoxia and LPS treatments on cytokine concentrations

Hypoxia had no effect on plasma TNF-α concentrations (Figure 6.3A). However, when combined with LPS treatment, there was a significant increase from 0.69 ± 0.52 ng/ml to 84.79 ± 3.84 ng/ml within the first hour. Thereafter, concentrations remained elevated significantly compared to pre-treatment values for the remainder of the experiment. When compared to LPS alone, the effects of the combined LPS and hypoxia treatment on plasma TNF-α concentrations were found to occur 30 minutes earlier (Figure 6.3A). However, the maximal response for increased TNF-α concentration was not different between these two groups.

LPS treatment, with or without hypoxia, increased plasma IL-6 concentrations (Figure 6.3B). In lambs that received the combined LPS/hypoxia treatment, the response was evident within 2 hours post-LPS administration, one hour earlier than that found for LPS alone. For both LPS groups, IL-6 concentrations continued to increase, with maximum levels found at 6 hours post-LPS treatment (Figure 6.3B). These maximum levels were significantly different between the two groups, with levels increasing from undetectable to 4.52 ± 0.58 ng/ml in LPS-treated lambs and from undetectable to 5.47 ± 0.22 ng/ml in the combined LPS and hypoxia-treated lambs. Interestingly, although the combined hypoxia and LPS treatment yielded greater IL-6 responses than LPS alone, the treatment of hypoxia alone had no effect on plasma IL-6 (Figure 6.3B).

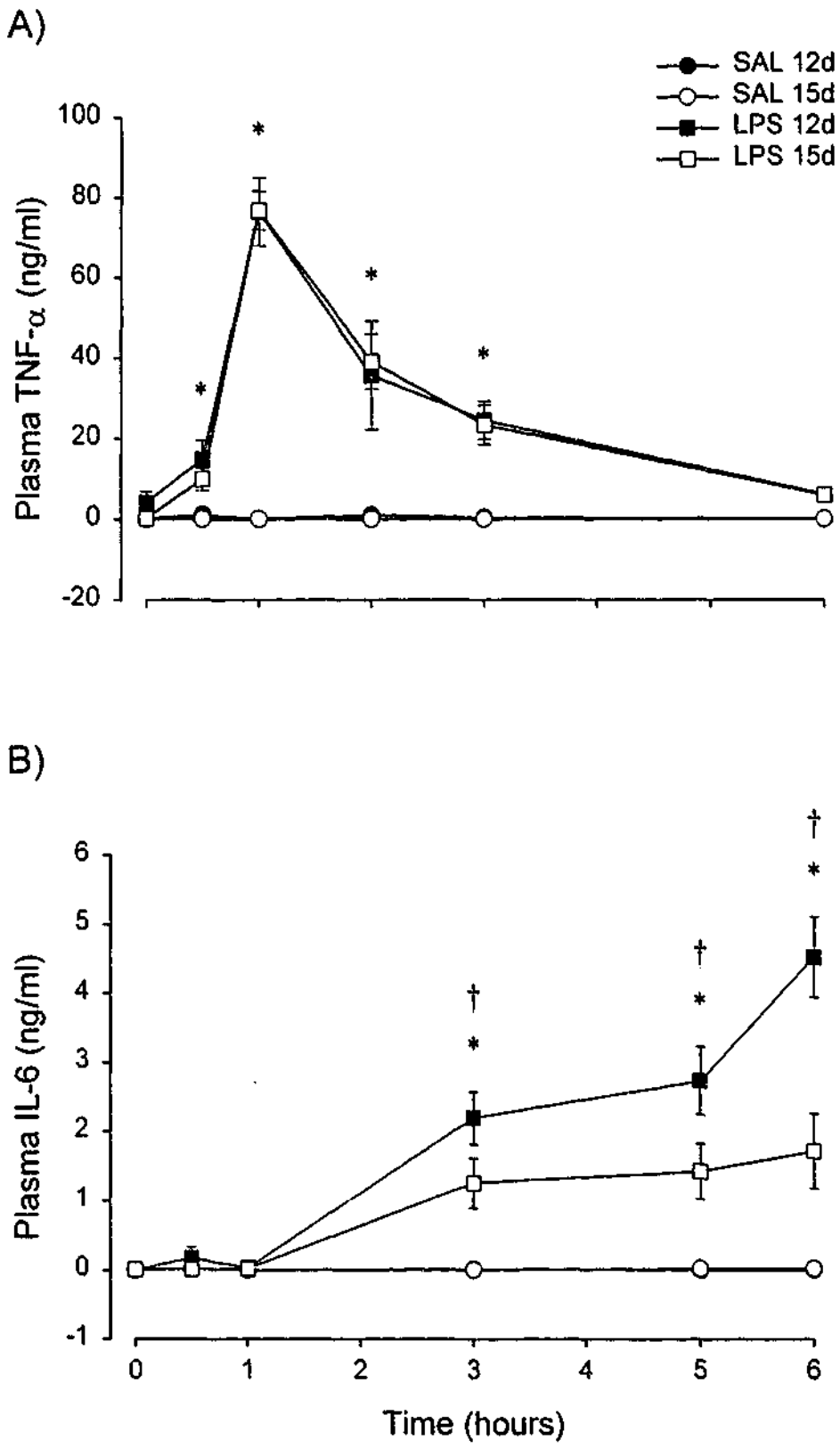


Figure 6.2: Effect of LPS treatment on TNF-α and IL-6 concentrations

Effect of saline or LPS treatment on plasma TNF-α concentrations (A). Effect of saline or LPS treatment on plasma IL-6 concentrations (B). * $P<0.05$ between saline and LPS-treated lambs at 12 and 15 days of age; † $P<0.05$ between LPS-treated lambs at 12 and 15 days of age. Values are $n=4$ for all groups. Data are expressed as mean ± S.E.M. Note the different y-axis scales between Figure A&B.

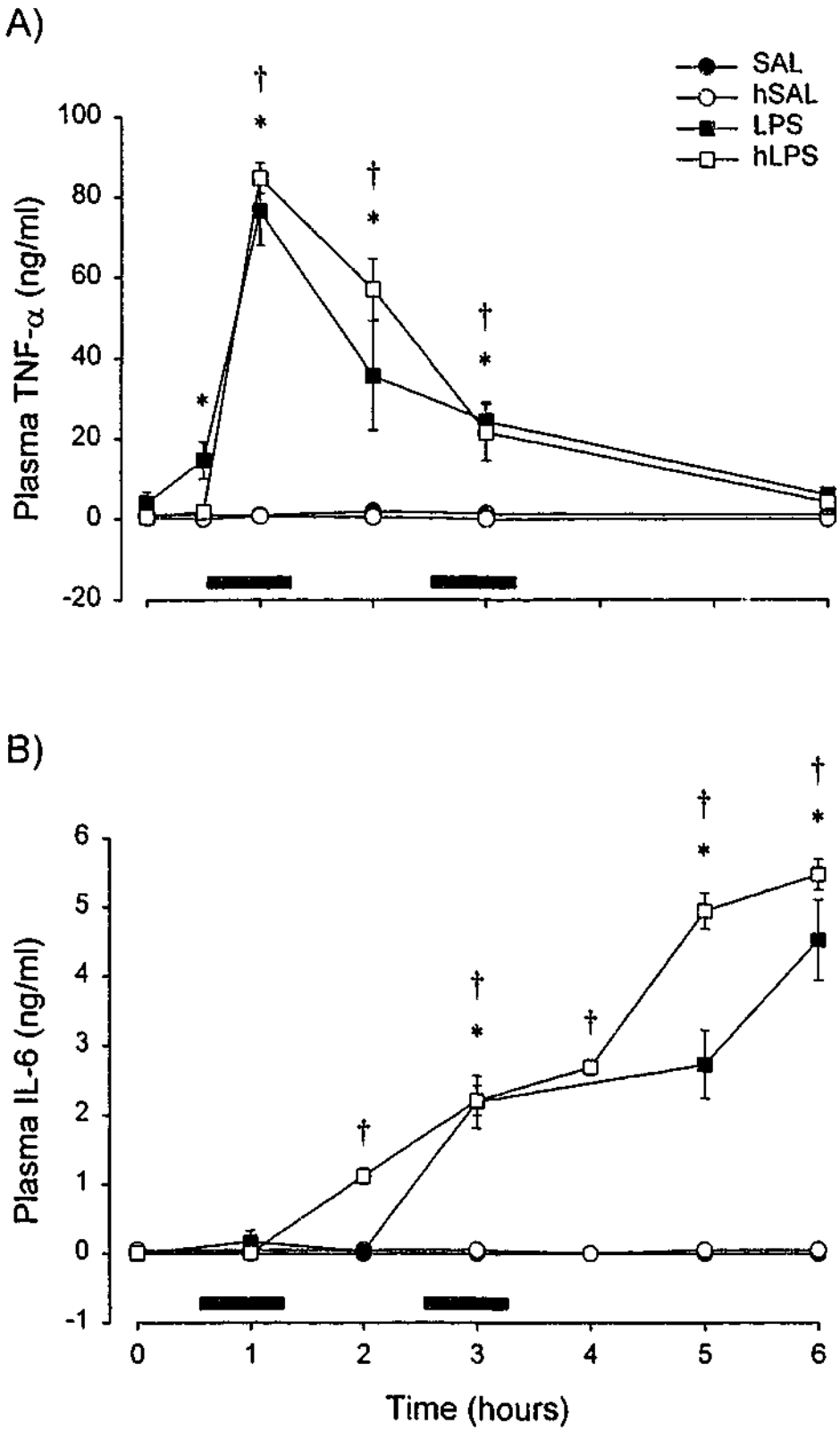


Figure 6.3: Effect of LPS and hypoxia treatments on TNF-α and IL-6 concentrations

Effect of SAL, hSAL, LPS and hLPS treatments on plasma TNF-α concentrations (A). Effect of SAL, hSAL, LPS and hLPS treatments on plasma IL-6 concentrations (B). * $P<0.05$ for LPS-treated lambs compared to time (0); † $P<0.05$ for hLPS-treated lambs compared to time (0). Values are $n=4$ for all groups. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia. Note the different y-axis scales between Figure A&B.

6.3 Discussion

The primary finding of this study is that a combination of LPS and hypoxia increased the concentration of IL-6, but not TNF-α, in plasma samples above that observed with LPS alone. In contrast, hypoxia alone was found to have no effect on either of the cytokines measured, despite reported increases in the incidence of sleep (see Chapters 4 and 5). As allopregnanolone was found to increase in response to hypoxia treatment, these findings suggest that the increased sleep observed in response to hypoxia may involve increased neurosteroids but not increased somnogenic cytokines. The increased somnogenesis observed in lambs treated with LPS (Chapters 3 and 5) may be attributed to a coordinated response between pro-inflammatory cytokines and neurosteroids, however, the involvement of other somnogenic agents cannot be ruled out.

6.3.1 Cytokine responses to LPS treatment

The effect of LPS administration on the host is mediated by the release of cytokines, both systemically and within the brain (Froen *et al.*, 2002). In the present study, the dose of LPS used (0.7μg/kg) was sufficient to cause a rapid and transient release of TNF-α, followed by a more sustained increase of IL-6 in the plasma of lambs. This is consistent with previous work that has reported increases in pro-inflammatory cytokine concentrations in mice (Zuckerman *et al.*, 1991) and humans (McCann *et al.*, 1994) exposed to LPS.

It has been reported that the inhibition of TNF-α production is a trigger for the synthesis and release of IL-6 (Vedder *et al.*, 1999; McWaters *et al.*, 2000). Increased IL-6 and allopregnanolone concentrations may be endogenous inhibitors of cerebral and systemic TNF-α production (Ghezzi *et al.*, 2000; McWaters *et al.*, 2000). In the present study, concentrations of TNF-α reached a maximum 1 hour following LPS treatment and decreased thereafter (Figure 6.2A). During this time, plasma allopregnanolone concentrations had also increased significantly, but remained elevated for the duration of the experiment (Chapter 3). IL-6 concentrations were increased significantly 3 hours following LPS treatment (Figure 6.2B), 2 hours later than those of plasma TNF-α and allopregnanolone. These data suggest, that with the current protocol, TNF-α is or may be inhibited by an endogenous increase in plasma allopregnanolone and that this inhibition causes the release and subsequent rise in IL-6 in plasma. However, further studies are

required to address the role of allopregnanolone, and neurosteroids in general, in the profile of cytokine responses.

Pro-inflammatory cytokines, such as TNF-α and IL-6, are important mediators of the physiological effects of LPS, including behavioural changes (Turnbull & Rivier, 1998). For example, exogenous administration of TNF-α has been found to increase the incidence of NREM sleep (Krueger *et al.*, 1998) and inhibition of this cytokine attenuates various sleep responses (Takahashi *et al.*, 1999). Administration of IL-6 has been reported to elicit fever without an associated increase in sleep (Opp *et al.*, 1989). Studies performed for this thesis found that LPS administration in the lamb increased the incidence of sleep, but this did not occur until 2-3 hours after LPS administration (see Chapter 3), a time when TNF-α levels were returning to control values and IL-6 levels were increasing. Therefore, it is possible that the actions of LPS treatment on sleep-wake profiles are either a result of the persisting effect of TNF-α even after it has been removed from the circulation (Tracey & Cerami, 1994) or a result of the actions of both TNF-α and allopregnanolone.

The effect of repeated LPS exposures on cytokine responses was also investigated. It was found that the TNF-α response was not affected by repeated LPS treatments. In contrast, plasma IL-6 concentrations were found to decrease in response to the second challenge of LPS, despite elevated TNF-α levels. This suggests that, in the current model of bacterial infection, TNF-α may not be required for the stimulation of IL-6 production and release. Other pro-inflammatory cytokines, such as IL-1 (Dinarello, 1992), may be the essential regulators.

6.3.2 Cytokine responses to LPS and hypoxia treatments

Hypoxia and LPS, both stimulators of the neurosteroid response in brain tissue, have been reported to alter cytokine expression. Ziesche *et al.* (1996) investigated the effects of LPS and hypoxia treatment alone, and in combination, on IL-6 mRNA expression in cultured pulmonary arteries. That study reported that treatment with LPS or hypoxia alone increased IL-6 mRNA expression significantly. Further, when these treatments were combined, a greater increase in the transcription of IL-6 mRNA occurred. To the best of my knowledge, the present study is the only one that has investigated the effects of LPS and hypoxia together on cytokine expression. Although Ziesche *et al.* (1996) assessed

mRNA expression, their results can be compared with some of the findings from the current study. The reported increase in IL-6 mRNA expression following hypoxia exposure alone (Ziesche *et al.*, 1996) was not matched by an increase in IL-6 concentration in the current study. The different effects of hypoxia exposure on cytokine mRNA expression and plasma cytokine concentrations between the studies could be due to a number of methodology differences, such as the longer duration of hypoxia used by Ziesche *et al.* (1996; days compared to minutes). Further, the increased mRNA expression may not translate into increased protein content. However, the level of hypoxia used in the studies presented in this thesis was adequate to raise IL-6 concentrations in lambs treated with the combined LPS/hypoxia regime above that of LPS alone. Perhaps the cellular changes occurring as a result of the LPS challenge are compounded by the presence of hypoxia used in these studies.

TNF-α concentrations in plasma were similar in samples from lambs that were treated with LPS alone, or the combination of LPS and hypoxia. There was no effect of hypoxia alone, suggesting that this stressor is not sufficient to elicit an increase in this cytokine. Interestingly, hypoxia alone did increase the incidence of sleep and allopregnanolone concentration in the brain (see Chapter 5). The increased sleep resulting from hypoxia may be due to increased allopregnanolone concentration in the brain, or from an increase of other somnogenic cytokines such as IL-1.

6.3.3 Summary

The response of the CNS to infectious challenge engages a wide range of physiological systems that include alterations in the expression of cytokines, neurosteroids and behaviour. Although the pro-inflammatory cytokines TNF-α and IL-6 have been reported to have some involvement in the repertoire of sickness behaviour, there has been difficulty establishing specific role(s) for these cytokines due to their complex interactions and overlapping biological activities (Leon *et al.*, 1998). Results from the current study demonstrate that treatment with LPS increases both TNF-α and IL-6 concentrations in plasma from lambs. Similar results were found for TNF-α when LPS was combined with hypoxia, while an additional effect was found for IL-6 concentrations. Surprisingly, no effect of hypoxia alone was observed for either cytokine measured. Since both stressors alone and in combination produced increases in allopregnanolone concentrations in the brain and increases in the incidence of sleep, it is suggested that there is a role for

allopregnanolone, along with pro-inflammatory cytokines, in regulating the somnogenic responses to stressful stimuli. TNF-α appears to be an unlikely candidate for affecting somnogenesis after a hypoxia exposure. Other somnogenic cytokines, such as IL-1, which has been shown to be involved in the induction of both TNF-α and IL-6 synthesis (Dinarello, 1992), may play critical roles.

Chapter 7: General discussion

This thesis has focused primarily on the contribution of neurosteroids to behavioural changes in lambs that are induced by stressful stimuli. Neurosteroids have emerged recently as key factors involved in many CNS disorders, such as depression and epilepsy (review Mellon & Griffin, 2002). Although neurosteroids, in particular allopregnanolone, have been identified as having unique somnogenic and sedative properties, the studies reported in this thesis are the first to document the potential somnogenic role of neurosteroids in sleep-related illnesses, particularly those that occur during the period of development (early post-natal) during which sleep occupies the greatest proportion of time (Hobson & Pace-Schott, 2002). The clinical focus of this thesis was to ascertain whether the somnogenic effects of neurosteroids contribute to the aetiology of SIDS. Since infection and hypoxia have been implicated as possible causes for SIDS deaths, these stressors were used and their effects on sleep-wake behaviour and neurosteroid concentrations were determined.

7.1 Allopregnanolone has a major role in regulating CNS activity in the lamb

The present studies investigated the effects of different treatments on neurosteroid concentrations in lambs. Results from studies performed in Chapter 3 reveal for the first time that allopregnanolone is present in higher concentrations in the brain of lambs between 12 and 21 days of age than in plasma. Previous studies investigating the effects of

stress on neurosteroid concentrations have primarily used the adult rat model. Since neurosteroids influence the CNS by binding to $GABA_A$ receptors, and are able to alter sleep-wake behaviour (Lancel *et al.*, 1997; review Muller-Preuss *et al.*, 2002), the use of a more precocial species for this study was required if parallels were to be drawn between the results obtained and the human infant. Plasma allopregnanolone concentrations were found to decrease with advancing age. The cause of this is still unknown and its significance remains to be elucidated, however, it is unlikely to be due to a change in precursor availability, as plasma progesterone concentrations were low or undetectable at all ages examined. Analysis of neurosteroid concentration in the brain at 20 days of age revealed that allopregnanolone concentrations differ dramatically between regions. While allopregnanolone was detected in all regions examined, higher concentrations were found in the pons, thalamus/hypothalamus and cerebellum, and lower concentrations in the midbrain and temporal cortex. This difference in regional distribution is likely to be due to variation in enzyme expression between brain regions with P450scc and 5α-reductase, two enzymes crucial for the synthesis of allopregnanolone, displaying marked regional differences in expression in lambs of similar age (Nguyen *et al.*, 2003).

7.2 LPS increases allopregnanolone concentrations in plasma and brain tissue along with the incidence of sleep

Infection has long been associated with a wide range of behavioural changes including increased sleepiness and drowsiness, both part of the host defence mechanism. In Chapter 3, LPS *E-Coli* was used to mimic the effects of infection by eliciting a wide range of physiological and behavioural responses in the lamb, supporting previous work (Turrin *et al.*, 2001). In addition, this study found that the increased incidence of sleep in young lambs treated with LPS (0.7 μg/kg) was associated with an increase in the concentration of allopregnanolone in both plasma and brain tissue.

The increase in plasma allopregnanolone following LPS treatment occurred without an increase in either pregnenolone or progesterone, suggesting that these precursors were not required for the synthesis of allopregnanolone. However, there was a large increase in plasma cortisol concentration indicating that activation of the HPA axis occurred with this treatment (Faggioni *et al.*, 1995; Takeuchi *et al.*, 1997). As LPS was able to stimulate the activity of the adrenal gland, it is most likely that this steroidogenic organ was responsible for the increase in allopregnanolone observed in the plasma of these lambs, as gonadal

steroidogenesis would be minimal in these sexually immature lambs and unlikely to contribute to allopregnanolone in the circulation.

Allopregnanolone concentration increased in the majority of brain regions examined, however, the degree of change differed substantially between areas. This suggests there is considerable regional variation in the *in situ* production of this steroid. In addition, the increase in allopregnanolone in some regions of the brain, including the cortex and midbrain, was greater relative to that found in plasma suggesting that the central and peripheral responses of neurosteroids are regulated independently. Although it has been reported previously that the brain is capable of producing neurosteroids (review Compagnone & Mellon, 2000), this is the first study that supports the role of local production in the lamb brain. The mechanisms through which LPS produces these changes in allopregnanolone concentration in the brain are still unknown, however, it is unlikely, for the reasons outlined above, that increased allopregnanolone is the result of increased precursor delivery from the periphery. Up-regulation of neurosteroidogenic enzyme gene expression or activity by LPS treatment is more likely, but further studies to measure the potential mechanisms through which LPS alters neurosteroid expression are required.

Several mechanisms, including increased allopregnanolone concentration may contribute to the increased somnolence observed in lambs following LPS treatment. It has been reported that the conversion of progesterone to allopregnanolone, and its subsequent actions at the $GABA_A$ receptor, produce sedation and somnogenesis (Lancel *et al.*, 1997; review Muller-Preuss *et al.*, 2002). The increased sleep observed following LPS treatment might be due to elevated allopregnanolone, since there was no detectable progesterone. Although allopregnanolone has been shown to modulate $GABA_A$ receptor function positively in the lamb brain (Crossley *et al.*, 2000), it has not been determined that the levels found in this study are sufficient to cause increased drowsiness and sleep. Further experiments in which similar concentrations of allopregnanolone are infused into the brain need to be performed. The somnogenic capacity of neurosteroids in response to LPS treatment could be tested by administering finasteride, a 5α-reductase inhibitor, along with LPS, to determine if the incidence of sleep is inhibited or reduced.

7.3 Hypoxia increases the incidence of sleep but only in the presence of increased brain allopregnanolone concentrations

Lambs exposed to moderate hypoxia (Chapter 4) displayed similar responses to those observed following LPS treatment (Chapter 3), i.e. increases in the incidence of sleep and the concentration of allopregnanolone in many of the brain regions examined. Surprisingly, the significant increase in plasma cortisol concentration, indicating activation of the HPA axis, was not accompanied by a change in plasma allopregnanolone. The absence of a response in plasma allopregnanolone could be due to a number of factors from a shift in the utilisation of precursors to the formation of other neurosteroids such as TH-DOC. Alternatively, since neurosteroids ave been implicated as having neuroprotective functions (review Compagnone & Mellon, 2000; review Mellon & Griffin, 2002), a greater metabolism of allopregnanolone may have occurred under these hypoxic conditions. Despite there being no change in plasma allopregnanolone concentration in response to hypoxia, the observed increase in brain allopregnanolone concentration following hypoxia does provide additional support for the differential regulation of neurosteroidogenesis between the brain and plasma in lambs.

7.4 Combined LPS and hypoxia treatments have an additive effect on brain allopregnanolone concentrations but not on sleep

The individual effects of LPS and hypoxia on sleep-wake architecture have been well documented (Johnston *et al.*, 1998; Schiffelholz & Lancel, 2001). However, little is known about specific CNS substance(s) that may be involved in augmenting the behaviour. Therefore, a primary aim of this thesis was to identify neurosteroids as potential contributors to the somnolence induced by these stressful stimuli. Since LPS and hypoxia were both found to increase the incidence of sleep and increase allopregnanolone concentration in the brain, further studies (Chapter 5) examined the potential additive effects of these stressors on neurosteroid concentrations and sleep-wake behaviour.

The results from experiments performed in Chapter 5 show that when LPS and hypoxia treatments were combined, greater increases in brain allopregnanolone concentration were observed for most regions examined, with no change in the incidence of sleep, when compared to LPS alone. Although allopregnanolone concentration in the

thalamus/hypothalamus, a key brain region that regulates sleep, was not affected by the individual treatments, there was a significant increase in response to the combined treatments. Therefore, it was reasonable to expect that there would be an additional increase in the incidence of sleep. As discussed in Chapter 5, this lack of an additive action of the combined treatments on the degree of somnolence could result from the already low levels of wakefulness, which could not be reduced any further. Hypoxia has been reported to affect arousal thresholds during sleep (Johnston *et al.*, 1998). The current studies measured the incidence of sleep-wake behaviour, therefore, an overall picture of the somnogenic capacity of neurosteroids has not been obtained. Further studies that include measurements of arousal thresholds are required.

7.5 TNF-α does not contribute to somnolence produced by hypoxia

The primary line of defence in response to infection is the endogenous production and release of cytokines which, in turn, can initiate a range of physiological responses including increased sleep (Dantzer, 2001). The possible contribution of pro-inflammatory cytokines along with neurosteroids to the somnolence observed following LPS and/or hypoxia treatments was investigated. Since allopregnanolone has been reported to inhibit TNF-α expression (Ghezzi *et al.*, 2000), it was hypothesised that the large increase in allopregnanolone concentration in response to the combined LPS and hypoxia treatments would reduce or inhibit the increase in TNF-α concentration and, subsequently, attenuate the effect on sleep as was reported in Chapter 5. The combined treatment of LPS and hypoxia produced an additive effect on the increased levels of IL-6, however, this was not found for TNF-α. This supports the potential inhibitory role of allopregnanolone on TNF-α concentrations. The treatment of hypoxia alone did not affect the concentration of either cytokine suggesting that TNF-α (in this case) does not contribute to the somnolence observed following hypoxia exposure. Other pro-inflammatory cytokines, such as IL-1β, which have been shown to induce somnolence and pro-inflammatory cytokine synthesis (Dinarello, 1992), may play more important roles during stress-induced somnogenesis.

7.6 Neurosteroids may contribute to the aetiology of SIDS

SIDS remains one of the leading causes of death in infants less than one year of age. In Australia, it accounts for approximately 140 deaths per year, or at least two deaths per week, making further studies into the causes of this syndrome crucial. SIDS is widely considered to be a phenomenon related to sleep. Infection (Kerr *et al.*, 2000) and hypoxia (Jones *et al.*, 2003) have been implicated as possible mechanisms contributing to these deaths. This thesis hypothesised that neurosteroids might provide a critical link between the stress of infection and/or hypoxia and the eventual death of these infants. The data obtained suggest that the young lamb brain is particularly susceptible to even mild challenges of bacterial infection and hypoxia. While it might be argued that increased neurosteroid production is part of an adaptive response to stressful challenges, it is also possible, considering the findings of the combined LPS and hypoxia study (Chapter 5), that large increases in neurosteroids may dampen appropriate arousal responses (Figure 7.1). While it cannot be demonstrated conclusively that neurosteroids are a key link responsible for SIDS deaths, the results of the present studies do provide support for the involvement of a neurosteroid mechanism. Future studies would need to examine arousal thresholds in response to LPS and/or hypoxia, as well as in response to neurosteroid administration. If neurosteroids are capable of dampening arousal mechanisms, several steroidal compounds and commercially available drugs that have been shown to inhibit neurosteroid production could be used to prevent the increase in neurosteroid concentrations in the brain. In so doing, these drugs may reduce the sleepiness of infants during infectious and or hypoxia challenges and, hence, reduce the risk of SIDS.

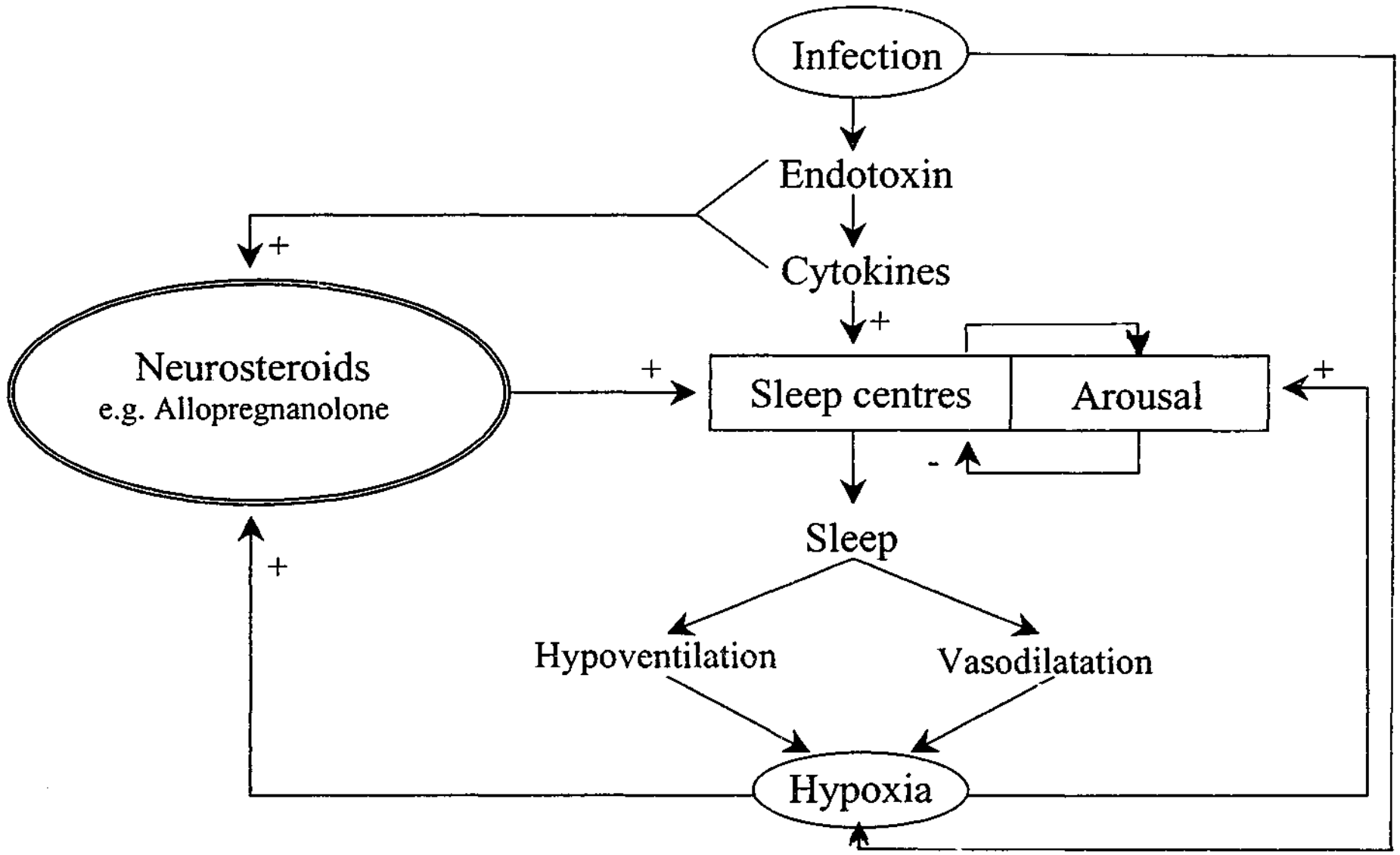


Figure 7.1: Possible pathways whereby neurosteroids inhibit arousal mechanisms

7.7 Concluding remarks

In summary, the findings of this thesis provide insight into the regulation of neurosteroid concentrations in response to LPS and hypoxia treatments, both of which are common factors contributing to disease. The results suggest that neurosteroids may contribute to the somnolence experienced as a result of LPS and hypoxia exposure, however, it remains to be elucidated whether the response is protective or detrimental to the newborn. Further studies assessing the effects of neurosteroids on arousal mechanisms may shed some light on the possible role of this unique group of compounds in contributing to SIDS deaths.

Chapter 8: Appendices

8.1 Birth and post-mortem weights of lambs used in this thesis

Gestational ages and birth weights of lambs were not different from each other (Table 8.1). Daily weight gain was reduced significantly in both LPS-treated groups, compared to saline controls (Table 8.1). Hypoxia alone had no effect on this parameter. Post-mortem, lambs treated with LPS alone weighed significantly ($P<0.05$) less and had smaller brain and adrenal weights than saline-treated controls (Table 8.1). Hypoxia exposure had no effect on body and tissue weights regardless of saline or LPS treatment. Significant ($P<0.05$) differences were found between the two LPS-treated groups for all parameters examined, except body weight. However, when organ weights were corrected for body weight, the significant effect of LPS treatment alone on organ weights was lost.

Table 8.1: Birth and post-mortem weights of lambs used in this thesis

	Treatment			
Parameter	***SAL (n=5)***	***hSAL (n=6)***	***LPS (n=7)***	***hLPS (n=7)***
Gestational age (days)	146.40 ± 1.60	145.83 ± 0.50	146.14 ± 0.34	144.29 ± 0.94
Birth weight (kg)	5.16 ± 0.32	4.11 ± 0.34	4.25 ± 0.11	4.51 ± 0.41
Daily weight gain (kg)	0.29 ± 0.02 ^{c}	0.25 ± 0.01 ^{bc}	0.18 ± 0.02 ^{a}	0.23 ± 0.01 ^{b}
Weight at post-mortem (kg)	10.34 ± 0.42 ^{b}	9.35 ± 0.51 ^{ab}	7.91 ± 0.33 ^{a}	9.33 ± 0.62 ^{ab}
Brain weight (g)	69.15 ± 2.40 ^{b}	66.22 ± 2.23 ^{b}	53.65 ± 3.80 ^{a}	65.45 ± 2.54 ^{b}
Right adrenal weight (g)	0.67 ± 0.03 ^{b}	0.61 ± 0.01 ^{ab}	0.52 ± 0.04 ^{a}	0.64 ± 0.03 ^{b}
Left adrenal weight (g)	0.68 ± 0.03 ^{b}	0.63 ± 0.01 ^{ab}	0.54 ± 0.05 ^{a}	0.66 ± 0.04 ^{b}

Letters indicate significant (P<0.05) differences between treatment groups. Data are expressed as mean ± S.E.M.

8.2 Additional results from Chapter 5

8.2.1 Effect of treatment on temperature and plasma iron concentrations

Saline treatment, with or without hypoxia, had no effect on rectal temperature despite significant differences in basal values between normoxic and hypoxic lambs (Figure 8.1). LPS treatment, on the other hand, produced the characteristic biphasic increase in rectal temperature, with or without hypoxia (Figure 8.1). The peak febrile response was significantly (P<0.05) higher for LPS alone (1.30 ± 0.34 °C) than for lambs treated with LPS and hypoxia (0.89 ± 0.19 °C), however, the LPS-induced temperature profiles for these two groups of lambs overall were not different from each other.

Plasma iron concentrations decreased in response to LPS treatment with (~28 %) or without (~29 %) hypoxia, 6 hours after LPS administration (Figure 8.2). Saline treatment, with or without hypoxia, had no effect.

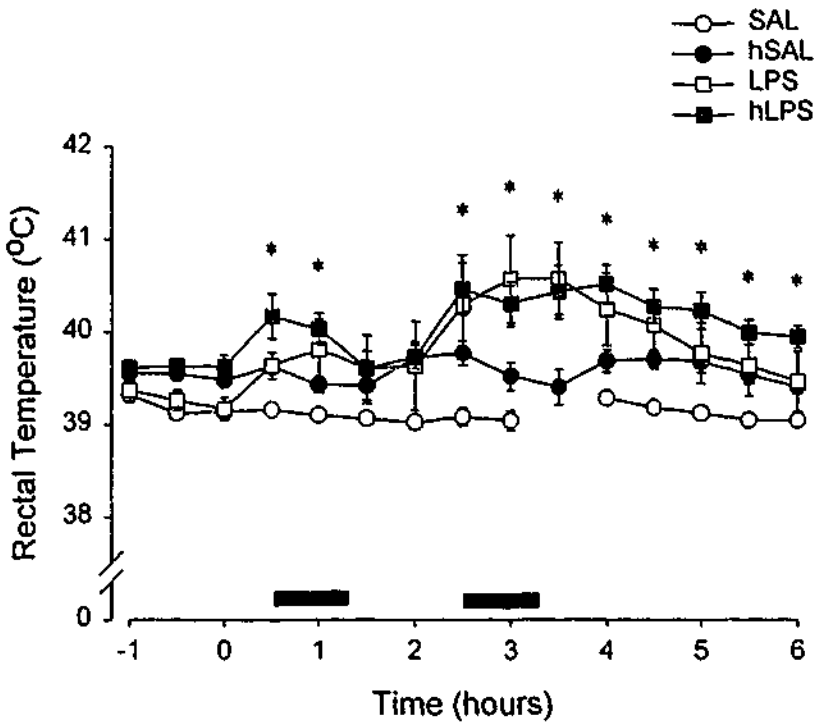


Figure 8.1: Effect of treatment on rectal temperature

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) and hLPS (n=7) treatments on rectal temperature. * P<0.05 for LPS-treated lambs compared to time (0). Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

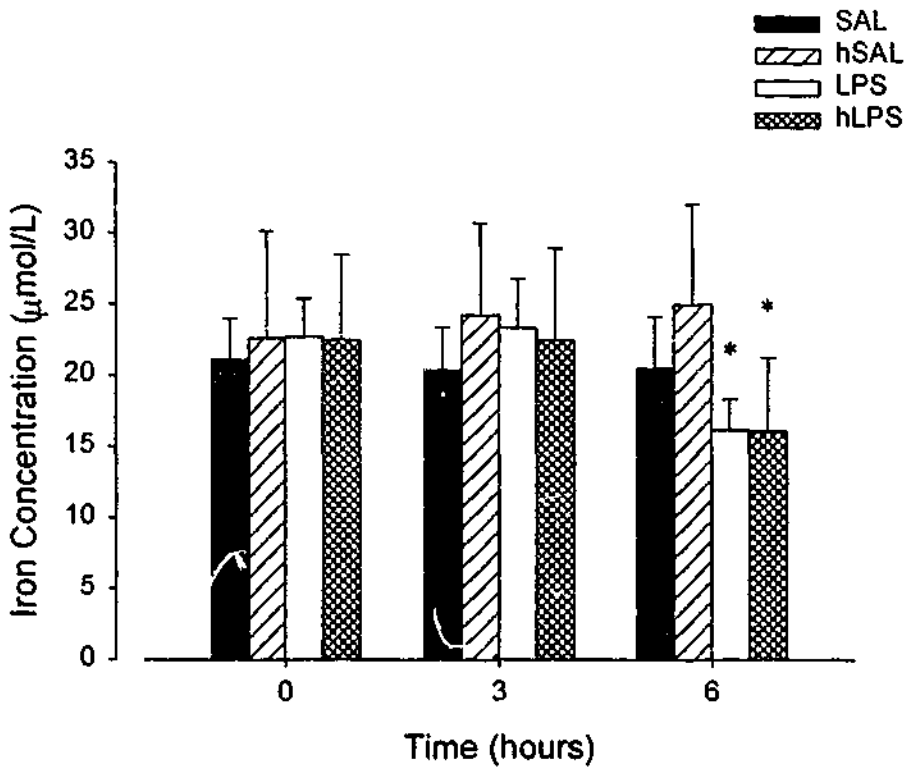


Figure 8.2: Effect of treatment on plasma iron concentrations

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) and hLPS (n=7) treatments on plasma iron concentrations. * P<0.05 for LPS-treated lambs compared to time (0). Data are expressed as mean ± S.E.M.

8.2.2 Effect of treatment on blood gases and pHa

Hypoxia exposure resulted in significant reductions in SaO_2, PaO_2 and $PaCO_2$ (Figure 8.3 A-C). The changes observed were not different between saline and LPS-treated lambs and were not different between the two hypoxia exposures. SaO_2 and PaO_2 were decreased significantly by ~50 % and ~71 %, respectively, during each hypoxia episode but returned to control values between episodes and within 15 minutes of the end of the second hypoxia episode (Figure 8.3 A&B). $PaCO_2$ was reduced significantly by ~33 % during each hypoxia challenge (Figure 8.3C), however, $PaCO_2$ concentrations did not return to control levels between episodes for either group. $PaCO_2$ remained significantly lower than control values (pre-treatment) for the remainder of the experiment. Changes in arterial pH were observed with increases ($P<0.05$) during each hypoxia episode that were similar for both saline and LPS-treated lambs (Figure 8.3D). Haemoglobin was not affected by hypoxia treatment. Saline treatment had no effect on any parameter measured, whilst LPS alone resulted in small but significant decreases in O_2 saturation (Figure 8.3A) and PaO_2 (Figure 8.3B) at 0.5 hours, and an increase in $PaCO_2$ (Figure 8.3C) at 1 hour post-LPS treatment. Normoxic lambs treated with LPS showed no change in pHa (Figure 8.3D) or Hb.

8.2.3 Effect of treatment on plasma glucose and lactate concentrations

Saline, with or without hypoxia, had no effect on plasma glucose concentrations for the duration of the experiment (Figure 8.4A). Glucose concentrations increased in both LPS-treated lamb groups 1 hour after LPS injection. Thereafter, concentrations decreased and remained lower than pre-treatment values for the remainder of the experiment (Figure 8.4A). Lactate concentrations for lambs treated with saline followed by hypoxia showed an increase of ~73 % for both episodes with concentrations returning to normal between and after the hypoxia challenges (Figure 8.4B). The increases in plasma lactate concentration were similar between the individual hypoxia and LPS treatments with concentrations increasing 1 hour after saline or LPS injection and returning to control values by 6 hours. The combined treatment of LPS followed by hypoxia resulted in a further increase in lactate concentration than that found for either treatment alone (Figure 8.4B). Lactate concentrations were still elevated at 6 hours post-LPS treatment for this group. Saline treatment in normoxic lambs had no effect on plasma lactate concentrations (Figure 8.4B).

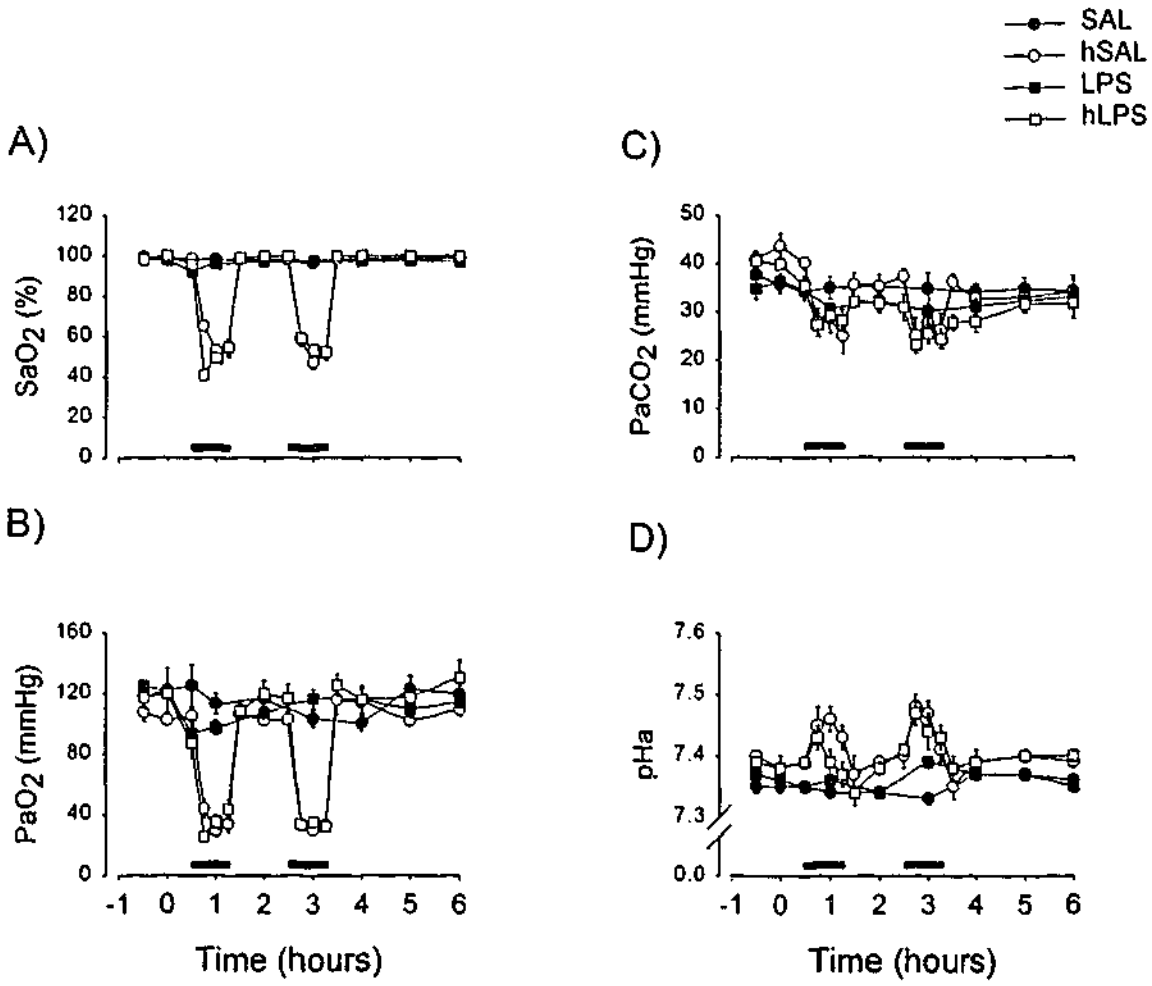


Figure 8.3: Effect of treatment on blood gases and pHa

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) and hLPS (n=7) treatments on SaO_2 (A), PaO_2 (B), $PaCO_2$ (C) and pHa (D). Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

8.2.4 Effect of treatment on cardiorespiratory parameters

Figure 8.5D summarises the effect of the different treatments on heart rate. Saline treatment alone had no effect on heart rate, whereas all other groups had variable responses to treatment. Heart rate increased in a biphasic manner with peak responses observed at 2 and 4.5 hours following saline or LPS injection. Heart rate remained elevated for the duration of the experiment in all groups except saline controls. Blood pressure was significantly higher in hypoxic lambs compared to normoxic lambs for the course of the experiment (Figure 8.5B). However, despite some variability, there was no effect of treatment on blood pressure. Respiratory effort, although variable, was not affected by any treatment over time (Figure 8.5C).

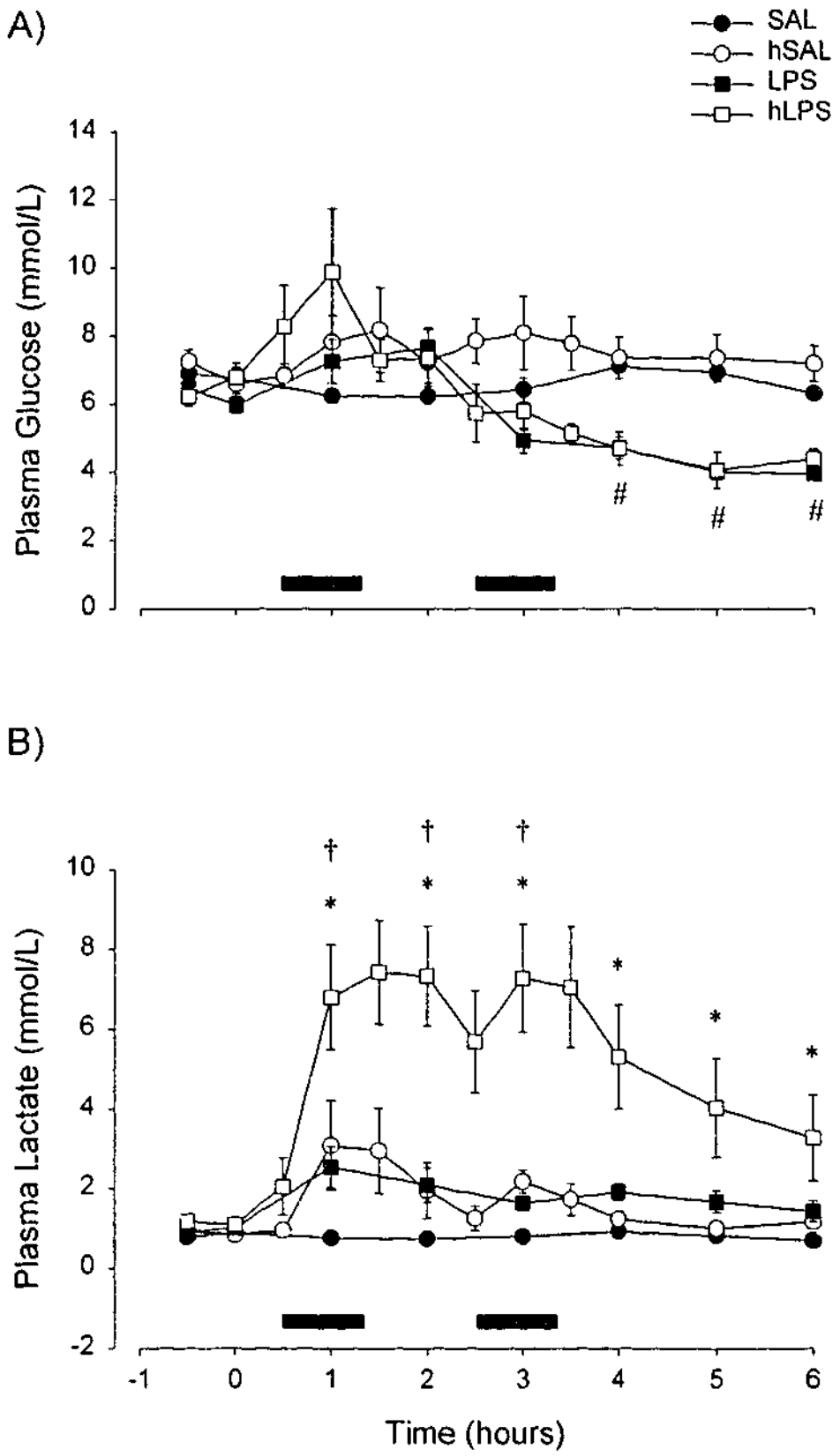


Figure 8.4: Effect of treatment on plasma glucose and lactate concentrations

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) and hLPS (n=7) treatments on glucose (A) and lactate (B) concentrations. * P<0.05 between SAL and hSAL; † P<0.05 between LPS and hLPS; # P<0.05 for LPS-treated lambs compared to time (0). Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia. Note the different scales between Figures A&B.

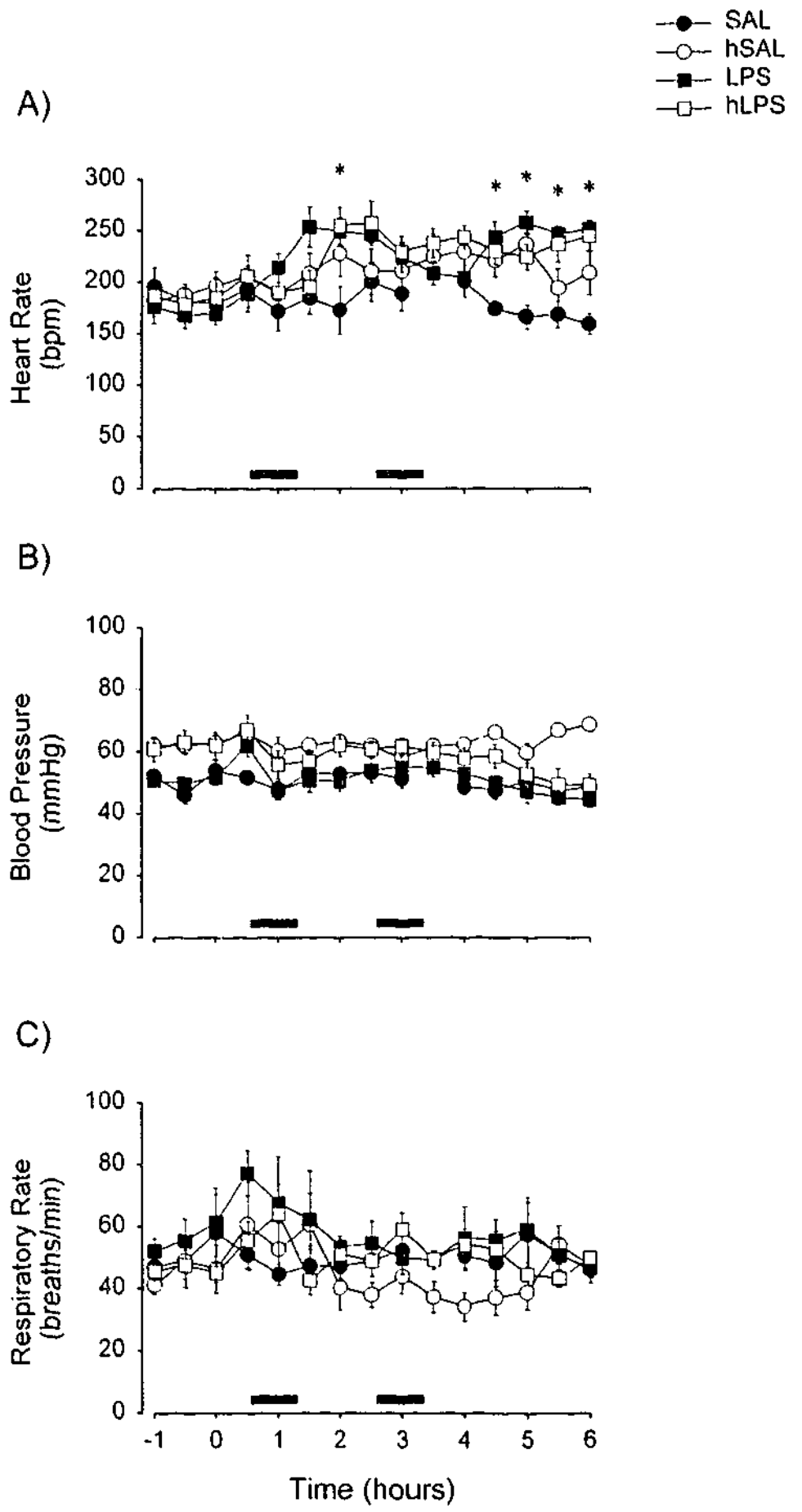


Figure 8.5: Effect of treatment on cardiorespiratory parameters

Effect of SAL (n=5), hSAL (n=6), LPS (n=7) and hLPS (n=7) treatments on heart rate (A), blood pressure (B) and respiratory rate (C). * P<0.05 for all treatment groups compared to saline controls. Data are expressed as mean ± S.E.M. *Black bars* represent periods of hypoxia.

8.2.5 Effect of treatment on somnogenic behaviour over time

Lambs that were treated with hypoxia alone showed a significant ($P<0.05$) decrease in the incidence of wakefulness compared to pre-treatment levels (Figure 8.6A). The reduction occurred prior to the first challenge and was maintained until the second challenge, upon which there was a further reduction. The reduced incidence of wakefulness during the first hypoxia challenge was associated with concomitant increases in QS (Figure 8.7B) and IS (Figure 8.9B). AS did not change from pre-treatment values during this period (Figure 8.8B). The additional reduction in wakefulness during the second hypoxia challenge was associated with increases in the incidences of QS and AS. No change in the incidence of IS was observed for this period.

LPS treatment alone induced a variable response in wakefulness for the duration of the experiment (Figure 8.6C). LPS treatment resulted in a reduced incidence of wakefulness that was evident at 0.5 hour post-LPS administration. A further reduction 3 hours post-LPS administration was also found. The decrease in the incidence of wakefulness due to LPS treatment was associated with concomitant increases in QS (Figure 8.7C) and IS (Figure 8.9C). No change in AS was observed following LPS treatment (Figure 8.8C). The combined treatment of LPS and hypoxia reduced the incidence of wakefulness significantly for both hypoxia episodes (Figure 8.6D). The level of reduction in wakefulness was not different between the two challenges, however, a further reduction was observed following the second challenge when compared to pre-treatment levels. The reduced wakefulness was associated with an increase in QS only (Figure 8.7D) that was similar between each challenge. During each episode of hypoxia, AS (Figure 8.8D) and IS (Figure 8.9D) were not different to pre-treatment control levels in LPS-treated lambs.

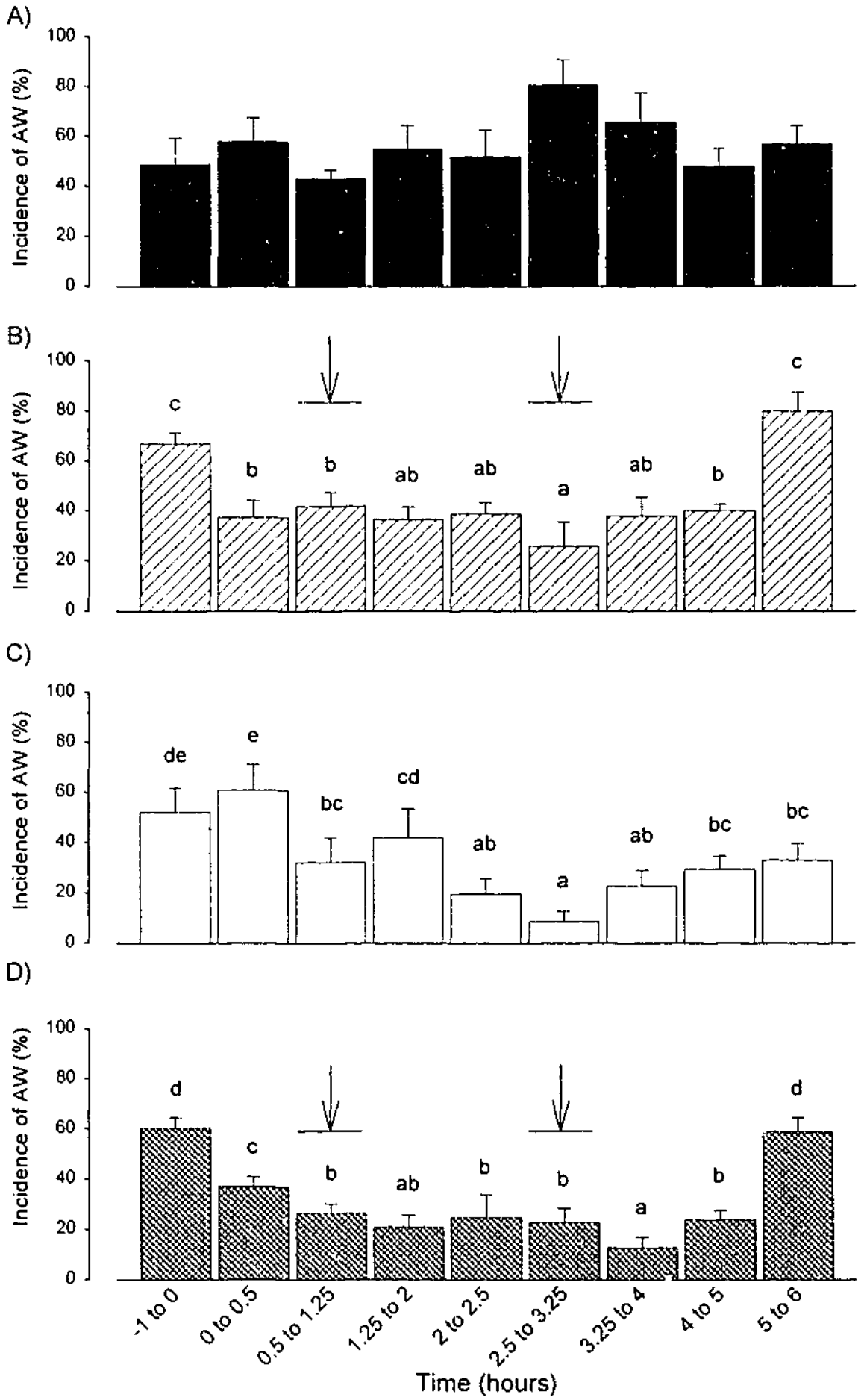


Figure 8.6: Effect of treatment on wakefulness

Effect of SAL (A; n=5), hSAL (B; n=6), LPS (C; n=7) or hLPS (D; n=7) treatments on the incidence of wakefulness (AW) in variable epochs. Letters indicate significant (P<0.05) differences between epochs within each group. Data are expressed as mean ± S.E.M. Arrows represent periods of hypoxia.

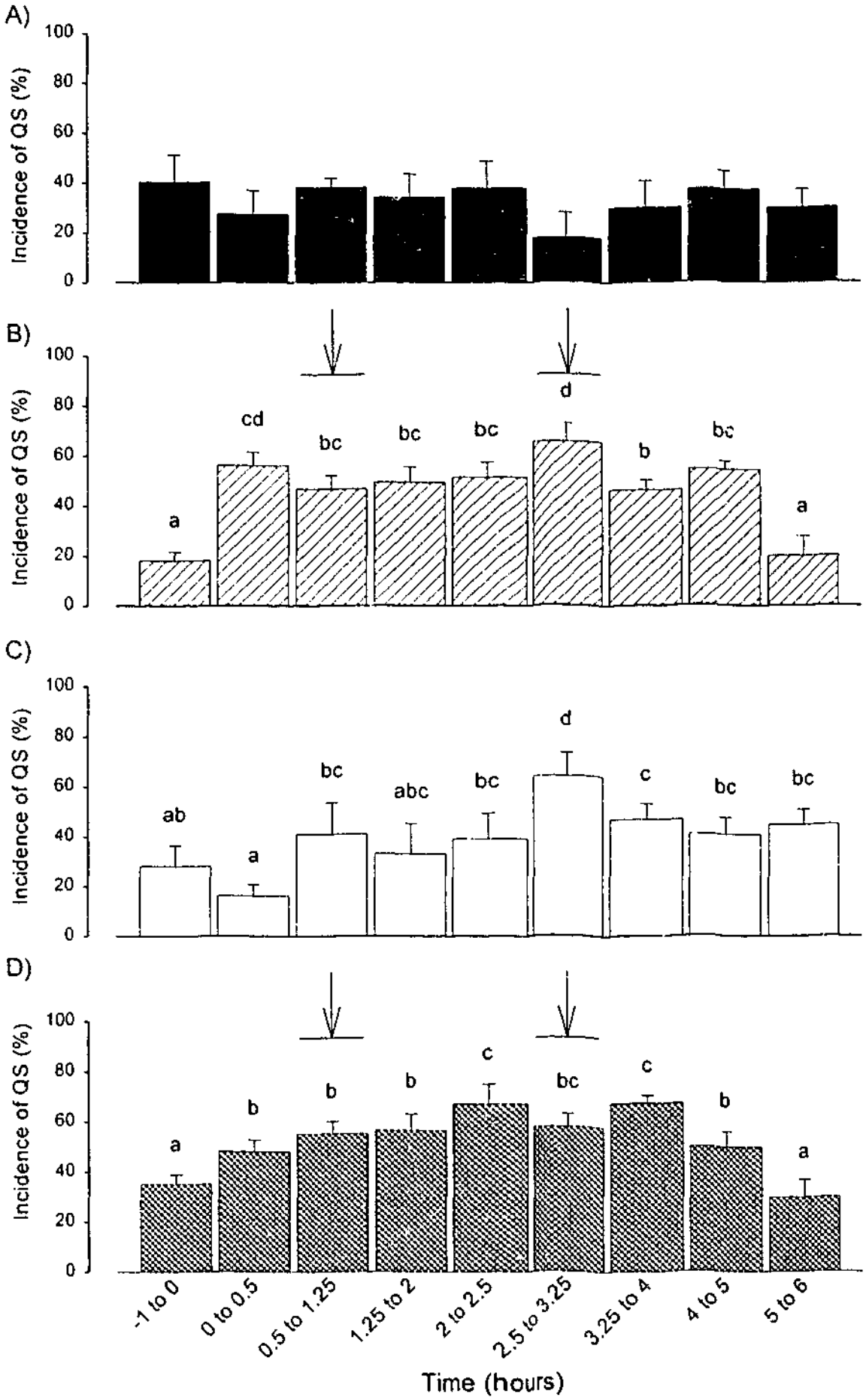


Figure 8.7: Effect of treatment on quiet sleep

Effect of SAL (A: n=5), hSAL (B; n=6), LPS (C; n=7) or hLPS (D; n=7) treatments on the incidence of quiet sleep (QS) in variable epochs. Letters indicate significant (P<0.05) differences between epochs within each group. Data are expressed as mean ± S.E.M. Arrows represent periods of hypoxia.

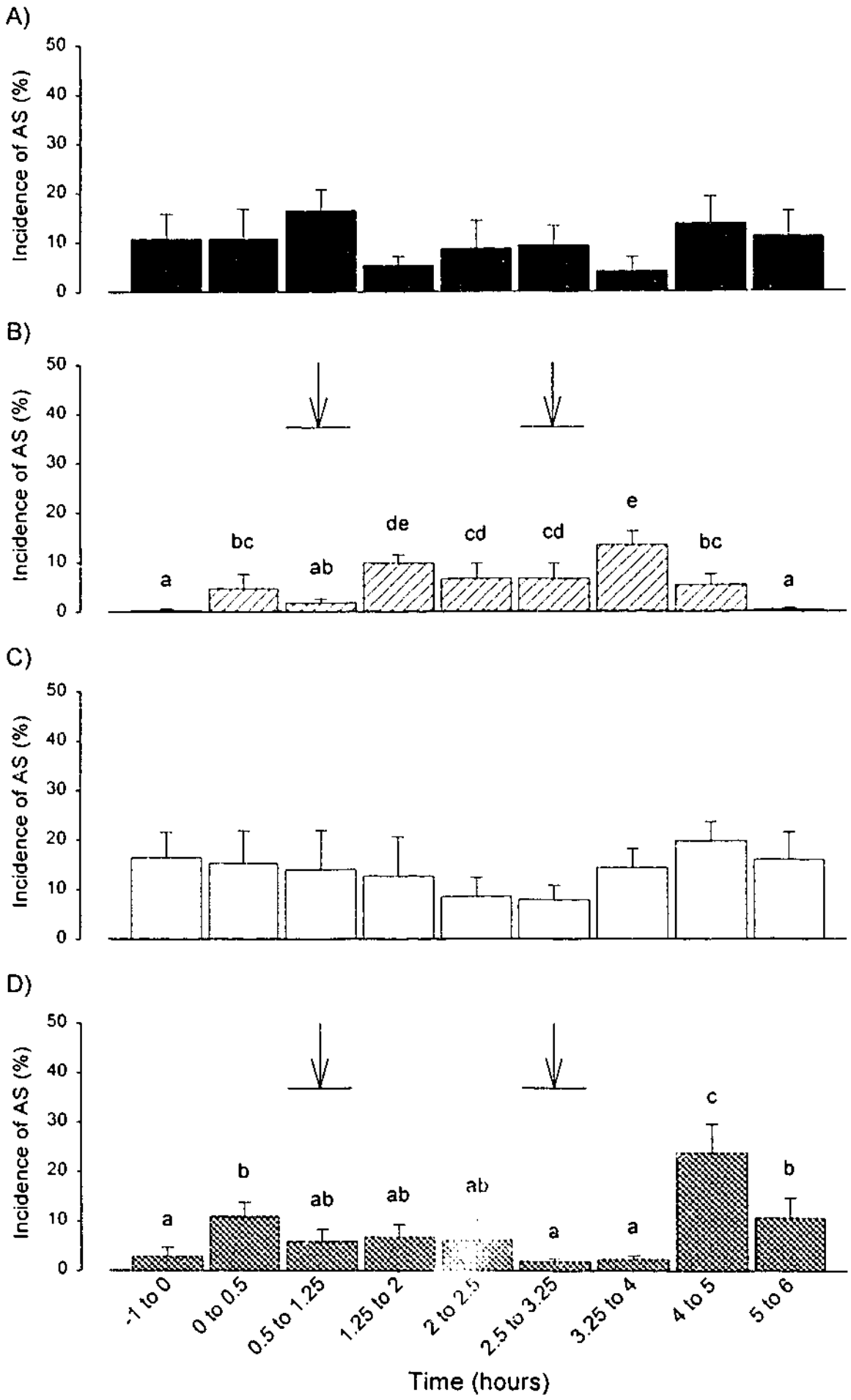


Figure 8.8: Effect of treatment on active sleep

Effect of SAL (A; n=5), hSAL (B; n=6), LPS (C; n=7) or hLPS (D; n=7) treatments on the incidence of active sleep (AS) in variable epochs. Letters indicate significant (P<0.05) differences between epochs within each group. Data are expressed as mean ± S.E.M. Arrows represent periods of hypoxia.

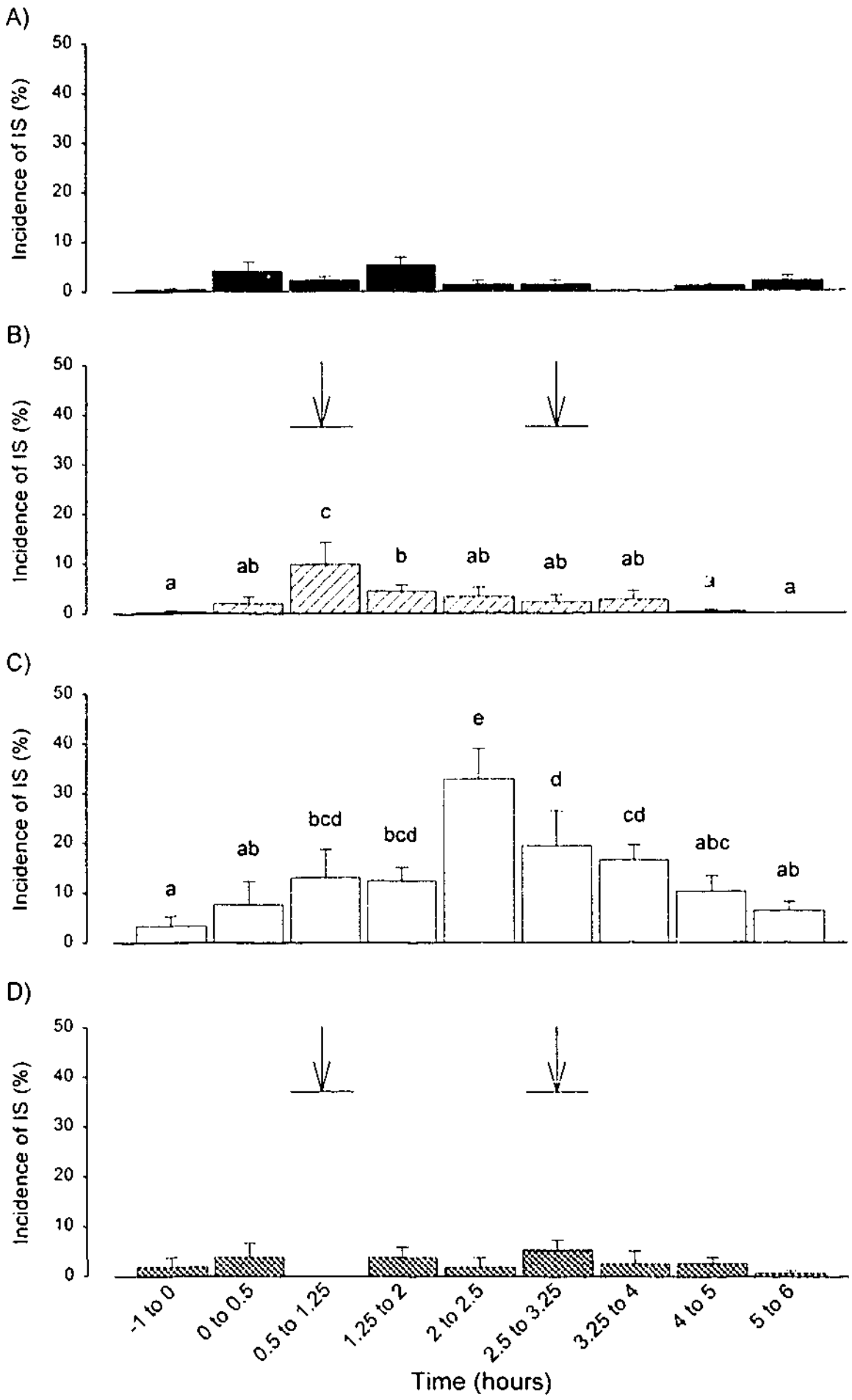


Figure 8.9: Effect of treatment on indeterminate sleep

Effect of SAL (A; n=5), hSAL (B; n=6), LPS (C; n=7) or hLPS (D; n=7) treatments on the incidence of indeterminate sleep (IS) in variable epochs. Letters indicate significant (P<0.05) differences between epochs within each group. Data are expressed as mean ± S.E.M. Arrows represent periods of hypoxia.

Table 8.2: Effects of treatment on physiological parameters, prior to tissue collection

	Treatment			
Parameter	***SAL (n=5)***	***hSAL (n=6)***	***LPS (n=7)***	***hLPS (n=7)***
Temperature (^{o}C)	39.06 ± 0.07	39.63 ± 0.10	40.84 ± 0.42*†	40.06 ± 0.19*†
Iron ($\mu mol/L$)	10.51 ± 2.79	10.59 ± 1.30	13.54 ± 3.20	13.23 ± 3.09
Glucose (mmol/L)	6.30 ± 0.18	8.10 ± 1.08*†	4.67 ± 0.35	5.80 ± 0.56
Lactate (mmol/L)	0.80 ± 0.08	1.75 ± 0.70*†	2.46 ± 0.82*†	4.71 ± 1.83*†
Heart rate (bpm)	184.00 ± 8.35	234.94 ± 16.60*†	216.57 ± 14.80*	204.57 ± 13.12
Blood pressure (mmHg)	54.53 ± 1.28	63.27 ± 4.20	54.67 ± 1.87	61.68 ± 2.51
Respiratory rate (breaths/min)	44.20 ± 5.26	49.57 ± 5.17	58.00 ± 5.15	58.74 ± 8.66

Data are expressed as mean ± S.E.M for the period 3 hours after saline or LPS administration, just prior to tissue collection. Saline alone had no effect on any parameter measured. Hypoxia treatment alone resulted in hypoglycaemia and tachycardia. LPS treatment alone resulted in hyperthermia and tachycardia. The combined treatment of LPS and hypoxia resulted in hyperthermia. All treatments, except for saline control, also increased plasma lactate concentrations. * $P<0.05$ within each treatment compared to its own time (0; data not shown); † $P<0.05$ between treatment groups compared to saline only.

8.3 Additional discussion from Chapter 5

8.3.1 Temperature and plasma iron responses to combined hypoxia and LPS treatments

The biphasic hyperthermic effects of LPS treatment have been described in Chapter 3. The rise in temperature is a result of endogenous pyrogen release which, in turn, precipitates the release of prostaglandins and cytokines (Romanovsky & Blatteis, 1995; Miller *et al.*, 1997). Hypoxia, on the other hand, results in a reduction in body temperature (Almeida *et al.*, 1999). Therefore, treatment with LPS or hypoxia has opposing effects on the thermal regulation of the host. In this study, hypoxic saline-treated lambs were found to have a slight reduction in body temperature that occurred within thirty minutes of each episode, although this change was not significant. When LPS and hypoxia treatments were combined, the biphasic hyperthermic effect that is characteristic of LPS treatment was still evident. However, the mean increase in rectal temperature was reduced significantly for these lambs compared to LPS alone. This observation suggests that the degree of hypoxia used was sufficient to slightly reduce the severity of the febrile response to LPS.

Plasma iron, a peripheral marker of fever (Goelst & Laburn, 1991), was found to decrease in LPS-treated lambs, with or without hypoxia, 6 hours following LPS administration. This reduction is consistent with a previous study, which reported that a reduced iron concentration in response to infectious challenge is beneficial in minimising bacterial growth (Kluger & Rothenburg, 1979). Hypoxia, on the other hand, has been reported to have opposing effects to LPS by increasing circulating iron concentrations (Raja *et al.*, 1990). However, this was not found in the current study and may reflect the moderate degree of hypoxia used in this study.

8.3.2 Blood and plasma responses to hypoxia and LPS treatments

In this study, moderate hypoxia was induced in lambs that received either saline or LPS treatment (Figure 8.3). Blood gases and pHa were similar between treatment groups and between episodes of hypoxia. The combined treatment had no additional effect on any parameter measured and, likewise, the second hypoxia challenge had no additive effect on the values obtained during the first challenge for these parameters. Although LPS alone produced an initial O_2 desaturation (at 30 minutes post-LPS administration; see Section 3.2.2.2), this was found to have no additional effect on SaO_2 levels in lambs treated with

LPS and hypoxia, suggesting that LPS in this case does not contribute to the hypoxic conditions experienced by the lambs. Thus, lambs that were exposed to two episodes of hypoxia were found to be hypoxaemic, hypocapnic and hypocarbic. These lambs were also tachycardic indicating that they had an increased cardiac output that contributed to the reduced PaO_2 since there was no evidence of an increased respiratory rate. As reported in Chapter 4, hypoxic lambs also experienced an increased pHa. Along with the decreased $PaCO_2$, the increase in pHa is consistent with the induction of respiratory alkalosis.

Lactate levels increased in response to both hypoxia and LPS challenges. The hyperlactaemia observed following hypoxia treatment supports findings from a previous study (Parer, 1998). This study, however, reported the increase in lactate as a consequence of anaerobic metabolism that was associated with a reduced pHa. As this was not found in this study, or in the studies of Chapter 4, the mechanisms involved in producing the increased lactate remain unclear. LPS alone increased lactate concentrations to a similar degree as those observed with hypoxia alone, without the significant changes in oxygenation. This finding supports previous work by Gore *et al.* (1996), who reported that lactate accumulation during septicaemia is a consequence of a significant increase in the rate of pyruvate production rather than a lack of tissue oxygenation. Therefore, a potential increase in pyruvate production due to LPS treatment, in association with a reduced oxygenation due to hypoxia treatment, could account for the additional increase in plasma lactate concentration in lambs treated with LPS and hypoxia combined.

8.4 Individual animal data for LPS studies

Table 8.3: The histories of saline-treated and LPS-treated lambs

Animal #	*Treatment*	*Sex*	*Single/Twin*	*Birth weight (kg)*	*Daily weight gain (kg)*
7	SAL	M	S	4.40	0.32
8	SAL	M	S	5.70	0.31
9	SAL	M	T	4.50	0.26
10	SAL	M	S	6.00	0.33
11	SAL	F	S	5.20	0.22
4	LPS	M	T	3.75	0.21
5	LPS	M	T	4.25	0.17
6	LPS	F	S	4.30	0.24
12	LPS	F	T	4.40	0.14
13	LPS	M	T	4.15	0.12
14	LPS	M	S	4.70	0.17
15	LPS	F	T	4.20	0.19

Data from this table are used in Chapters 3, 4, and 5. Abbreviations are as follows: SAL=saline treatment; LPS=endotoxin treatment; F=female; M=male; S=single; T=twin.

8.5 Individual animal data for hypoxia studies

Table 8.4: The histories of hypoxia-treated lambs

Animal #	*Treatment*	*Sex*	*Single/Twin*	*Birth weight (kg)*	*Daily weight gain (kg)*
102	hSAL	M	T	5.90	0.30
104	hSAL	F	T	3.80	0.21
106	hSAL	M	T	4.30	0.28
108	hSAL	F	T	3.65	0.22
110	hSAL	M	T	3.50	0.25
112	hSAL	F	T	3.50	0.22
101	hLPS	M	T	4.05	0.21
103	hLPS	M	T	4.40	0.25
105	hLPS	M	T	3.10	0.18
107	hLPS	F	T	4.70	0.24
109	hLPS	M	S	5.60	0.24
111	hLPS	M	S	6.35	0.22
113	hLPS	F	T	3.45	0.29

Data from this table are used in Chapters 4 and 5. Abbreviations are as follows: hSAL=saline followed by hypoxia treatment; hLPS=endotoxin followed by hypoxia treatment; F=female; M=male; S=single; T=twin.

8.6 Ontogenic expression of allopregnanolone in sheep

The following table contains results from a brief investigation of plasma allopregnanolone concentrations in fetal, neonatal and adult sheep. Plasma concentrations of allopregnanolone were high in fetal samples, but decreased with postnatal age up to 8 weeks of age. Although allopregnanolone concentrations appeared to increase at 2 years of age, this result was not significantly ($P<0.05$) different to that at 8 weeks of age. These results are consistent with those reported by Petratos *et al.* (2000) and Nguyen *et al.* (2003).

Table 8.5: Ontogeny expression of allopregnanolone

Age	*Concentration (nmol/l.)*
113-121 GA (n=4)	64.15 ± 2.57 [a]
140-145 GA (n=3)	62.14 ± 1.54 [a]
1 week (n=4)	57.84 ± 1.57 [b]
2 weeks (n=4)	48.23 ± 1.88 [c]
3 weeks (n=4)	39.31 ± 2.3 [d]
8 weeks (n=5)	18.43 ± 1.13 [e]
2 years (n=3)	24.87 ± 3.17 [e]

Allopregnanolone concentrations in fetal, newborn and adult sheep. Letters indicate significant difference ($P<0.05$) between age groups. Numbers of animals examined are in parentheses. Data are expressed as mean ± S.E.M. GA = Gestational age.

References

AKWA, Y., YOUNG, J., KABBADJ, K., SANCHO, M. J., ZUCMAN, D., VOURC'H, C., JUNG-TESTAS, I., HU, Z. Y., LE GOASCOGNE, C., JO, D. H. & ET AL. (1991). Neurosteroids: biosynthesis, metabolism and function of pregnenolone and dehydroepiandrosterone in the brain. *J Steroid Biochem Mol Biol* **40,** 71-81.

ALMEIDA, M. C., CARNIO, E. C. & BRANCO, L. G. (1999). Role of nitric oxide in hypoxia inhibition of fever. *J Appl Physiol* **87,** 2186-90.

ALOISI, F. (2001). Immune function of microglia. *Glia* **36,** 165-79.

BAILEY, C. D., BRIEN, J. F. & REYNOLDS, J. N. (1999). Neurosteroid modulation of the GABAA receptor in the developing guinea pig cerebral cortex. *Brain Res Dev Brain Res* **113,** 21-8.

BARBACCIA, M. L., ROSCETTI, G., TRABUCCHI, M., AMBROSIO, C. & MASSOTTI, M. (1992). Cyclic AMP-dependent increase of steroidogenesis in brain cortical minces. *Eur J Pharmacol* **219,** 485-6.

BARBACCIA, M. L., ROSCETTI, G., TRABUCCHI, M., CUCCHEDDU, T., CONCAS, A. & BIGGIO, G. (1994). Neurosteroids in the brain of handling-habituated and naive rats: effect of CO_2 inhalation. *Eur J Pharmacol* **261,** 317-20.

BARBACCIA, M. L., ROSCETTI, G., TRABUCCHI, M., MOSTALLINO, M. C., CONCAS, A., PURDY, R. H. & BIGGIO, G. (1996). Time-dependent changes in rat brain neuroactive steroid concentrations and GABAA receptor function after acute stress. *Neuroendocrinology* **63,** 166-72.

BAULIEU, E. E. (1991). Neurosteroids: a new function in the brain. *Biol Cell* **71,** 3-10.

BAULIEU, E. E. (1998). Neurosteroids: a novel function of the brain. *Psychoneuroendocrinology* **23,** 963-87.

BAUMANN, H. & GAULDIE, J. (1994). The acute phase response. *Immunol Today* **15,** 74-80.

BERNARDI, F., SALVESTRONI, C., CASAROSA, E., NAPPI, R. E., LANZONE, A., LUISI, S., PURDY, R. H., PETRAGLIA, F. & GENAZZANI, A. R. (1998). Aging is associated with changes in allopregnanolone concentrations in brain, endocrine glands and serum in male rats. *Eur J Endocrinol* **138,** 316-21.

BERRY, R. B. & GLEESON, K. (1997). Respiratory arousal from sleep: mechanisms and significance. *Sleep* **20,** 654-75.

BETTELHEIM, K. A., GOLDWATER, P. N., DWYER, B. W., BOURNE, A. J. & SMITH, D. L. (1990). Toxigenic Escherichia coli associated with sudden infant death syndrome. *Scand J Infect Dis* **22,** 467-76.

BICEGO, K. C., STEINER, A. A., GARGAGLIONI, L. H. & BRANCO, L. G. (2002). Is lactate a mediator of hypoxia-induced anapyrexia? *Pflugers Arch* **444,** 810-5.

BISSONNETTE, J. M. (2000). Mechanisms regulating hypoxic respiratory depression during fetal and postnatal life. *Am J Physiol* **278,** R1391-400.

BLACK, P. H. (2002). Stress and the inflammatory response: A review of neurogenic inflammation. *Brain Behav Immun* **16,** 622-53.

BLACKWELL, C. C., SAADI, A. T., RAZA, M. W., STEWART, J. & WEIR, D. M. (1992). Susceptibility to infection in relation to SIDS. *J Clin Pathol* **45,** 20-4.

BLACKWELL, C. C., WEIR, D. M. & BUSUTTIL, A. (1999). Infection, inflammation and sleep: more pieces to the puzzle of sudden infant death syndrome (SIDS). *Apmis* **107,** 455-73.

BLATTEIS, C. M. (2000). The afferent signalling of fever. *J Physiol* **526 Pt 3,** 470.

BLATTEIS, C. M., HALES, J. R., FAWCETT, A. A. & MASHBURN, T. A., JR. (1988). Fever and regional blood flows in wethers and parturient ewes. *J Appl Physiol* **65,** 165-72.

BLATTEIS, C. M., HALES, J. R., MCKINLEY, M. J. & FAWCETT, A. A. (1987). Role of the anteroventral third ventricle region in fever in sheep. *Can J Physiol Pharmacol* **65,** 1255-60.

BLATTEIS, C. M., MASHBURN, T. A., JR. & AHOKAS, R. A. (1981). Fever and trace metal changes in endotoxin-challenged neonates. *Pflugers Arch* **389,** 177-9.

BLATTEIS, C. M. & SEHIC, E. (1997). Circulating pyrogen signaling of the brain. A new working hypothesis. *Ann N Y Acad Sci* **813,** 445-7.

BLUTHE, R. M., LAYE, S., MICHAUD, B., COMBE, C., DANTZER, R. & PARNET, P. (2000). Role of interleukin-1beta and tumour necrosis factor-alpha in lipopolysaccharide-induced sickness behaviour: a study with interleukin-1 type 1 receptor-deficient mice. *Eur J Neurosci* **12,** 4447-56.

BOCKING, A. D., MCMILLEN, I. C., HARDING, R. & THORBURN, G. D. (1986). Effect of reduced uterine blood flow on fetal and maternal cortisol. *J Dev Physiol* **8,** 237-45.

BOPST, M., HAAS, C., CAR, B. & EUGSTER, H. P. (1998). The combined inactivation of tumor necrosis factor and interleukin-6 prevents induction of the major acute phase proteins by endotoxin. *Eur J Immunol* **28,** 4130-7.

BROT, M. D., AKWA, Y., PURDY, R. H., KOOB, G. F. & BRITTON, K. T. (1997). The anxiolytic-like effects of the neurosteroid allopregnanolone: interactions with GABA(A) receptors. *Eur J Pharmacol* **325,** 1-7.

CARMICHAEL, L., SADOWSKY, D., OLSON, D., CHALLIS, J. & RICHARDSON, B. (1997). Activation of the fetal hypothalamic-pituitary-adrenal axis with prolonged and graded hypoxemia. *J Soc Gynecol Investig* **4,** 8-14.

CELOTTI, F., MELCANGI, R. C. & MARTINI, L. (1992). The 5 alpha-reductase in the brain: molecular aspects and relation to brain function. *Front Neuroendocrinol* **13,** 163-215.

CHAI, Z., GATTI, S., TONIATTI, C., POLI, V. & BARTFAI, T. (1996). Interleukin (IL)-6 gene expression in the central nervous system is necessary for fever response to lipopolysaccharide or IL-1 beta: a study on IL-6-deficient mice. *J Exp Med* **183,** 311-6.

COCEANI, F., BISHAI, I., ENGELBERTS, D., HOUSE, R. V. & ADAMSON, S. L. (1995). Response of newborn and adult sheep to pyrogens: relation between fever and brain eicosanoid changes. *Brain Res* **700,** 191-204.

COHEN, G., MALCOLM, G. & HENDERSON-SMART, D. (1997). Ventilatory response of the newborn infant to mild hypoxia. *Pediatr Pulmonol* **24,** 163-72.

COMPAGNONE, N. A. & MELLON, S. H. (2000). Neurosteroids: biosynthesis and function of these novel neuromodulators. *Front Neuroendocrinol* **21,** 1-56.

CONCAS, A., MOSTALLINO, M. C., PORCU, P., FOLLESA, P., BARBACCIA, M. L., TRABUCCHI, M., PURDY, R. H., GRISENTI, P. & BIGGIO, G. (1998). Role of brain allopregnanolone in the plasticity of gamma-aminobutyric acid type A receptor in rat brain during pregnancy and after delivery. *Proc Natl Acad Sci U S A* **95,** 13284-9.

CORPECHOT, C., ROBEL, P., AXELSON, M., SJOVALL, J. & BAULIEU, E. E. (1981). Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. *Proc Natl Acad Sci U S A* **78,** 4704-7.

CORPECHOT, C., SYNGUELAKIS, M., TALHA, S., AXELSON, M., SJOVALL, J., VIHKO, R., BAULIEU, E. E. & ROBEL, P. (1983). Pregnenolone and its sulfate ester in the rat brain. *Brain Res* **270,** 119-25.

CRAWLEY, B. A., MORRIS, J. A., DRUCKER, D. B., BARSON, A. J., MORRIS, J., KNOX, W. F. & OPPENHEIM, B. A. (1999). Endotoxin in blood and tissue in the sudden infant death syndrome. *FEMS Immunol Med Microbiol* **25,** 131-5.

CROSSLEY, K. J., WALKER, D. W., BEART, P. M. & HIRST, J. J. (2000). Characterisation of GABA(A) receptors in fetal, neonatal and adult ovine brain: region and age related changes and the effects of allopregnanolone. *Neuropharmacology* **39,** 1514-22.

DAMIANISCH, K., RUPPRECHT, R. & LANCEL, M. (2001). The influence of subchronic administration of the neurosteroid allopregnanolone on sleep in the rat. *Neuropsychopharmacology* **25,** 576-84.

DANTZER, R. (2001). Cytokine-induced sickness behavior: where do we stand? *Brain Behav Immun* **15,** 7-24.

DARNALL, R. A., CURRAN, A. K., FILIANO, J. J., LI, A. & NATTIE, E. E. (2001). The effects of a GABA(A) agonist in the rostral ventral medulla on sleep and breathing in newborn piglets. *Sleep* **24,** 514-27.

DARNAUDERY, M., PALLARES, M., BOUYER, J. J., LE MOAL, M. & MAYO, W. (1999). Infusion of neurosteroids into the rat nucleus basalis affects paradoxical sleep in accordance with their memory modulating properties. *Neuroscience* **92,** 583-8.

DAVIDSON, J., ABUL, H. T., MILTON, A. S. & ROTONDO, D. (2001). Cytokines and cytokine inducers stimulate prostaglandin E2 entry into the brain. *Pflugers Arch* **442,** 526-33.

DENT, G. W., SMITH, M. A. & LEVINE, S. (1999). The ontogeny of the neuroendocrine response to endotoxin. *Brain Res Dev Brain Res* **117,** 21-9.

DINARELLO, C. A. (1984). Interleukin-1 and the pathogenesis of the acute-phase response. *N Engl J Med* **311,** 1413-8.

DINARELLO, C. A. (1992). Role of interleukin-1 in infectious diseases. *Immunol Rev* **127,** 119-46.

DOBROVOLSKAIA, M. A. & VOGEL, S. N. (2002). Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes Infect* **4,** 903-14.

DUNN, A. J. (1993). Role of cytokines in infection-induced stress. *Ann N Y Acad Sci* **697,** 189-202.

EGAN, P. J., ROTHEL, J. S., ANDREWS, A. E., SEOW, H. F., WOOD, P. R. & NASH, A. D. (1994). Characterization of monoclonal antibodies to ovine tumor necrosis factor-alpha and development of a sensitive immunoassay. *Vet Immunol Immunopathol* **41,** 259-74.

ELMQUIST, J. K., SCAMMELL, T. E. & SAPER, C. B. (1997). Mechanisms of CNS response to systemic immune challenge: the febrile response. *Trends Neurosci* **20,** 565-70.

ENGEL, S. R. & GRANT, K. A. (2001). Neurosteroids and behavior. *Int Rev Neurobiol* **46,** 321-48.

ERRIDGE, C., BENNETT-GUERRERO, E. & POXTON, I. R. (2002). Structure and function of lipopolysaccharides. *Microbes Infect* **4,** 837-51.

FADALTI, M., PETRAGLIA, F., LUISI, S., BERNARDI, F., CASAROSA, E., FERRARI, E., LUISI, M., SAGGESE, G., GENAZZANI, A. R. & BERNASCONI, S. (1999). Changes of serum allopregnanolone levels in the first 2 years of life and during pubertal development. *Pediatr Res* **46,** 323-7.

FAGGIONI, R., FANTUZZI, G., VILLA, P., BUURMAN, W., VAN TITS, L. J. & GHEZZI, P. (1995). Independent down-regulation of central and peripheral tumor necrosis factor production as a result of lipopolysaccharide tolerance in mice. *Infect Immun* **63,** 1473-7.

FAGIOLI, I. (2002). Mental activity during sleep. *Sleep Med Rev* **6,** 307-20.

FANTAZIA, D. (1983). Physiologic basis of host susceptibility. *JOGN Nurs* **12,** 27s-30s.

FANTUZZI, G., DI SANTO, E., SACCO, S., BENIGNI, F. & GHEZZI, P. (1995). Role of the hypothalamus-pituitary-adrenal axis in the regulation of TNF production in mice. Effect of stress and inhibition of endogenous glucocorticoids. *J Immunol* **155,** 3552-5.

FEWELL, J. E. & BAKER, S. B. (1987). Arousal from sleep during rapidly developing hypoxemia in lambs. *Pediatr Res* **22,** 471-7.

FEWELL, J. E. & KONDURI, G. G. (1989). Influence of repeated exposure to rapidly developing hypoxaemia on the arousal and cardiopulmonary response to rapidly developing hypoxaemia in lambs. *J Dev Physiol* **11,** 77-82.

FEWELL, J. E., RICCIUTI, F., KONDO, C. S. & DASCALU, V. (1991). Fever in young lambs: temperature, metabolic and cardiorespiratory responses to a small dose of bacterial pyrogen. *J Dev Physiol* **15,** 229-35.

FILIANO, J. J. & KINNEY, H. C. (1994). A perspective on neuropathologic findings in victims of the sudden infant death syndrome: the triple-risk model. *Biol Neonate* **65,** 194-7.

FLETCHER, E. C., LESSKE, J., BEHM, R., MILLER, C. C., 3RD, STAUSS, H. & UNGER, T. (1992). Carotid chemoreceptors, systemic blood pressure, and chronic episodic hypoxia mimicking sleep apnea. *J Appl Physiol* **72,** 1978-84.

FLOOD, J. F., MORLEY, J. E. & ROBERTS, E. (1992). Memory-enhancing effects in male mice of pregnenolone and steroids metabolically derived from it. *Proc Natl Acad Sci U S A* **89,** 1567-71.

FRANK, C. & SAGRATELLA, S. (2000). Neuroprotective effects of allopregnenolone on hippocampal irreversible neurotoxicity in vitro. *Prog Neuropsychopharmacol Biol Psychiatry* **24,** 1117-26.

FROEN, J. F., MUNKEBY, B. H., STRAY-PEDERSEN, B. & SAUGSTAD, O. D. (2002). Interleukin-10 reverses acute detrimental effects of endotoxin-induced inflammation on perinatal cerebral hypoxia-ischemia. *Brain Res* **942,** 87-94.

GABAY, C. & KUSHNER, I. (1999). Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* **340,** 448-54.

GANDOLFO, G., SCHERSCHLICHT, R. & GOTTESMANN, C. (1994). Benzodiazepines promote the intermediate stage at the expense of paradoxical sleep in the rat. *Pharmacol Biochem Behav* **49,** 921-7.

GENAZZANI, A. R., BERNARDI, F., STOMATI, M., MONTELEONE, P., LUISI, S., RUBINO, S., FARZATI, A., CASAROSA, E., LUISI, M. & PETRAGLIA, F. (2000). Effects of estradiol and raloxifene analog on brain, adrenal and serum allopregnanolone content in fertile and ovariectomized female rats. *Neuroendocrinology* **72,** 162-70.

GENAZZANI, A. R., PETRAGLIA, F., BERNARDI, F., CASAROSA, E., SALVESTRONI, C., TONETTI, A., NAPPI, R. E., LUISI, S., PALUMBO, M., PURDY, R. H. & LUISI, M. (1998). Circulating levels of allopregnanolone in humans: gender, age, and endocrine influences. *J Clin Endocrinol Metab* **83,** 2099-103.

GESSLER, P., NEBE, T., BIRLE, A., HAAS, N. & KACHEL, W. (1996). Neutrophil respiratory burst in term and preterm neonates without signs of infection and in those with increased levels of C-reactive protein. *Pediatr Res* **39,** 843-8.

GHEZZI, P., SANTO, E. D., SACCO, S., FODDI, C., BARBACCIA, M. L. & MENNINI, T. (2000). Neurosteroid levels are increased in vivo after LPS treatment and negatively regulate LPS-induced TNF production. *Eur Cytokine Netw* **11,** 464-9.

GOELST, K. & LABURN, H. (1991). Response of body temperature and serum iron concentration to repeated pyrogen injection in rabbits. *Pflugers Arch* **417,** 558-61.

GOELST, K., MITCHELL, D. & LABURN, H. (1992). Fever responses in newborn lambs. *Pflugers Arch* **421,** 299-301.

GOLDWATER, P. N. (1992). Reappraisal of the SIDS enigma: an epidemiological and clinicopathological approach. *J Paediatr Child Health* **28,** S21-5.

GONZALEZ-VIDAL, M. D., CERVERA-GAVIRIA, M., RUELAS, R., ESCOBAR, A., MORALI, G. & CERVANTES, M. (1998). Progesterone: protective effects on the cat hippocampal neuronal damage due to acute global cerebral ischemia. *Arch Med Res* **29,** 117-24.

GORE, D. C., JAHOOR, F., HIBBERT, J. M. & DEMARIA, E. J. (1996). Lactic acidosis during sepsis is related to increased pyruvate production, not deficits in tissue oxygen availability. *Ann Surg* **224,** 97-102.

GUNTHEROTH, W. G. & SPIERS, P. S. (2002). The triple risk hypotheses in sudden infant death syndrome. *Pediatrics* **110,** e64.

HADID, R., SPINEDI, E., GIOVAMBATTISTA, A., CHAUTARD, T. & GAILLARD, R. C. (1996). Decreased hypothalamo-pituitary-adrenal axis response to neuroendocrine challenge under repeated endotoxemia. *Neuroimmunomodulation* **3,** 62-8.

HAENEY, M. (1994). Infection determinants at extremes of age. *J Antimicrob Chemother* **34 Suppl A,** 1-9.

HARRISON, L. M., MORRIS, J. A., TELFORD, D. R., BROWN, S. M. & JONES, K. (1999). The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. *FEMS Immunol Med Microbiol* **25,** 19-28.

HAYAISHI, O. (2000). Molecular mechanisms of sleep-wake regulation: a role of prostaglandin D2. *Philos Trans R Soc Lond B Biol Sci* **355,** 275-80.

HENDERSON-SMART, D. J. & READ, D. J. (1979). Reduced lung volume during behavioral active sleep in the newborn. *J Appl Physiol* **46,** 1081-5.

HEUMANN, D. & ROGER, T. (2002). Initial responses to endotoxins and Gram-negative bacteria. *Clin Chim Acta* **323,** 59-72.

HILLIQUIN, P. (1995). Biological markers in inflammatory rheumatic diseases. *Cell Mol Biol* **41,** 993-1006.

HOBSON, J. A. & PACE-SCHOTT, E. F. (2002). The cognitive neuroscience of sleep: neuronal systems, consciousness and learning. *Nat Rev Neurosci* **3,** 679-93.

HOBSON, J. A., STICKGOLD, R. & PACE-SCHOTT, E. F. (1998). The neuropsychology of REM sleep dreaming. *Neuroreport* **9,** R1-14.

HOLZBAUER, M. (1976). Physiological aspects of steroids with anaesthetic properties. *Med Biol* **54,** 227-42.

JACOBSON, N. A., LADLE, D. R. & LEPHART, E. D. (1997). Aromatase cytochrome P450 and 5 alpha-reductase in the amygdala and cortex of perinatal rats. *Neuroreport* **8,** 2529-33.

JAKEMAN, K. J., RUSHTON, D. I., SMITH, H. & SWEET, C. (1991). Exacerbation of bacterial toxicity to infant ferrets by influenza virus: possible role in sudden infant death syndrome. *J Infect Dis* **163,** 35-40.

JIANG, N., CHOPP, M., STEIN, D. & FEIT, H. (1996). Progesterone is neuroprotective after transient middle cerebral artery occlusion in male rats. *Brain Res* **735,** 101-7.

JOHNSON, R. W. (2002). The concept of sickness behavior: a brief chronological account of four key discoveries. *Vet Immunol Immunopathol* **87,** 443-50.

JOHNSON, R. W., CURTIS, S. E., DANTZER, R., BAHR, J. M. & KELLEY, K. W. (1993). Sickness behavior in birds caused by peripheral or central injection of endotoxin. *Physiol Behav* **53,** 343-8.

JOHNSTON, R. V., GRANT, D. A., WILKINSON, M. H. & WALKER, A. M. (1998). Repetitive hypoxia rapidly depresses arousal from active sleep in newborn lambs. *J Physiol* **510,** 651-9.

JOHNSTON, R. V., GRANT, D. A., WILKINSON, M. H. & WALKER, A. M. (1999). Repetitive hypoxia rapidly depresses cardio-respiratory responses during active sleep but not quiet sleep in the newborn lamb. *J Physiol* **519,** 571-9.

JONES, K. L., KROUS, H. F., NADEAU, J., BLACKBOURNE, B., ZIELKE, H. R. & GOZAL, D. (2003). Vascular endothelial growth factor in the cerebrospinal fluid of infants who died of sudden infant death syndrome: evidence for antecedent hypoxia. *Pediatrics* **111,** 358-63.

JUNG-TESTAS, I., HU, Z. Y., BAULIEU, E. E. & ROBEL, P. (1989). Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. *Endocrinology* **125,** 2083-91.

JUNG-TESTAS, I., RENOIR, J. M., GASC, J. M. & BAULIEU, E. E. (1991). Estrogen-inducible progesterone receptor in primary cultures of rat glial cells. *Exp Cell Res* **193,** 12-9.

KADLECOVA, O., ANOCHINA, I. P., BAUER, V., MASEK, K. & RASKOVA, H. (1972). Effect of Escherichia coli endotoxin on temperature and sleep cycles of rats. *J Infect Dis* **126,** 179-81.

KAHN, A., GROSWASSER, J., SOTTIAUX, M., KELMANSON, I., REBUFFAT, E., FRANCO, P., DRAMAIX, M. & WAYENBERG, J. L. (1994). Prenatal exposure to cigarettes in infants with obstructive sleep apneas. *Pediatrics* **93,** 778-83.

KANOH, S., NISHIO, A. & KAWASAKI, H. (1977). Studies on pyrogenic tolerance to bacterial lipopolysaccharide in rabbits. *Biken J* **20,** 69-75.

KARNI, A., TANNE, D., RUBENSTEIN, B. S., ASKENASY, J. J. & SAGI, D. (1994). Dependence on REM sleep of overnight improvement of a perceptual skill. *Science* **265,** 679-82.

KASTING, N. W., VEALE, W. L. & COOPER, K. E. (1979). Development of fever in the newborn lamb. *Am J Physiol* **236,** R184-7.

KEHOE, P., MALLINSON, K., MCCORMICK, C. M. & FRYE, C. A. (2000). Central allopregnanolone is increased in rat pups in response to repeated, short episodes of neonatal isolation. *Brain Res Dev Brain Res* **124,** 133-6.

KERR, J. R., AL-KHATTAF, A., BARSON, A. J. & BURNIE, J. P. (2000). An association between sudden infant death syndrome (SIDS) and Helicobacter pylori infection. *Arch Dis Child* **83,** 429-34.

KHISTI, R. T., CHOPDE, C. T. & JAIN, S. P. (2000). Antidepressant-like effect of the neurosteroid 3alpha-hydroxy-5alpha-pregnan-20-one in mice forced swim test. *Pharmacol Biochem Behav* **67,** 137-43.

KINNEY, H. C., BURGER, P. C., HARRELL, F. E., JR. & HUDSON, R. P., JR. (1983). 'Reactive gliosis' in the medulla oblongata of victims of the sudden infant death syndrome. *Pediatrics* **72,** 181-7.

KLUGER, M. J. (1980). Fever. *Pediatrics* **66,** 720-4.

KLUGER, M. J., KOZAK, W., LEON, L. R., SOSZYNSKI, D. & CONN, C. A. (1998). Fever and antipyresis. *Prog Brain Res* **115,** 465-75.

KLUGER, M. J. & ROTHENBURG, B. A. (1979). Fever and reduced iron: their interaction as a host defense response to bacterial infection. *Science* **203,** 374-6.

KOCHAN, I., KVACH, J. T. & WILES, T. I. (1977). Virulence-associated acquisition of iron in mammalian serum by Escherichia coli. *J Infect Dis* **135,** 623-32.

KOENIG, H. L., SCHUMACHER, M., FERZAZ, B., THI, A. N., RESSOUCHES, A., GUENNOUN, R., JUNG-TESTAS, I., ROBEL, P., AKWA, Y. & BAULIEU, E. E. (1995). Progesterone synthesis and myelin formation by Schwann cells. *Science* **268,** 1500-3.

KONSMAN, J. P., PARNET, P. & DANTZER, R. (2002). Cytokine-induced sickness behaviour: mechanisms and implications. *Trends Neurosci* **25,** 154-9.

KOPP, E. B. & MEDZHITOV, R. (1999). The Toll-receptor family and control of innate immunity. *Curr Opin Immunol* **11,** 13-8.

KORNEYEV, A. & COSTA, E. (1996). Allopregnanolone (THP) mediates anesthetic effects of progesterone in rat brain. *Horm Behav* **30,** 37-43.

KRONFOL, Z. & REMICK, D. G. (2000). Cytokines and the brain: implications for clinical psychiatry. *Am J Psychiatry* **157,** 683-94.

KRUEGER, J. M., FANG, J., TAISHI, P., CHEN, Z., KUSHIKATA, T. & GARDI, J. (1998). Sleep. A physiologic role for IL-1 beta and TNF-alpha. *Ann N Y Acad Sci* **856,** 148-59.

KRUEGER, J. M. & KARNOVSKY, M. L. (1987). Sleep and the immune response. *Ann N Y Acad Sci* **496,** 510-6.

KRUEGER, J. M., OBAL, F., JR., OPP, M., TOTH, L., JOHANNSEN, L. & CADY, A. B. (1990). Somnogenic cytokines and models concerning their effects on sleep. *Yale J Biol Med* **63,** 157-72.

KRUEGER, J. M., OBAL, F. J., FANG, J., KUBOTA, T. & TAISHI, P. (2001). The role of cytokines in physiological sleep regulation. *Ann N Y Acad Sci* **933,** 211-21.

KRUEGER, J. M., PAPPENHEIMER, J. R. & KARNOVSKY, M. L. (1982). Sleep-promoting effects of muramyl peptides. *Proc Natl Acad Sci U S A* **79,** 6102-6.

KRUGERS, H. J., KNOLLEMA, S., KEMPER, R. H., TER HORST, G. J. & KORF, J. (1995). Down-regulation of the hypothalamo-pituitary-adrenal axis reduces brain damage and number of seizures following hypoxia/ischaemia in rats. *Brain Res* **690,** 41-7.

KUMON, Y., KIM, S. C., TOMPKINS, P., STEVENS, A., SAKAKI, S. & LOFTUS, C. M. (2000). Neuroprotective effect of postischemic administration of progesterone in spontaneously hypertensive rats with focal cerebral ischemia. *J Neurosurg* **92,** 848-52.

LACROIX, S., FEINSTEIN, D. & RIVEST, S. (1998). The bacterial endotoxin lipopolysaccharide has the ability to target the brain in upregulating its membrane CD14 receptor within specific cellular populations. *Brain Pathol* **8,** 625-40.

LADURELLE, N., EYCHENNE, B., DENTON, D., BLAIR-WEST, J., SCHUMACHER, M., ROBEL, P. & BAULIEU, E. (2000). Prolonged intracerebroventricular infusion of neurosteroids affects cognitive performances in the mouse. *Brain Res* **858,** 371-9.

LAFLAMME, N. & RIVEST, S. (2001). Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J* **15,** 155-63.

LANCEL, M. (1997). The GABA(A) agonist THIP increases non-REM sleep and enhances non-REM sleep-specific delta activity in the rat during the dark period. *Sleep* **20,** 1099-104.

LANCEL, M. (1999). Role of GABAA receptors in the regulation of sleep: initial sleep responses to peripherally administered modulators and agonists. *Sleep* **22,** 33-42.

LANCEL, M., FAULHABER, J., HOLSBOER, F. & RUPPRECHT, R. (1996). Progesterone induces changes in sleep comparable to those of agonistic GABAA receptor modulators. *Am J Physiol* **271,** E763-72.

LANCEL, M., FAULHABER, J., SCHIFFELHOLZ, T., ROMEO, E., DI MICHELE, F., HOLSBOER, F. & RUPPRECHT, R. (1997). Allopregnanolone affects sleep in a benzodiazepine-like fashion. *J Pharmacol Exp Ther* **282,** 1213-8.

LAPCHAK, P. A., CHAPMAN, D. F., NUNEZ, S. Y. & ZIVIN, J. A. (2000). Dehydroepiandrosterone sulfate is neuroprotective in a reversible spinal cord ischemia model: possible involvement of GABA(A) receptors. *Stroke* **31,** 1953-6; discussion 7.

LAUBER, M. E. & LICHTENSTEIGER, W. (1996). Ontogeny of 5 alpha-reductase (type 1) messenger ribonucleic acid expression in rat brain: early presence in germinal zones. *Endocrinology* **137,** 2718-30.

LE GOASCOGNE, C., ROBEL, P., GOUEZOU, M., SANANES, N., BAULIEU, E. E. & WATERMAN, M. (1987). Neurosteroids: cytochrome P-450scc in rat brain. *Science* **237,** 1212-5.

LEE, P. C., STRUVE, M., LEWIS, S. M. & RAFF, H. (2002). Neonatal hypoxia in the rat: effects on exocrine pancreatic development. *J Pediatr Gastroenterol Nutr* **34,** 542-7.

LEON, L. R., KOZAK, W., RUDOLPH, K. & KLUGER, M. J. (1999). An antipyretic role for interleukin-10 in LPS fever in mice. *Am J Physiol* **276,** R81-9.

LEON, L. R., WHITE, A. A. & KLUGER, M. J. (1998). Role of IL-6 and TNF in thermoregulation and survival during sepsis in mice. *Am J Physiol* **275,** R269-77.

LEWIS, K. W. & BOSQUE, E. M. (1995). Deficient hypoxia awakening response in infants of smoking mothers: possible relationship to sudden infant death syndrome. *J Pediatr* **127,** 691-9.

LINDER, M. C. & MUNRO, H. N. (1973). Iron and copper metabolism during development. *Enzyme* **15,** 111-38.

LINDGREN, C., LIN, J., GRAHAM, B. S., GRAY, M. E., PARKER, R. A. & [illegible]ELL, H. W. (1996). Respiratory syncytial virus infection enhances the response to laryngeal chemostimulation and inhibits arousal from sleep in young lambs. *Acta Paediatr* **85,** 789-97.

LUDDENS, H. & KORPI, E. R. (1995). Biological function of GABAA/benzodiazepine receptor heterogeneity. *J Psychiatr Res* **29,** 77-94.

MAJEWSKA, M. D. (1992). Neurosteroids: endogenous bimodal modulators of the GABAA receptor. Mechanism of action and physiological significance. *Prog Neurobiol* **38,** 379-95.

MAJEWSKA, M. D., HARRISON, N. L., SCHWARTZ, R. D., BARKER, J. L. & PAUL, S. M. (1986). Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* **232,** 1004-7.

MARKS, G. A., SHAFFERY, J. P., OKSENBERG, A., SPECIALE, S. G. & ROFFWARG, H. P. (1995). A functional role for REM sleep in brain maturation. *Behav Brain Res* **69,** 1-11.

MCCANN, S. M., KARANTH, S., KAMAT, A., LES DEES, W., LYSON, K., GIMENO, M. & RETTORI, V. (1994). Induction by cytokines of the pattern of pituitary hormone secretion in infection. *Neuroimmunomodulation* **1,** 2-13.

MCCORMICK, C. M., KEHOE, P., MALLINSON, K., CECCHI, L. & FRYE, C. A. (2002). Neonatal isolation alters stress hormone and mesolimbic dopamine release in juvenile rats. *Pharmacol Biochem Behav* **73,** 77-85.

MCCULLOCH, K., BROUILLETTE, R. T., GUZZETTA, A. J. & HUNT, C. E. (1982). Arousal responses in near-miss sudden infant death syndrome and in normal infants. *J Pediatr* **101,** 911-7.

MCKUSKEY, R. S., MCKUSKEY, P. A., URBASCHEK, R. & URBASCHEK, B. (1984). Species differences in Kupffer cells and endotoxin sensitivity. *Infect Immun* **45,** 278-80.

McWaters, P., Hurst, L., Chaplin, P. J., Collins, R. A., Wood, P. R. & Scheerlinck, J. P. (2000). Characterisation of monoclonal antibodies to ovine interleukin-6 and the development of a sensitive capture ELISA. *Vet Immunol Immunopathol* **73,** 155-65.

Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388,** 394-7.

Melcangi, R. C., Celotti, F., Ballabio, M., Castano, P., Massarelli, R., Poletti, A. & Martini, L. (1990). 5 alpha-reductase activity in isolated and cultured neuronal and glial cells of the rat. *Brain Res* **516,** 229-36.

Melcangi, R. C., Celotti, F. & Martini, L. (1994). Progesterone 5-alpha-reduction in neuronal and in different types of glial cell cultures: type 1 and 2 astrocytes and oligodendrocytes. *Brain Res* **639,** 202-6.

Mellon, S. H. (1994). Neurosteroids: biochemistry, modes of action, and clinical relevance. *J Clin Endocrinol Metab* **78,** 1003-8.

Mellon, S. H. & Deschepper, C. F. (1993). Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. *Brain Res* **629,** 283-92.

Mellon, S. H. & Griffin, L. D. (2002). Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol Metab* **13,** 35-43.

Mensah-Nyagan, A. G., Do-Rego, J. L., Beaujean, D., Luu-The, V., Pelletier, G. & Vaudry, H. (1999). Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacol Rev* **51,** 63-81.

Miller, A. J., Luheshi, G. N., Rothwell, N. J. & Hopkins, S. J. (1997). Local cytokine induction by LPS in the rat air pouch and its relationship to the febrile response. *Am J Physiol* **272,** R857-61.

Mok, W. M., Bukusoglu, C. & Krieger, N. R. (1993). 3 alpha-hydroxy-5 alpha-pregnan-20-one is the only active anesthetic steroid in anesthetized mouse brain. *Steroids* **58,** 112-4.

Monteleone, P., Luisi, S., Tonetti, A., Bernardi, F., Genazzani, A. D., Luisi, M., Petraglia, F. & Genazzani, A. R. (2000). Allopregnanolone concentrations and premenstrual syndrome. *Eur J Endocrinol* **142,** 269-73.

Morris, J. A. (1999). The common bacterial toxins hypothesis of sudden infant death syndrome. *FEMS Immunol Med Microbiol* **25,** 11-7.

Moss, I. R. (2000). Respiratory responses to single and episodic hypoxia during development: mechanisms of adaptation. *Respir Physiol* **121,** 185-97.

MOSS, T. J., JAKUBOWSKA, A. E., MCCRABB, G. J., BILLINGS, K. & HARDING, R. (1995). Ventilatory responses to progressive hypoxia and hypercapnia in developing sheep. *Respir Physiol* **100,** 33-44.

MULLER-PREUSS, P., RUPPRECHT, R. & LANCEL, M. (2002). The effects of the neuroactive steroid 3 alpha,5 alpha-THDOC on sleep in the rat. *Neuroreport* **13,** 487-90.

MULLINGTON, J., KORTH, C., HERMANN, D. M., ORTH, A., GALANOS, C., HOLSBOER, F. & POLLMACHER, T. (2000). Dose-dependent effects of endotoxin on human sleep. *Am J Physiol Regul Integr Comp Physiol* **278,** R947-55.

NAEYE, R. L. (1988). Sudden infant death syndrome, is the confusion ending? *Mod Pathol* **1,** 169-74.

NASH, A. D., BRANDON, M. R. & BELLO, P. A. (1992). Effects of tumour necrosis factor-alpha on growth hormone and interleukin 6 mRNA in ovine pituitary cells. *Mol Cell Endocrinol* **84,** R31-7.

NGUYEN, P. N., BILLIARDS, S. S., WALKER, D. W. & HIRST, J. J. (2002). Changes in Allopregnanolone concentrations and neurosteroidogenic enzyme expression in the IUGR fetus. *Ceske Gynekologie* **67,** 28.

NGUYEN, P. N., BILLIARDS, S. S., WALKER, D. W. & HIRST, J. J. (2003). Changes in 5α-pregnane steroids and neurosteroidogenic enzyme expression in the perinatal sheep. *Pediatric Research* **53 (*In Press*).**

NICOL, M. B., HIRST, J. J. & WALKER, D. (1999). Effects of pregnanolone on behavioural parameters and the responses to GABA(A) receptor antagonists in the late gestation fetal sheep. *Neuropharmacology* **38,** 49-63.

NICOL, M. B., HIRST, J. J., WALKER, D. & THORBURN, G. D. (1997). Effect of alteration of maternal plasma progesterone concentrations on fetal behavioural state during late gestation. *J Endocrinol* **152,** 379-86.

NICOL, M. B., HIRST, J. J. & WALKER, D. W. (2001). Effect of finasteride on behavioural arousal and somatosensory evoked potentials in fetal sheep. *Neurosci Lett* **306,** 13-6.

NITZ, D. & SIEGEL, J. M. (1997). GABA release in the locus coeruleus as a function of sleep/wake state. *Neuroscience* **78,** 795-801.

NOAH, T. L., HENDERSON, F. W., WORTMAN, I. A., DEVLIN, R. B., HANDY, J., KOREN, H. S. & BECKER, S. (1995). Nasal cytokine production in viral acute upper respiratory infection of childhood. *J Infect Dis* **171,** 584-92.

OPP, M., OBAL, F., JR., CADY, A. B., JOHANNSEN, L. & KRUEGER, J. M. (1989). Interleukin-6 is pyrogenic but not somnogenic. *Physiol Behav* **45,** 1069-72.

O'REILLY, B., VANDER, A. J. & KLUGER, M. J. (1988). Effects of chronic infusion of lipopolysaccharide on food intake and body temperature of the rat. *Physiol Behav* **42,** 287-91.

OZAWA, Y. & TAKASHIMA, S. (2002). Developmental neurotransmitter pathology in the brainstem of sudden infant death syndrome: a review and sleep position. *Forensic Sci Int* **130 Suppl,** S53-9.

PARER, J. T. (1998). Effects of fetal asphyxia on brain cell structure and function: limits of tolerance. *Comp Biochem Physiol A Mol Integr Physiol* **119,** 711-6.

PARKES, D. G., COGHLAN, J. P. & SCOGGINS, B. A. (1995). Pyrogenic stimulation of vascular resistance in conscious sheep. *Life Sci* **57,** 599-608.

PATCHEV, V. K., MONTKOWSKI, A., ROUSKOVA, D., KORANYI, L., HOLSBOER, F. & ALMEIDA, O. F. (1997). Neonatal treatment of rats with the neuroactive steroid tetrahydrodeoxycorticosterone (THDOC) abolishes the behavioral and neuroendocrine consequences of adverse early life events. *J Clin Invest* **99,** 962-6.

PAUL, S. M. & PURDY, R. H. (1992). Neuroactive steroids. *FASEB J* **6,** 2311-22.

PENNING, D. H., GRAFE, M. R., HAMMOND, R., MATSUDA, Y., PATRICK, J. & RICHARDSON, B. (1994). Neuropathology of the near-term and midgestation ovine fetal brain after sustained in utero hypoxemia. *Am J Obstet Gynecol* **170,** 1425-32.

PETRATOS, S., HIRST, J. J., MENDIS, S., ANIKIJENKO, P. & WALKER, D. W. (2000). Localization of p450scc and 5alpha-reductase type-2 in the cerebellum of fetal and newborn sheep. *Brain Res Dev Brain Res* **123,** 81-6.

PHILLIPSON, E. A. & SULLIVAN, C. E. (1978). Arousal: the forgotten response to respiratory stimuli. *Am Rev Respir Dis* **118,** 807-9.

PLATA-SALAMAN, C. R., OOMURA, Y. & KAI, Y. (1988). Tumor necrosis factor and interleukin-1 beta: suppression of food intake by direct action in the central nervous system. *Brain Res* **448,** 106-14.

PUIA, G., SANTI, M. R., VICINI, S., PRITCHETT, D. B., PURDY, R. H., PAUL, S. M., SEEBURG, P. H. & COSTA, E. (1990). Neurosteroids act on recombinant human GABAA receptors. *Neuron* **4,** 759-65.

PURDY, R. H., MOORE, P. H., JR., RAO, P. N., HAGINO, N., YAMAGUCHI, T., SCHMIDT, P., RUBINOW, D. R., MORROW, A. L. & PAUL, S. M. (1990). Radioimmunoassay of 3 alpha-hydroxy-5 alpha-pregnan-20-one in rat and human plasma. *Steroids* **55,** 290-6.

PURDY, R. H., MORROW, A. L., MOORE, P. H., JR. & PAUL, S. M. (1991). Stress-induced elevations of gamma-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc Natl Acad Sci U S A* **88,** 4553-7.

RAINA, N., MATSUI, J. & JEEJEEBHOY, K. N. (2000). Nutritional and metabolic effects of the endotoxin bacterial lipopolysaccharide in orally and parenterally fed rats. *Am J Clin Nutr* **71,** 835-43.

RAJA, K. B., DUANE, P. & PETERS, T. J. (1990). Effects of turpentine-induced inflammation on the hypoxic stimulation of intestinal Fe3+ absorption in mice. *Int J Exp Pathol* **71,** 785-9.

RAZA, M. W. & BLACKWELL, C. C. (1999). Sudden infant death syndrome, virus infections and cytokines. *FEMS Immunol Med Microbiol* **25,** 85-96.

READ, P. A., HORNE, R. S., CRANAGE, S. M., WALKER, A. M., WALKER, D. W. & ADAMSON, T. M. (1998). Dynamic changes in arousal threshold during sleep in the human infant. *Pediatr Res* **43,** 697-703.

REYES, T. M. & SAWCHENKO, P. E. (2002). Involvement of the arcuate nucleus of the hypothalamus in interleukin-1-induced anorexia. *J Neurosci* **22,** 5091-9.

RICE, G. E., JENKIN, G. & THORBURN, G. D. (1986). Comparison of particle-associated progesterone and oxytocin in the ovine corpus luteum. *J Endocrinol* **108,** 109-16.

RICHARDSON, B. S. & BOCKING, A. D. (1998). Metabolic and circulatory adaptations to chronic hypoxia in the fetus. *Comp Biochem Physiol A Mol Integr Physiol* **119,** 717-23.

RODRIGUEZ GIL, D. J., VIAPIANO, M. S. & FISZER DE PLAZAS, S. (2000). Acute hypoxic hypoxia transiently reduces GABA(A) binding site number in developing chick optic lobe. *Brain Res Dev Brain Res* **124,** 67-72.

ROMANOVSKY, A. A. & BLATTEIS, C. M. (1995). Biphasic fever: what triggers the second temperature rise? *Am J Physiol* **269,** R280-6.

ROTH, J., MCCLELLAN, J. L., KLUGER, M. J. & ZEISBERGER, E. (1994). Attenuation of fever and release of cytokines after repeated injections of lipopolysaccharide in guinea-pigs. *J Physiol* **477,** 177-85.

ROTONDO, D., ABUL, H. T., MILTON, A. S. & DAVIDSON, J. (1988). Pyrogenic immunomodulators increase the level of prostaglandin E2 in the blood simultaneously with the onset of fever. *Eur J Pharmacol* **154,** 145-52.

RUPPRECHT, R., HAUSER, C. A., TRAPP, T. & HOLSBOER, F. (1996). Neurosteroids: molecular mechanisms of action and psychopharmacological significance. *J Steroid Biochem Mol Biol* **56,** 163-8.

RUPPRECHT, R. & HOLSBOER, F. (1999). Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci* **22,** 410-6.

SAGAR, S. M. (1994). The functional neuroanatomy of the acute-phase response. *Ann N Y Acad Sci* **739,** 282-91.

SAYERS, N. M., DRUCKER, D. B. & GRENCIS, R. K. (1995). Cytokines may give insight into mechanisms of death in sudden infant death syndrome. *Med Hypotheses* **45,** 369-74.

SCHIFFELHOLZ, T. & LANCEL, M. (2001). Sleep changes induced by lipopolysaccharide in the rat are influenced by age. *Am J Physiol* **280,** R398-403.

SCHLAFER, D. H., YUH, B., FOLEY, G. L., ELSSASER, T. H., SADOWSKY, D. & NATHANIELSZ, P. W. (1994). Effect of Salmonella endotoxin administered to the pregnant sheep at 133-142 days gestation on fetal oxygenation, maternal and fetal adrenocorticotropic hormone and cortisol, and maternal plasma tumor necrosis factor alpha concentrations. *Biol Reprod* **50,** 1297-302.

SCHOLZ, H. (2003). The adrenal response of neonates to hypoxia. *Am J Physiol* **284,** R76-7.

SCHROEDL, W., FUERLL, B., REINHOLD, P., KRUEGER, M. & SCHUETT, C. (2001). A novel acute phase marker in cattle: lipopolysaccharide binding protein (LBP). *J Endotoxin Res* **7,** 49-52.

SELYE, H. (1941). Anaesthetic effect of steroid hormones. *Exp Biol Med* **46,** 116-21.

SELYE, H. (1942). Correlations between the chemical structure and the pharmacological actions of the steroids. *Endocrinology* **30,** 437-53.

SERRA, M., PISU, M. G., LITTERA, M., PAPI, G., SANNA, E., TUVERI, F., USALA, L., PURDY, R. H. & BIGGIO, G. (2000). Social isolation-induced decreases in both the abundance of neuroactive steroids and GABA(A) receptor function in rat brain. *J Neurochem* **75,** 732-40.

SHANKS, N., LAROCQUE, S. & MEANEY, M. J. (1995). Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity to stress. *J Neurosci* **15,** 376-84.

SHANKS, N., WINDLE, R. J., PERKS, P. A., HARBUZ, M. S., JESSOP, D. S., INGRAM, C. D. & LIGHTMAN, S. L. (2000). Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. *Proc Natl Acad Sci U S A* **97,** 5645-50.

SIDI, D., KUIPERS, J. R., TEITEL, D., HEYMANN, M. A. & RUDOLPH, A. M. (1983). Developmental changes in oxygenation and circulatory responses to hypoxemia in lambs. *Am J Physiol* **245,** H674-82.

SIEGHART, W. (1992). GABAA receptors: ligand-gated Cl- ion channels modulated by multiple drug-binding sites. *Trends Pharmacol Sci* **13,** 446-50.

SINGH, B. & ATWAL, O. S. (1997). Ultrastructural and immunocytochemical study of the pulmonary intravascular macrophages of Escherichia coli lipopolysaccharide-treated sheep. *Anat Rec* **247,** 214-24.

SMITH, S. S. (2002). Withdrawal properties of a neuroactive steroid: implications for GABA(A) receptor gene regulation in the brain and anxiety behavior. *Steroids* **67,** 519-28.

SONTI, G., ILYIN, S. E. & PLATA-SALAMAN, C. R. (1996). Anorexia induced by cytokine interactions at pathophysiological concentrations. *Am J Physiol* **270,** R1394-402.

STEEL, D. M. & WHITEHEAD, A. S. (1994). The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol Today* **15,** 81-8.

STEIGER, M., SENN, M., ALTREUTHER, G., WERLING, D., SUTTER, F., KREUZER, M. & LANGHANS, W. (1999). Effect of a prolonged low-dose lipopolysaccharide infusion on feed intake and metabolism in heifers. *J Anim Sci* **77,** 2523-32.

STEINSCHNEIDER, A. (1975). Nasopharyngitis and prolonged sleep apnea. *Pediatrics* **56,** 967-71.

STERIADE, M. & MCCARLEY, R. W. (1990). *Brainstem control of sleep and wakefulness*, New York: Plenum Press.

STONESTREET, B. S., OCAMPO, S. S. & OH, W. (1998). Reductions in cardiac output in hypoxic young pigs: systemic and regional perfusion and oxygen metabolism. *J Appl Physiol* **85,** 874-82.

STONESTREET, B. S., PETERSSON, K. H., SADOWSKA, G. B., PETTIGREW, K. D. & PATLAK, C. S. (1999). Antenatal steroids decrease blood-brain barrier permeability in the ovine fetus. *Am J Physiol* **276,** R283-9.

SUFFREDINI, A. F., FANTUZZI, G., BADOLATO, R., OPPENHEIM, J. J. & O'GRADY, N. P. (1999). New insights into the biology of the acute phase response. *J Clin Immunol* **19,** 203-14.

SULLIVAN, F. M. & BARLOW, S. M. (2001). Review of risk factors for sudden infant death syndrome. *Paediatr Perinat Epidemiol* **15,** 144-200.

SZETO, H. H. & HINMAN, D. J. (1985). Prenatal development of sleep-wake patterns in sheep. *Sleep* **8,** 347-55.

TAKAHASHI, S., KAPAS, L., FANG, J. & KRUEGER, J. M. (1999). Somnogenic relationships between tumor necrosis factor and interleukin-1. *Am J Physiol* **276,** R1132-40.

TAKEUCHI, Y., KIKUSUI, T., KIZUMI, O., OHNISHI, H. & MORI, Y. (1997). Pathophysiological changes evoked by lipopolysaccharide administration in goats. *J Vet Med Sci* **59,** 125-7.

THRANE, P. S., MAEHLEN, J., STOLTENBERG, L. & BRANDTZAEG, P. (1995). Retrograde axonal cytokine transport: a pathway for immunostimulation in the brain inducing hypoxia and sudden infant death? *Med Hypotheses* **44,** 81-4.

TORTEROLO, P., MORALES, F. R. & CHASE, M. H. (2002). GABAergic mechanisms in the pedunculopontine tegmental nucleus of the cat promote active (REM) sleep. *Brain Res* **944,** 1-9.

TOTH, L. A. & CHAUDHARY, M. A. (1998). Developmental alterations in auditory arousal from sleep in healthy and virus-infected cats. *Sleep* **21,** 143-52.

TOTH, L. A. & KRUEGER, J. M. (1989). Effects of microbial challenge on sleep in rabbits. *FASEB J* **3,** 2062-6.

TRACEY, K. J. & CERAMI, A. (1994). Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu Rev Med* **45,** 491-503.

TRIANTAFILOU, M. & TRIANTAFILOU, K. (2002). Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. *Trends Immunol* **23,** 301-4.

TURNBULL, A. V. & RIVIER, C. L. (1998). Intracerebroventricular passive immunization. I. The effect of intracerebroventricular administration of an antiserum to tumor necrosis factor-alpha on the plasma adrenocorticotropin response to lipopolysaccharide in rats. *Endocrinology* **139,** 119-27.

TURRIN, N. P., GAYLE, D., ILYIN, S. E., FLYNN, M. C., LANGHANS, W., SCHWARTZ, G. J. & PLATA-SALAMAN, C. R. (2001). Pro-inflammatory and anti-inflammatory cytokine mRNA induction in the periphery and brain following intraperitoneal administration of bacterial lipopolysaccharide. *Brain Res Bull* **54,** 443-53.

TURRIN, N. P. & PLATA-SALAMAN, C. R. (2000). Cytokine-cytokine interactions and the brain. *Brain Res Bull* **51,** 3-9.

UZUNOVA, V., SHELINE, Y., DAVIS, J. M., RASMUSSON, A., UZUNOV, D. P., COSTA, E. & GUIDOTTI, A. (1998). Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. *Proc Natl Acad Sci U S A* **95,** 3239-44.

VEDDER, H., SCHREIBER, W., YASSOURIDIS, A., GUDEWILL, S., GALANOS, C. & POLLMACHER, T. (1999). Dose-dependence of bacterial lipopolysaccharide (LPS) effects on peak response and time course of the immune-endocrine host response in humans. *Inflamm Res* **48,** 67-74.

VERTES, R. P. & PERRY, G. W. (1993). Sudden infant death syndrome: a theory. *Neurosci Biobehav Rev* **17,** 305-12.

VITKOVIC, L., BOCKAERT, J. & JACQUE, C. (2000). "Inflammatory" cytokines: neuromodulators in normal brain? *J Neurochem* **74,** 457-71.

VREUGDENHIL, A. C., DENTENER, M. A., SNOEK, A. M., GREVE, J. W. & BUURMAN, W. A. (1999). Lipopolysaccharide binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. *J Immunol* **163,** 2792-8.

WHYTE, R. I., WARREN, H. S., GREENE, E., GLENNON, M. L., ROBINSON, D. R. & ZAPOL, W. M. (1989). Tolerance to low-dose endotoxin in awake sheep. *J Appl Physiol* **66,** 2546-52.

WILSON, C. B. (1986). Immunologic basis for increased susceptibility of the neonate to infection. *J Pediatr* **108,** 1-12.

WRIGHT, S. D., RAMOS, R. A., TOBIAS, P. S., ULEVITCH, R. J. & MATHISON, J. C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249,** 1431-3.

XAIO, H., BANKS, W. A., NIEHOFF, M. L. & MORLEY, J. E. (2001). Effect of LPS on the permeability of the blood-brain barrier to insulin. *Brain Res* **896,** 36-42.

XIAO, Q., SUGUIHARA, C., HEHRE, D., DEVIA, C., HUANG, J. & BANCALARI, E. (2000). Effects of GABA receptor blockade on the ventilatory response to hypoxia in hypothermic newborn piglets. *Pediatr Res* **47,** 663-8.

YOLKEN, R. & MURPHY, M. (1982). Sudden infant death syndrome associated with rotavirus infection. *J Med Virol* **10,** 291-6.

ZIESCHE, R., PETKOV, V., WILLIAMS, J., ZAKERI, S. M., MOSGOLLER, W., KNOFLER, M. & BLOCK, L. H. (1996). Lipopolysaccharide and interleukin 1 augment the effects of hypoxia and inflammation in human pulmonary arterial tissue. *Proc Natl Acad Sci U S A* **93,** 12478-83.

ZUCKERMAN, S. H., EVANS, G. F. & BUTLER, L. D. (1991). Endotoxin tolerance: independent regulation of interleukin-1 and tumor necrosis factor expression. *Infect Immun* **59,** 2774-80.

ZWAIN, I. H. & YEN, S. S. (1999). Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. *Endocrinology* **140,** 3843-52.

ADDENDUM

Page 20, line 18 - delete "The following sections describe…its precursor progesterone" and replace with "The following sections describe the brain regions that regulate wakefulness and somnogenesis, as well as the somnogenic capabilities of the neurotransmitter GABA and neurosteroids, with particular attention to the role of allopregnanolone and its precursor progesterone."
Page 20, line 22 - insert the following:

1.5.3.1 Regulation of wakefulness
"The state of wakefulness is dependent upon the activity of the brainstem reticular formation, an extensive network of neurons, extending throughout the core of the medulla, pons and midbrain (Steriade and McCarley, 1990). This area receives and integrates information from visceral, somatic and sensory inputs, and exerts widespread effects over most cortical fields through neural projections to the thalamus and hypothalamus (Coenen, 1998). The regulation of wakefulness and arousal through specific neural connections is maintained by a series of neurotransmitters. For example, noradrenergic projections from the locus coeruleus (LC), and serotonergic projections from the dorsal raphe (DR) nucleus (both located in the hindbrain) are associated with arousal (Pace-Schott and Hobson, 2002). The transition between wakefulness and sleep occurs when the availability of these neurotransmitters decreases through deactivation of the reticular formation. The posterior hypothalamus is also involved in the process of behavioural arousal through the actions of histamine, an amine transmitter located in the tuberomammillary nucleus (TMN) (Pace-Schott & Hobson, 2002). As seen with the brainstem reticular formation, the transition from behavioural arousal to somnogenesis occurs through the reduced activity of these histaminergic cells (Pace-Schott & Hobson, 2002).

1.5.3.2 Regulation of sleep
The regulation of sleep requires a widely distributed neuronal network extending from the forebrain through to caudal regions of the brainstem. Although the mechanisms underlying the establishment of sleep are still not completely understood it has been postulated that the ventrolateral preoptic (VLPO) area of the anterior hypothalamus is a 'sleep-promoting' region of the brain (Sherin *et al.*, 1996). The initiation of sleep is thought to result from a reciprocal GABAergic and histaminergic-mediated interaction between the sleep-promoting effects of the VLPO and the wake-promoting effects of the posterior hypothalamus, respectively (Pace-Schott & Hobson, 2002). Neurons in the VLPO produce GABA, as well as galanin, an inhibitory neuropeptide (Sherin *et al.*, 1996). The activity of these neurons is proportional to the quantity of sleep and these neurons project to the arousal systems of the brainstem (LC and DR nucleus) and to the TMN, located in the posterior hypothalamus (Pace-Schott & Hobson, 2002).

The VLPO can be subdivided into two regions, the 'tightly clustered' or the 'diffuse' region (Pace-Schott and Hobson, 2002). These classifications are based upon the distribution pattern of neurons and each has its own postulated somnogenic effect. That is, neurons that are tightly clustered have been found to have projections that extend to the TMN (Pace-Schott and Hobson, 2002) and have been suggested as selectively promoting NREM sleep (Lu *et al.*, 2000). On the other hand, neurons that have a diffuse pattern have projections that extend to the brainstem (including the LC and DR nucleus) and may influence the incidence of REM sleep (Lu *et al.*, 2000 & Pace-Schott and Hobson, 2002). The summary provided on tis more concerned with the actions of neurosteroids. Therefore the following section will describe in more detail the studies that have investigated the somnogenic effects of GABA and its receptor $GABA_A$.

Page 24, line 14- replace "Therefore…postnatal period in the sheep" with "Therefore the first aim of this thesis was to determine the levels of neurosteroids, both centrally and peripherally, during the early post-natal period in the sheep."
Page 24, line 23 - delete last two sentences of final paragraph and replace with "Since allopregnanolone has somnogenic properties, it is hypothesised that allopregnanolone concentrations will increase in response to infection and hypoxic challenge, resulting in increased sleep. Therefore the specific aims of the studies presented in Chapters 3 and 4 was to assess the effects of an inflammatory challenge (i.e. endotoxin) and hypoxia, respectively, on the incidence of sleep-wake behaviour and neurosteroid levels in both plasma and brain tissue. The aim of the study presented in Chapter 5 was to compare the effects of the individual stressors with a combined endotoxin/hypoxia treatment on sleep-wake behaviour and neurosteroid concentrations. Whilst the study presented in Chapter 6 aimed at determining the effects of the stressors on circulating pro-inflammatory cytokine levels."
Page 35, line 17 - replace "… directly into the nebuliser...generated smaller particles that were easier to atomise" with "… directly into the nebuliser allowing for smaller sample volumes to be used"
Page 40, line 5 - delete "This assessment…being assayed" and replace with "This assessment determined that 48 hours, although no different to 72 hours, was optimal for all samples to achieve equilibrium prior to being assayed. It also meant that the process of homogenising, extracting and assaying could all be performed within a week."
Page 57, line 4 - replace "…has been proposed as a risk factor in SIDS" with "…has been suggested as a contributor to the aetiology of SIDS."
Page 58, line 31- replace the first sentence of the third paragraph with "Therefore the first aim of this chapter was to assess if the young lamb is able to initiate a neurosteroid response to an inflammatory challenge, and if so, whether this occurs along with increased levels of sleep."
Page 61, line 19 - insert sentence "Each concentration was tested on each animal in a random fashion, which therefore excluded the possibility of any age affect."

Page 67, line 2 - insert sentence "All cardiorespiratory parameters were measured on a minute-by-minute basis averaged over 5, 10 or 30 minute intervals and analysed. As there were no differences between the three analyses, the data presented is a representation of the effects of LPS averaged over 30 minute intervals."
Page 68, line 22 - insert "...duration of the experiment, *including the 1 hour epoch immediately following the return of lambs to their mothers*"
Page 82, line 6 - insert sentence "These changes in blood gases, although mild, are likely to be a consequence of the well established injurious effect, including increased pulmonary permeability and oedema, that gram negative bacterium, has on the lung (Stephens *et al.*, 1988; Redl *et al.*, 1990)."
Page 82, line 11 - insert "LPS treatment has also been reported to alter cardiovascular properties including decreasing cardiac output and increasing systemic vascular resistance (Goto *et al.*, 1988). In this study however, a moderate biphasic..."
Page 83, line 15 - replace "...it has not yet been established that LPS treatment actually up-regulates either gene expression..." with "no study has investigated the effects of LPS on gene expression..."
Page 84, line 16 - insert paragraph "The VLPO, located in the anterior hypothalamus has been postulated as being the 'sleep-centre' of the mammalian brain (Sherin *et al.*, 1996). In the current study, this area would have been included in the diencephalon, an area that did not show an increase in allopregnanolone concentration following LPS treatment. This is not to say however, that LPS through the actions of allopregnanolone had no contribution to the overall somnogenic effects observed. It may be that a possible increase in allopregnanolone concentration localised to the anterior hypothalamus was not detected due to the use of combined tissue, particularly a tissue sample that included the posterior hypothalamus, an area important for the regulation of wakefulness. Other areas that are also involved in the generation of sleep, such as the midbrain and cortex, did however, show significant increases in allopregnanolone concentration. Therefore, it is possible that the 2-3 fold..."
Page 84, line 16 - replace "...no study has reported increased sleepiness following the direct infusion..." with "...no study has investigated the somnogenic effects of infusing similar concentrations of allopregnanolone into the brain"
Page 84, line 29 - insert sentence "In addition, as there was no development of tolerance in the behavioural effects observed in lambs treated with LPS, we can only speculate based on the hypothesis presented for this chapter, that there was also no effect of tolerance on the responses of central allopregnanolone concentrations to this stressor. Although this issue would have ideally been addressed with the measurement of central allopregnanolone levels at the conclusion of each experiment, this would have required killing additional animals."
Page 85, line 19 - insert sentence "Although not considered to be the direct cause for SIDS death, the use of the endotoxin, LPS, gives an insight into the possible role of an infectious/inflammatory challenge in the aetiology of SIDS."
Page 96, line 2 - insert sentence "Cardiorespiratory parameters were again analysed over 5, 10 and 30 minute intervals as reported in Chapter 3. As there were no significant differences between the analyses, for ease of interpretation, the data below is a representation of the effects of hypoxia averaged over 30 minute intervals."
Page 98, line 10 - delete Section 4.2.5.1 and replace with "To gain an understanding of the immediate short-term effects of hypoxia, analysis of data using intermittent epoch lengths was used. These results are presented in Figures 4.6 and 4.7. Control treatment had no effect on wakefulness or on any of the sleep states (Figure 4.6; *black bars*). In contrast, lambs that were exposed to a single episode of hypoxia showed a significant reduction in wakefulness (Figure 4.6A; *hatched bars*) and an increased incidence of quiet sleep (Figure 4.6B; *hatched bars*) that occurred well before the onset of hypoxia. These lambs were also found to experience much higher levels of wakefulness in the first epoch when compared to control lambs (Figure 4.6A). It is unlikely that the application of the hood contributed to this higher level of wakefulness, as the incidence of wakefulness was not significantly different between the 30 minutes prior to hood application and the 30 minutes following hood application (data not shown). Therefore, the observation of a reduced incidence of wakefulness before hypoxia was introduced may simply be a consequence of the initial high levels of wakefulness in these hypoxic lambs (Figure 4.6A; *hatched bars*). When examining the epochs immediately before, during and after hypoxia, it can be seen that hypoxia had no affect on the incidence of wakefulness (Figure 4.6A; *hatched bars*). However there did appear to be a slight reduction in the incidence of quiet sleep (Figure 4.6B; *hatched bars*) that was associated with an increased incidence of active sleep (Figure 4.6C; *hatched bars*) immediately following hypoxia, but again this was not significant. Indeterminate sleep remained unaffected throughout the experiment (Figure 4.6D; *hatched bars*).

The incidence of wakefulness in lambs to be treated with repeated episodes of hypoxia was reduced significantly within the first hour post-saline administration (Figure 4.7A; *hatched bars*). As observed in single hypoxia experiments, the level of wakefulness was significantly higher than controls (Figure 4.6A) and therefore may have contributed to this affect (again the application of the hood was not found to contribute to the increased levels of wakefulness observed). Examination of the data surrounding the first episode of hypoxia found no change in the incidence of wakefulness immediately before, during or after hypoxia (Figure 4.7A; *hatched bars*). The incidence of quiet sleep (Figure 4.7B; *hatched bars*) also remained unaffected by the first hypoxic challenge. Active sleep was found to significantly increase following the cessation of hypoxia (Figure 4.7C; *hatched bars*) whilst the incidence of indeterminate sleep was found to significantly increase during the first episode of hypoxia (Figure 4.7D; *hatched bars*). The second episode of hypoxia also appeared to have no immediate affect on the incidence of wakefulness, that is, there was no differences found

immediately before, during or after hypoxia exposure (Figure 4.7A; *hatched bars*). However, when comparing the levels of wakefulness between each hypoxia challenge, it was found that there was a significant reduction during the second episode (Figure 4.7A; *hatched bars*). This reduction in wakefulness during the second episode of hypoxia, as compared to the first, occurred with a concomitant increase in the incidence of quiet sleep during the second hypoxia challenge (Figure 4.7B; *hatched bars*). Active sleep remained unaffected during the second challenge, however, as found with the first challenge, levels increased following the cessation of hypoxia (Figure 4.7C; *hatched bars*). Indeterminate sleep remained unaffected during the second episode of hypoxia (Figure 4.7D; *hatched bars*)."

Page 108, line 8 - delete "…variability and lack of clarity of results…" and replace with "…variability, most likely due to the small sample size (n=4) of results…"

Page 108, line 18 - insert paragraph "The incidence of sleep-wake behaviour was also not different to those observed in the earlier repeated hypoxia experiment. That is, there was no immediate effect of hypoxia on the incidence of wakefulness during the first episode, however there was a significant reduction during the second (as compared to the first). This finding occurred concomitantly with an increase in the incidence of quiet sleep during the second episode. Active sleep again was found to increase immediately following the first episode of hypoxia, whilst there was no change in the incidence of indeterminate sleep. Overall, lambs exposed to repeated episodes of hypoxia again exhibited an increased incidence of quiet sleep that was associated with a concomitant decrease in wakefulness. Active sleep and indeterminate sleep were not affected."

Page 114, line 6 – delete "This reduces O_2 demand…and cardiac output (Almeida *et al.*, 1999; Bicego *et al.*, 2002)" and replace with "This in turn reduces energy requirements by lowering metabolite demand. In addition, the lower body temperature shifts the O_2 dissociation curve to the left ultimately reducing the supply of O_2 release to the tissues (Almeida *et al.*, 1999; Bicego *et al.*, 2002)."

Page 114, line 22 – delete the following sentence "The reduction in PaO_2 as a consequence of hypoxia…" and replace with "The reduction in PaO_2 during periods of hypoxia…"

Page 114, line 23 - delete "However, as there was no evidence…increase in gas exchange took place to compensate for the hypoxia" and replace with "However, as there was no evidence of increased respiratory rate, it may be that there was an increase in tidal volume leading to an increase in ventilation. In addition, there may have also been an increase in pulmonary blood flow and recruitment within the lung."

Page 114, line 25 – delete sentence and replace with "However, as there was no evidence of increased respiratory rate, other factors such as increased pulmonary blood flow or increased tidal volume may have occurred to compensate for the hypoxia."

Page 114, line 28 - delete sentence and replace with "The decreased $PaCO_2$ was therefore a reflection of increased ventilation (relative to that required to maintain normal $PaCO_2$ levels) in response to the hypoxia."

Page 115, line 10 - replace "The greater effect of a single hypoxia…is sufficient to induce such dramatic changes" with "The greater effect of a single hypoxia episode on plasma lactate levels in these lambs may be a result of the longer duration of the single episode (1 hour) compared to the repeated episodes (45 minutes each), particularly when plasma lactate concentrations at 3 hours where not significantly different between the two hypoxic groups. However, it is unclear whether this length of time (i.e 15 minutes) is sufficient to induce such dramatic changes."

Page 117, line 21 - insert paragraph "Although neurosteroids, due to their lipophilic nature, are able to cross the blood-brain-barrier, the source of plasma neurosteroids is largely restricted to the adrenal glands and gonads in the fetus (Nguyen, *personal communication*) and adult (Smith, 2002). In the fetus, adrenalectomy markedly reduces plasma, but not brain allopregnanolone concentrations (Nguyen *et al.*, *personal communication*). In addition, a study performed by Barbaccia and colleagues (1996) where rats were exposed to CO_2, found that inhalation of CO_2 induced increases in brain and plasma samples, the time dependence of which varied. That is, allopregnanolone in plasma peaked within 30 mins following the stress whilst, brain allopregnanolone concentrations showed a slower and longer-lasting increase. A similar conclusion was also drawn by Bernardi *et al.* (1998). In this study these authors examined the possible age-related variations in allopregnanolone content in brain tissue and plasma. From their work, they found that there were opposite variations in allopregnanolone concentration between the brain and plasma. Thus they too concluded that the brain is independent of circulating allopregnanolone. Finally, studies in which gonadectomy and/or adrenalectomy was performed revealed that allopregnanolone remained in the nervous system, despite reduced or no evidence of peripheral allopregnanolone concentrations (Corpechot *et al.*, 1981; Corpechot *et al.*, 1983). Therefore, the available data, including those reported in this thesis support the concept of differential and independent regulation of neurosteroidogenesis in the peripheral and central nervous systems. The concentration of steroids in the brain and the effects of adrenalectomy suggest that it is unlikely that the brain is a source of plasma allopregnanolone."

Page 117, line 23 - delete the first paragraph and replace with "Hypoxia can have major implications for CNS activity in infants and adults, depending on severity. It can alter brain activity (Moss, 2000) and arousal responses (Vertes & Perry, 1993), and has been suggested as a factor contributing to SIDS deaths (see Ozawa & Takashima, 2002). In the current study, when analysing the data in variable epoch lengths over the course of the experiment (Figure 4.6), it was found that a single episode of hypoxia had no immediate effect (that is during and after the hypoxia challenge) on the incidence of sleep-wake behaviour. Similarly, when examining the somnogenic effects of this treatment in blocks of time (i.e. between 0 and 3 hours etc.), there was again no effect on wakefulness. However, overall there was a significant increase

in quiet sleep compared to controls (Figure 4.8). The increased quiet sleep was most likely offset by a tendency for active sleep to decrease during the same time periods and therefore ultimately did not produce any affect on the incidence of wakefulness. Despite the differences between the two types of analyses, it is apparent from this study that a single episode of hypoxia does not significantly alter the architecture of sleep-wake behaviour in young lambs. This is in contrast to a repeated episode of hypoxia where there were more variable immediate effects. The incidence of wakefulness was significantly reduced during the second episode of hypoxia when compared to the first (Figure 4.7). This reduction was associated with a concomitant increase in quiet sleep, supporting some of the overall findings (Figure 4.9). When inspecting the data from the period 0.5-3.5 hours (Figure 4.9B), it can be seen from Figure 4.7A, that this period includes pre-treatment levels of wakefulness that were not significantly different to controls, thereby reducing the confounding effects of the high levels of wakefulness. Despite the difficulty in analysing the data from lambs treated to hypoxia, it does appear that overall, the incidence of sleep is increased significantly in lambs that had been exposed to two bouts of hypoxia."

Page 118, line 13 – insert sentence "However, as observed in the previous chapter with LPS administration, hypoxia did not elicit an increase in allopregnanolone concentrations in the diencephalon, the tissue encompassing the anterior hypothalamus, or VLPO. As discussed in Chapter 3, this finding does not exclude the possibility that there was an increase in allopregnanolone in this sleep-promoting region. On the basis of these studies it is clear that future work should isolate the anterior hypothalamus to be able to conclusively determine whether allopregnanolone specifically targets this region, or whether the somnogenic effects of hypoxia, and likewise LPS are a result of increased allopregnanolone levels in other sleep-promoting areas."

Page 118, line 16 - delete sentence and replace with "However, studies by Johnston *et al.* (1998, 1999) have shown that arousal responses in lambs do differ to those observed in human infants. That is, in the lamb, thresholds are higher during active sleep when compared to quiet sleep. In our studies, the incidence of active sleep was found to increase significantly following the cessation of hypoxia. It is possible that elevated neurosteroid concentrations in association with increased active sleep following hypoxia exposure may increase arousal thresholds in lambs. However it is also possible that these mechanisms increase arousal thresholds in human neonates and may increase the risk of SIDS by altering sleep-wake behaviour."

Page 118, line 31 - replace "Repeated hypoxia...habituation between exposures" with "Repeated hypoxia challenges resulted in more defined and conclusive behavioural responses without any evidence of habituation between exposures, again suggesting no effect of tolerance on central allopregnanolone responses (see Chapter 3 for further discussion)."

Page 130, line 7 - insert sentence "The effect of the combined LPS and hypoxia treatment on the incidence of sleep-wake behaviour during this final experiment was not different to those reported initially (data not shown). That is, there was a significant reduction in the incidence of wakefulness during the first episode that was sustained through to euthanasia. This reduction in wakefulness occurred concomitantly with an increase in quiet sleep, whilst active sleep and indeterminate sleep remained unaffected."

Page 136, line 10 - delete "It was surprising to find...behavioural state any further" and replace with "It was surprising to find that there was no additional effect on somnogenesis when hypoxia and LPS treatments were combined, compared to LPS treatment alone. There was however a greater increase in brain allopregnanolone content in the thalamus/hypothalamus and midbrain, regions that are designated sleep-promoting areas. Considering that allopregnanolone has been reported as having somnogenic properties (Korneyev & Costa, 1996; Lancel *et al.*, 1997), it was expected that the combined treatment regime would result in further reductions in the incidence of wakefulness and, hence, increase the incidence of sleep compared to the individual stressors. The fact that the combined LPS and hypoxia treatment produced a significant increase in allopregnanolone levels in the thalamus/hypothalamus suggests that this treatment may have been strong enough to elicit changes in the anterior hypothalamus. Since there was no additional somnogenic effect however, it is possible that the concentration of allopregnanolone had risen to a point where its effect was maximal. Likewise, it is also possible that due to the already low levels of wakefulness produced as a result of the LPS treatment (see Section 8.2.5), the addition of hypoxia may not have been able to depress this behavioural state any further. In both cases however, these points require further investigation, as we are unaware of any study(s) that have focused on these areas of question."

Page 139, line 14 - insert sentence "The pro-inflammatory cytokines, IL-1β, TNF-α and IL-6 have been implicated as key mediators in this process of inflammation (Krueger *et al.*, 1990; Leon *et al.*, 1998). Although it is understood that IL-1β has an essential role in the mediation of the APR and somnogenesis (Krueger *et al.*, 1990), other pro-inflammatory cytokines such as TNF-α and IL-6 have also been found to have somnogenic properties. For example, administration of TNF-α in rabbits, has been reported to produce large increases in the incidence of NREM sleep (Krueger *et al.*, 1998), whilst large increases in IL-6 have been associated with excessive daytime sleepiness in humans (Vgontzas & Chrousos, 2002)".

Page 139, line 32 - replace "Since pro-inflammatory cytokines have somnogenic properties..." with "Since TNF-α and IL-6 have been reported to have somnogenic properties..."

Page 140, line 3 – replace "Therefore, it is possible...cytokine cascade..." with "Therefore, it is hypothesised that their combination may induce an uncontrolled cytokine cascade..."

Page 144, line 16 - replace "...concentrations remained elevated significantly compared to pre-treatment values for the remainder of the experiment" with "...concentrations remained elevated significantly compared to pre-treatment values at 2 and 3 hours post-LPS administration, but returned to control values at the completion of the experiment."
Page 151, line 3 - replace "...focused primarily on the contribution of neurosteroids to behavioural..." with "...focused primarily on the association between neurosteroids and behavioural..."
Page 154, line 17 - insert sentence "Future studies investigating adrenal concentrations of allopregnanolone and its precursors would be of interest to determine whether there is increased metabolism of allopregnanolone under hypoxic conditions (as opposed to inflammation) or whether other potent neurosteroids are formed preferentially."
Page 155, line 9 - insert "It was not until most animal studies had been completed that the potential importance of arousal in these studies was realised, therefore further studies..."
Page 163, line 8 - insert sentence "The effects of LPS and/or hypoxia treatments on cardiorespiratory patterns over 30 minute intervals are presented in Figure 8.5 (analysis of data over 5 and 10 minute intervals provided no additional information)."

Additional references to be included;
Page 179 – insert reference after Coceani *et al.*, (1995);
COENEN, A. M. (1998). Neuronal phenomena associated with vigilance and consciousness: from cellular mechanisms to electrographic patterns. *Conscious Cogn* **7,** 42-53.
Page 182 – insert reference after Gore *et al.*, (1996);
GOTO, M., GRIFFIN, A. J., CHIEMMONGKOLTIP, P., ONOUCHI, Z. & BERNHEIM, B. (1988). Endotoxin shock in newborn dogs: serial haemodynamic studies. *J Lab Clin Med* **112,** 109-17.
Page 187 – insert reference after Lindgren *et al.*, (1996);
LU, J., GRECO, M. A., SHIROMANI, P. & SAPER, C. B. (2000). Effect of lesions of the ventrolateral preoptic nucleus on NREM and REM sleep. *J Neurosci* **20,** 3830-42.
Page 190 – insert reference after Ozawa & Takashima (2002);
PACE-SCHOTT, E. F. & HOBSON, J. A. (2002). The neurobiology of sleep: genetics, cellular physiology and subcortical networks. *Nat Rev Neurosci* **3,** 591-605.
Page 191 – insert reference after Read *et al.*, (1998);
REDL, H., SCHLAG, G. & LAMCHE, H. (1990). TNF- and LPS-induced changes of lung vascular permeability: Studies in unaesthetised sheep. *Circ Shock* **31,** 183-92.
Page 192 – insert reference after Shanks *et al.*, (2000);
SHERIN, J. E., SHIROMANI, P. J., MCCARLEY, R. W. & SAPER, C. B. (1996). Activation of ventrolateral preoptic neurons during sleep. *Science* **271,** 216-9.
Page 193 – insert reference after Steinschneider (1975);
STEPHENS, K. E., ISHIZAKA, A., LARRICK, J. W. & RAFFIN, T. A. (1988). Tumor necrosis factor causes increased pulmonary permeability and oedema. Comparison to septic acute lung injury. *Am Rev Respir Dis* **137,** 1364-70.
Page 194 - insert reference after Vertes & Perry (1993);
VGONTZAS, A. N. & CHROUSOS, G. P. (2002). Sleep, the hypothalamic-pituitary-adrenal axis, and cytokines: multiple interactions and disturbances in sleep disorders. *Endocrinol Metab Clin* **31,** 15-36.