ADRENOMEDULLARY REGULATION DURING INTRAUTERINE STRESS IN THE FETAL SHEEP

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" 'Forty-two!' yelled Loonquwal. 'Is that all you've got to show for seven and a half million years' work?'

'I checked it very thoroughly,' said the computer, 'and that quite definitely is the answer. I think the problem, to be quite honest with you, is that you've never actually known what the question is.'

'But it was the Great Question! The Ultimate Question of Life, the Universe and Everything,' howled Loonquwal.

'Yes,' said Deep Thought with the air of one who suffers fools gladly, 'but what actually is it?' "

Douglas Adams

The Hitch Hiker's Guide to the Galaxy (1979)

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ABSTRACT

Whilst the mechanisms mediating catecholamine secretion from the fetal adrenal medulla in response to physiological stress are relatively well characterised, the regulation of catecholamine synthesis in the fetal adrenal has yet to be investigated. The primary aims of this thesis were therefore to examine the impacts of development and acute and chronic stress upon the gene expression of two of the key catecholamine synthetic enzymes, tyrosine hydroxylase (TH) and phenylethanolamine *N*-methyltransferase (PNMT), in the adrenal medulla of the fetal sheep.

Adrenal TH mRNA expression was found to be maximal coincident with the establishment of functional splanchnic innervation of the adrenal whilst PNMT mRNA expression peaked in late gestation coincident with the prepartum surge in adrenal glucocorticoid output. Acute fetal hypoxia resulted in a decrease in the expression of adrenal TH mRNA and an increase in PNMT mRNA expression, both before and after development of functional adrenal innervation. These changes were related to the degree of change in fetal arterial PO_2. The changes in adrenal TH and PNMT mRNA expression with hypoxia after the development of adrenal innervation were attenuated by the nicotinic receptor antagonist, hexamethonium, indicating that they were neurally mediated.

There is an increase in cortisol secretion from the fetal sheep adrenal in response to stress and before delivery. Intrafetal infusion of cortisol to mimic the prepartum cortisol rise, at a stage in gestation when fetal cortisol levels are low, resulted in a significant and specific decline in PNMT mRNA and protein expression in the fetal adrenal.

Placental restriction of fetal growth also resulted in a specific decrease in adrenal PNMT mRNA expression in late gestation and there was a direct correlation between PNMT mRNA expression and mean gestational arterial PO_2.

In summary, there is differential regulation of adrenal catecholamine synthetic enzyme gene expression during development and in response to neurogenic and hormonal stimulation. Catecholamine synthetic enzyme gene expression is also differentially regulated by hypoxia prior to adrenal innervation. Intrauterine growth

retardation and inappropriate fetal exposure to excess glucocorticoids exert specific suppressive effects upon adrenal PNMT mRNA expression which may manifest themselves as impaired responses to physiological stress in the fetus and neonate.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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PUBLICATIONS ARISING FROM THIS THESIS

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RELATED PUBLICATIONS

ADAMS, M.B., SIMONETTA, G., MCMILLEN, I.C. (1996) The non-neurogenic catecholamine response of the fetal adrenal to hypoxia is dependent on voltage sensitive Ca^{2+} channels. *Developmental Brain Research*, **94**, 182-189.

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COMMONLY USED ABBREVIATIONS

A

AADC	aromatic amino acid decarboxylase
ABC	avidin-biotin-peroxidase complex
ACE	angiotensin converting enzyme
ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
Ad	adrenaline
Ang II	angiotensin II
ANOVA	analysis of variance
AP-1	activator protein 1
ATF1	activating transcription factor 1
ATP	adenosine triphosphate

B C

b	bovine
bp	base pair(s)
BPt	tetrahydrobiopterine
BAT	brown adipose tissue
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine 3',5'-monophosphate
CGRP	calcitonin gene related peptide
CRE	cyclic AMP response element
CREB	cAMP response element binding protein
CRF	corticotrophin releasing factor
CTP	cytidine triphosphate
CV	coefficient of variation

D E

d	days
DAB	3,3-diaminobenzadine tetrahydrochloride
DAG	diacylglycerol
DBH	dopamine β hydroxylase
DHBA	3,4 dihydroxybenzylamine
DMPP	dimethylphenylpiperazinium
DNA	deoxyribonucleic acid
DOPA	dihydroxyphenylalanine

ΔPO_2	experimentally induced change in fetal arterial PO_2
E	embryological day
ECD	electrochemical detection
EDTA	ethylenediamine tetraacetic acid
Enk	enkephalin
EPO	erythropoietin

F G H

F_iO_2	fractional inspired oxygen
FGF	fibroblast growth factor
g	gravitational acceleration
GR	glucocorticoid type II receptor
GRE	glucocorticoid response element
GSH	reduced glutathione
GSSG	oxidised glutathione
h	hours
Hex	hexamethonium
HIF-1	hypoxia inducible factor 1
HIP	hypoxia induced proteins
HIPBS	hypoxia induced protein binding sequence
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
HPX	hypophysectomised

I K L M N

ICER	inducible cAMP early represser
IP_3	inositol trisphosphate
ir	immunoreactive
IUGR	intrauterine growth retardation
iv	intravenous
kb	kilobases
K_i	Inhibitory constant
K_m	Michaelis constant
L	lumbar
LB	Luria-Bertani
Leu	Leucine
mAChR	muscarinic acetylcholine receptor
Met	Methionine
min	minutes
mRNA	messenger ribonucleic acid
nAChR	nicotinic acetylcholine receptor

NAd	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
NGF	nerve growth factor
NGS	normal goat serum
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide tyrosine
NSF	*N*-ethylmaleimide sensitive fusion protein

O P R

OD	optical density
ODS	octadecylsilylsilica
P-450$_{c17}$	P-450 17α-hydroxylase / *C*17-*C*20 lyase
P-450$_{c21}$	P-450 21-hydroxylase
P-450$_{SCC}$	P-450 cholesterol side chain cleavage
PACAP	pituitary adenylate cyclase activating peptide
P_aCO_2	partial pressure of CO_2 in arterial blood
P_aO_2	partial pressure of O_2 in arterial blood
PBS	phosphate buffered saline
PCA	perchloric acid
PEnk A	proenkephalin A
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PNMT	phenylethanolamine *N*-methyltransferase
POMC	proopiomelanocortin
PR	placental restriction
RAS	renin angiotensin system
ROI	reactive oxygen intermediate
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	room temperature

S T

SA	sympathoadrenal
SAM	*S*-adenosylmethionine
S_aO_2	oxygen saturation of arterial blood
SCG	superior cervical ganglion
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SNAP	soluble NSF attachment proteins

SNARE	SNAP receptors
SSC	saline sodium citrate
SSPE	saline sodium phosphate EDTA
T	thoracic
TBE	tris-borate EDTA
TH	tyrosine hydroxylase
TPA	12-*O*-tetradecanoylphorbal 13-acetate
TTX	tetrodotoxin

U V

UCP 1	uncoupling protein 1
VAMP	vesicle membrane associated protein
VEGF	vascular associated growth factor
VIP	vasoactive intestinal polypeptide
V_{max}	maximal velocity

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1. ADRENOMEDULLARY CATECHOLAMINE SECRETION, SYNTHESIS, AND ACTIONS IN THE FETUS

1.1 INTRODUCTION

It is well established that catecholamines synthesised in the adrenal medulla of the fetus are secreted in response to an array of intrauterine stressors. The subsequent increase in circulating catecholamine concentrations initiate and coordinate a number of physiological responses in the fetus which are essential for survival. The fetal adrenal glands, which are proportionally larger than those of the adult, are a key source of catecholamines during physiological stress due to the functional immaturity of the peripheral sympathetic nervous system.

Despite the documented importance of adrenomedullary catecholamines in the response to stress *in utero*, our current understanding of how catecholamine secretion and biosynthesis in the fetal adrenal medulla are regulated during acute and chronic stress remains poor. The current review briefly summarises the structural and functional development of the adrenal gland and then discusses the regulation of catecholamine biosynthesis and secretion in the adult adrenal medulla. The crucial role that the adrenal medulla and catecholamines play in the fetal response to physiological stress and the transition to extrauterine life is then considered in detail. The main focus of this review is the concluding examination of the current state of knowledge on the regulation of adrenomedullary catecholamine synthesis and secretion in the adrenal medulla of the fetus.

1.2 ANATOMY AND EMBRYOLOGY OF THE ADRENAL GLAND

1.2.1 THE ADRENAL MEDULLA

The embryological origins of the adrenal medulla lie in ectodermal neural crest cells which migrate ventrally from the apex of the neural tube to the dorsal aorta where they aggregate and differentiate to form sympathetic neurones, or to the adrenal gland primordia where they differentiate to form chromaffin cells (Coupland, 1980; Hammond & Yntema, 1947; LeDouarin & Dupin, 1993; Turkel & Itabashi, 1974). The migratory primitive chromaffin cells invade the medial side of the developing adrenal cortical anlage and pass between the cortical cells and as the medulla occupy the centre of the gland (Boshier *et al.*, 1989*a*; Coupland,

1980; Coupland & Tomlinson, 1989; Robinson *et al.*, 1979*b*).

In the developing mammalian adrenal medulla, three types of cells can be observed depending on the stage of development (Coupland, 1965; Coupland & Weakley, 1968; El Maghraby & Lever, 1980; Hervonen, 1971). The first type are the primitive sympathetic migratory neurones which are known as sympathogonia (Coupland, 1965) and are totipotent being capable of differentiating into either sympathetic neurones or chromaffin cells depending on migratory route (LeDouarin, 1980; LeDouarin & Dupin, 1993) and the levels of environmental factors such as the presence of glucocorticoids (Anderson & Michelson, 1989; Doupe *et al.*, 1985; Michelsohn & Anderson, 1992), nerve growth factor (NGF) (Anderson & Axel, 1986; Doupe *et al.*, 1985; Grothe *et al.*, 1985) and fibroblast growth factors (Anderson, 1993; Blottner *et al.*, 1989). These cells are characterised by a small rounded nucleus with only a very thin peripheral rim of cytoplasm (Coupland, 1965). The second type of cell are the phaeochromoblasts with large elongated nuclei and a cytoplasm which is devoid of catecholamine secretory granules (Coupland, 1965). The third and final cell type is the mature 'chromaffin' cell or phaeochromocyte which possesses a smaller ovoid nucleus and contain catecholamine storing granules with the cytoplasm giving a positive staining reaction to chromic acid due to the oxidation of the catecholamines to melanin (Coupland, 1965).

Whilst the development of the mammalian adrenal medulla displays a common sequence of epigenetic events amongst a number of species, the timing of these events varies quite markedly between species (Boshier *et al.*, 1989*a;* Elfvin, 1967; Hervonen, 1971; Weakley & Coupland, 1965; Wilburn *et al.*, 1986). In the developing adrenal gland of the fetal sheep small groups of migratory chromaffin cells have been reported to be found amongst the cortical cells of the adrenal anlagen from as early as 40 d gestation (term = 147 ± 3 d gestation) (Robinson *et al.*, 1979*b*). At 53 d gestation, whorls and columns of migratory sympathochromaffin cells containing phaeochromoblasts and phaeochromocytes are evident amongst the cortical cells (Boshier *et al.*, 1989*a*). At this stage of medullary development the phaeochromocytes exclusively possess asymmetric electron dense granules, characteristic of noradrenaline-containing vesicles (Boshier *et al.*, 1989*a*). By 80-100 d gestation the separation of the medulla from

the cortex is complete (Boshier *et al.*, 1989*a;* McMillen *et al.*, 1988; Robinson *et al.*, 1979*b*). The medulla itself is found to contain two populations of chromaffin cells, the outer juxtacortical medullary cells which are columnar in appearance and the central medullary cells which are polygonal (Boshier *et al.*, 1989*a;* McMillen *et al.*, 1988). The juxtacortical medullary cells are arranged in palisades adjacent to blood sinusoids interdigitating with the adrenal cortex and enclosing the central medullary cells (Boshier *et al.*, 1989*a;* McMillen *et al.*, 1988). Both the juxtacortical and central medullary cells possess noradrenaline containing granules (Boshier *et al.*, 1989*a*). The juxtacortical medullary cells maintain their relationship with the corticomedullary blood vessels over the rest of gestation but increasingly exhibit the presence of adrenaline containing vesicles with some cells having almost exclusively adrenaline containing vesicles (Boshier *et al.*, 1989*a*). Central medullary cells increase in number over the rest of gestation but show little change ultrastructurally, possessing only noradrenaline containing granules (Boshier *et al.*, 1989*a*). In late gestation the medullary cells become more discretely organised with groups of cells being enclosed by connective tissue sheaths in which blood vessels and innervating axons (Boshier *et al.*, 1989*a*). The detailed morphometric studies of Boshier *et al.* (1989*a*) demonstrated that there are two periods of adrenomedullary growth with the major increase occurring over the period of migration of chromaffin cells into the adrenal gland between 53 d-100 d gestation. A second phase of growth occurring between 130 and 144 d gestation which appears to be associated with functional maturation of the adrenal gland (Boshier *et al.*, 1989*a*).

Aside from the adrenal medulla, phaeochromoblasts and phaeochromocytes also gather on both sides of the aorta forming paraganglia with well developed sinusoidal capillary networks indicative of active endocrine glands (Phillipe, 1983). This extra-adrenal chromaffin tissue, which has been studied in a number of mammalian species, is unique to the fetus and degenerates in early postnatal life (Brundin, 1966; DeGallardo *et al.*, 1974; Phillipe, 1983). The paraganglia which contain and secrete predominantly noradrenaline and dopamine and lack adrenaline, display biochemical and histological maturation earlier than the chromaffin tissue of the adrenal medulla (Coupland, 1980; Phillipe, 1983). In species such as man, dog, cat, rabbit, and guinea pig the paraganglia are a major source of catecholamines in fetal life with relative catecholamine levels being

greater than those observed in the adrenal gland (Brundin, 1966; DeGallardo *et al.*, 1974; Eranko *et al.*, 1966; Phillipe, 1983). In the fetal sheep, however, the paraganglia are much less prominent as the amount of extra-adrenal chromaffin tissue and their catecholamine stores (approximately 1 % of that in the adrenal) are minor in relation to other species (Jones *et al.*, 1987). Interestingly, selective destruction of the adrenal medulla (demedullation) in fetal sheep induces a 4-5 fold increase in para-aortic body mass and a four-fold increase in their catecholamine stores, suggesting a compensatory role for this tissue in the sheep (Jones *et al.*, 1987).

1.2.2 THE ADRENAL CORTEX

The adrenal cortex in the adult consists of three functionally and histologically distinct zones; the zona glomerulosa, zona fasciculata, and zona reticularis. The outermost and thinnest layer of the adrenal cortex, the zona glomerulosa, is composed of discontinuous subcapsular aggregates of small, lipid poor, cells (McNicol, 1992; Soffer *et al.*, 1961). The most substantial component of the of the adrenal cortex, the zona fasciculata, consists of parallel columns of large, lipid rich, cells which extend from the zona glomerulosa to the inner zona reticularis (McNicol, 1992; Soffer *et al.*, 1961). The cells of the zona reticularis are compact with little lipid storage and quite frequently contain the pigment, lipofuscin (McNicol, 1992; Soffer *et al.*, 1961). The cells of the zona glomerulosa, zona fasciculata, and zona reticularis synthesise and secrete mineralocorticoid, glucocorticoid, and androgenic hormones, respectively (McNicol, 1992).

The mammalian adrenal cortex has its embryological origins in the mesoderm arising from mesenchymal tissue adjacent to the coelomic epithelium lying close to the urogenital ridge (McNicol, 1992; Soffer *et al.*, 1961). Mesothelial cells between the root of the mesentery and the developing gonad undergo proliferation and subsequently penetrate the underlying mesenchyme (Sadler, 1985). These cells then differentiate to form the adrenocortical masses which are invaded during development by migrating sympathochromaffin cells which will form the adrenal medulla (Robinson *et al.*, 1979*b;* Sadler, 1985; Soffer *et al.*, 1961). Adrenocortical development in the sheep differs somewhat from the human as there is no unique 'fetal zone' or 'X-zone' which predominates in fetal life and regresses shortly after

birth to be replaced by the definitive cortex (McNicol, 1992; Soffer *et al.*, 1961). The definitive permanent cortex is the only zone present in the fetal sheep adrenal gland during development (Boshier & Holloway, 1989*b;* Robinson *et al.*, 1979*b*).

In the fetal sheep the developing adrenal gland can be clearly identified from as early as 28 d gestation (Wintour *et al.*, 1975). Between 30-60 d gestation a single type of immature cortical cell with a round or oval nuclei containing several nucleoli, sparse smooth endoplasmic reticulum, and mitochondria with mixed lamellar and vesicular cristae, can be identified in the adrenal anlagen (Robinson *et al.*, 1979*b*). Gene expression of the cytochrome P-450 steroidogenic enzymes: P-450 cholesterol side-chain cleavage ($P\text{-}450_{SCC}$), P-450 17α-hydroxylase / *C*17-*C*20 lyase ($P\text{-}450_{c17}$), and P-450 21-hydroxylase ($P\text{-}450_{c21}$) has been established in the immature cortical cells (Tangalakis *et al.*, 1989). Despite the fact that these immature cells possess little smooth endoplasmic reticulum and mitochondria where many key steroid synthesising cells are found in mature adrenal cells, they secrete significant amounts of aldosterone and cortisol *in vitro* in response to stimulation with angiotensin II (Ang II) and adrenocorticotrophic hormone (ACTH) (Glickman & Challis, 1980; Wintour, 1984; Wintour *et al.*, 1975). In fact the adrenal glands at this stage secrete relatively greater amounts of cortisol in response to ACTH stimulation than at any other period during gestation (Glickman & Challis, 1980; Wintour *et al.*, 1975). These findings correlate with the demonstration of the presence of steroids in the fetal circulation as early as 60 d gestation (Wintour *et al.*, 1975).

From 53 d-60 d gestation, there is morphological evidence for the development of zonation within the adrenal gland. Within the subcapsular outer region of the cortex, groups of cells are observed to be arranged in rounded cap-like aggregations possessing a connective tissue frame work typical of the zona glomerulosa (Boshier & Holloway, 1989*b;* Robinson *et al.*, 1979*b*). Dispersed amongst the developing glomerulosa are cords of homogeneous cortical cells running centripetally to the centre of the gland, with clumps of migratory sympathochromaffin cells frequently observed amongst these cortical cells (Boshier & Holloway, 1989*b;* Robinson *et al.*, 1979*b*). From 80-120 d gestation there is clear differentiation of the zona glomerulosa and the zona fasciculata cells (Boshier & Holloway, 1989*b;* Robinson *et al.*, 1979*b*). The cells of the

glomerulosa are arranged into well organised glomus-like aggregations surrounded by subcapsular loose connective tissue (Boshier & Holloway, 1989*b*). This connective tissue marked the site of entry of the cortical blood vessels which either ramified in the zona glomerulosa or rapidly branched into anastomosing sinusoids within the zona fasciculata (Boshier & Holloway, 1989*b*). The glomerulosa cells at this stage exhibit features consistent with mature cells in that they are found to possess large amounts of smooth endoplasmic reticulum and mitochondria with mainly lamellar cristae (Robinson *et al.*, 1979*b*). In contrast to the glomerulosa cells, the cells in the inner zone of the cortex are still immature displaying sparse amounts of smooth endoplasmic reticulum, few ribosomes, and mitochondria with mixed lamellar and vesicular cristae (Robinson *et al.*, 1979*b;* Webb, 1980). Most of the migratory chromaffin cells have accumulated in the centre of the adrenal gland at this stage and there is a well defined cortico-medullary junction (Boshier & Holloway, 1989*b*).

The establishment of morphological zonation of the adrenal cortex is also associated with the development of a functional zonation. From 80 d gestation P-450_{c21} and P-450_{scc} mRNA expression is found in both the zona fasciculata and zona glomerulosa. Labelling for P-450_{c17} mRNA, which is exclusively involved in the synthesis of cortisol, is restricted to the cortical cells within the zona fasciculata (Tangalakis *et al.*, 1989). The expression of these steroidogenic enzymes, most notably P-450_{scc} and P-450_{c17}, is relatively low during this period when compared to earlier and later periods in gestation (Tangalakis *et al.*, 1989). This decline in steroidogenic enzyme gene expression coincides with a functional quiescence of the cortex; ACTH stimulation of fetal cortisol secretion is relatively inefficient and Ang II and K^+ are unable to stimulate aldosterone secretion (Challis & Brooks, 1989; Glickman & Challis, 1980; Siegel & Fischer, 1980; Wintour *et al.*, 1979; Wintour *et al.*, 1975).

In late gestation from 120 d onwards there is morphological maturation of the cells in the inner cortex, with the appearance of more spherical mitochondria with vesicular cristae and well developed smooth endoplasmic reticulum (Robinson *et al.*, 1979*b*). Over the later stages of gestation from 130 d onwards there is a dramatic increase in adrenal cortical size which is primarily due to rapid growth of the zona fasciculata (Boshier & Holloway, 1989*b;* Robinson *et al.*, 1979*b*). The

growth of the fasciculata region is due to both hypertrophy and hyperplasia of the secretory cells (Boshier & Holloway, 1989*b*). Gene expression of the steroidogenic enzymes P-450$_{c21}$, P-450$_{scc}$, and P-450$_{c17}$ within the adrenal cortex also increases dramatically in late gestation (Tangalakis *et al.*, 1989). The increase in growth of the zona fasciculata coincides with increased ACTH output from the fetal pituitary, increased responsiveness of the fetal cortex to ACTH stimulation, and a dramatic surge in cortisol output from the adrenal gland (Challis & Brooks, 1989; Wintour, 1984).

There is no evidence for the development of the zona reticularis in the sheep up to early postnatal life (Boshier & Holloway, 1989*b;* Robinson *et al.*, 1979*b*).

1.2.3 INNERVATION OF THE ADRENAL GLAND

1.2.3.1 Adrenomedullary innervation

Cholinesterase staining and nerve degeneration studies confirm that the majority of the nerve fibres which project to the mammalian adrenal gland are pre-ganglionic cholinergic sympathetic fibres arising from the splanchnic nerve (MacFarland & Davenport, 1941; Robinson *et al.*, 1977*b*). Retrograde tracer studies reveal that the pre-ganglionic sympathetic fibres which innervate the adrenal gland arise ipsilaterally from the intermediolateral horn of the spinal cord between thoracic level 3 (T3) and lumbar level 2 (L2) with the majority arising from T8-T11 (Kesse *et al.*, 1988; Parker *et al.*, 1993; Pyner & Coote, 1994). The pattern of pre-ganglionic sympathetic innervation within the mammalian adrenal gland appears to demonstrate a common pattern between species. Cholinergic fibres from the capsular network run radially through the cortex with very little branching until reaching the adrenal medulla whereupon there is extensive ramification to form a dense medullary plexus with numerous axons running between chromaffin secretory cells with frequent synapses (MacFarland & Davenport, 1941; Parker *et al.*, 1993; Robinson *et al.*, 1977*b*).

1.2.3.2 Adrenocortical innervation

Despite the fact that the adrenal cortex is poorly innervated in relation to the adrenal medulla, ultrastructural studies identify axon terminals adjacent to cortical cells (Robinson *et al.*, 1977*b;* Unsicker, 1971). There is a body of evidence to

suggest that splanchnic nerve stimulation can enhance ACTH induced glucocorticoid output from the adrenal cortex (Edwards & Jones, 1987; Edwards *et al.*, 1986). The peptide neurotransmitter, corticotrophin releasing factor (CRF) modulates steroidogenesis in the adrenal gland of the calf (Jones & Edwards, 1990) and is present along with its receptors in the adrenal glands of a number of species (Aguilera *et al.*, 1991; Bruhn *et al.*, 1987; Hashimoto *et al.*, 1984; Rundle *et al.*, 1988). Immunocytochemical and retrograde tracer techniques reveal that the splanchnic nerve contains CRF-positive fibres which project from the intermediolateral cell column of the spinal cord at levels T5-T13 (Li & McDonald, 1997; Pomerantz *et al.*, 1996) and stimulation of the splanchnic nerve elicits an increase in the concentration of CRF in adrenal venous blood (Edwards & Jones, 1988; Plotsky *et al.*, 1990).

1.2.3.3 Postganglionic sympathetic innervation

The vast majority of the nerve fibres projecting to the adrenal gland have cell bodies in the spinal cord confirming their pre-ganglionic nature, although small numbers of post ganglionic sympathetic nerves are evident synapsing in the sympathetic ganglia chain or in the suprarenal ganglion (Celler & Schram, 1981; Kesse *et al.*, 1988; Parker *et al.*, 1993). Post-ganglionic sympathetic fibres containing catecholamines have been found using fluorescence histochemistry to run in close association with vascular supply of the gland (Robinson *et al.*, 1977*b*). Post-ganglionic sympathetic fibres do not degenerate following transection of the splanchnic nerve but stripping the adventitia of the arteries supplying the adrenal gland does result in a reduction in the number of these fibres (Robinson *et al.*, 1977*b*). It therefore appears that the post-ganglionic sympathetic fibres innervate blood vessels within the gland (Carlson *et al.*, 1992; Carlson *et al.*, 1990; Robinson *et al.*, 1977*b;* Unsicker, 1971).

1.2.3.4 Sensory innervation

There is also a significant sensory innervation of the mammalian adrenal gland with afferent nerves being identified in a number of species (Mohamed *et al.*, 1988; Parker *et al.*, 1993; Zhou *et al.*, 1991*b*). The majority of the afferent nerve supply to the adrenal gland via the splanchnic nerve has cell bodies lying ipsilaterally in the dorsal root ganglion of the spinal cord with the highest

proportions of ganglia between T9-T10 (Mohamed *et al.*, 1988; Parker *et al.*, 1993; Zhou *et al.*, 1991*b*). Most sensory nerve fibre endings are found in the capsular region or in association with capillaries in both the adrenal cortex and medulla (Parker *et al.*, 1993). The role of sensory innervation in the adrenal gland is unclear but is likely to involve regulation of adrenal blood flow and modulation of secretory output (Khalil *et al.*, 1986; Livett *et al.*, 1990; Niijima & Winter, 1968; Parker *et al.*, 1993).

1.2.3.5 Intrinsic innervation

The adrenal gland also appears to have an intrinsic innervation with ganglion cells having been identified within the gland with their numbers being species specific (Coupland & Holmes, 1958; Migally, 1979; Mikhail & Mahran, 1965; Parker *et al.*, 1993; Unsicker *et al.*, 1978*b;* Watanabe *et al.*, 1990). In the most studied species, the rat, two populations of ganglion cells have been identified. One population which has been termed Type I ganglion cells by Holgert and colleagues (1996*a*) are relatively large and exhibit properties consistent with a post-ganglionic, noradrenergic phenotype (Dagerlind *et al.*, 1990; Oomori *et al.*, 1994; Pelto-Huikko, 1989). These cells are probably derived from neural crest cells which invaded the cortical anlage but unlike the chromaffin cells were exposed to a different set of environmental factors (e.g. NGF) (Anderson, 1993; Doupe *et al.*, 1985) which led to their differentiation into neurones. Type II ganglion cells, whose origins have yet to be determined, are smaller, have a non-classical peptidergic transmitter phenotype, and express nitric oxide synthase (NOS) (Dun *et al.*, 1993; Holgert *et al.*, 1995*a;* Holzwarth, 1984). Intra-adrenal ganglion cells have been demonstrated to have fibres projecting to cortical, medullary, and capsular regions of the gland with a number appearing in close approximation with blood vessels and chromaffin cells (Afework *et al.*, 1994; Holgert *et al.*, 1996*a;* Kondo *et al.*, 1986; Pelto-Huikko, 1989). Type I ganglion cells also have projections which have been described to leave the adrenal gland via the splanchnic nerve (Dagerlind *et al.*, 1995). Little is known about the physiological function of these intrinsic ganglion cells but they may act to regulate adrenal blood flow and also secretory output from the gland and in the case of the type I ganglion cells may act as a feedback system.

1.2.3.6 Ontogeny of adrenal innervation

Functional innervation of the rat adrenal gland, and in particular the medulla, by the preganglionic, cholinergic, splanchnic nerve does not occur until after the first week of postnatal life. Ultrastructural studies reveal a dramatic increase in the number of synapses from splanchnic nerve fibres on chromaffin cells occurring at 10 d postnatally (Lau *et al.*, 1988) which correlates with an increase in the number of acetylcholinesterase immunoreactive fibres within the gland at this stage (Holgert *et al.*, 1994). These structural observations are corroborated by functional studies which demonstrate that insulin induced hyperglycaemia, which requires functional splanchnic innervation of the adrenal gland to stimulate catecholamine secretion, does not elicit a full catecholamine response in the rat until postnatal day 10 (Slotkin & Seidler, 1988). In contrast to pre-ganglionic sympathetic innervation, sensory innervation of the rat adrenal gland already appears quite mature at postnatal day 2 stage (Holgert *et al.*, 1994) as do type II ganglion neurones (Holgert *et al.*, 1996*a*), whereas type 1 ganglion neurones display a developmental pattern similar to that of the extrinsic innervation (Holgert *et al.*, 1996*a;* Holgert *et al.*, 1994).

Unlike the rat, functional splanchnic innervation to the sheep adrenal medulla occurs prior to birth. Ultrastructural studies reveal the absence of sympathetic innervation to the adrenal medulla at 53 d gestation, however, by 100 d gestation axonal profiles are present amongst the chromaffin cells but synapses are absent (Boshier *et al.*, 1989*a*). At 130 d, synapses between the axons and chromaffin cells of the medulla are present, increasing in frequency over late gestation and the early post-partum period (Boshier *et al.*, 1989*a*). Comline and Silver (1961) found significant increases in adrenal venous catecholamine concentrations of anaesthetised, fetal lambs could only be elicited by electrical stimulation of the splanchnic nerve from 125 d gestation, concurring with the structural observations of Boshier et al. (1989). Currently there is no available information on the development of sensory and intra-adrenal ganglion cells in the fetal sheep adrenal.

1.2.4 VASCULATURE

The adrenal gland exhibits an unusually high degree of vascularity, with the arterial blood supply to the mammalian adrenal gland arising from a number of sources (Harrison & McDonald, 1966; Harrison & Hoey, 1960; Vinson & Hinson, 1992). In the sheep the sources of arterial blood supply exhibit variability amongst breeds but primarily arise from the lumbar arteries and renal arteries, with contributions from the coeliac axis, and anterior mesenteric artery (Harrison & McDonald, 1966).

The arterial supply to the adrenal divides repeatedly into smaller arterioles in and just below the connective tissue of the capsule, forming a plexus which consists of an extensive capillary network. The capillaries of this subcapsular network then form an extensively cross connecting network of thin-walled blood vessels, termed sinusoids, which supply the zona glomerulosa. Continuous with this network is a straighter centripetal network of sinusoids which pass through and supply the zona fasciculata. The latter network as it reaches the inner parts of the cortex converges and forms numerous cross connecting channels in the deeper regions of the cortex, giving rise to the extensive reticular network in the zona reticularis (Harrison, 1951; Harrison & Asling, 1955; Vinson *et al.*, 1985). The continuous networks of vessels which supply the cortex converge and empty into the larger sinusoids present within the corticomedullary and medullary regions, such that the medulla is directly exposed to blood which has passed through the adrenal cortex (Harrison & Hoey, 1960; Vinson & Hinson, 1992; Vinson *et al.*, 1985). The medullary sinusoids then drain into medullary veins (Vinson *et al.*, 1985).

Aside from the cortical effluent which drains into the medullary sinusoids, the medulla also receives blood directly from arteries known as arteriae medullae (Coupland & Selby, 1976; Merklin, 1962; Vinson & Hinson, 1992; Vinson *et al.*, 1985). The presence of arteriae medullae within the adrenal gland is species specific with relatively few being observed in the rat adrenal gland (Vinson *et al.*, 1985) whilst substantially greater numbers are found in cat and bovine adrenals (Coupland & Selby, 1976). The arteriae medullae derive from branches of the large capsular arteries which pass directly through the adrenal cortex before entering the medulla and branching into arterioles and capillaries. These vessels

then empty into the medullary veins which in turn empty into the central adrenal vein, where all venous effluent drains from the adrenal gland (Vinson *et al.*, 1985). The adrenal venous effluent subsequently discharges into either the renal vein (left adrenal) or directly into the inferior vena cava (right adrenal) (Soffer *et al.*, 1961; Vinson & Hinson, 1992).

There is evidence to suggest that the blood flow to the adrenal cortex and the adrenal medulla can be independently regulated. Under basal conditions the majority of blood flow to the adrenal gland has been found to be to the medulla by the use of microsphere and microradiographic techniques (Carter *et al.*, 1993; Harrison & Hoey, 1960). Cortical and medullary blood flow have been found in the adult dog to be independently regulated during haemorrhagic hypotension (Breslow *et al.*, 1987), hypoxic hypoxia (Nishijima *et al.*, 1989), and splanchnic nerve stimulation (Breslow *et al.*, 1987). In the fetal sheep radiolabelled microsphere studies have revealed that ACTH administration elicits a preferential increase in adrenal cortical blood flow (Carter *et al.*, 1993). The mechanisms underlying the independent regulation of cortical and medullary blood flow within the adrenal gland have yet to be elucidated, however, it has been speculated that alterations in the tone of the muscular arterioles in the sub capsular plexus control the blood flow to the thin walled sinusoids (Vinson *et al.*, 1985).

In the fetal sheep, wide anastomosing sinusoids arising from thin walled vessels in the adrenal capsule and leading to the centre of the gland are observed as early as 53 d gestation where the vasculature occupies a relatively large proportion of the cortex (Boshier & Holloway, 1989*b*). By 100 d gestation there is ramification of a number of the cortical blood vessels within the zona glomerulosa to form the glomerulosa network, however, the majority of the vessels branch within the zona fasciculata to form somewhat narrower anastomosing sinusoids which recombine into larger vessels at the corticomedullary junction (Boshier & Holloway, 1989*b*). At this stage of development, the volume density of the vasculature within the zona fasciculata falls significantly due to the re-organisation of the vasculature, cellular proliferation, and functional zonation within the cortex (Boshier & Holloway, 1989*b*). Separation of the medulla from the cortex is complete at 100 d gestation with the relative volume of the medulla occupied by blood vessels

increasing over mid to late gestation as vascularisation of the medulla continues (Boshier *et al.*, 1989*a*).

1.3 THE ADULT ADRENAL MEDULLA

1.3.1 ADRENOMEDULLARY CATECHOLAMINE SYNTHESIS AND STORAGE

1.3.1.1 Synthesis

Catecholamines are synthesised in the chromaffin cells of the adrenal gland from the dietary amino acid, tyrosine, as illustrated in Figure 1.1 (for reviews see Kirshner, 1975; Ungar and Phillips, 1983). The initial step in the synthesis of adrenomedullary catecholamines is the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) by the enzyme tyrosine hydroxylase [TH; tyrosine 3-monooxygenase; tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] (Levitt *et al.*, 1965; Nagatsu *et al.*, 1964). TH is a mixed function oxidase which requires molecular oxygen and utilises a tetrahydrobiopterine as a co-substrate (Brenneman & Kaufman, 1964; Fitzpatrick, 1993; Joh *et al.*, 1969). The tetrahydropteridine co-substrate is oxidised to dihydrobiopteridine in the conversion of tyrosine to DOPA (Fitzpatrick, 1993; Joh *et al.*, 1969), with dihydropteredine reductase (EC 1.6.99.7) and nicotinamide adenine dinucleotide phosphate (NADPH) being required to regenerate the pool of the reduced form of the biopterine (Brenneman & Kaufman, 1964; Musacchio, 1969; Shiman *et al.*, 1970). Tyrosine hydroxylase is the rate limiting enzyme in the catecholamine synthetic pathway (Levitt *et al.*, 1965), as TH has a substantially lower specific activity than any of the other catecholamine synthetic enzymes and the pool of tyrosine is greater than that of any of the other catecholamine synthetic enzyme substrates (Nagatsu *et al.*, 1964). Tyrosine hydroxylase is located in the soluble fractions of adrenal medullary homogenates indicating its presence in the cytoplasm of the chromaffin cells (Laudron & Belpaire, 1968; Sabban & Goldstein, 1984; Wurzburger & Musacchio, 1971).

DOPA is subsequently decarboxylated by DOPA decarboxylase, also known as aromatic L-amino acid decarboxylase (AADC; EC 4.1.1.28), to form dopamine (Kirshner, 1975). AADC is not specific for catecholamine synthesis as it is involved in the decarboxylation of a number of aromatic L-amino acids (Lovenberg

et al., 1962) and appears to have a wide distribution in a number of tissues (Christenson *et al.*, 1970; Kirshner, 1975; Sourkes, 1966). Like TH, AADC activity in the chromaffin cell is found in the water-soluble fraction of adrenal homogenates indicating that its located in the cytosol of chromaffin cells (Laudron & Belpaire, 1968; Sabban & Goldstein, 1984).

Dopamine is actively transported into the chromaffin granules and converted to noradrenaline by dopamine β-hydroxylase [DBH; 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] (Kirshner, 1975; Kirshner, 1962; Levin *et al.*, 1960). DBH is the only enzyme in the catecholamine biosynthetic pathway located within the chromaffin granules with all of the other enzymes situated in the cytoplasm of the chromaffin cells (Laudron, 1975; Sabban & Goldstein, 1984). DBH is present in the chromaffin granules in one of two similar forms, either bound to the internal membrane or free in the internal matrix of the chromaffin granule (Helle *et al.*, 1984; Joh & Hwang, 1987). The relative proportions of the enzyme which are free and membrane bound vary between species (Ciaranello *et al.*, 1975; Gagnon *et al.*, 1976*b*) with the free form probably being derived from a membrane bound precursor which undergoes proteolysis (Helle *et al.*, 1984; Joh & Hwang, 1987). Like TH, DBH is a mixed function oxidase and it catalyses the oxidation of dopamine to noradrenaline and requires molecular oxygen and utilises ascorbate as a co-factor (Dilberto & Allen, 1981; Levin *et al.*, 1960).

The final step in the catecholamine biosynthetic pathway is the *N*-methylation of noradrenaline which passively permeates into the cytosol from chromaffin granules, to adrenaline (Kirshner, 1975; Ungar & Phillips, 1983). This is achieved by transfer of the *S*-methyl group from *S*-adenosylmethionine to the primary nitrogen group of noradrenaline by the enzyme phenylethanolamine *N*-methyltransferase [PNMT; *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.28].

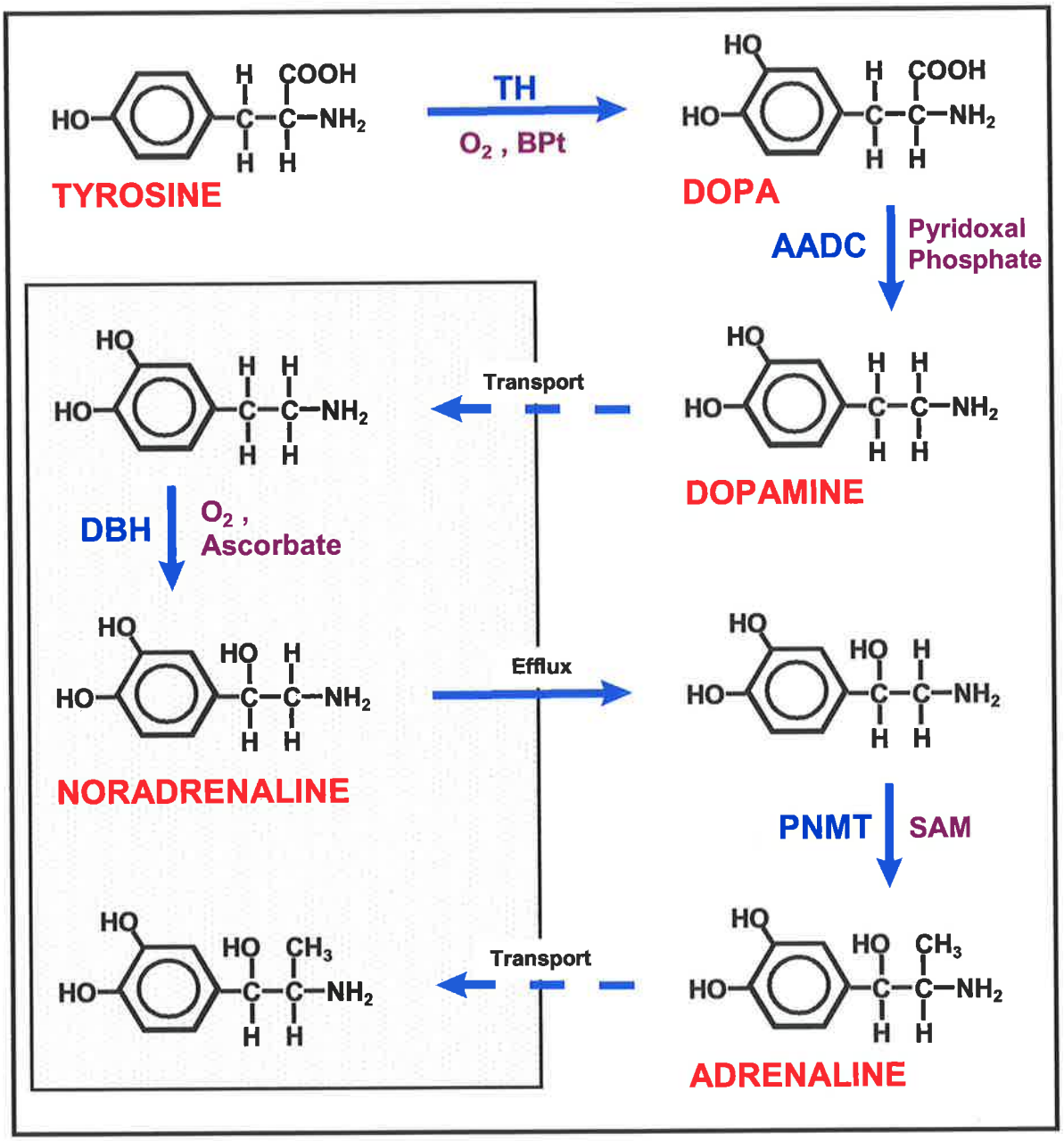


Figure 1.1 Biosynthetic pathway of adrenomedullary catecholamines

Schematic diagram of the synthesis of catecholamines within the chromaffin cells of the adrenal medulla, with substrates and products, enzymes, and co-factors indicated. Shaded area represents chromaffin granule with the unshaded area depicting the cytosol of the chromaffin cell. DOPA, dihydroxyphenylalanine; TH, Tyrosine Hydroxylase; AADC, aromatic amino acid decarboxylase; DBH, dopamine β–hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase; BPt, tetrahydrobiopterine; SAM, *S*-adenosylmethionine.

1.3.1.2 Storage

1.3.1.2.1 Chromaffin granules

The adrenomedullary catecholamines are stored in the chromaffin cells within specialised vesicles known as chromaffin granules. Chromaffin granules are membrane bound spheres which have been identified to contain a number of components including, catecholamines, nucleotides (predominantly ATP), ascorbic acid, calcium, a family of acidic glycoproteins known as the chromogranins, a number of neuropeptides predominantly consisting of the enkephalins and their precursors and neuropeptide tyrosine, and DBH (soluble form) (Winkler *et al.*, 1986; Winkler & Westhead, 1980). The chromaffin granule membrane exhibits a characteristically high lipid to protein ratio with large amounts of cholesterol, and the phospholipid, lysolecithin, present in the membrane (Frischenschlager *et al.*, 1983; Winkler, 1976). The lipids are arranged in a bimolecular leaflet to form the membrane of the granule (Winkler & Westhead, 1980) and within the membrane are a number of enzymes some of which have been identified using 2D gel electrophoresis, whilst others are known to be present due to their functional identification. Amongst these membrane bound proteins are DBH (membrane bound form), cytochrome b_{561}, phosphotidylinositol kinase, NADH dehydrogenase, and a Mg^{2+}-ATPase (H^+ translocating) as well as transporters for catecholamines, adenine nucleotides, and a Ca^{2+} / Na^+ exchanger (Winkler *et al.*, 1986; Winkler & Westhead, 1980).

1.3.1.2.2 Chromaffin granule synthesis

The chromaffin granules are synthesised by the Golgi bodies and when the newly formed granules are pinched off from the Golgi stacks they already contain chromogranins, and neuropeptides which have been incorporated into the granule within the Golgi region (Winkler *et al.*, 1987). These immature granules are less dense than mature granules and they gradually accumulate catecholamines, ATP, and calcium (Winkler, 1977; Winkler *et al.*, 1987). The synthesis rate of secretory proteins in the chromaffin granules is significantly greater than membrane bound proteins suggesting that there is recycling of the chromaffin granule membranes following exocytosis (Ungar & Phillips, 1983; Winkler, 1977). The use of specific antigens against chromaffin granule membrane components which can be

visualised by immunofluorescence has revealed that during stimulation these antigens appear on the surface of the cell membrane consistent with exocytosis (Lingg *et al.*, 1983; Phillips *et al.*, 1983). After stimulation, the chromaffin granule membrane antigens are retrieved into the cell and are initially found in close proximity to the Golgi region and finally in newly formed chromaffin granules consistent with recycling of the chromaffin granule membranes (Lingg *et al.*, 1983; Phillips *et al.*, 1983).

1.3.1.2.3 Catecholamine transport and storage

The concentration of catecholamines within chromaffin granules has been estimated to be around 20 000 times greater than the cytosolic concentration (Phillips & Apps, 1980; Ungar & Phillips, 1983). The large concentration between granular and cytosolic catecholamines is maintained by a secondary active transport mechanism (Njus *et al.*, 1981; Phillips, 1982). The interior of isolated chromaffin granules is acidic and has been estimated to be pH 5.7 with the membrane potential of the granule being around +60 mV in respect to that across the cytosol (Johnson & Scarpa, 1976; Johnson & Scarpa, 1979; Njus *et al.*, 1978; Ungar & Phillips, 1983). The acidic nature of the granule and the positive membrane potential arise from the translocation of H^+ across the chromaffin granule membrane by the membrane bound H^+-ATPase which utilises the hydrolysis of cytoplasmic Mg-ATP to drive electrogenic proton transport (Johnson *et al.*, 1982; Njus *et al.*, 1981; Winkler *et al.*, 1986). Transport of the catecholamines into the granule is directly coupled to the proton gradient by a translocator which exchanges a proton for one uncharged catecholamine molecule (Marron *et al.*, 1983; Ramu *et al.*, 1983; Winkler *et al.*, 1986).

Catecholamines are able to passively leak out of the chromaffin granules down their concentration gradient into the cytosol which allows noradrenaline to be methylated to adrenaline by the enzyme PNMT (Corcoran *et al.*, 1984) as outlined previously. A steady state equilibrium exists between the levels of cytosolic and granular catecholamines such that an increase in granular catecholamine content results in an increased efflux from the granule, whereas an increased cytoplasmic catecholamine level stimulates uptake into the chromaffin granule such that the granules act as buffers of cytoplasmic catecholamine content in the resting cell

(Ungar & Phillips, 1983). Adrenaline and noradrenaline secreting chromaffin cells are identifiable by ultrastructural differences and immunohistochemical techniques. The presence or absence of PNMT in chromaffin cells determines their adrenaline synthesising capacity. In the sheep adrenal the expression of PNMT is confined to the peripheral juxtacortical region of the medulla which interdigitate with the cortex and also to a degree around the central adrenal vein and sinusoids (McMillen *et al.*, 1988). This organisation of adrenaline and noradrenaline synthesising chromaffin cells is similar to that observed in the bovine adrenal gland (Livett *et al.*, 1982).

1.3.1.2.4 Chromaffin granules and catecholamine synthesis

As outlined previously the chromaffin granules contain both a soluble form of DBH and a membrane bound form. The presence of relatively large amounts of the DBH cofactor ascorbic acid within the chromaffin granule allows the conversion of dopamine to noradrenaline to occur (Dilberto & Allen, 1981; Ingebretsen *et al.*, 1980). During the conversion of dopamine to noradrenaline the co-factor, ascorbic acid is oxidised to semidehydroasorbate (Dilberto & Allen, 1981). In order to maintain the pool of the co-factor for noradrenaline synthesis, semidehydroascorbate must be reduced to regenerate ascorbic acid (Dilberto & Allen, 1981; Winkler *et al.*, 1986). This reduction process requires electrons and the presence of the heme-containing cytochrome b_{561} as the most abundant membrane protein within the chromaffin granule seems to provide the most obvious means for the transportation of electrons across the chromaffin granule membrane (Njus *et al.*, 1983; Srivastava *et al.*, 1984) to allow this process to occur (Tsubaki *et al.*, 1997; Winkler & Westhead, 1980). The midpoint redox potential of cytochrome b_{561} at the acidic pH of the chromaffin granule is consistent with a role in the reduction of oxidised forms of ascorbate (Winkler *et al.*, 1986).

1.3.1.2.5 Chromaffin granules and calcium homeostasis

The chromaffin granules also store an unusually high amount of calcium with estimates of 20-40 m*M* having been reported (Bulenda & Gratzl, 1985; Phillips *et al.*, 1977; Reiffen & Gratzl, 1986). Indeed the majority of calcium in the bovine adrenal medulla is reported to be located within the chromaffin granules (Phillips

et al., 1977). Whilst the granules may have a high calcium content the free calcium concentrations are only in the low micromolar range (≈ 4 μ*M*), with this anomaly being attributed to the binding of calcium by the acidic granule matrix proteins, the chromogranins (Bulenda & Gratzl, 1985; Reiffen & Gratzl, 1986; Ungar & Phillips, 1983). Acetylcholine (ACh) induced secretion from perfused bovine adrenal glands results in an increase in calcium content within the chromaffin granules (Serck-Hanssen & Christiansen, 1973) suggesting that they play an important role in scavenging cytosolic Ca^{2+} following stimulus induced entry into the cell (Ungar & Phillips, 1983). Calcium appears to enter the vesicle via an electroneutral Na^{+} / Ca^{2+} exchanger present within the membrane of the chromaffin granule (Krieger-Bauer & Gratzl, 1982; Phillips, 1981; Winkler, 1987; Yoon & Sharp, 1985).

1.3.2 ADRENOMEDULLARY NEUROPEPTIDES

A number of neuropeptides and non-classical neurotransmitters are also present within the chromaffin cells, nerve fibres, and intra-adrenal ganglion cells of the adrenal medulla.

1.3.2.1 Enkephalins

The most abundant neuropeptides and the first to be positively identified, within the adrenal medulla, are the enkephalin-containing opioid peptides (Schultzberg *et al.*, 1978*b;* Winkler *et al.*, 1986). Enkephalin (Enk) containing peptides are established to be present within the adrenaline containing chromaffin cells and preganglionic splanchnic nerve fibres in the adrenal glands of several species (Dagerlind *et al.*, 1993; Kondo & Yui, 1984; Livett *et al.*, 1982; McMillen *et al.*, 1988; Pelto-Huikko *et al.*, 1985; Schultzberg *et al.*, 1978*a*). Enkephalin containing peptides within the adrenal medulla are derived from a 263 amino acid, 30 kDa precursor molecule, the gene for which was first cloned from bovine adrenal medulla by Noda and co-workers (1982). Subsequent studies derived the peptide sequence of the enkephalin-containing precursor molecule, termed Proenkephalin A (PEnk A), which contains four copies of [Met]enkephalin, one copy of [Leu]enkephalin, and one copy each of [Met]enkephalyl-Arg^{6}-Phe^{7} (MERF) and [Met]enkephalyl-Arg^{6}-Gly^{7}-Leu^{8} (MERGL) (Comb *et al.*, 1982; Gubler *et al.*, 1982). Proenkephalin A is cleaved by trypsin-like and carboxypeptidase B-like proteases

(Evangelista *et al.*, 1982; Hook, 1984; Wallace *et al.*, 1982) within the chromaffin granule to form a number of smaller enkephalin-containing peptides ranging in size from > 20 kDa to < 1 kDa, as well as free enkephalin peptides 5-8 amino acids in length (Coulter *et al.*, 1990*a;* Fleminger *et al.*, 1983; Fleminger *et al.*, 1984; Kojima *et al.*, 1982; Patey *et al.*, 1985; Patey *et al.*, 1984).

1.3.2.2 Neuropeptide tyrosine

Neuropeptide tyrosine (NPY) is a 36 amino acid regulatory peptide which has a wide distribution in the central and peripheral nervous system being found in sites such as the hypothalamus, cerebral cortex, hippocampus, brainstem, and in particular within noradrenergic sympathetic neurones (Hokfelt *et al.*, 1983; Jhanwar-Uniyal *et al.*, 1993; Lundberg *et al.*, 1983; Lundberg *et al.*, 1984; Warnes *et al.*, 1998; Woodhams *et al.*, 1985). It is therefore not surprising that NPY has also been identified in the adrenal gland within both the chromaffin cells and the type I intra-adrenal ganglion cells and their processes (Allen *et al.*, 1983; Dagerlind *et al.*, 1990; Fried *et al.*, 1991; Kondo, 1985; Kuramoto *et al.*, 1986; Oomori *et al.*, 1994; Pelto-Huikko, 1989; Varndell *et al.*, 1984), which are considered to be analogous to post-ganglionic sympathetic neurones. Next to the enkephalin-containing peptides, NPY is the second most abundant neuropeptide found within the chromaffin cells of the adrenal medulla (Winkler *et al.*, 1986).

1.3.2.3 Vasoactive intestinal polypeptide

Vasoactive Intestinal Polypeptide (VIP) was one of the first neuropeptides to have been established to be secreted from the adrenal medulla (Said, 1976). Immunohistochemical localisation of VIP within the adrenal gland reveals that it is predominantly located in type II intra-adrenal ganglion cells as well as nerve fibres which are distributed in networks around medullary chromaffin cells and also in the cortex and capsule usually in association with vascular structures (Holgert *et al.*, 1996*a;* Holzwarth, 1984; Kondo *et al.*, 1986; Linnoila *et al.*, 1980; Pelto-Huikko, 1989; Yoshikawa *et al.*, 1990). The VIP immunopositive fibres within the adrenal gland consist of fibres which project into the splanchnic nerve and are presumably sensory in origin, as well as projections from type II intra-adrenal ganglion cells (Holgert *et al.*, 1996*a;* Holzwarth, 1984; Linnoila *et al.*, 1980). VIP is not generally co-stored with catecholamines in bovine or rat adrenomedullary

chromaffin cells (Holzwarth, 1984; Kondo, 1985; Pelto-Huikko, 1989; Yoshikawa *et al.*, 1990) although there does appear to be species differences with VIP immunoreactivity being detected within the chromaffin cells of the frog adrenal gland (Leboulenger *et al.*, 1983). VIP immunoreactivity which has been reported to exist in human phaeochromocytoma cells and cultured bovine chromaffin cells (Eiden *et al.*, 1982; Gozes *et al.*, 1983; Siegel *et al.*, 1985) and is found to be associated with the transformation of the chromaffin cell phenotype to the neurone-like phenotype and culture conditions (Eiden *et al.*, 1983; Siegel *et al.*, 1985; Tischler *et al.*, 1985; Tischler *et al.*, 1984).

1.3.2.4 Nitric oxide

Nitric oxide (NO) is a novel neurotransmitter as it is a highly labile, free radical gas which diffuses to its target sites after synthesis rather than being released from synaptic vesicles by exocytosis (Dawson & Snyder, 1994). Nitric oxide is synthesised by the calmodulin dependent enzyme, nitric oxide synthase (NOS), which oxidises L-arginine utilising NADPH and O_2 to form L-citrulline and NO (Stuehr *et al.*, 1991; White & Marletta, 1992). Due to its highly labile nature, the localisation of NO in tissue is demonstrated indirectly by the presence of NOS immunoreactivity or by NADPH-diaphorase staining which is found to be co-localised with NOS in neurones. Indeed, it is believed that NOS is responsible for the NADPH diaphorase staining reaction within neurones (Dawson *et al.*, 1991; Hope *et al.*, 1991). There are isoforms of NOS that are structurally, functionally, and immunologically distinct, which include inducible isoforms found in macrophages (iNOS) and neurones (nNOS) as well as the constitutively expressed NOS in vascular endothelial cells (eNOS) (Marletta, 1993). nNOS immunoreactivity and NADPH diaphorase staining has been established to be present in nerve fibres amongst adrenomedullary chromaffin cells and also in fibres running in the subcapsular region and zona glomerulosa quite often in association with vascular structures (Afework *et al.*, 1994; Dun *et al.*, 1993; Holgert *et al.*, 1995*a;* Holgert *et al.*, 1996*a;* Marley *et al.*, 1995*a*). These fibres arise from preganglionic sympathetic nerve fibres originating from the intermediolateral column of the spinal cord and the type II intra-adrenal ganglion cells where NOS is co-localised with VIP (Blottner & Baumgarten, 1992; Holgert *et al.*, 1995*a;* Holgert *et al.*, 1996*a*).

1.3.2.5 Other neuropeptides

Other neuropeptides present within the adrenal medulla include Substance P, which is present in capsaicin-sensitive sensory nerve fibres which can be retrogradely traced back through the splanchnic nerve synapsing in the dorsal root ganglion (Parker *et al.*, 1993; Zhou *et al.*, 1991*b*). Calcitonin gene related peptide (CGRP) is also observed in sensory nerve fibres within the mature adrenal gland (Holgert *et al.*, 1994; Pelto-Huikko, 1989). Pituitary Adenylate Cyclase Activating Peptide (PACAP), a novel peptide molecule which has similar actions to VIP but with greater potency, is also found within the adrenal gland predominantly in nerve fibres which are probably sensory in origin (Arimura & Shioda, 1995; Dun *et al.*, 1996; Tabarin *et al.*, 1994).

1.3.3 REGULATION OF ADRENAL CATECHOLAMINE SECRETION

1.3.3.1 Neurogenic catecholamine secretion

1.3.3.1.1 Secretory pathway

Physiological stressors in the mature animal elicit catecholamine secretion from the adrenal medulla via reflex stimulation of the pre-ganglionic, cholinergic splanchnic nerve which innervates the chromaffin cells. Electrical stimulation of the splanchnic nerves of a number of species including the sheep (Comline & Silver, 1961), cat (Marley & Prout, 1965), dog (Malmejac, 1964), cow (Bloom *et al.*, 1988; Edwards *et al.*, 1980), and rat (Wakade, 1988; Wakade, 1981) evokes substantial increases in the output of adrenal catecholamines. The reflex nature of adrenomedullary catecholamine secretion in response to physiological stressors is demonstrated further by the observations that adrenomedullary catecholamine secretion in response to insulin induced hypoglycaemia can be abolished by transection of the splanchnic nerve (Seidler & Slotkin, 1986*b;* Slotkin & Seidler, 1988). In the case of acute hypoxaemia, pharmacological blockade of cholinergic transmission or sectioning of the spinal cord at the level of the fifth and sixth cervical spines, prevents the catecholamine secretory response of the adrenal gland (Lee *et al.*, 1987; Seidler & Slotkin, 1985; Seidler & Slotkin, 1986*b*). Furthermore, chemoreceptor stimulation by perfusion of vascularly isolated carotid bifurcations with hypoxic blood and baroreceptor stimulation by the selective

reduction of carotid sinus pressure stimulates adrenomedullary catecholamine secretion in both cats and dogs (Critchley *et al.*, 1980).

Splanchnic output to the adrenal medulla is regulated by distinct regions within the hypothalamus and brainstem (Goadsby, 1985; Robinson *et al.*, 1983*b*) which have been established by trans-neuronal labelling studies to have inputs into the intermediolateral neurones from which the splanchnic nerve arises (Strack *et al.*, 1989; Wesselingh *et al.*, 1989). Carotid body chemoreceptor and carotid sinus baroreceptor sensory afferent fibres project to the brainstem via the carotid sinus nerve (Donoghue *et al.*, 1984; Eyzaguirre & Zapata, 1984; Fidone & Gonzalez, 1986; Finley & Katz, 1992). Sectioning of the afferent sensory carotid sinus nerve results in an attenuation of the increase in splanchnic nerve activity and catecholamine secretion which occurs in response to asphyxia and acute hypoxaemia (Biesold *et al.*, 1989; Jensen & Hanson, 1995; Stein *et al.*, 1998). It is interesting to note that during long term hypoxia, however, sectioning of the carotid sinus nerve does not affect the adrenomedullary catecholamine response (Dalmaz *et al.*, 1994; Soulier *et al.*, 1997). This finding raises the possibility that other peripheral chemosensory paraganglia (Martin-Body *et al.*, 1986; Sinclair, 1987) may become involved in reflex adrenomedullary activation in the absence of input from the primary oxygen sensors in the carotid body (Dalmaz *et al.*, 1994). Central mechanisms may also be involved as neurones within the ventrolateral medulla are found to be oxygen sensitive, although their ability to regulate sympathoadrenal output is not clear (Soulier *et al.*, 1997; Sun & Reis, 1994).

Acetylcholine (ACh) secreted from splanchnic nerve terminals binds to nicotinic receptors (nAChR) and / or muscarinic receptors (mAChR) on the chromaffin cell membrane to elicit catecholamine secretion. The classic studies of Feldberg and colleagues (1934) first demonstrated the predominantly cholinergic nature of neural transmission within the adrenal medulla, via the splanchnic nerve to stimulate catecholamine secretion. The relative proportions of nAChR and mAChR present on chromaffin cells varies between species. For instance, catecholamine secretion from the bovine adrenal medulla is essentially mediated by nicotinic stimulation (Fisher *et al.*, 1981; Livett, 1984), whereas both nAChR and mAChR activation stimulate adrenomedullary catecholamine secretion in the cat (Rubin & Miele, 1968), dog (Critchley *et al.*, 1986), and rat (Wakade &

Wakade, 1983). In the chicken , catecholamine secretion is under the exclusive control of mAChR (Knight & Baker, 1986). In the perfused sheep adrenal gland the majority of the catecholamine secretory response to ACh is abolished by the nicotinic antagonist hexamethonium (Butler *et al.*, 1995), indicating that catecholamine secretion in this species is predominantly under nAChR control.

1.3.3.1.2 Nicotinic receptor stimulation

1.3.3.1.2.1 Calcium dependence

Nicotinic receptor activation appears to stimulate catecholamine secretion by a mechanism which is dependent on the presence of extracellular Ca^{2+} (Harish *et al.*, 1987; Holz *et al.*, 1982; Kao & Schneider, 1986; Knight & Kesteven, 1983; Malhotra *et al.*, 1988*a*). Nicotinic stimulation of catecholamine secretion markedly facilitates uptake of the radioactive calcium isotope $^{45}Ca^{2+}$ by the adrenal medulla (Douglas & Poisner, 1962; Malhotra *et al.*, 1988*b;* Wakade *et al.*, 1986). Burgoyne and colleagues have examined the temporal and spatial changes of intracellular Ca^{2+} flux in isolated bovine chromaffin cells loaded with the Ca^{2+}-sensitive fluorescent dye, Fura-2, in response to a number of agonists (Cheek *et al.*, 1989; O'Sullivan *et al.*, 1989). Both depolarisation with high K^+ or exposure to nicotine results in a large and transient rise in cytosolic Ca^{2+} levels in the subplasmalemmal region of the chromaffin cell which then sweeps throughout the cell in a manner compatible with the influx of extracellular Ca^{2+} through membrane channels (Cheek *et al.*, 1989; O'Sullivan *et al.*, 1989).

1.3.3.1.2.2 Ionic events

Electrophysiological data concerning the effects of nicotinic receptor stimulation on chromaffin cells was first provided by Douglas and colleagues (1967). These investigators found that ACh and nicotine depolarised isolated gerbil chromaffin cells in which transmembrane potential was measured by intracellular micro-electrodes (Douglas *et al.*, 1967). More detailed electrophysiological studies on isolated bovine and guinea pig chromaffin cells using the patch clamp technique (Hamill *et al.*, 1981) established the presence of ACh- and nicotine-activated ion channels which are similar in many respects to the nAChR-activated ion channels observed in ganglion cells (Fenwick *et al.*, 1982*a;* Inoue & Kuriyama, 1991). Both

the depolarisation of the chromaffin cell membrane and inward depolarising currents associated with the opening of ACh-activated channels occur in a dose-dependent manner over a range of ACh concentrations (Douglas *et al.*, 1967; Fenwick *et al.*, 1982*a*). The ACh induced depolarisation of chromaffin cells is reliant on the presence of extracellular Na^+ (Brandt *et al.*, 1976) and stimulation of catecholamine secretion by carbachol stimulates $^{22}Na^+$ uptake into chromaffin cells, an action which is antagonised by the nicotinic receptor antagonist, hexamethonium (Wada *et al.*, 1984). Furthermore, neurotoxins which specifically block ion flux via nAChR-activated channels abolish nicotinic stimulation of both Ca^{2+} uptake and catecholamine secretion from chromaffin cells by inhibiting Na^+ influx through nAChR channels (Wada *et al.*, 1989*a;* Wada *et al.*, 1989*b*).

ACh is able to induce the formation of action potentials in chromaffin cells (Biales *et al.*, 1976; Brandt *et al.*, 1976; Fenwick *et al.*, 1982*a;* Kidokoro & Ritchie, 1980). There is a significant correlation between the opening of ACh-sensitive channels in chromaffin cells and the formation of action potentials (Fenwick *et al.*, 1982*a*). In fact, the opening of a single ACh-sensitive channel appears to be sufficient to trigger the formation of an action potential in chromaffin cells, a phenomenon which is probably related to the high input resistance of the cell (Fenwick *et al.*, 1982*a*). Administration of the voltage-sensitive Na^+ channel antagonist, tetrodotoxin (TTX), to chromaffin cells blocks action potential formation and attenuates ACh induced catecholamine release (Biales *et al.*, 1976; Brandt *et al.*, 1976; Holman *et al.*, 1994; Kidokoro & Ritchie, 1980). Patch clamp studies directly demonstrate voltage-sensitive Na+ channels, which display similar properties to those of nerve cells, to be present within chromaffin cells (Fenwick *et al.*, 1982*b*). It therefore appears that nAChR activation depolarises the chromaffin cell by the entrance of cations, predominantly Na^+, through receptor-linked channels which can then stimulate action potential formation by the opening of voltage-sensitive Na^+ channels.

The depolarisation of the chromaffin cell by Na^+ influx stimulated by the activation of nAChR, facilitates the movement of extracellular Ca^{2+} across the membrane of the chromaffin cell via the opening of voltage-sensitive Ca^{2+} channels (Wada *et al.*, 1985). Voltage-sensitive Ca^{2+} channels are established in patch clamp studies to be present in isolated bovine chromaffin cells (Bossu *et al.*, 1991; Fenwick *et*

al., 1982*b*). At least two types of voltage-sensitive Ca^{2+} channel are identifiable based on their inactivation characteristics, with properties similar to those of N- and L-type currents identified in sympathetic neurones (Bossu *et al.*, 1991). The L-type current is a slowly decaying current which is sensitive to dihydropyridine blockade, whilst the N-type current is fast decaying and sensitive to ω-conotoxin (Bossu *et al.*, 1991; Hirning *et al.*, 1988). The slowly decaying L-type Ca^{2+} current is primarily responsible for catecholamine secretion from adrenomedullary chromaffin cells as dihydropyridine L-type Ca^{2+} channel antagonists markedly attenuate the catecholamine secretion induced by nicotinic stimulation and depolarisation (Boarder *et al.*, 1987; Harish *et al.*, 1987; Lopez *et al.*, 1989; Owen *et al.*, 1989).

1.3.3.1.2.3 Adenylate cyclase activation

Stimulation of the adrenal medulla gland or isolated chromaffin cells with nicotinic cholinoceptor agonists such as acetylcholine, nicotine, carbachol, or dimethylphenylpiperazinium (DMPP), results in a rise in the levels of the second messenger adenosine 3' :5'-cyclic monophosphate (cAMP) in a number of species including rat, cat, dog, and cow (Anderson *et al.*, 1992; Guidotti & Costa, 1973; Jaanus & Rubin, 1974; Morita *et al.*, 1987; Pocotte *et al.*, 1986; Tsujimoto *et al.*, 1980). In bovine adrenal chromaffin cells administration of the specific muscarinic agonist, Acetyl-β-methylcholine, does not elicit any change in cAMP levels, nor does it modulate the nicotinic response, indicating that in this species at least the rise in cAMP levels is purely mediated by nicotinic mechanisms (Anderson *et al.*, 1992). The increase in cAMP production mediated by nicotinic receptor stimulation in bovine chromaffin cells has been found to be dependent on the presence of extracellular Ca^{2+} (Anderson *et al.*, 1992; Keogh & Marley, 1991; Pocotte *et al.*, 1986). Furthermore this response is also inhibited by the calmodulin antagonist, W7 (N-(6-aminohexyl)-5-chloro-1-napthalene sulphonamide HCl) and trifluoperazine, hence it appears that the cAMP response to nicotinic receptor stimulation is mediated by a Ca^{2+} / calmodulin-sensitive adenylate cyclase (Anderson *et al.*, 1992).

The generation of cAMP during nicotinic stimulation of chromaffin cells may function to regulate catecholamine secretion. In perfused adrenal glands and

adrenal chromaffin cells treatment with cAMP analogues, forskolin, or phosphodiesterase inhibitors which increase intracellular cAMP levels, stimulates catecholamine secretion as well as potentiating secretion evoked by nicotinic agonists (Higgins & Berg, 1988; Morita *et al.*, 1985; Morita *et al.*, 1995*b*). It has been reported that cAMP dependent processes increase ionic conductance changes in chromaffin cells, possibly by phosphorylation of the nAChR channel or by inhibition of Na^+, K^+-ATPase (Higgins & Berg, 1988; Morita *et al.*, 1995*b;* Norenberg *et al.*, 1995).

1.3.3.1.3 Muscarinic receptor stimulation

1.3.3.1.3.1 Calcium dependence

Whilst nicotinic receptor stimulation of catecholamine secretion depends on the presence of extracellular Ca^{2+}, muscarinic receptor stimulation of catecholamine release in species such as the rat and guinea pig does not require extracellular Ca^{2+} (Harish *et al.*, 1987; Malhotra *et al.*, 1988*b;* Misbahuddin & Oka, 1988). Bovine chromaffin cells loaded with quin-2 exhibit increases in intracellular Ca^{2+} levels in response to muscarine and methacholine, even in the absence of extracellular Ca^{2+}, implying the utilisation of intracellular Ca^{2+} stores (Cheek & Burgoyne, 1985; Kao & Schneider, 1986; Kao & Schneider, 1985). The effects of muscarinic receptor stimulation on the mobilisation of intracellular Ca^{2+} stores in chromaffin cells was confirmed in a later study utilising video imaging and the fluorescent probe, fura-2 (O'Sullivan *et al.*, 1989). When bovine chromaffin cells were exposed to muscarinic agonists there was a delayed rise in intracellular calcium levels which originated from an internal region, possibly the endoplasmic reticulum (O'Sullivan *et al.*, 1989).

1.3.3.1.3.2 Calcium mobilisation

Mobilisation of intracellular Ca^{2+} stores requires a signal to the intracellular Ca^{2+} stores. In bovine adrenomedullary slices exposed to ACh there is an increase in phosphatidylinositol and phosphatidate turnover as indicated by ^{32}P incorporation (Trifaro, 1969). The increase in phosphatidylinositol metabolism stimulated by acetylcholine in the chromaffin cells was subsequently found to be due to muscarinic receptor activation (Adnan & Hawthorne, 1981; Fisher *et al.*, 1981;

Forsberg *et al.*, 1986; Malhotra *et al.*, 1988*b*). The increase in phosphatidylinositol metabolism is consistent with mAChR, G-protein mediated, activation of membrane bound enzyme phospholipase C which subsequently cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1984; Berridge *et al.*, 1983; Eberhard & Holz, 1987; Hulme *et al.*, 1990; Nishizuka, 1984). One of the products of PIP_2 metabolism, IP_3, has been shown to release intracellular stores of calcium in other cell types (Joseph *et al.*, 1984; Prentki *et al.*, 1984; Steb *et al.*, 1983). In both bovine and rat adrenomedullary chromaffin cells the muscarine induced increase in intracellular Ca^{2+} levels is associated with an increase in phosphoinositide breakdown (Eberhard & Holz, 1987; Malhotra *et al.*, 1988*b*). Stoehr and associates (Stoehr *et al.*, 1986) administered IP_3 to permeabilsed bovine chromaffin cells to allow intracellular access to the second messenger and showed that IP_3 elicited an increase in intracellular free calcium levels in the presence of calcium free suspension buffer.

Most of the studies of muscarinic receptor stimulation of calcium release have been to a great extent carried out in bovine chromaffin cells. In this species muscarinic stimulation does not elicit catecholamine release and indeed the increase in cytosolic levels of Ca^{2+} achieved in response to muscarinic stimulation are below the threshold required to stimulate exocytosis (Kao & Schneider, 1986; Kao & Schneider, 1985). The density of muscarinic receptors on the plasma membrane of bovine chromaffin cells is around an order of magnitude lower than the number observed on the adrenomedullary chromaffin cells of the rat (Kayaalp & Neff, 1979*a;* Kayaalp & Neff, 1979*b*), a species in which muscarinic receptor stimulation does elicit catecholamine secretion (Harish *et al.*, 1987; Wakade & Wakade, 1983). A difference in muscarinic receptor density may therefore explain the greater release of intracellular Ca^{2+} stores in the rat and hence secretion of catecholamines.

1.3.3.1.3.3 Muscarinic modulation of nicotinic stimulation

Whilst muscarinic stimulation alone is not sufficient to stimulate catecholamine secretion from bovine chromaffin cells it must be borne in mind that acetylcholine release from the splanchnic nerve stimulates both nicotinic and muscarinic

receptors. There is evidence that muscarinic receptor activation can modulate nicotinic receptor function, although it is unclear as to whether this modulation is positive or negative (Adnan & Hawthorne, 1981; Derome *et al.*, 1981; Forsberg *et al.*, 1986). Muscarinic stimulation of bovine chromaffin cells elicits increases in cyclic GMP levels (Derome *et al.*, 1981; Yanagihara *et al.*, 1979) as well as inhibiting cAMP formation (Fernando *et al.*, 1991). Cyclic GMP analogues inhibit nicotinically mediated catecholamine secretion from chromaffin cells (Derome *et al.*, 1981; Swilem *et al.*, 1983). On the other hand, the small rise in cytosolic Ca^{2+} levels stimulated by IP_3 can contribute to the larger nAChR mediated rise to stimulate catecholamine exocytosis (Kao & Schneider, 1985). Another product of phosphoinositide metabolism, DAG, is known to act along with Ca^{2+} to activate protein kinase C (PKC) in chromaffin cells (Terbush & Holz, 1986). Activators of protein kinase C, such as the tumour promoting phorbal esters, have been found to enhance the Ca^{2+} dependent release of catecholamines from adrenomedullary chromaffin cells (Knight & Baker, 1983; Pocotte *et al.*, 1985; Wakade *et al.*, 1986).

1.3.3.1.4 Cellular mechanisms

Both nicotinic and muscarinic receptor stimulation result in an increase in cytosolic free calcium levels as outlined above. Douglas and Rubin (1961; 1963) established that acetylcholine induced catecholamine secretion from perfused cat adrenal glands is dependent on the presence of calcium. Acetylcholine was also found to stimulate ^{45}Ca uptake in the adrenal medulla (Douglas & Poisner, 1962; Douglas & Poisner, 1961). These experimental findings led to the proposal that Ca^{2+} was the pivotal link in stimulus-secretion coupling in catecholamine secretion from the adrenal medulla (for review see Douglas, 1975).

At the cellular level, it was observed in bovine chromaffin cells loaded with the fluorescent Ca^{2+} indicator, quin-2, acetylcholine stimulates an increase in intracellular calcium levels (Knight & Kesteven, 1983). Direct evidence of the vital role which Ca^{2+} plays in catecholamine secretion was obtained in studies utilising 'leaky' chromaffin cells with membranes which had been permeabilised through exposure to strong electric fields (Baker & Knight, 1978), or by treatment with detergents such as digitonin (Dunn & Holz, 1983; Wilson & Kirshner, 1983) or saponin (Brooks & Treml, 1983). The exocytotic release of chromaffin granule

contents from permeabilised chromaffin cells, as evidenced by parallel increases in the secretion of high and low molecular weight chromaffin granule contents, is stimulated by simply raising Ca^{2+} in the suspension buffer to micromolar levels (Baker & Knight, 1978; Brooks & Treml, 1983; Dunn & Holz, 1983; Wilson & Kirshner, 1983). Whilst the stimulatory effect of Ca^{2+} on chromaffin granule exocytosis is independent of cholinergic stimuli it does require the presence of Mg-ATP (Baker & Knight, 1978), implicating the possible involvement of protein phosphorylation in the secretory process (Burgoyne, 1984).

1.3.3.1.5 Exocytosis

The mechanisms by which an increase in intracellular Ca^{2+} levels stimulates exocytosis of the chromaffin granules remains to be fully elucidated. One of the key events in exocytosis is the docking of secretory vesicles to the plasma membrane of the cell to allow membrane fusion and the release of vesicle contents to occur (Burgoyne, 1984; Smith *et al.*, 1973). This docking has been established in rat and bovine neurones to be due to the formation of a fusion particle consisting of a number of proteins which combine to join the secretory vesicle to the acceptor membrane (Bennett *et al.*, 1992; Sollner *et al.*, 1993). *N*-ethylmaleimide-sensitive fusion protein (NSF) is an important component of the fusion particle and in its absence, transport vesicles merely accumulate at the acceptor membrane (Malhotra *et al.*, 1988*c*). NSF requires the presence of other cytoplasmic factors known as soluble NSF attachment proteins (SNAPs) to allow vesicle fusion to proceed (Clary *et al.*, 1990; Sollner *et al.*, 1993). There is also a further requirement for membrane bound co-factors on both the secretory vesicle and the plasma membrane to allow for targeted docking of the synaptic vesicle to specific areas of the plasma membrane (Robinson *et al.*, 1996). Two such membrane bound synaptic proteins which have been identified to function as SNAP receptors (SNAREs) are the vesicle associated membrane protein (VAMP) also known as synaptobrevin, and syntaxin, a protein which is found in the plasma membrane (Sollner *et al.*, 1993). NSF, SNAP, and the SNAREs bind to form a characteristic 20 S fusion particle (Sollner *et al.*, 1993) which results in the docking of the secretory vesicle with the plasma membrane. NSF is established to be a functional ATP-ase (Tagaya *et al.*, 1993) and the hydrolysis of Mg-ATP by this component of the fusion engine leads to a disassembly of the complex which

appears to be an intrinsic step in membrane fusion (Sollner *et al.*, 1993; Wilson *et al.*, 1992).

All of the synaptic components which are required for the formation of the fusion engine in brain tissue have also been identified in chromaffin cells including synaptobrevin / VAMP, syntaxin, α-SNAP, SNAP-25, and NSF (Burgoyne & Williams, 1997; Hodel *et al.*, 1994; Roth & Burgoyne, 1994). Indeed, this fusion engine is functional in chromaffin cells as administration of exogenous α-SNAP to chromaffin cells stimulates exocytosis while the administration of tetanus toxin which cleaves synaptobrevin / VAMP, blocks the calcium induced exocytosis from chromaffin cells (Hohne-Zell *et al.*, 1994; Livett, 1993). As all of the components required for formation of the fusion engine are already present within chromaffin cells it then follows that resting chromaffin granules must be prevented from reaching the docking zone with the plasma membrane. It has been demonstrated using electron microscopy that chromaffin granules are restrained within a three dimensional filamentous network within intact chromaffin cells (Kondo *et al.*, 1982). Substances which are found to disrupt the cortical actin network within chromaffin cells stimulate the exocytosis of chromaffin granules (Roth & Burgoyne, 1995). Furthermore, it is interesting that the chromaffin granule membrane contains on its cytoplasmic side the protein α-actinin which binds the cytoskeletal component, F-actin (Aunis *et al.*, 1980; Bader & Aunis, 1983; Burgoyne, 1984). The binding of chromaffin granule α-actinin to F-actin is Ca^{2+}-dependent and increasing the levels of Ca^{2+} to levels commensurate with those observed to stimulate exocytosis results in dissociation of the two components (Fowler & Pollard, 1982*a;* Fowler & Pollard, 1982*b*).

1.3.3.2 Non-cholinergic regulation of adrenal catecholamine secretion

As previously described, the adrenal gland contains a number of neuropeptides which are expressed in chromaffin cells, splanchnic nerve fibres, intra-adrenal ganglion cells, or a combination of the three. The exact function of these neuropeptides within the adrenal gland has yet to be fully determined but it is thought that they, along with other peptidergic hormones, may act as non-cholinergic transmitters to regulate catecholamine secretion and adrenal blood

flow. Non-cholinergic transmitters may influence catecholamine secretion either directly, or indirectly by the modulation of cholinergic transmitter actions.

1.3.3.2.1 Experimental evidence

Convincing evidence for a major physiological role of non-cholinergic transmitters in regulating adrenomedullary function comes from studies of retrogradely perfused rat (Malhotra & Wakade, 1987*a;* Malhotra *et al.*, 1988*b;* Wakade, 1988; Wakade *et al.*, 1991) and bovine adrenal glands (Marley *et al.*, 1993). Wakade and colleagues reported that catecholamine secretion from the perfused rat adrenal increased in a linear fashion in response to transmural electrical stimulation at frequencies between 0.5 and 3 Hz, as cholinergic fibres were recruited, reaching a plateau at 10 Hz (Wakade, 1981). Similarly, Marley and colleagues established that catecholamine secretion from the perfused bovine adrenal gland increased linearly in response to continuous field stimulation from 2 to 10 Hz, and then subsequently plateaued (Marley *et al.*, 1993). The profile of adrenomedullary catecholamine secretion in response to field stimulation of the perfused bovine gland *in vitro* (Marley *et al.*, 1993) closely resembled that previously reported for stimulation of the peripheral end of the splanchnic nerve *in vivo* for the conscious calf (Edwards, 1982; Edwards *et al.*, 1980).

The cholinergic contribution to the catecholamine secretory response of the perfused adrenal gland to electrical stimulation was examined by using a combination of nicotinic and muscarinic receptor antagonists (Malhotra & Wakade, 1987*a;* Marley *et al.*, 1993; Wakade, 1988). At high frequency stimulation (10 Hz) nicotinic and muscarinic receptor blockade combined reduced catecholamine secretion from perfused rat and bovine adrenals by 80 % and 75 %, respectively. At low frequency stimulation (0.5-2 Hz) the catecholamine secretory response in the presence of nicotinic and muscarinic blockade is only reduced by 35 % in the rat adrenal, yet it is abolished in the bovine adrenal (Malhotra & Wakade, 1987*a;* Marley *et al.*, 1993; Wakade, 1988). The residual catecholamine secretion which occurred in response to electrical stimulation despite nicotinic and muscarinic receptor blockade was attributed to a non-cholinergic component of adrenal nerve stimulation (Malhotra & Wakade, 1987*a;* Marley *et al.*, 1993). Therefore the non cholinergic component predominates at low frequency stimulation in the rat,

representing about 65 % of secretion at 0.5 Hz, and 20 % of secretion at 10 Hz (Malhotra & Wakade, 1987*a;* Wakade, 1988). The non-cholinergic component associated with low frequency electrical stimulation of the rat adrenal gland is capable of maintaining catecholamine secretion for up to several hours, whereas more transient responses are observed at the higher frequencies associated with cholinergic stimulation (Wakade, 1988). In contrast to the rat, non-cholinergic secretion is more prominent at high frequency stimulation in the bovine adrenal gland, representing 25 % of the total secretion at 10 Hz, whilst being absent at 2 Hz (Marley *et al.*, 1993). Bursts of high frequency electrical stimulation of the peripheral end of the splanchnic nerve have been reported to stimulate the release of a number of peptidergic transmitters in the calf (Bloom *et al.*, 1988; Edwards & Jones, 1993*b*).

1.3.3.2.2 Potential non-cholinergic transmitters

Of all the neuropeptides and non-classical transmitters known to be present within the adrenal medulla the roles of VIP, PACAP, Substance P, NPY, Enk-containing peptides, Angiotensin II, and NO in the regulation of adrenomedullary function are probably the best characterised.

1.3.3.2.2.1 VIP and PACAP

Both VIP and the VIP-like secretin peptide, PACAP, which shares a 70 % sequence homology with VIP (Arimura, 1998), are released from the adrenal gland in response to splanchnic nerve stimulation (Bloom *et al.*, 1988; Wakade *et al.*, 1992; Wakade *et al.*, 1991). VIP and PACAP directly stimulate or facilitate cholinergic catecholamine secretion in a dose dependent manner from the adrenomedullary chromaffin cells of a number of species, both *in vitro* and *in vivo* (Cheung & Holzwarth, 1986; Chowdhury *et al.*, 1994; Edwards & Jones, 1993*a;* Edwards & Jones, 1994; Gaspo *et al.*, 1997; Guo & Wakade, 1994; Isobe *et al.*, 1993; Lamouce *et al.*, 1999; Malhotra & Wakade, 1987*b;* Perrin *et al.*, 1995; Watanabe *et al.*, 1995; Wilson, 1988). Receptors for VIP and PACAP belong to the VIP receptor family and have been demonstrated to be present on the chromaffin cells of the adrenal medulla by immunohistochemical, radioligand binding, *in situ* hybridisation, and pharmacological studies (Arimura, 1998; Arimura & Shioda, 1995; Gaspo *et al.*, 1997; Hashimoto *et al.*, 1993; Magistretti *et al.*,

1988; Moller & Sundler, 1996; Shivers *et al.*, 1991; Wakade *et al.*, 1991). PACAP is a substantially more potent stimulator of catecholamine release than is VIP (Guo & Wakade, 1994; Wakade *et al.*, 1992; Wakade *et al.*, 1991; Watanabe *et al.*, 1995) which indicates that the actions of these peptides in the adrenal medulla is predominantly mediated by the PAC_1 receptor which has a high affinity for PACAP and a relatively low affinity for VIP (Gaspo *et al.*, 1997; Hashimoto *et al.*, 1993; Moller & Sundler, 1996). PACAP and VIP interact with chromaffin cells to stimulate increases in cAMP formation, phosphoinositide metabolism (depending on species), release of intracellular calcium stores, and calcium influx (PACAP only); effects which could all contribute to the actions of these peptides on catecholamine secretion (Babinski *et al.*, 1996; Bunn *et al.*, 1990; Isobe *et al.*, 1993; Malhotra *et al.*, 1989; Malhotra *et al.*, 1988*b;* O'Farrell & Marley, 1997; Perrin *et al.*, 1995; Tanaka *et al.*, 1998; Watanabe *et al.*, 1992; Wilson, 1988). Interestingly, VIP receptor antagonists are able to specifically block non-cholinergic catecholamine secretion during low frequency electrical stimulation of the rat adrenal gland, implicating VIP or PACAP as the major non-cholinergic transmitter in this species (Guo & Wakade, 1994; Wakade *et al.*, 1991). Aside from their role as neurotransmitters and neuromodulators within the adrenal gland both VIP and PACAP are established to be potent vasodilators, markedly increasing adrenal blood flow in a number of species (Bloom *et al.*, 1987; Edwards & Jones, 1994; Hinson *et al.*, 1994; Lamouce *et al.*, 1999).

1.3.3.2.2.2 Substance P

Substance P also appears to regulate adrenomedullary catecholamine secretion and has been reported to be released from the adrenal gland in response to physiological stress *in vivo* (Vaupel *et al.*, 1988). The role of substance P in adrenomedullary catecholamine secretion, unlike that of VIP and PACAP, appears to be purely as a neuromodulator of cholinergic stimulation via the nicotinic receptor (Livett & Marley, 1993; Livett & Zhou, 1991). Substance P itself, is without effect on adrenomedullary catecholamine secretion but has complex quantitative and temporal bimodal effects on nicotinic catecholamine secretion; generally being facilitatory at low concentrations and inhibitory at high concentrations (Boksa & Livett, 1984; Khalil *et al.*, 1988*a;* Khalil *et al.*, 1988*b;* Livett *et al.*, 1979; Zhou *et al.*, 1990*b;* Zhou *et al.*, 1991*a*). Destruction of

substance P nerve fibres in the adrenal gland by capsaicin pretreatment in rats, results in a failure of the perfused gland *in vitro* to maintain adrenal catecholamine secretion in response to prolonged electrical stimulation, an effect which can be reversed by substance P administration (Zhou & Livett, 1990; Zhou *et al.*, 1990*a*). Similarly capsaicin pretreatment reduces the capacity of the adrenal to maintain neurogenic catecholamine secretion in response to a number of physiological stressors in the anaesthetised rat (Khalil *et al.*, 1986). These data suggest that substance P released from sensory nerve fibres plays an important role in maintaining adrenal catecholamine secretion during prolonged stimulation (Livett *et al.*, 1990). Pharmacological studies have determined that the actions of substance P on catecholamine secretion are mediated by a novel, specific binding site on adrenomedullary chromaffin cells which is distinct from previously characterised tachykinin receptors, NK_1, NK_2, and NK_3 (Geraghty *et al.*, 1990; Khalil *et al.*, 1988*b;* Livett & Marley, 1993). The actions of substance P appear to occur at the level of the nicotinic receptor ionophore as substance P is not a competitive antagonist for nicotinic receptor binding, nor does it affect catecholamine secretion induced by events which bypass the nicotinic receptor-ionophore complex (Boksa & Livett, 1984; Livett & Marley, 1993). Systemic administration of substance P is found to increase adrenal gland blood flow (Yeo *et al.*, 1984) and like VIP, substance P increases flow in isolated perfused adrenals although less potently (Hinson *et al.*, 1994).

1.3.3.2.2.3 NPY and the opioid peptides

NPY and the Enk-containing opioid peptides represent the most abundant neuropeptides within the adrenal medulla being present in both the chromaffin cells and adrenal innervation (as outlined in 1.3.2). Both NPY and Enk-containing peptides may be co-secreted with catecholamines in response to physiological stress, splanchnic nerve stimulation, or nicotinic cholinergic stimulation (Bloom *et al.*, 1988; Briand *et al.*, 1990; Cheng *et al.*, 1992; Damase-Michel *et al.*, 1994; Damase-Michel *et al.*, 1993; Gaumann & Yaksh, 1988; Hexum *et al.*, 1987; Kruger *et al.*, 1995; Livett *et al.*, 1981; Lundberg *et al.*, 1986; Wilson *et al.*, 1982). In addition, NPY receptors and a number of subtypes of opioid peptide receptor are found to be present on adrenomedullary chromaffin cells (Bunn *et al.*, 1988; Castanas *et al.*, 1985*a;* Castanas *et al.*, 1985*b;* Dumont & Lemaire, 1984;

Wahlestedt *et al.*, 1992; Wharton *et al.*, 1993). NPY inhibits nicotinically evoked catecholamine secretion from adrenomedullary chromaffin cells, apparently by the activity of Y_3 receptors which inhibit cAMP formation following nicotinic stimulation (Boksa, 1990; Higuchi *et al.*, 1988; Norenberg *et al.*, 1995; Shimoda *et al.*, 1993; Wahlestedt *et al.*, 1992). Opioid peptides, like NPY, possess inhibitory actions on catecholamine secretion, attenuating catecholamine secretion from adrenomedullary chromaffin cells in response to nicotinic and splanchnic nerve stimulation (Chen *et al.*, 1989; Critchley *et al.*, 1988; Kumakura *et al.*, 1980; Marley *et al.*, 1986; Saiani & Guidotti, 1982). Opioid peptides seem to exert their inhibitory influence on catecholamine secretion by reducing inward Ca^{2+} currents during stimulation as well as potentiating a Ca^{2+}-dependent K^+ current (Albillos *et al.*, 1996; Twitchell & Rane, 1994). The inhibitory effect of opioid peptides on adrenomedullary catecholamine secretion may also be mediated via presynaptic inhibition of neurotransmitter release from the preganglionic splanchnic nerve which also possesses opioid receptors (Araujo & Collier, 1987; Bunn *et al.*, 1988; Chen *et al.*, 1989; Konishi *et al.*, 1979). Enk-containing peptides are found to be moderate adrenal vasodilators (Hinson *et al.*, 1994), whilst NPY is a potent vasoconstrictor and causes a reduction in adrenal blood flow both *in vitro* and *in vivo* (Hinson *et al.*, 1994; Nilsson, 1991).

1.3.3.2.2.4 Angiotensin II

The potent octapeptide vasoconstrictor hormone, Ang II, and its analogues have long been known to be efficacious stimulators of catecholamine secretion from both the adrenal gland and isolated chromaffin cells (Bunn & Marley, 1989; Feldberg & Lewis, 1964; O'Sullivan & Burgoyne, 1989; Peach, 1971; Powis & O'Brien, 1991; Stachowiak *et al.*, 1990*c;* Wong *et al.*, 1990). Ang II appears to be partly responsible for mediating catecholamine secretion during haemorrhage or hypotension, indicating it may play an important physiological role in regulating adrenomedullary function in response to cardiovascular challenge (Livett & Marley, 1993). Furthermore the adrenal gland appears to be capable of generating its own Ang II as all of the components of an intrinsic renin-angiotensin system (RAS) are present within the gland including angiotensinogen, renin, and angiotensin converting enzyme (ACE) which is expressed in the membranes of chromaffin cells (Dzau *et al.*, 1987; Laliberte *et al.*, 1987; Phillips *et al.*, 1993;

Racz *et al.*, 1992; Strittmatter *et al.*, 1986). Autoradiographic and ligand binding studies have detailed the presence of large numbers of AT_1 receptors in the adrenal medulla which mediate the actions of angiotensin II on catecholamine secretion (Balla *et al.*, 1991; Marley *et al.*, 1989; Wong *et al.*, 1990). The secretion of catecholamines from adrenomedullary chromaffin cells in response to Ang II may be due to a combination of events which occur, including IP_3 generation, PKC activation, release of Ca^{2+} from IP_3-sensitive intracellular stores, and an influx of extracellular Ca^{2+} (Bunn *et al.*, 1990; Burgoyne, 1991; McMillian *et al.*, 1992; Stauderman & Pruss, 1990; Stauderman & Pruss, 1989; Tuominen *et al.*, 1991) Despite the fact that the rise in intracellular Ca^{2+} due to cellular influx is relatively minor compared to that released from intracellular stores (McMillian *et al.*, 1992; O'Sullivan & Burgoyne, 1989; Stauderman & Pruss, 1989) it is the former event which appears to be most important for secretion (Bunn & Marley, 1989; McMillian *et al.*, 1992; O'Sullivan & Burgoyne, 1989). Ang II has been found to stimulate Ca^{2+} influx into the chromaffin cell by activation of a novel ω-conotoxin-sensitive and dihydropyridine-insensitive Ca^{2+} channels (McMillian *et al.*, 1992).

1.3.3.2.2.5 Nitric oxide

The presence of neuronal isoform of NOS within the preganglionic splanchnic nerve fibres and ganglion cells in the adrenal medulla has led to investigators seeking a role for the labile transmitter, NO, in regulating catecholamine secretion (Blottner & Baumgarten, 1992; Breslow *et al.*, 1992; Dun *et al.*, 1993; Marley *et al.*, 1995*a*). NO has been established to play a role in many important functions including vasodilatation, smooth muscle relaxation, neurotransmission, neuromodulation, and long term hippocampal potentiation (Dawson & Snyder, 1994; Huizinga *et al.*, 1992; Lonart *et al.*, 1992; Montague *et al.*, 1994). NO produces a number of its actions, in particular vasodilatation, by activation of soluble guanylate cyclase which leads to an elevation intracellular cyclic GMP levels (Chen & Schofield, 1995; Dawson & Snyder, 1994; Moncada *et al.*, 1991). The adrenal medulla is capable of producing cGMP in response to the NO donors, sodium azide and sodium nitroprusside (Dohi *et al.*, 1983; O'Sullivan & Burgoyne, 1990). Perfused canine and bovine adrenal glands secrete cGMP in response to electrical stimulation (Dohi *et al.*, 1983; Marley *et al.*, 1995*a*) and cGMP levels are

increased by a NO dependent pathway in the cat adrenal medulla following electrical stimulation (Moro *et al.*, 1993). The effects of cGMP or NO on modulating catecholamine secretion from isolated chromaffin cells chromaffin cells are inconclusive with reports of inhibition, enhancement, or no effect (Derome *et al.*, 1981; O'Sullivan & Burgoyne, 1990; Uchiyama *et al.*, 1994). Studies in the perfused adrenal gland demonstrate no effect of NO donors or NOS inhibitors on adrenomedullary catecholamine secretion in response to electrical stimulation despite marked actions on cGMP synthesis (Marley *et al.*, 1995*a;* Moro *et al.*, 1993). Therefore it appears that NO plays a minor role if any, in regulating adrenomedullary catecholamine secretion (Breslow *et al.*, 1992; Marley *et al.*, 1995*a;* Moro *et al.*, 1993). The actions of NO in the adrenal gland most probably involve the regulation of vascular tone and blood flow in the gland. Indeed, NOS inhibitors are reported to decrease basal adrenomedullary blood flow as well as attenuating the vasodilatory response of the medulla to splanchnic nerve stimulation (Breslow, 1992; Breslow *et al.*, 1992).

1.3.4 REGULATION OF ADRENOMEDULLARY CATECHOLAMINE BIOSYNTHESIS

Stimulation of adrenomedullary catecholamine secretion by both physiological stress and pharmacological stimulation results in a compensatory increase in the synthesis of adrenomedullary catecholamines to replenish depleted stores (Ciaranello *et al.*, 1975; Fluharty *et al.*, 1985; Patrick & Kirshner, 1971*b;* Stachowiak *et al.*, 1986; Ungar & Phillips, 1983). Therefore catecholamine synthesis in the adrenal medulla appears to be tightly regulated and is coupled to the secretion of catecholamines such that it is able to respond to increased demands (Wakade *et al.*, 1988). The 'stimulation-secretion-synthesis coupling' of adrenomedullary catecholamines occurs by short term and long term processes (Fluharty *et al.*, 1983; Fluharty *et al.*, 1985; Ungar & Phillips, 1983). Short term, rapid regulation of adrenomedullary catecholamine synthesis occurs through alterations in the activity of synthetic enzymes whereas long term regulation involves the modulation of enzyme levels within the adrenal gland (Fluharty *et al.*, 1985; Ungar & Phillips, 1983). These mechanisms may function together or separately in a stimulus specific manner (Fluharty *et al.*, 1983; Fluharty *et al.*, 1985).

1.3.4.1 Short term regulation of catecholamine biosynthesis

Immediate, short term, regulation of the rate of catecholamine synthesis are achieved by alterations in the activity of the rate limiting enzyme, TH (Levitt *et al.*, 1965; Nagatsu *et al.*, 1964; Ungar & Phillips, 1983).

1.3.4.1.1 Negative feedback inhibition

TH activity can be regulated by negative feedback inhibition from its end products, the catecholamines (Kumer & Vrana, 1996; Ungar & Phillips, 1983). Catecholamines can compete with the pteridine co-factor for binding to the free enzyme, and hence inhibit the activity of the enzyme in a reversible manner (Kumer & Vrana, 1996; Udenfriend *et al.*, 1965; Ungar & Phillips, 1983). This mechanism of TH regulation may only play a fine tuning role in regulating catecholamine synthesis given that the large buffering capacity of the chromaffin granules makes it unlikely that cytosolic catecholamine levels would fluctuate very much (Ungar & Phillips, 1983).

Aside from the classical kinetic inhibition of TH by its end products, it has been determined that catecholamines, in particular dopamine, can complex with Fe^{3+} within the active site of the enzyme in an almost irreversible manner (Andersson *et al.*, 1988; Andersson *et al.*, 1992; Okuno & Fujisawa, 1991; Okuno & Fujisawa, 1985). The TH-dopamine complex is highly stable and displays reduced activity (Okuno & Fujisawa, 1991). Both forms of negative feedback inhibition can be reversed by phosphorylation of the enzyme (Andersson *et al.*, 1992; Kumer & Vrana, 1996).

1.3.4.1.2 Tyrosine hydroxylase phosphorylation

Neural and hormonal stimulation of catecholamine release can increase the activity of TH by phosphorylation of the enzyme via second messenger systems (for review see Kumer & Vrana, 1996). TH is phosphorylated at distinct sites by a number of protein kinases including cAMP-dependent kinase (PKA), Ca^{2+} / calmodulin-dependent kinase (CaM-PKII), mitogen activated protein (MAP) kinases, Protein Kinase G (PKG) and PKC (George *et al.*, 1989; Goncalves *et al.*, 1997; Pocotte *et al.*, 1985; Roskoski *et al.*, 1987; Sutherland *et al.*, 1993). The phosphorylation sites of tyrosine hydroxylase are found to be clustered within the

first 40 amino acids of the N-terminal regulatory domain at serine residues Ser^{8}, Ser^{19}, Ser^{31}, and Ser^{40} with each being phosphorylated in a specific manner by various protein kinases (Campbell *et al.*, 1986; Haycock, 1993; Haycock, 1990). Of all the phosphorylation sites, Ser^{40} is the most promiscuous being phosphorylated by a number of well characterised second messenger systems, especially PKA (Campbell *et al.*, 1986; Funakoshi *et al.*, 1991; Goncalves *et al.*, 1997; Roskoski *et al.*, 1987; Sutherland *et al.*, 1993).

The mechanisms by which phosphorylation increases the activity of TH remains controversial as the relationship between phosphorylation at different sites and activation of the enzyme *in vivo* has yet to be established (Kumer & Vrana, 1996). Phosphorylation of Ser^{40} appears to be the major contributor to TH activation *in vitro* although there is evidence that Ser^{19} and Ser^{31} may also be involved (Bobrovskaya *et al.*, 1998; Haycock *et al.*, 1992; Haycock & Wakade, 1992). Phosphorylation of TH has been reported to increase enzyme activity by reducing the Michaelis constants (K_m) of the enzyme for its pteridine cofactor and tyrosine, as well as increasing the inhibitory constant (K_i) for catecholamine feedback inhibitors (Daubner *et al.*, 1992; Zigmond *et al.*, 1989). Therefore there is a more efficient use of the substrates and cofactors by the enzyme and less susceptibility to negative feedback (Zigmond *et al.*, 1989). There is also an increase in the maximal velocity (V_{max}) of TH when the catecholamine bound form of the enzyme is phosphorylated which does not occur for the free form, indicating that the form of the enzyme influences its activation characteristics (Bailey *et al.*, 1989; Daubner & Fitzpatrick, 1993; Kumer & Vrana, 1996)

ACh released from splanchnic nerve terminals is capable of stimulating a number of second messenger systems within chromaffin cells by causing depolarisation and increasing intracellular Ca^{2+} levels, with consequent increases in cAMP and DAG (see 1.3.3). ACh, nicotine, depolarisation, and Ca^{2+} are found to increase TH activity by affecting multiple site phosphorylation of the enzyme (Dunkley *et al.*, 1996; Goncalves *et al.*, 1997; Haycock, 1993; Haycock *et al.*, 1982; Marley *et al.*, 1995*b;* Niggli *et al.*, 1984; Pocotte *et al.*, 1986; Powis *et al.*, 1996; Waymire *et al.*, 1988). Stimulation of catecholamine secretion with non-cholinergic secretagogues such as Ang II, VIP, and PACAP also increase phosphorylation and activity of the enzyme via the activation of various second messenger systems (see 1.3.3.2,

Bobrovskaya *et al.*, 1998; Haycock, 1996; Houchi *et al.*, 1994; Olasmaa *et al.*, 1992; Stachowiak *et al.*, 1990*c;* Waymire *et al.*, 1991).

1.3.4.2 Long term regulation of catecholamine biosynthesis

Long term regulation of catecholamine biosynthesis occurs through regulation of the levels of the catecholamine synthesising enzymes within the adrenal gland. A number of neural and hormonal factors have been shown to impact on catecholamine synthetic enzyme expression within the adrenal medulla, and it has only been relatively recently that some understanding of the molecular mechanisms underlying these processes has been gained.

1.3.4.2.1 Trans-synaptic enzyme induction

Sustained and intense neurogenic stimulation of adrenal catecholamine secretion by stressors such as hypoglycaemia, hypotension, cold exposure, and the administration of 6-hydroxydopamine or reserpine, elicit a prolonged increase in the enzymatic activities of TH, DBH, and to a lesser extent, PNMT (Ciaranello & Black, 1971; Fluharty *et al.*, 1985; Kvetnansky *et al.*, 1971; Molinoff *et al.*, 1970; Patrick & Kirshner, 1971*b;* Thoenen *et al.*, 1970). This long term increase in enzymatic activity is associated with an increase in the amount of immunologically detectable enzyme molecules present (Ciaranello *et al.*, 1975; Ciaranello *et al.*, 1976; Hoeldtke *et al.*, 1974; Weisberg *et al.*, 1989; Wong *et al.*, 1993). Protein synthesis inhibition as well as enzyme incorporation and turnover studies indicate that the increased levels of catecholamine synthetic enzymes following neurogenic stimulation are due to enhanced *de novo* synthesis (Chuang & Costa, 1974; Ciaranello *et al.*, 1975; Gagnon *et al.*, 1976*a;* Patrick & Kirshner, 1971*b;* Thoenen *et al.*, 1969; Ungar & Phillips, 1983). Adrenal denervation prevents the increase in catecholamine synthetic enzyme activity which occurs in response to many stressors and prevents the recovery of adrenomedullary catecholamine stores previously depleted by reserpine or hypoglycaemia (Fluharty *et al.*, 1985; Patrick & Kirshner, 1971*a;* Patrick & Kirshner, 1971*b;* Thoenen *et al.*, 1970; Thoenen *et al.*, 1969). Enzyme activity following denervation and the recovery of depleted catecholamine stores can be stimulated by cholinergic agonists such as ACh and nicotine (Fossom *et al.*, 1991*a;* Patrick & Kirshner, 1971*a*). Therefore trans-synaptic factors derived from the splanchnic nerve appear to play an

important role in the long term regulation of catecholamine synthetic enzyme activity.

The enhanced synthesis of adrenomedullary catecholamine synthetic enzymes in response to prolonged neural stimulation appears to be due to an upregulation of gene expression (DeCristofaro & LaGamma, 1994; Faucon Biguet *et al.*, 1991; Schalling *et al.*, 1988; Stachowiak *et al.*, 1985; Stachowiak *et al.*, 1986; Weisberg *et al.*, 1989; Wessel & Joh, 1992; Wong & Wang, 1994). Furthermore, cholinergic stimulation of adrenomedullary and immortalised PC12 chromaffin cells elicits increases in the expression of TH, DBH, and PNMT mRNAs (Craviso *et al.*, 1992; Evinger *et al.*, 1994; Hiremagalur *et al.*, 1993; Jahng *et al.*, 1997; Stachowiak *et al.*, 1988*a;* Stachowiak *et al.*, 1990*b*). Chromaffin cells from many species possess both nicotinic and muscarinic cholinergic receptors and both types of receptor have been reported to differentially regulate the gene expression of a number of catecholamine synthetic enzymes (Chen *et al.*, 1996; Craviso *et al.*, 1992; Evinger *et al.*, 1994; Hiremagalur *et al.*, 1993). Nuclear run-off assays and promoter region-reporter gene construct studies reveal that cholinergic stimulation of gene expression is attributable in large part to an augmented transcription of catecholamine synthetic enzyme genes (Craviso *et al.*, 1992; Craviso *et al.*, 1995; Evinger *et al.*, 1994; Fossom *et al.*, 1991*b;* Hemmick *et al.*, 1995; Tonshoff *et al.*, 1997). Transcriptional regulation of gene expression requires the interaction between regulatory proteins (*trans*-acting transcription factors) and sequences within the gene (*cis*-acting elements) (Maniatis *et al.*, 1987). Trans-synaptic stimulation of catecholamine synthetic enzyme gene transcription is likely to be mediated by the interaction of second messenger systems with *trans*-acting regulatory proteins. Events which occur downstream from cholinergic receptor activation that contribute to second messenger system activation, such as depolarisation and increased intracellular calcium levels, are in themselves sufficient to bring about selective changes in TH, DBH, and PNMT mRNA expression (Hwang & Lee, 1995; Kilbourne *et al.*, 1992; Kilbourne & Sabban, 1990; Livett *et al.*, 1990; Stachowiak *et al.*, 1994; Stachowiak *et al.*, 1990*c;* Tschernitz *et al.*, 1995; Wan *et al.*, 1991*b*). Two second messenger systems likely to play a key role in the cholinergic stimulation of catecholamine synthetic enzyme gene expression are cAMP dependent PKA, and PKC (see 1.3.3).

1.3.4.2.1.1 cAMP

A distinct feature of nicotinic stimulation of the adrenal gland *in vivo* (Guidotti & Costa, 1973) and both adrenomedullary and PC12 chromaffin cells *in vitro* (Baizer & Weiner, 1985; Eiden *et al.*, 1984; Olasmaa *et al.*, 1992) is an increase in cAMP formation. Cyclic AMP binds cooperatively to two sites on the regulatory sub-unit of PKA releasing the active catalytic sub-unit (Sassone-Corsi, 1995). Protein kinase A activators, such as forskolin, and stable cAMP analogues are capable of increasing the expression of TH and DBH mRNAs in both adrenomedullary and PC12 chromaffin cells (Hwang & Joh, 1993; Hwang *et al.*, 1994; Hwang *et al.*, 1997; Kim *et al.*, 1994; Kim *et al.*, 1993*b;* McMahon & Sabban, 1992; Stachowiak *et al.*, 1990*b*). There are conflicting reports as to whether or not forskolin or cAMP analogues can induce PNMT mRNA expression in chromaffin cells (Hwang *et al.*, 1994; Hwang *et al.*, 1997; Livett *et al.*, 1990; Stachowiak *et al.*, 1994; Stachowiak *et al.*, 1990*b;* Wan *et al.*, 1991*a*). In studies which report upregulated PNMT mRNA expression in response to forskolin or cAMP analogues, the effect was relatively minor when compared to the induction of DBH and TH mRNAs (Hwang *et al.*, 1994; Hwang *et al.*, 1997; Stachowiak *et al.*, 1994; Stachowiak *et al.*, 1990*b*). The effects of forskolin and cAMP analogues are specifically mediated by PKA activation as the specific inhibitor, *N*-[2-(*p*-bromocinnamylamine)ethyl]-5-isoquinolinesulfonamide (H-89), prevents the induction of catecholamine synthetic enzyme mRNA expression by forskolin (Hwang *et al.*, 1997) and TH and DBH mRNA induction by cyclic AMP is abolished in PKA-deficient PC12 cell lines (Kim *et al.*, 1994; Kim *et al.*, 1993*b*). Both nicotinic stimulation and PKA activation demonstrate similar non-additive patterns of catecholamine synthetic enzyme mRNA induction, implicating a common transduction pathway for both stimuli (Craviso *et al.*, 1995; Hiremagalur *et al.*, 1993; Hwang *et al.*, 1994; Stachowiak *et al.*, 1990*c*). In addition, the induction of TH mRNA expression and gene transcription by nicotine is not observed in PKA-deficient PC12 cells (Hiremagalur *et al.*, 1993). Non-cholinergic secretagogues such as PACAP and VIP, which also stimulate cAMP formation and hence PKA activation in chromaffin cells also have stimulatory effects on catecholamine synthetic enzyme mRNA expression (Isobe *et al.*, 1996; Olasmaa *et al.*, 1992; Park *et al.*, 1999; Rius *et al.*, 1994; Tonshoff *et al.*, 1997).

Aside from their role in nicotinic induction of gene expression, cAMP dependent mechanisms also appear to be important in the maintenance of the basal expression of adrenomedullary TH, DBH, and PNMT mRNA. PKA-deficient PC12 cell lines exhibit reduced basal levels of TH and DBH gene transcription when compared with wild type PC12 cells (Kim *et al.*, 1994; Kim *et al.*, 1993*a;* Kim *et al.*, 1993*b*). In isolated adrenomedullary chromaffin cells the inhibition of PKA activity with H-89 profoundly inhibits the basal expression of TH, DBH, and PNMT mRNAs (Hwang *et al.*, 1997).

Analysis of the 5'-promoter regions of the TH (Best *et al.*, 1995; Coker *et al.*, 1988; D'Mello *et al.*, 1989; Harrington *et al.*, 1987; Piech-Dumas & Tank, 1999) and DBH genes (Afar *et al.*, 1996; Kobayashi *et al.*, 1989; McMahon & Sabban, 1992; Shaskus *et al.*, 1992) has established the presence of functional *cis*-acting cAMP response element (CRE) motifs. A family of *trans*-acting CRE binding proteins including CRE binding protein (CREB) and activating transcription factor 1 (ATF1) are phosphorylated by PKA, whereupon they dimerise and bind to the CRE motifs to stimulate transcription (Sassone-Corsi, 1995). Transfection assays using PC12 and human neuroblastoma SK-N-BE(2) cell lines have established that mutations and deletions of the CRE motif in both the rat TH gene and human DBH gene 5' promoter regions effectively impair transcriptional activity of the promoter in response to nicotine and cAMP (Hiremagalur *et al.*, 1993; Kim *et al.*, 1993*a*; Kim *et al.*, 1994). Western blot analysis of nuclear protein extracts from PC12 cells reveals an enhanced expression of the *trans*-acting CREB protein following PKA activation by forskolin (Tinti *et al.*, 1996). Gel supershift assays of nuclear protein extracts from PC12 cells using oligonucleotides containing the consensus CRE sequence of the TH promoter show that antiserum for both CREB and ATF1 are able to produce supershifted bands indicating an interaction between these transcription factors and the TH-CRE (Tinti *et al.*, 1996). Furthermore, PC12 cells stably transfected with CREB antisense mRNA expression vectors display reduced levels of CREB protein and mRNA expression and there is a failure of cAMP to induce TH promoter-reporter gene construct expression (Piech-Dumas & Tank, 1999). DNase1 footprinting analysis and DNA binding assays also implicate the involvements of CREB and ATF1 in stimulating DBH gene transcription via the CREs in the 5' proximal promoter region of the DBH gene (Afar *et al.*, 1996; Seo *et al.*, 1996).

The 5'-promoter regions of the PNMT genes of a number of species have not been reported to contain a consensus CRE sequence motif (Morita *et al.*, 1992; Ross *et al.*, 1990; Sasaoka *et al.*, 1989), which may explain the relatively poor inducibility of PNMT mRNA expression in response to PKA activation and nicotine administration (Hwang *et al.*, 1994; Hwang *et al.*, 1997; Livett *et al.*, 1990; Stachowiak *et al.*, 1990*b;* Wan *et al.*, 1991*a*). PKA may regulate PNMT expression by a novel CRE sequence motif, or via the activation of a CRE independent pathway (Hwang *et al.*, 1997). There is increasing evidence of functional "cross-talk" between second messenger systems (Cambier *et al.*, 1987; Hoeffler *et al.*, 1989; Kim *et al.*, 1997; Masquilier & Sassone-Corsi, 1992; Narindrasorasak *et al.*, 1987; Yoshimasa *et al.*, 1987) such that activated PKA might indirectly regulate PNMT gene expression via interaction at a number of possible levels with a separate second messenger system.

1.3.4.2.1.2 Protein kinase C

Nicotinic and muscarinic receptor activation stimulate protein kinase C activity in chromaffin cells by enhanced phosphoinositide metabolism mediated by calcium influx and G-protein activation, respectively (Akaike *et al.*, 1993; Eberhard & Holz, 1987; Malhotra *et al.*, 1989; Terbush *et al.*, 1988). Phorbol esters, such as 12-*O*-tetradecanoylphorbal 13-acetate (TPA), which are also potent stimulators of PKC activity are found to stimulate the expression of TH and PNMT mRNA expression in adrenomedullary and PC12 chromaffin cells (Stachowiak *et al.*, 1990*c;* Terbush *et al.*, 1988; Vyas *et al.*, 1990; Wan *et al.*, 1991*a*). Thus PKC may play a role in the trans-synaptic induction of catecholamine synthetic enzyme genes.

The 5'-promoter region of the TH gene contains an AP-1 genomic regulatory elements (Icard-Liepkalns *et al.*, 1992; Stachowiak *et al.*, 1990*a*). The regulation of gene expression by the AP-1 element involves interactions with *trans*-acting homodimers or heterodimers formed between fos and jun leucine zipper DNA-binding proteins (Chiu *et al.*, 1988; Ransone & Verma, 1990). The expression of fos and jun is induced in catecholaminergic cells both *in vivo* and *in vitro* by a wide variety of neural stimuli including hypoglycaemia, reserpine treatment, nicotinic and muscarinic stimulation, and protein kinase C activation (Craviso *et al.*, 1995; Faucon Biguet *et al.*, 1991; Goc *et al.*, 1992; Icard-Liepkalns *et al.*, 1992;

Koistinaho, 1991; Pelto-Huikko *et al.*, 1995; Stachowiak *et al.*, 1990*a;* Stachowiak *et al.*, 1990*d;* Wessel & Joh, 1992). Gel shift analysis strongly implicates the involvement of fos and jun protein binding to TH AP-1 sites in the phorbol ester induced stimulation of TH gene transcription (Goc *et al.*, 1992; Icard-Liepkalns *et al.*, 1992). Furthermore point mutagenesis and deletion analysis of AP-1 binding sites in the TH promoter establish their necessity for phorbol ester mediated upregulation of TH gene transcription (Goc & Stachowiak, 1994; Icard-Liepkalns *et al.*, 1992). Down regulation of PKC by prior incubation of PC18 chromaffin cells with TPA inhibits the induction of transfected TH promoter expression by muscarinic stimulation (Chen *et al.*, 1996). Additionally, muscarinic stimulation of PC18 cells increases binding of nuclear proteins to TH AP-1 sites and the expression of minimal TH promoter constructs containing an AP-1 site (Chen *et al.*, 1996). Hence muscarinic stimulation of a PKC / AP-1 pathway is potentially involved in the regulation of TH gene expression. Nicotinic stimulation of chromaffin cells also induces the binding of nuclear proteins including c-fos, to AP-1 binding sites in the promoter region of the TH gene, indicating a potential role for PKC activation of AP-1 transcription factors in the nicotinic regulation of TH gene expression (Stachowiak *et al.*, 1990*a*). The major pathway involved in nicotinically mediated TH gene transcription appears, however, to be the PKA / CRE pathway (Craviso *et al.*, 1995; Hiremagalur *et al.*, 1993).

Egr-1 is a zinc finger DNA binding protein which is also a member of the immediate early gene family of transcription factors and is expressed in both the adrenal gland and PC12 cells (Ebert *et al.*, 1994; Gashler *et al.*, 1993). Egr-1 expression can be induced in the adrenal medulla *in vivo* in response to reflex neural stimulation by reserpine and metrazole, and in PC12 cells *in vitro* by treatment with phorbol 12-myristate 13-acetate (PMA) and cholinergic agonists (Ebert *et al.*, 1994; Morita *et al.*, 1996; Morita *et al.*, 1995*a*). Consensus DNA binding sequences for Egr-1 are present in the 5' promoter region of the PNMT gene in close proximity to the transcription initiation site (Ebert *et al.*, 1994). Co-transfection assays of RS1, a PC12 derived cell line, with a PNMT promoter-reporter gene construct and either a functional or non functional Egr-1 expression construct, reveal that Egr-1 is able to stimulate the expression of both the reporter gene construct and endogenous PNMT mRNA (Ebert *et al.*, 1994; Ebert & Wong, 1995). Gel mobility shift assays show that the increased PNMT promoter activity

in the presence of Egr-1 is associated with the binding of Egr-1 protein to its consensus element within the promoter. With regards to protein kinase C, PMA treatment of RS1 cells increases the expression of transfected PNMT promoter-reporter gene constructs in a manner which correlates with the degree of Erg-1 mRNA induction (Morita *et al.*, 1995*a*). Mutations of the Egr-1 binding site of PNMT promoter-reporter gene constructs effectively negate the stimulatory effect of phorbol esters on reporter gene expression (Morita *et al.*, 1995*a*).

1.3.4.2.2 Hormonal regulation of catecholamine biosynthesis

1.3.4.2.2.1 Glucocorticoids

As outlined previously the adrenal cortex synthesises and secretes a number of steroid hormones (McNicol, 1992). The intimate relationship which the adrenal medulla shares with the adrenal cortex in terms of both anatomical proximity and circulatory supply would be indicative of regulatory influence of the cortex on the medulla. In species for which noradrenaline, rather than adrenaline, is the predominant adrenomedullary catecholamine the relationship between the medulla and the cortex is not as obvious (Weiner, 1975). Early endocrine ablation and replacement experiments seeking a functional link between the adrenal medulla and the cortex clearly demonstrated that glucocorticoids synthesised by the zona fasciculata of the adrenal cortex play a role in the maintenance of the activity of a number of catecholamine synthetic enzymes. These catecholamine synthetic enzymes included TH (Wurtman & Axelrod, 1966), DBH (Ciaranello *et al.*, 1975; Ciaranello *et al.*, 1976) and in particular, PNMT (Wurtman & Axelrod, 1966; Wurtman & Axelrod, 1965).

Of all the adrenal catecholamine synthetic enzymes, PNMT activity, is the most dependent upon the presence of glucocorticoids. In the adult rat, hypophysectomy (HPX) which eliminates pituitary ACTH secretion and reduces adrenal corticosterone output, also dramatically decreases both adrenaline content and PNMT activity within the adrenal gland (Jiang *et al.*, 1989; Pohorecky & Rust, 1968; Wurtman & Axelrod, 1966). Administration of corticosterone or the potent synthetic glucocorticoid, dexamethasone, reverses the fall in PNMT activity which occurs following HPX in the rat (Jiang *et al.*, 1989; Pohorecky & Rust, 1968; Wurtman & Axelrod, 1966). This is blocked by protein synthesis inhibitors

indicating that glucocorticoids act to stimulate PNMT synthesis (Wurtman & Axelrod, 1966). Double label glutamic acid incorporation experiments have shown that HPX also results in an increased degradation of PNMT, resulting in a fall in activity (Ciaranello, 1978). Glucocorticoids are also found to inhibit the enhanced degradation of the PNMT enzyme which occurs as a consequence of HPX, by stabilising the enzyme co-substrate, SAM (Ciaranello, 1978; Wong *et al.*, 1985; Wong *et al.*, 1992).

At the gene level a number of studies have found that glucocorticoid administration to HPX rats increases PNMT mRNA expression (Evinger *et al.*, 1992; Jiang *et al.*, 1989). This stimulation of adrenal PNMT mRNA expression was determined by nuclear run off assays to be due to increased transcription of the PNMT gene and when adrenal mRNAs are translated *in vitro*, adrenals from HPX rats treated with dexamethasone exhibit higher levels of immunoprecipitable PNMT than those of saline treated animals (Evinger *et al.*, 1992). Analysis of the PNMT gene has revealed the presence of consensus sequences for the glucocorticoid response element (GRE) in the 5' upstream regulatory / promoter sequence (Batter *et al.*, 1988; Kaneda *et al.*, 1988; Morita *et al.*, 1992). Functional experiments using PNMT-chloramphenicol acetyltransferase reporter gene constructs show that this site is functional in the presence of glucocorticoids (Ross *et al.*, 1990).

Many of the studies in the HPX rat found that glucocorticoids could only restore PNMT activity but could not elevate it above control levels (Ciaranello, 1978; Pohorecky & Rust, 1968; Pohorecky & Wurtman, 1968). It was considered that the portal nature of the adrenal gland whereby the medulla of the adrenal is bathed in the glucocorticoid rich effluent of the cortex containing corticosterone concentrations estimated to be as high as 10^{-5} M (Jones *et al.*, 1977) probably meant that the stimulatory effect of endogenous glucocorticoids on PNMT activity was already maximal. Wong et al. (1992), however found that supraphysiological doses of dexamethasone and the specific glucocorticoid type II receptor agonist, RU 28362, at doses up to 1000 μg / day increased PNMT mRNA expression by 10-20 fold in normal intact rats (Wong *et al.*, 1992). Additionally glucocorticoids appear to play a crucial role in the induction of adrenal PNMT activity and mRNA expression which occurs in response to immobilisation stress in rats. HPX

prevents the increase in PNMT activity and mRNA in response to immobilisation an effect which can be reversed by ACTH administration (Kvetnansky *et al.*, 1995). Downregulation of endogenous adrenal glucocorticoid synthesis by the prior administration of exogenous glucocorticoids also prevents the induction of PNMT mRNA expression produced by immobilisation (Kvetnansky *et al.*, 1995).

In primary cultures of isolated bovine chromaffin cells which have been removed from any cortical influence, dexamethasone, is a powerful inducer of PNMT activity with an EC_{50} for PNMT mRNA expression and activity induction of 1-10 nM (Kelner & Pollard, 1985; Wan & Livett, 1989). Bovine chromaffin cells have been found to possess high affinity glucocorticoid binding receptors with a K_d of around 1 nM (Kelner & Pollard, 1985) and the specific glucocorticoid type II receptor antagonist RU 38486 is found to block the induction of PNMT activity (Betito *et al.*, 1992). Binding of glucocorticoids to type II glucocorticoid receptors present in chromaffin cells results in dimerisation and transport into the nucleus where the complex interacts with glucocorticoid response elements on the PNMT gene to stimulate transcription (Wan & Livett, 1989; Wright *et al.*, 1993).

1.3.4.2.2.2 Angiotensin II

Consistent with its ability to stimulate catecholamine secretion and tyrosine hydroxylase activity the peptide hormone Ang II also plays a role in the regulation of catecholamine synthetic enzyme gene expression within the adrenal medulla. Stable analogues of Ang II are able to stimulate the expression and synthesis of PNMT and TH mRNA in adrenomedullary chromaffin cells at nanomolar doses (Cahill *et al.*, 1996; Stachowiak *et al.*, 1990*a;* Stachowiak *et al.*, 1990*c*). This effect of Ang II on TH and PNMT gene expression is mediated by specific AT receptors as its actions are abolished by the AT receptor antagonist, saralasin (Stachowiak *et al.*, 1990*c*). PKC is activated in chromaffin cells in response to Ang II, and both prior down regulation of PKC activity with phorbol esters or inhibition with sphingosine prevent Ang II mediated increases in TH and PNMT mRNA expression (Stachowiak *et al.*, 1990*c*). Therefore Ang II may regulate PNMT and TH mRNA gene expression via a PKC mediated stimulation of both Egr-1 (Morita *et al.*, 1995*a*) and AP-1 *trans*-activation factors (Goc *et al.*, 1992), respectively.

In the case of TH, treatment of adrenomedullary chromaffin cells with Ang II induces the protein and mRNA expression of a number of AP-1 factors including c-fos and c-jun via a PKC mediated mechanism (Stachowiak *et al.*, 1990*a;* Stachowiak *et al.*, 1990*d*). Concomitant with the elevation of AP-1 factors in chromaffin cells by Ang II there is also enhanced binding of nuclear proteins, including c-fos, from chromaffin cells to AP-1 sites on the TH gene promoter (Stachowiak *et al.*, 1990*a*). Deletion of an AP-1 element between base pairs -269 and -194 of the bovine TH promoter substantially reduces Ang II induction of the promoter-reporter gene construct (Goc & Stachowiak, 1994). The mechanisms of Ang II induction of PNMT mRNA expression are yet to be determined, however the inductive effect of Ang II can be blocked by the protein synthesis inhibitor, cycloheximide (Cahill *et al.*, 1996). Therefore PNMT mRNA induction by Ang II requires ongoing protein synthesis indicating that the *de novo* synthesis of transcriptional factors is required. This finding would be consistent with Ang II induction of Egr-1 to stimulate PNMT gene expression

1.4 THE FETAL ADRENAL MEDULLA

1.4.1 PHYSIOLOGICAL ROLE OF ADRENOMEDULLARY CATECHOLAMINES IN THE FETUS

1.4.1.1 Catecholamine secretion in response to stress in utero

In mature animals the activation of sympathetic neurones is a key factor in the physiological response to stressful stimuli. The role of the adrenal medulla as a source of catecholamines during fetal life is particularly important, as in a number of mammals peripheral sympathetic innervation of end organs does not become fully competent until late fetal or early neonatal life (Comline & Silver, 1966; Erath *et al.*, 1982; Gootman *et al.*, 1979; Lebowitz *et al.*, 1972; Pappano, 1977; Smith *et al.*, 1982).

1.4.1.1.1 Acute stress

1.4.1.1.1.1 Hypoxaemia

In the fetal lamb and neonatal rat, hypoxaemia stimulates an increase in both plasma noradrenaline and adrenaline levels (Cheung, 1990; Iwamoto *et al.*, 1989;

Jones & Robinson, 1983; Robinson *et al.*, 1977*a;* Shaul *et al.*, 1989; Widmark *et al.*, 1989). In fetal sheep in which adrenal venous effluent was collected directly during asphyxia or hypoxaemia, there are marked increases in catecholamine output (Cohen *et al.*, 1991; Cohen *et al.*, 1984; Comline & Silver, 1966; Comline & Silver, 1961). The changes in peripheral plasma catecholamine levels during hypoxaemia tend to be qualitative reflections of changes which occur in the adrenal venous plasma with the latter displaying greater sensitivity to hypoxaemia (Cohen *et al.*, 1991). In neonatal rats exposed to hypoxia (7-9 % F_iO_2) there is a significant depletion of adrenomedullary catecholamine stores (Seidler & Slotkin, 1985; Seidler & Slotkin, 1986*b;* Shaul *et al.*, 1989). Furthermore, selective adrenal demedullation in the fetal sheep by the injection of acid formalin into the adrenal medulla, or bilateral adrenalectomy, totally abolishes the hypoxia (30-50% decrease in fetal arterial PO_2) induced rise in plasma adrenaline levels and markedly attenuates the noradrenaline secretory response (Jones *et al.*, 1988; Simonetta *et al.*, 1996*a*). It has clearly been demonstrated that the fetal and neonatal adrenal medulla secretes catecholamines in response to hypoxaemia even prior to the establishment of functional innervation to the gland by a direct, non-neurogenic mechanism (Adams *et al.*, 1996; Cheung, 1990; Comline & Silver, 1966; Comline & Silver, 1961; Seidler & Slotkin, 1985; Seidler & Slotkin, 1986*a;* Seidler & Slotkin, 1986*b;* Slotkin & Seidler, 1988). With the advent of functional innervation to the adrenal gland the non-neurogenic response to hypoxaemia is replaced by the reflex neurogenic stimulation observed in the mature animal (Cheung, 1990; Comline & Silver, 1966; Slotkin & Seidler, 1988). Therefore the adrenal medulla is clearly a vital source of catecholamines in the fetus and neonate during episodes of acute hypoxaemia.

1.4.1.1.1.2 Hypoglycaemia

In the fetal sheep and neonatal rat, hypoglycaemia is also able to stimulate adrenomedullary catecholamine release. In the late gestation sheep fetus insulin-induced hypoglycaemia selectively increases adrenaline levels in adrenal venous outflow (Cohen *et al.*, 1991). The selective nature of hypoglycaemia stimulated adrenomedullary catecholamine secretion is also observed in rats where insulin-induced hypoglycaemia preferentially depletes adrenaline content in the adrenal gland (Lau *et al.*, 1987). Whilst insulin itself under euglycaemic conditions is

unable to stimulate adrenomedullary catecholamine secretion (Cohen *et al.*, 1991; Young *et al.*, 1984), it has been found to stimulate the peripheral sympathetic nervous system (Liang *et al.*, 1982; Morgan *et al.*, 1993; Rowe *et al.*, 1981). This observation may explain the observed increases in plasma noradrenaline levels which occur in response to insulin-induced hypoglycaemia in the fetal sheep (Papparella *et al.*, 1994). Adrenomedullary catecholamine secretion in response to hypoglycaemia is reliant on the presence of functional splanchnic innervation to the adrenal gland. In the neonatal rat catecholamine secretion from the adrenal medulla does not occur in response to hypoglycaemia until after the first week of life (Seidler & Slotkin, 1986*b;* Slotkin & Seidler, 1988), a time which coincides with the establishment of functional splanchnic synapses within the adrenal medulla (Lau *et al.*, 1988). The selective secretion of adrenaline from the adrenal medulla in response to hypoglycaemia in late (127-140 d) gestation fetal sheep and neonatal rats is also characteristic of reflex neurogenic stimulation (Cohen *et al.*, 1991; Lau *et al.*, 1987).

1.4.1.1.1.3 Labour and parturition

A profound increase in fetal catecholamine levels is found to occur in umbilical arterial blood and neonatal blood at birth in humans (Eliot *et al.*, 1980; Lagercrantz & Bistoletti, 1973; Nakai & Yamada, 1978; Nylund *et al.*, 1979; Padbury *et al.*, 1982). Circulating catecholamine levels far in excess of the levels observed in adults during heavy exercise have been reported for newborn infants following an uneventful delivery (Lagercrantz & Slotkin, 1986). The large umbilical artery : umbilical vein gradient in catecholamine concentrations which exists in newborn infants (Lagercrantz & Bistoletti, 1973) demonstrates that the catecholamine surge is of fetal not maternal origin. In agreement with the fetal origin of the catecholamine surge at birth is the observation that there is very little placental transfer of catecholamines with the placenta being a major site of catecholamine clearance (Bzoskie *et al.*, 1995; Jones & Robinson, 1975; Parvez & Parvez, 1974). A similar catecholamine response to parturition has also been established to occur in the fetal sheep (Eliot *et al.*, 1981; Padbury *et al.*, 1987*a;* Padbury *et al.*, 1985). The elevation in plasma catecholamine levels occurs during the last few hours of spontaneous labour prior to delivery in catheterised fetal sheep (Eliot *et al.*, 1981). On top of the catecholamine surge which occurs during labour in the

fetal sheep there are further increases in noradrenaline and adrenaline levels in response to both delivery and cutting of the umbilical cord (Padbury *et al.*, 1985). Bilateral adrenalectomy in the fetal sheep with compensatory glucocorticoid replacement resulted in a blunted noradrenaline surge and an abolition of the adrenaline surge in response to birth (Padbury *et al.*, 1987*a*). Therefore it appears that the fetal adrenal glands are a major source of circulating catecholamines during labour and birth.

1.4.1.1.2 Chronic stress

1.4.1.1.2.1 Clinical observations

Intrauterine growth retardation (IUGR) is a relatively common fetal condition (de Onis *et al.*, 1998; Villar & Belizan, 1982) which results in disproportionately high levels of perinatal morbidity and mortality (Divon *et al.*, 1998; Knutzen & Sher, 1982; Minior & Divon, 1998; Newton *et al.*, 1987). IUGR is associated with a number of factors including pre-eclampsia and maternal smoking (Peters *et al.*, 1983). Ultrasonographic measurements of midgestational placental volume reveal that reduced placental volumes are highly predictive of IUGR at term (Wolf *et al.*, 1989). Furthermore, the functional surface area of the placenta available for oxygen and nutrient exchange is significantly reduced in pre-eclamptic pregnancies and in cases of idiopathic fetal IUGR (Boyd & Scott, 1985). Maternal smoking is linked to smaller placentas, which display reduced capillary number, and increased diffusional distances across villi membranes (Robinson *et al.*, 1995). Hence IUGR is generally considered to arise from a chronic deficiency of maternal oxygen and nutrient supply to the fetus via the placenta.

The high incidence of perinatal asphyxia observed amongst IUGR infants is suggestive of pre-existing hypoxia (Cassady, 1981; Cetrulo & Freeman, 1977). Chronic intra-uterine hypoxaemia is also inferred by increased packed cell volume, red blood cell numbers, and erythropoietin concentrations observed in IUGR infants (Robinson, 1979; Robinson *et al.*, 1985). Cordocentesis studies which allow for ultrasound guided blood sampling from the umbilical cord of undisturbed human fetuses *in utero* reveal that IUGR fetuses are frequently hypoxaemic (Economides *et al.*, 1991; Soothill *et al.*, 1987). Depending on the severity of the hypoxaemia, IUGR fetuses may also exhibit hypercapnia, respiratory and

metabolic acidosis, and increased lactate levels due to decreased oxidative metabolism (Economides *et al.*, 1991; Soothill *et al.*, 1987). Erythrocyte numbers increase in hypoxaemic IUGR fetuses due to elevated fetal erythropoietin concentrations, a compensatory response which occurs as early as 24-26 weeks gestation (Snijders *et al.*, 1993; Soothill *et al.*, 1987; Thomas *et al.*, 1983).

The IUGR infant is also at significantly greater risk of hypoglycaemia than appropriately grown infants at birth (Minior & Divon, 1998; Robinson, 1979). Cordocentesis of IUGR fetuses shows that these fetuses are often hypoglycaemic, with the degree of hypoglycaemia closely correlating with the severity of hypoxaemia present (Economides *et al.*, 1991; Soothill *et al.*, 1987). Attendant with a reduction in blood glucose levels, IUGR fetuses are also hypoinsulinaemic (Economides *et al.*, 1991). Cortisol levels are elevated in the cord blood of IUGR fetuses (Economides *et al.*, 1991; Economides *et al.*, 1988) which would serve to counter the decrease in blood glucose levels by stimulating fetal gluconeogenesis in late gestation (Fowden *et al.*, 1993; Townsend *et al.*, 1991).

As previously outlined, both acute hypoxaemia and hypoglycaemia stimulate catecholamine secretion from the fetal adrenal medulla. The clinical evidence presented above demonstrates that the IUGR fetus is frequently both chronically hypoxaemic and hypoglycaemic. Levels of catecholamines and their metabolites in amniotic fluid are increased in IUGR pregnancies, which is consistent with increased fetal sympathoadrenal tone (Divers *et al.*, 1981; Lagercrantz *et al.*, 1980; Puolakka *et al.*, 1984).

1.4.1.1.2.2 Experimental observations

There are several models of IUGR in experimental animals which induce growth retardation by restricting oxygen and nutrient supply to the fetus. A number of these models involve impeding the utero-placental or umbilico-placental circulation by a variety of methods including uterine artery and terminal aorta occlusion (Boyle *et al.*, 1996; Kollee *et al.*, 1979; Lafeber *et al.*, 1985), and placental embolisation with microspheres (Block *et al.*, 1990; Clapp *et al.*, 1981; Murotsuki *et al.*, 1997). Another approach is to restrict placental implantation. This is achieved in sheep by the surgical removal of the majority of visible endometrial caruncles from the uterus of the ewe prior to pregnancy. (Alexander, 1964). The

excision of these placental implantation sites, which are the anlagen of the maternal cotyledons, limits the number of individual placentomes formed restricting both placental size and fetal growth (Alexander, 1964; Robinson *et al.*, 1979*a*). Fetuses in experimental models of growth retardation also demonstrate hypoxaemia, hypercapnia, hyperlacticaemia, hypoglycaemia, hypoinsulinaemia, and increased cortisol levels (Clapp *et al.*, 1980; Gagnon *et al.*, 1994; Robinson *et al.*, 1985; Simonetta *et al.*, 1997; Sug Tang *et al.*, 1992; Unterman *et al.*, 1993).

Elevated fetal circulating catecholamine levels have been reported in a number of paradigms of experimental IUGR (Gagnon *et al.*, 1994; Gu *et al.*, 1985; Hooper *et al.*, 1990; Jones & Robinson, 1983; Simonetta *et al.*, 1997). Basal levels of both noradrenaline and adrenaline are elevated in the placentally restricted (PR) fetal sheep (Jones & Robinson, 1983; Simonetta *et al.*, 1997). Basal plasma noradrenaline levels in the both the normal and PR ovine fetus are inversely related to arterial PO_2 and pH (Simonetta *et al.*, 1997). Basal plasma adrenaline levels which are also inversely related to arterial PO_2 in the normal fetal sheep, display a paradoxical positive relationship to arterial PO_2 in hypoxaemic PR fetuses (Simonetta *et al.*, 1997). This finding may imply that adrenaline secretion despite being greater under basal conditions in the PR fetal sheep may be compromised in response to further stressful stimuli. Indeed, the adrenaline secretory response to acute hypoxaemia (9% Maternal F_iO_2 for 60 min) in PR fetal sheep is attenuated compared to the response which occurs in normal fetuses, whilst the noradrenaline response is enhanced (Jones & Robinson, 1983). Furthermore, the effects of IUGR on adrenaline secretion may persist postnatally, as adrenaline secretion from the adrenal gland in response to acute hypoxia is abolished in growth retarded neonatal rat pups (Shaul *et al.*, 1989). It therefore appears that adrenaline secretion is impaired with IUGR despite an elevation in plasma cortisol levels which as previously noted has a stimulatory effect of PNMT activity and synthesis in the adult.

The source of the increase in basal circulating catecholamine levels in response to IUGR has yet to be fully determined. In the normal fetal sheep basal noradrenaline levels are not significantly affected by adrenal demedullation but are substantially reduced following peripheral sympathectomy by guanethidine administration (Jones *et al.*, 1987). Adrenal demedullation alone is sufficient to

eliminate adrenaline from the circulation under basal conditions (Jones *et al.*, 1987). Therefore under basal conditions the adrenal medulla is the major source of adrenaline whilst "overspill" from sympathetic terminals appears to be primarily responsible for basal noradrenaline levels in the fetal sheep. Infusion of tyramine, which acts to selectively displace catecholamines from sympathetic nerve terminals (Jones *et al.*, 1987; Lewis & Sischo, 1985; Schuijers *et al.*, 1986), stimulates a greater overall noradrenaline increase in PR fetal sheep than in their respective controls (Simonetta *et al.*, 1997). The proportional increase in noradrenaline secretion in response to tyramine infusion, however, is not different between PR and control fetuses (Simonetta *et al.*, 1997). This finding would be consistent with the increased basal plasma noradrenaline levels associated with PR being derived from increased sympathetic "overspill" (Simonetta *et al.*, 1997). Further support of this hypothesis can be derived from the observation that IUGR neonatal rat pups also have elevated cardiac sympathetic neuronal activities under basal conditions (Shaul *et al.*, 1989).

Chronic stress associated with IUGR is therefore associated with long term changes in basal circulating catecholamine levels and responsiveness to subsequent acute stressors. These changes may reflect long term alterations in fetal catecholamine secretion, synthesis, and metabolism. It is unknown to what degree any of these determinants of peripheral catecholamine levels is altered in the fetal adrenal gland in response to chronic stress.

1.4.1.2 Physiological actions of catecholamines in the fetus and neonate

The fetus grows and matures in an intrauterine environment in which temperature is maintained and oxygen as well as other metabolic substrates required for growth and survival are continuously supplied via the placenta. As detailed above, this intrauterine environment, however, can be disrupted during pregnancy by conditions such as maternal anaemia, maternal undernutrition, placental insufficiency, placental abruption, pre-eclampsia and cord occlusions, such that the fetus may experience potentially threatening shortages of oxygen and / or nutrient supply. Furthermore, at the time of parturition the fetus must prepare for the transition to extrauterine life which entails obtaining its own oxygen via air breathing, generating its own body heat, and utilising its own energy stores. The

consequent increase in plasma catecholamine levels due to birth and shortages of metabolic substrates, particularly oxygen, elicit a number of cardiovascular, metabolic, respiratory, and thermogenic responses which act to promote fetal and neonatal survival (Lagercrantz & Slotkin, 1986; Padbury, 1989; Phillipe, 1983; Slotkin & Seidler, 1988).

1.4.1.2.1 Cardiovascular actions

1.4.1.2.1.1 Fetal cardiovascular responses to hypoxaemia

The cardiovascular response of the fetus and neonate to acute hypoxia and asphyxia have been extensively examined due to the array of physiological and pathophysiological situations which can lead to reduced fetal oxygenation (for review see Giussani *et al.*, 1994). The cardiovascular responses of the fetal sheep to hypoxaemia are dependent upon gestational age. Prior to 102 d gestation there is a sustained increase in heart rate, and little change in arterial pressure (Boddy *et al.*, 1974; Iwamoto *et al.*, 1989). In older fetuses however, there is a progressive increase in arterial pressure, and transient bradycardia (Boddy *et al.*, 1974; Cohn *et al.*, 1974; Giussani *et al.*, 1993; Jones & Robinson, 1975; Robinson *et al.*, 1977*a*).

Microsphere studies reveal that hypoxaemia in fetal sheep stimulates a strategic redistribution of blood flow to vital organs, including the brain, heart, and adrenal glands (Cohn *et al.*, 1974; Iwamoto *et al.*, 1989; Peeters *et al.*, 1979; Rudolph, 1985; Yaffe *et al.*, 1987). The redistribution of blood flow in older sheep fetuses occurs at the expense of blood flow to the skeletal muscle, pulmonary, cutaneous, gastrointestinal, and renal circulations (Cohn *et al.*, 1974; Peeters *et al.*, 1979; Rudolph, 1985; Yaffe *et al.*, 1987). In fetuses younger than 100 d gestation, blood flow is only compromised in the pulmonary and placental circulations, suggesting along with a lack of change in arterial pressure, that hypoxaemia elicits its effects on blood flow distribution in the very young fetus by direct local vascular actions (Iwamoto *et al.*, 1989). The circulatory redistribution which occurs during acute hypoxia is found to be maintained during chronic hypoxaemia (Bocking *et al.*, 1988). A strategic redistribution of blood flow similar to that which occurs in the fetal sheep has also been reported during asphyxia in the primate fetus (Behrman *et al.*, 1970).

1.4.1.2.1.2 Catecholamines and haemodynamic responses to hypoxaemia

Like hypoxaemia, intrafetal infusions of physiological levels of noradrenaline and adrenaline stimulate an increase of arterial blood pressure in the fetal sheep (Cheung & Brace, 1988; Jones & Ritchie, 1978*a;* Miyake *et al.*, 1991; Padbury *et al.*, 1987*c*). α-adrenoreceptor stimulation by catecholamines appears to be primarily responsible for the increase in blood pressure (Jones & Ritchie, 1978*a*) and causes a redistribution of cardiac output which mirrors that observed during hypoxaemia (Barrett *et al.*, 1972).

The hypoxia-induced increase in arterial pressure and reduction in peripheral blood flow due to vasoconstriction, is sensitive to blockade with the α-adrenoreceptor antagonist, phentolamine (Brace & Cheung, 1987; Giussani *et al.*, 1993; Lewis *et al.*, 1980; Paulick *et al.*, 1991; Reuss *et al.*, 1982). The α-adrenergic vasoconstriction induced by hypoxia is stimulated by catecholamines derived from both sympathetic vasomotor innervation and the adrenal medulla. Abolition of post-ganglionic sympathetic input to the femoral artery by sectioning of the sciatic nerve depresses the reduction in femoral blood flow which occurs in response to asphyxia, whilst noradrenaline infusion is able to reproduce the response (Dawes *et al.*, 1968). Interruption of the chemoreceptor reflex arc, which stimulates sympathoadrenal outflow during hypoxia, by carotid body and vagal denervation blunts the increases in circulating catecholamine levels and femoral artery resistance as well as the decrease in peripheral blood flow (Giussani *et al.*, 1994; Giussani *et al.*, 1993; Itskovitz *et al.*, 1991; Jansen *et al.*, 1989; Jensen & Hanson, 1995; Stein *et al.*, 1998). A slower increase in femoral resistance during hypoxaemia which persists despite chemoreceptor denervation may be due to direct stimulation of catecholamine release from the adrenal medulla (Giussani *et al.*, 1993).

Chemical destruction of the peripheral sympathetic nervous system in the fetal sheep by the infusion of 6-hydroxydopamine or guanethidine, does not substantially alter the blood pressure or catecholamine secretory responses to hypoxaemia (Jones *et al.*, 1988; Lewis & Sischo, 1985). Hence circulating catecholamines derived from the adrenal medulla during hypoxia play a key role in the circulatory responses which occur during hypoxia. In further support of this

conclusion, adrenal demedullation of 127-138 d fetal sheep abolishes both the increase in peripheral catecholamine levels and mean arterial pressure which occurs in response to hypoxaemia (Jones *et al.*, 1988).

1.4.1.2.1.3 Catecholamines and cardiac responses to hypoxaemia

The initial slowing of heart rate in late gestation fetal sheep is a vagal reflex as it is antagonised by atropine and abolished by vagotomy (Boddy *et al.*, 1974; Giussani *et al.*, 1993; Jansen *et al.*, 1989). The reflex is not due to baroreceptor activation stimulated by the increase in arterial pressure as it precedes the rise in pressure and can be stimulated independently of it (Giussani *et al.*, 1993; Lewis *et al.*, 1980; Parer *et al.*, 1980). Chemoreceptor denervation by sectioning of the carotid sinus nerve eliminates the bradycardic response to hypoxia indicating that it is a carotid body reflex (Giussani *et al.*, 1993; Itskovitz *et al.*, 1991). In addition to eliminating the transient bradycardia, atropine infusion during hypoxia elicits an increase in heart rate analogous to that observed in fetuses < 102 d gestation (Boddy *et al.*, 1974; Giussani *et al.*, 1994; Giussani *et al.*, 1993; Iwamoto *et al.*, 1989).

Despite the initial bradycardia which occurs in response to fetal hypoxaemia heart rate gradually increases back to baseline levels during hypoxaemia (Boddy *et al.*, 1974; Giussani *et al.*, 1993; Jones & Robinson, 1975). Both the recovery of heart rate from the initial bradycardia and an increase in cardiac contractility which occur during hypoxaemia are blocked by the β-adrenoreceptor antagonist, propranolol (Court *et al.*, 1984; Evers *et al.*, 1981; Robinson *et al.*, 1977*a*). Furthermore infusion of physiological levels of adrenaline stimulate an increase in heart rate in the fetal sheep which is also antagonised by propranolol (Jones & Ritchie, 1978*a;* Jones & Robinson, 1975; Robinson *et al.*, 1977*a*). Demedullation of the fetal sheep also prevents the recovery of heart rate from the initial vagal inhibition during hypoxaemia (Jones *et al.*, 1988). Therefore circulating catecholamines released during hypoxaemia act at cardiac β-adrenoreceptors to overcome the vagal inhibition of heart rate and maintain cardiac output (Court *et al.*, 1984; Evers *et al.*, 1981; Jones & Robinson, 1975; Robinson *et al.*, 1977*a*).

The neonatal rat which lacks a functionally mature peripheral sympathetic at birth and is reliant on adrenomedullary catecholamines, possesses specialised cardiac

α-adrenoreceptors (Slotkin & Seidler, 1988). These receptors are unique to the immature animal and are gradually replaced by the cardiac β-adrenoreceptors which are associated with mature animals (Slotkin *et al.*, 1986). Catecholamines released from the adrenal medulla during the exposure of the neonatal rat to hypoxia prevents any significant changes in cardiac conduction characteristics from occurring (Seidler *et al.*, 1987; Slotkin & Seidler, 1988). The presence of the α-adrenoreceptor antagonist, phenoxybenzamine, is associated with the development of marked atrio-ventricular conduction defects and a progressive decline in sinus rhythm during hypoxia leading to cardiac arrest (Seidler *et al.*, 1987). Elevated levels of the cardiac enzyme ornithine decarboxylase, indicative of cardiac tissue damage, are also associated with α-adrenergic blockade during hypoxaemia (Slotkin *et al.*, 1987; Slotkin & Seidler, 1988).

1.4.1.2.1.4 Catecholamines and cardiovascular adaptations at birth

At birth the fetus must undergo remarkable circulatory changes to adapt to neonatal life. Birth, umbilical cord cutting, inflation of the lungs, and increased pulmonary oxygenation results in the closure of fetal shunts as well as increases in blood pressure, peripheral vascular resistance, heart rate, cardiac output, and left ventricular dominance (Padbury, 1989; Rudolph *et al.*, 1988). Catecholamines released from the adrenal medulla during labour and birth facilitate a number of these events (Padbury & Martinez, 1988).

A decrease in pulmonary resistance occurs at delivery, predominantly due to inflation of the lungs and a local dilator effect of oxygenation (Rudolph *et al.*, 1988). As a consequence of increased pulmonary blood flow there is a shift to left ventricular dominance with a greater return of blood to the left atrium and increased atrial pressure which leads to increased left ventricular output due to the Frank-Starling mechanism (Rudolph *et al.*, 1988). The consequent increase in cardiac output is further augmented by an increase in heart rate (Padbury *et al.*, 1987*a*). Large increases in the isovolemic measure of left ventricular contractility, peak derivative of pressure with respect to time (*dP / dt*), are established to occur at delivery (Padbury *et al.*, 1987*a*). Although the left ventricular *dP / dt* is directly related to heart rate, the increase in *dP / dt* is unable to be accounted for by heart rate alone, indicating that there is enhanced left ventricular contractility also

contributing to increased cardiac output at birth (Padbury *et al.*, 1987*a*). Abolition of the catecholamine surge which occurs at birth by adrenalectomy eliminates the rapid increase in heart rate following delivery and also markedly attenuates the rise in *dP / dt* and left ventricular output (Padbury *et al.*, 1987*a*). Peripheral sympathectomy on the other hand does not significantly affect these variables of cardiac function at birth (Agata *et al.*, 1986). Adrenomedullary catecholamines, particularly adrenaline, appear to contribute to the increase in cardiac output at birth by stimulating heart rate and myocardial contractility through β-adrenergic mechanisms.

There is a marked redistribution of blood flow which occurs at birth with a diversion of blood flow from the periphery to central organs to protect the neonate from episodes of hypoxaemia during delivery and in the event of postnatal respiratory difficulties (Faxelius *et al.*, 1984; Lagercrantz & Slotkin, 1986). The elevation in peripheral vascular resistance which has been noted to occur in animal models of simulated birth would contribute to the redistribution of blood flow and the increase in blood pressure at birth (Rudolph *et al.*, 1988). Studies in the human infant comparing vaginal delivery and Caesarean section delivery have found that Caesarean section is associated with lower plasma catecholamine levels at birth and an increased peripheral blood flow (Faxelius *et al.*, 1984). Peripheral vascular resistance in the neonate in the first few hours of life was found to correlate with plasma noradrenaline levels (Faxelius *et al.*, 1984). Arterial pressure is depressed following delivery in lambs which are adrenalectomised (Padbury *et al.*, 1987*a*) but not after chemical sympathectomy (Agata *et al.*, 1986). Adrenalectomised newborn puppies are also found to be hypotensive and bradycardic (Geis *et al.*, 1975). These findings indicate that the sympathoadrenal system plays a key role in peripheral vasoconstriction and blood pressure regulation at birth and early neonatal life (Padbury & Martinez, 1988).

1.4.1.2.2 Effect of catecholamines on metabolism and growth

1.4.1.2.2.1 Fetal and neonatal metabolism

The fetus is primarily reliant on glucose as the main metabolic fuel although lactate, amino acids, free fatty acids, and ketones are utilised to varying degrees (Battaglia & Meschia, 1978; Jones & Rolph, 1985). There is an absence of active

glucose transport mechanisms in the placenta with glucose being supplied to the fetus via facilitated diffusion across the placenta (Hay, 1995). This process is dependent on a glucose concentration gradient with maternal concentrations being higher than those observed in the fetus (Economides *et al.*, 1991; Hay, 1995). A fall in maternal glucose concentrations will subsequently reduce glucose transport, making the fetus susceptible to hypoglycaemia (Battaglia & Meschia, 1978). Excess substrate can be stored by the fetus primarily in the form of liver glycogen, protein, and triglycerides, which are important for the development of energy autonomy at birth (Battaglia & Meschia, 1978; Jones & Rolph, 1985). The fetus, however, is not capable of substantial endogenous glucose production until relatively late in gestation due to deficiencies in gluconeogenic enzyme pathways which are induced in late gestation by increasing concentrations of glucocorticoids (Fowden *et al.*, 1993; Townsend *et al.*, 1991).

The elevation of plasma catecholamine levels, particularly adrenaline, which occurs in experimental animals in response to hypoglycaemia elicits a number of counterregulatory responses. Acute infusions of physiological levels of adrenaline into fetal sheep increases plasma glucose levels concurrent with decreased insulin secretion and increased availability of alternative metabolic substrates such as lactate, free fatty acids, and amino acids (Jones & Ritchie, 1978*b;* Padbury *et al.*, 1987*c;* Sperling *et al.*, 1984). Catecholamines are able to directly increase plasma glucose levels by stimulating glycogenolysis in the fetal liver, an action which is blocked by the β-adrenoreceptor antagonist, propranolol (Sperling *et al.*, 1984). Catecholamines also stimulate increases in the levels of the glycogenolytic hormone, glucagon (Jones & Ritchie, 1978*b;* Sperling *et al.*, 1984). Due to the high levels of catecholamines required to elicit glucagon secretion and the fact that the fetus is relatively glucagon resistant, it is more likely that the direct glycogenolytic actions of catecholamines prevail (Devaskar *et al.*, 1984; Padbury *et al.*, 1987*c*). The increased availability of alternative metabolic substrates such as lactate, free fatty acids, and amino acids which occurs in the fetus during catecholamine infusion is predominantly dependent upon β-adrenergic mechanisms (Jones & Ritchie, 1978*b*). The inhibition of insulin secretion by catecholamines is blocked by phentolamine, indicating this actions mediated by α-adrenergic mechanisms (Jones & Ritchie, 1978*b*). The inhibition of insulin

secretion and antagonism of its peripheral actions by catecholamines would decrease glucose and oxygen utilisation by insulin sensitive tissues such as skeletal muscle and increase the availability of the these substrates for the brain and myocardium (Felig *et al.*, 1979; Laakso *et al.*, 1992; Milley *et al.*, 1986).

Adrenal demedullation of the fetal sheep which abolishes the catecholamine surge in response to hypoxaemia, also eliminates the rise in plasma glucose and non-esterified fatty acids and prevents the decline in plasma insulin levels which also occur during hypoxaemia (Jones *et al.*, 1988). Furthermore adrenalectomy with glucocorticoid replacement severely attenuates the birth related increases in plasma glucose and free fatty acid concentrations in the lamb (Padbury *et al.*, 1987*a*). Catecholamines released from the adrenal medulla therefore play a vital role in energy homeostasis in the fetus as well as the development of energy autonomy in the neonate.

1.4.1.2.2.2 Fetal growth

Adaptations of the fetus to long term shortages in oxygen and nutrient availability frequently occur at the expense of fetal growth. The striking elevation of plasma catecholamine levels is a feature of experimental models of intrauterine growth retardation (Gagnon *et al.*, 1994; Gu *et al.*, 1985; Simonetta *et al.*, 1997). Chronic (8-12 d) infusions of both noradrenaline and adrenaline at physiological levels are able to produce growth retardation of the fetal sheep in the absence of hypoxaemia and nutrient deficit (Bassett & Hanson, 1998). This growth retardation occurs primarily due to a decrease in skeletal muscle growth as well as the growth of a number of organs, whilst brain and adrenal growth are maintained (Bassett & Hanson, 1998). The pattern of catecholamine-induced growth retardation is similar to those produced by a number of experimental models of growth retardation including placental restriction and placental embolisation (Gagnon *et al.*, 1994; Harding *et al.*, 1985). Interestingly pancreatectomy of the fetal sheep has similar effects on fetal growth as catecholamine infusion (Fowden *et al.*, 1986). This finding combined with the observation that there is a down regulation of the β-adrenoreceptor mediated catabolic actions of catecholamines with prolonged infusion (Basset & Symonds, 1998; Basset *et al.*, 1990; Bassett & Hanson, 1998), suggests that catecholamines may in part mediate their negative

effects on fetal growth indirectly by the suppression of fetal insulin secretion and activity (Bassett & Hanson, 1998). Catecholamines may also indirectly impact on fetal growth by their effects on the secretion of other hormones, such as ACTH and cortisol (Griguere *et al.*, 1981; Haidan *et al.*, 1998).

1.4.1.2.3 Respiratory actions

Perhaps the most dramatic transition which occurs at birth is the transition of the neonate to air breathing. In the fetal sheep liquid is secreted into the lungs at around 200-300 ml / 24 h and passes through the lungs in a manner analogous to that of air in the mature animal (Brown *et al.*, 1983; Lagercrantz & Slotkin, 1986). This phenomenon is vital for normal lung alveolar development in the fetus, however at birth this liquid must be absorbed and the lungs must secrete pulmonary surfactant into the alveoli in order to allow the lungs to function (Bland, 1988; Jobe, 1988). Intrafetal administration of adrenaline to the sheep halts lung liquid secretion and stimulates lung liquid absorption by a β-adrenergic dependent mechanism (Brown *et al.*, 1983; Lawson *et al.*, 1978; Walter & Olver, 1978) an observation also made with β-adrenoreceptor agonists in the fetal rabbit (Corbet *et al.*, 1977; Enhorning *et al.*, 1977).

In order to maintain the patency of the alveoli during air breathing the fetus must secrete pulmonary surfactant, a mixture of phospholipids, neutral lipids, cholesterol, and proteins, which act to reduce surface tension in the alveoli and prevent them from collapsing (King & Clements, 1972; Stahlman & Gray, 1978). Stimulation of β_2-adrenoreceptors in the fetal sheep and fetal rabbit lung with adrenaline and specific β_2-adrenoreceptor agonists elicits an increase in pulmonary phospholipid synthesis and also the levels of phospholipids in lung lavage fluid (Corbet *et al.*, 1977; Enhorning *et al.*, 1977; Lawson *et al.*, 1978). Surfactant secretion is also stimulated by β_2-adrenoreceptor activation in primary cultures of type II pneumocytes (Brown & Longmore, 1981; Sommers-Smith & Giannopoulos, 1983). Hence catecholamines, in particular adrenaline, via β_2-adrenoreceptor stimulation are able to stimulate an increase in pulmonary surfactant secretion and synthesis.

Glucocorticoids are well established to have biochemical maturational effects on fetal lungs (Liggins, 1994; Liggins & Schellenberg, 1988). Glucocorticoids may

interact with catecholamines to produce some of these maturational effects. For example the synthetic glucocorticoid, betamethasone, is able to induce the expression of β-adrenoreceptors in the fetal lung analogous to that which occurs spontaneously during development (Cheng *et al.*, 1980). Furthermore corticosteroid pretreatment potentiates surfactant release from cultures of type II pneumocytes (Smith, 1977).

Bilateral adrenalectomy of the fetal sheep with glucocorticoid replacement causes significant decreases in lung compliance, ventilatory efficiency, and alveolar surfactant levels following delivery (Padbury *et al.*, 1987*a*). Selective chemical demedullation also reduces alveolar surfactant content in neonatal rabbits (Padbury *et al.*, 1984). Whilst human data on the effects of adrenomedullary catecholamines on lung liquid absorption and surfactant synthesis and secretion cannot be directly obtained there are indirect data which underline catecholamine involvement. Newborns which are delivered by Caesarean section do not experience the substantial catecholamine surge which occurs in babies which are vaginally delivered and have a lower lung compliance after delivery (Faxelius *et al.*, 1983). Treatment of mothers with β_2-adrenoreceptor agonists in an effort to delay pre-term delivery also results in a significant reduction in the incidence of respiratory distress syndrome in prematurely delivered infants (Bergman, 1981; Boog *et al.*, 1975).

1.4.1.2.4 Thermogenic actions

At birth the infant goes from the womb in which temperature is maintained by maternal thermoregulatory mechanisms, to a relatively cold external environment in which the infant is particularly susceptible to heat loss due to its relatively large surface area to volume ratio (Lagercrantz & Slotkin, 1986). Therefore the neonate needs to be prepared to efficiently generate its own heat in order to maintain its body temperature.

The neonate of many species possess substantial amounts of a specialised thermogenic tissue known as brown adipose tissue (BAT) which is involved in non-shivering thermogenesis. BAT is predominantly located in perirenal adipose depots as well as retroperitoneal, pericardial, omental, and subcutaneous adipose depots, often in association with major blood vessels to facilitate heat transfer

(Casteilla *et al.*, 1987; Loncar, 1991; Trayhurn *et al.*, 1993). BAT develops *in utero* and is unique to the fetus and neonate of a number of species including ruminants, cats, and dogs before converting to a white adipose-like tissue, known as convertible adipose tissue, before the end of the first postnatal month (Casteilla *et al.*, 1987; Champigny *et al.*, 1991; Finn *et al.*, 1998; Loncar, 1991; Trayhurn *et al.*, 1993). In humans brown fat is present until early adolescence whilst it plays a role in thermogenesis and metabolic regulation in small rodents and insectivores throughout life (Loncar, 1991).

BAT has a high thermogenic activity due to the presence of a mitochondrial protein unique to this tissue, known as uncoupling protein 1 (UCP1) (Klaus *et al.*, 1991). UCP1 generates heat by dissipation of the mitochondrial proton gradient such that oxidative phosphorylation is 'uncoupled' with the oxidative energy is released as heat with little ATP formation (Klaus *et al.*, 1991; Nicholls & Locke, 1984). Thermogenesis is stimulated in BAT by catecholamines through the β-adrenergic stimulation of lipase which liberates free fatty acids from triglyceride stores which are subsequently activated to forms which can be used in mitochondrial respiration (Nicholls & Locke, 1984). A subsequent build up of fatty acids activates UCP1 resulting in the uncoupling of mitochondrial oxidative respiration from ATP formation with increased respiration and heat generation (Nicholls & Locke, 1984). UCP1 expression is transcriptionally regulated by β-adrenergic and thyroid hormone stimulation (Klaus *et al.*, 1994; Silva, 1988). Indeed mice which are lacking catecholamines due to genetic knockout of the gene which encodes the catecholamine synthetic enzyme, DBH, exhibit reduced expression of UCP1 mRNA in BAT as well as impaired thermoregulation (Thomas & Palmiter, 1997). Administration of β-adrenoreceptor agonists has also been found to induce UCP1 gene and protein expression in brown adipocyte cell lines and adult dogs as well as prolonging their expression in neonatal lambs (Champigny *et al.*, 1991; Klaus *et al.*, 1994; Nougues *et al.*, 1993).

Catecholamines, in particular noradrenaline, stimulate thermogenesis in the BAT of the newborn rabbit and sheep (Alexander & Stevens, 1980; Heim & Hull, 1968; Klein *et al.*, 1983). Endogenous catecholamines and cooling are relatively inefficient in stimulating thermogenesis in utero in the fetal lamb when compared to postnatal lambs (Hodgkin *et al.*, 1988; Schroder *et al.*, 1987). This appears due

to the inhibition of thermogenic activation of BAT by placental factors, as umbilical cord occlusion is able to stimulate thermogenesis (Gunn & Gluckman, 1989). Two substances produced by the placenta, adenosine and prostaglandins, have been found to have inhibitory effects on thermogenesis (Ball *et al.*, 1995; Gunn *et al.*, 1993). Whilst BAT in the sheep receives a dense sympathetic innervation (Alexander & Stevens, 1980), and there is a significant rise in free fatty acid concentrations at birth despite adrenalectomy, this rise is blunted indicating that circulating catecholamines enhance lipolysis (Padbury *et al.*, 1987*a*). Aside from blunted lipolysis, adrenalectomised lambs also display impaired non-shivering thermogenesis which can be restored in part by glucocorticoid replacement and in part by catecholamine infusion (Alexander & Bell, 1982).

In newborn humans cold exposure increases urinary noradrenaline secretion which occurs coincident with an increase in oxygen consumption, carbon dioxide, and respiratory quotient, indicative of aerobic catabolism of fat stores (Stern *et al.*, 1965). Direct evidence for the role of catecholamines in thermogenesis has been obtained in human infants which have been infused with noradrenaline and demonstrated increases plasma free fatty acid levels as well as core body temperature (Schiff *et al.*, 1966).

1.4.2 REGULATION OF CATECHOLAMINE SECRETION AND BIOSYNTHESIS IN THE FETAL ADRENAL MEDULLA

1.4.2.1 Ontogeny of adrenomedullary catecholamines and enkephalins

1.4.2.1.1 Catecholamines

The ontogeny of catecholamine biosynthesis within the adrenal gland has been well established in the rat. In this species, immunoreactivity for the catecholamine synthesising enzymes TH and DBH, as well as formaldehyde-induced catecholamine fluorescence has been detected in migrating sympathoadrenal precursor cells well before they have reached the adrenal anlage (Bohn *et al.*, 1981; Cochard *et al.*, 1979; Cochard *et al.*, 1978; Verhofstad *et al.*, 1979). TH, DBH, and catecholamine containing cells are observed in the sympathetic ganglion primordia at the level of the developing adrenal anlage in the caudal thoracic region at E12.5 (Bohn *et al.*, 1981; Cochard *et al.*, 1979; Cochard *et al.*,

1978). By E13.5 a number of these cells migrate ventrolaterally along the aorta and invade the developing adrenal anlage with scattered TH, DBH, and catecholamine containing cells identified in the adrenal primordium (Bohn *et al.*, 1981; Verhofstad *et al.*, 1979). Interestingly, despite the presence of TH, DBH, and catecholamines in adrenomedullary cells from the earliest stages of medullary development, PNMT immunoreactivity and mRNA expression has not been reported in adrenal chromaffin cells until much later in adrenal development at E15.5-E17 (Bohn *et al.*, 1981; Ehrlich *et al.*, 1989; Verhofstad *et al.*, 1985; Verhofstad *et al.*, 1979). PNMT immunoreactivity within the rat adrenal gland is weak and sparsely distributed at E17 but there are rapid and dramatic increases in both the distribution and intensity of PNMT immunoreactivity as gestation progresses (Bohn *et al.*, 1981).

Biochemical determinations of the catecholamine synthesising capacity of the fetal and postnatal rat adrenal strongly support the observations made using histochemical and immunohistochemical techniques (Bohn *et al.*, 1981; Coulter *et al.*, 1988; Verhofstad *et al.*, 1985; Verhofstad *et al.*, 1979). Catecholamines have been detected within the adrenal glands of the fetal rat from as early as E16 gestation, using high performance liquid chromatography coupled with electrochemical detection (Coulter *et al.*, 1988; Verhofstad *et al.*, 1985). This coincides with the formation of a distinct medulla within the adrenal gland of the developing fetus (Verhofstad *et al.*, 1979). The predominant catecholamine within the adrenal gland of the fetal rat is initially noradrenaline and this corresponds to the lack of PNMT expression within the adrenal gland (Bohn *et al.*, 1981; Coulter *et al.*, 1988; Verhofstad *et al.*, 1985; Verhofstad *et al.*, 1979). There is subsequently a dramatic increase in the level of adrenaline within the adrenal gland from the 17^{th}-18^{th} day of gestation onwards such that by E18-E20 adrenaline becomes the predominant catecholamine (Coulter *et al.*, 1988; Verhofstad *et al.*, 1985). The dramatic increase in adrenal adrenaline content coincides with the appearance and increase in PNMT immunoreactivity and enzymatic activity within the fetal adrenal medulla (Bohn *et al.*, 1981; Coulter *et al.*, 1988; Margolis *et al.*, 1966; Verhofstad *et al.*, 1985; Verhofstad *et al.*, 1979).

The ontogeny of catecholamine biosynthesis in the adrenal gland of the fetal sheep is somewhat less well defined than it is in the rat. Immunohistochemical

techniques have demonstrated the presence of two of the catecholamine synthetic enzymes, TH and PNMT in the adrenal gland of the fetal sheep from as early as 54 d gestation (Riley *et al.*, 1992). TH immunoreactivity was observed in cords of medullary cells present in the centre of the gland, whilst PNMT immunoreactivity was documented in small groups of medullary cells immediately adjacent to the developing zona fasciculata, and also in the central region of the gland (Riley *et al.*, 1992). The apparent catecholamine synthetic capacity of the fetal sheep adrenal gland at this early stage of gestation is in agreement with ultrastructural evidence of phaeochromocytes possessing granules characteristic of noradrenaline-containing vesicles within the adrenal gland at 53 d gestation (Boshier *et al.*, 1989*a*). With increasing gestational age, layers of chromaffin cells containing PNMT immunoreactivity and expressing PNMT mRNA, form in the region of the medulla principally underlying the zona fasciculata at the developing corticomedullary interface (McMillen *et al.*, 1988; Riley *et al.*, 1992; Wan *et al.*, 1989*a;* Wan *et al.*, 1989*b*). A morphometric study of adrenal growth by Coulter and colleagues (1989*b*) reported that the major increase in medullary growth occurs between 90 and 120 d gestation, and that the relative growth of the adrenomedullary noradrenaline (DBH-immunoreactive)- and adrenaline (DBH- and PNMT-immunoreactive)- synthesising areas appears to be synchronised during this rapid growth phase.

Catecholamines themselves are detectable in the adrenal venous blood of the fetal lamb at the earliest reported age of 80 d gestation (Comline & Silver, 1961), a finding which has been confirmed in isolated, retrogradely perfused fetal, adrenal glands (Butler *et al.*, 1995). In the fetal sheep, as is the case with other mammalian species such as the rat, rabbit, calf, and man; noradrenaline is initially the predominant adrenomedullary catecholamine with a subsequent maturational increase in adrenaline content occurring (Comline & Silver, 1966; Comline & Silver, 1961; Coulter *et al.*, 1988; Verhofstad *et al.*, 1985). Both adrenomedullary noradrenaline and adrenaline content are found to increase in the fetal sheep with progressing gestational age whilst the ratio of adrenaline to noradrenaline content within the adrenal gland also increases, exceeding one after 140 d gestation (Comline & Silver, 1966; Comline & Silver, 1961). Like the fetal rat, noradrenaline and adrenaline synthesis in the adrenal gland of the fetal sheep appears to be differentially regulated during gestation. The major increase in the noradrenaline

content of the fetal sheep adrenal occurs between 80 and 110 d gestation (ie. during the major phase of adrenomedullary growth) whereas the predominant increase in the adrenaline content of the adrenal occurs somewhat later, after 130 d gestation (Boshier & Holloway, 1989*b;* Comline & Silver, 1961; Coulter *et al.*, 1989*b*).

1.4.2.1.2 Enkephalins

Enk-containing peptides have been identified within the prenatal adrenal medulla of several species including human, primate, sheep, and rat (Dunlap *et al.*, 1985; Henion & Landis, 1990; Wilburn *et al.*, 1986). The ontogeny of Enk peptide expression has been characterised most extensively in the rat (Henion, 1992; Henion & Landis, 1990; Holgert *et al.*, 1995*a;* Holgert *et al.*, 1994; LaGamma *et al.*, 1989) and sheep (Coulter *et al.*, 1990*a;* Dunlap *et al.*, 1985; McMillen *et al.*, 1988; Wan *et al.*, 1989*a*). Both immunohistochemical and biochemical techniques have established that Enk peptide expression appears in the fetal rat adrenal medulla at E16 (Henion & Landis, 1990; LaGamma *et al.*, 1989). In the fetal sheep Enk-containing peptides have been reported within the adrenal medulla from 68-80 d, the earliest gestational ages studied (Coulter *et al.*, 1990*a;* Dunlap *et al.*, 1985; McMillen *et al.*, 1988).

The appearance of Enk-containing peptides within the chromaffin cells of the fetal rat adrenal at E16 coincides with the appearance of PNMT, and it is found that Enk expression is colocalised within PNMT expressing chromaffin cells (Henion & Landis, 1990). There is a subsequent dramatic increase in Enk peptide expression within the adrenal medulla between E16 and E20, which is followed by a marked decline in the first postnatal week of life (Henion & Landis, 1990; Holgert *et al.*, 1994). In the fetal sheep, Enk peptide and PEnk A mRNA expression is initially distributed homogeneously throughout the adrenal medulla from 80-110 d gestation (McMillen *et al.*, 1988; Wan *et al.*, 1989*a*). After 110 d, however, Enk-containing peptides and PEnk A mRNA expression is predominantly confined to the peripheral adrenaline-containing cells of the fetal sheep adrenal medulla (McMillen *et al.*, 1988; Wan *et al.*, 1989*a*). Along with the alterations in Enk-peptide localisation there is also a large increase in the total enkephalin content of the fetal adrenal between 70 and 120 d gestation which tends to decrease in later

gestation (Coulter *et al.*, 1990*a*). The ratio of high molecular weight forms to low molecular weight forms significantly decreases between 70-120 d gestation, indicative of increased precursor molecule processing, and this decrease is maintained over gestation (Coulter *et al.*, 1990*a*).

The appearance of Enk peptide expression in adrenal chromaffin cells of the fetal rat only after they have entered the developing adrenal anlage suggests that adrenocortical glucocorticoids may regulate Enk peptide expression in the developing adrenal (Henion & Landis, 1990). Glucocorticoids appear to be involved in the regulation of Enk peptide synthesis in mature adrenal chromaffin cells (LaGamma & Alder, 1987; Naranjo *et al.*, 1986; Stachowiak *et al.*, 1988*b;* Yoburn *et al.*, 1987). It has been found that the synthetic glucocorticoid, dexamethasone, increases Leu-Enk levels in cultures of embryonic rat adrenal chromaffin cells (Henion, 1992). In the sheep, fetal hypophysectomy at 105 and 118 d reduced both plasma cortisol and total adrenal Enk content in the 140-141 d fetal sheep (Coulter *et al.*, 1992; Simonetta *et al.*, 1996*b*). Furthermore, ACTH replacement in late gestation following fetal HPX significantly increased both plasma cortisol and total adrenal Enk content in the fetal sheep above the levels observed in saline infused fetuses (Coulter *et al.*, 1992).

A number of the changes in the localisation, synthesis, and post-translational processing of adrenal Enk-containing peptides occur in parallel with the development of functional splanchnic innervation (McMillen *et al.*, 1997). In the rat, a number of factors associated with cholinergic innervation are found to increase the levels of Enk-peptides in chromaffin cells. These factors include depolarisation (Henion, 1992) and cholinergic receptor stimulation (LaGamma *et al.*, 1989). Paradoxically, there is a decrease in adrenal Enk-containing peptide expression *in vivo* with the onset of splanchnic innervation to the gland (Henion & Landis, 1990; Holgert *et al.*, 1994). This decrease coincides with the appearance of Enk-peptide containing cholinergic nerve fibres within the adrenal medulla (Henion & Landis, 1990; Holgert *et al.*, 1994; Holgert *et al.*, 1996*b*). Adrenal denervation by the transection of the preganglionic cholinergic splanchnic nerve or by the administration of acetylcholinesterase antibodies results in an upregulation of enkephalin peptide expression in the chromaffin cells of the medulla (Henion, 1992; Holgert *et al.*, 1996*b;* Lewis *et al.*, 1981). Administration of the opioid

receptor antagonist, naloxone, mimics the effects of denervation whilst the administration of synthetic enkephalin analogues mimics the effect of innervation (Henion, 1992). Hence the developmental suppression of Enk expression in the adrenal gland of the rat with the onset of functional innervation appears to be due to the actions of opioid peptides within the splanchnic nerve.

A similar suppressive effect of adrenal innervation on Enk-containing peptide expression during development may also occur in the fetal sheep adrenal gland. Interestingly, HPX of the fetal sheep results in the continued expression of Enk-immunoreactivity in the central noradrenaline-containing region of the adrenal gland in late gestation (Coulter *et al.*, 1991; Coulter *et al.*, 1989*b*). Administration of ACTH to HPX fetuses whilst increasing adrenal Enk-containing peptide content, is unable to suppress this central medullary expression which normally disappears at around the time of innervation of the gland, with expression restricted to the outer adrenaline-containing region of the medulla (Coulter *et al.*, 1991; McMillen *et al.*, 1988). HPX of the fetal sheep results in a functional athyroidism (Mesiano *et al.*, 1987). Thyroid hormones play a crucial role in the development of functional splanchnic innervation to the adrenal (Lau *et al.*, 1988). Simonetta *et al.* (1996*c*) reported that continuous thyroxine replacement after fetal hypophysectomy resulted in a relative increase in the density of enkephalin staining in the peripheral adrenaline-containing region and a decrease in the central noradrenaline-containing region of the late gestation adrenal. This effect may be either a direct effect of thyroid hormones or more likely an indirect effect mediated by their actions on the development of splanchnic innervation (Lau *et al.*, 1988; Simonetta *et al.*, 1996*c*). Thyroxine replacement did not, however, restore either the total Enk content or free Enk content in the adrenals of HPX fetal sheep (Simonetta *et al.*, 1996*b*).

1.4.2.2 Neuropeptide regulation of adrenomedullary catecholamine secretion

There is currently very little information available on the role of adrenomedullary neuropeptides in the regulation of catecholamine secretion during stress in the fetus. The best characterised neuropeptides in this regard are the opioid peptides (McMillen *et al.*, 1997). Adrenalectomy does not alter the basal levels of Enk-

containing peptides in the circulation of the fetal sheep, indicating that under basal conditions the adrenal medulla is not a major source of circulating Enk-containing peptides (Simonetta *et al.*, 1993). During fetal hypoxia and asphyxia there are increases in the levels of circulating Enk-containing peptides (Coulter *et al.*, 1990*b;* Jones, 1992; Martinez *et al.*, 1990). These Enk-containing peptides may be derived from both the adrenal medulla and sympathetic nervous system as both adrenalectomy and chemical sympathectomy substantially blunt the response to hypoxia (Jones, 1992). In addition, moderate fetal hypoxaemia over 48 h has also been reported to increase PEnk A mRNA expression within the fetal sheep adrenal gland (Fraser *et al.*, 1997) indicating an increased synthesis of adrenal Enk-containing peptides in response to hypoxia.

The non-selective opioid receptor antagonist, naloxone, when administered to fetal sheep augments the elevations in plasma catecholamine levels which occur in response to both hypoxia and delivery (Martinez *et al.*, 1988; Padbury *et al.*, 1987*b*). The adrenal medulla is the predominant source of circulating catecholamines in response to these stressors (Jones *et al.*, 1988; Padbury *et al.*, 1987*a*), which indicates that intra-adrenal opioid peptides co-secreted with catecholamines during fetal stress act to modulate catecholamine secretion by suppressing their release (Martinez *et al.*, 1988; Padbury *et al.*, 1987*b;* Padbury *et al.*, 1989). Furthermore, naloxone augments the increases in heart rate, blood pressure, and blood flow redistribution which occur in response to asphyxia and delivery in the fetal sheep (Espinoza *et al.*, 1989; Padbury *et al.*, 1987*b*). These findings indicate that at the physiological level opioid peptides act to oppose the actions of catecholamines on the cardiovascular system, possibly by central mechanisms or by peripheral mechanisms via modulation of adrenomedullary catecholamine secretion or antagonism of their peripheral actions (Espinoza *et al.*, 1989; Padbury *et al.*, 1987*b;* Padbury *et al.*, 1989) .

1.4.2.3 Non-neurogenic catecholamine secretion

The adrenal medulla of the fetus and in some species, the neonate, possess the unique capacity to secrete catecholamines in direct response to hypoxia prior to the development of functional splanchnic innervation by a direct non-neurogenic mechanism (McMillen *et al.*, 1997).

1.4.2.3.1 Experimental evidence

The pioneering studies of Comline and Silver (Comline & Silver, 1961) first identified non-neurogenic secretion of adrenomedullary catecholamines in response to hypoxia. Using anaesthetised fetal sheep acutely exteriorised from the uterus, these investigators found that electrical stimulation of the splanchnic nerve first caused significant catecholamine release from the adrenal at 125 days gestation. Asphyxia induced by umbilical cord occlusion, however, produced significant increases in the catecholamine levels in the adrenal venous blood from as early as eighty days gestation (Comline & Silver, 1961). The asphyxia induced release of catecholamines which occurred prior to functional splanchnic innervation of the adrenal was not abolished by splanchnic nerve transection, spinal cord destruction, nor hexamethonium administration, and hence was non-neurogenic in origin (Comline & Silver, 1961). Comline and Silver (1961) also demonstrated that the non-neurogenic catecholamine response to asphyxia diminished with the onset of the development of functional splanchnic innervation to the adrenal medulla.

The methods used by Comline and Silver to identify non-neurogenic catecholamine secretion from the fetal adrenal, including the acute surgical procedures, anaesthesia, and extremely low oxygen tensions achieved during asphyxia (< 4-5 mmHg), constitute relatively unphysiological conditions (Comline & Silver, 1966; Comline & Silver, 1961). Cheung (1990) readdressed this question by examining the effects of moderate hypoxaemia, independently of asphyxia, on adrenomedullary catecholamine secretion from chronically catheterised fetal sheep *in utero*. Fetal hypoxia, induced by infusing nitrogen into the maternal trachea, produced a 60 % decline in fetal arterial PO_2 levels (Cheung, 1990). Arterial plasma catecholamine levels increased significantly in response to hypoxia in both 110-120 d old and 130-140 d old fetuses (Cheung, 1990). Hexamethonium administration attenuated the catecholamine secretory response to hypoxia in the 110-120 d fetuses and abolished the secretory response of the 130-140 d fetuses (Cheung, 1990). The results obtained by Cheung (1990) confirmed the non-neurogenic release of catecholamines reported by Comline and Silver (1961, 1966). A substantial component of the hypoxia induced release of catecholamines in the 110-120 d old fetal sheep used in the

study by Cheung (1990) was abolished by hexamethonium, indicating that the onset of functional innervation of the adrenal medulla occurs earlier than the 125 d gestation reported for anaesthetised fetuses by Comline and Silver (1961). Cheung (1990) also demonstrated that non-neurogenic catecholamine secretion in response to hypoxia was absent by 130 d gestation with secretion being solely mediated by neurogenic mechanisms.

A non-neurogenic stimulation of catecholamine secretion in response to hypoxia has also been documented in the neonatal rat and calf (Comline & Silver, 1966; Seidler & Slotkin, 1985; Seidler & Slotkin, 1986*b;* Slotkin & Seidler, 1988). In both of these species functional innervation of the adrenal medulla by the splanchnic nerve does not develop until postnatal life and hence the neonate is reliant on the non-neurogenic release of adrenomedullary catecholamines (Comline & Silver, 1966; Slotkin & Seidler, 1988). As is the case with the fetal sheep, the development of functional splanchnic innervation of the rat and calf is associated with the loss of the non-neurogenic secretion of catecholamines (Comline & Silver, 1966; Seidler & Slotkin, 1986*b*).

A factor(s) associated with the onset of functional innervation of the adrenal medulla appear to be directly responsible for the loss of the non-neurogenic secretion of adrenomedullary catecholamines in response to hypoxia (McMillen *et al.*, 1997). The non-neurogenic secretion of catecholamines persists in rats who have had the splanchnic nerves supplying the adrenal glands surgically transected at birth (Seidler & Slotkin, 1986*b;* Slotkin & Seidler, 1988). Conversely, acceleration of the development of splanchnic innervation to the adrenal gland by the administration of thyroid hormones results in a premature loss of non-neurogenic catecholamine secretion in the rat (Lau *et al.*, 1988; Slotkin & Seidler, 1988). Adrenomedullary chromaffin cells obtained from adult sheep and cultured in the absence of any neural influences regain the ability to secrete catecholamines in direct response to hypoxia (Cheung, 1989). Adrenal glands isolated from fetal sheep after the development of functional splanchnic innervation at 135-146 d and perfused *in vitro* in the absence of neural influence also secrete catecholamines in direct response to hypoxia (Adams *et al.*, 1996).

1.4.2.3.2 Cellular mechanisms

Seidler and Slotkin have measured chromaffin cell uptake capacity of [^{3}H] adrenaline, a function which is lost as chromaffin granule integrity is disrupted by exocytosis, in one day old rats exposed to hypoxia or treated with nicotine (Seidler & Slotkin, 1986*a*). Hypoxia produced a depression of chromaffin cell [^{3}H] adrenaline uptake and adrenomedullary catecholamine depletion equivalent to that observed for nicotine, which is known to stimulate the exocytotic secretion of adrenal catecholamines (Seidler & Slotkin, 1986*a*). This result suggested that the non-neurogenic catecholamine secretion which occurs from adrenomedullary chromaffin cells in response to hypoxia occurs via a regulated exocytotic process (Seidler & Slotkin, 1986*a*).

As outlined previously, a rise in intracellular calcium levels is a key step in the exocytotic release of catecholamines. An isolated, retrogradely perfused, adrenal gland preparation developed by Wakade and colleagues for a comprehensive series of studies examining the regulation of catecholamine secretion in the adult rat (Malhotra & Wakade, 1987*a;* Malhotra *et al.*, 1988*b;* Shukla & Wakade, 1991; Wakade, 1981; Wakade & Wakade, 1983) was modified and validated for the fetal sheep adrenal gland (Butler *et al.*, 1995) and used to examine the calcium dependence of non-neurogenic catecholamine secretion (Adams *et al.*, 1996). Fetal adrenal glands retrogradely perfused with hypoxic Krebs buffer (PO_2 ~ 45 mmHg) exhibit a sustained and reversible increase in catecholamine secretion which can be abolished by the absence of calcium from the perfusion solution (Adams *et al.*, 1996). Furthermore, the addition of the L-type, voltage-sensitive, calcium channel antagonist, Nifedipine, to the perfusion solution also abolishes the non-neurogenic catecholamine secretory response to hypoxia (Adams *et al.*, 1996). The extracellular calcium required for non-neurogenic catecholamine secretion during hypoxia therefore appears to enter the cell via voltage-sensitive calcium channels. Such a finding implies that the non-neurogenic stimulatory effect of hypoxia on catecholamine secretion is mediated by a depolarisation of the chromaffin cell membrane.

Subsequent studies on isolated chromaffin cells from other species have confirmed and extended the findings made in the retrogradely perfused, fetal

sheep adrenal gland. Primary cultures of adrenomedullary chromaffin cells obtained from neonatal and adult rats and exposed to moderate hypoxia (~ 40 mmHg) demonstrate significant increases in intracellular Ca^{2+} levels and catecholamine release which are blocked by L- and N-type Ca^{2+} channel antagonists (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997). Furthermore, when the resting membrane potential is measured in these cells it is found to depolarise in response to hypoxia (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997). Similarly, dispersed guinea-pig adrenomedullary chromaffin cells are also found to secrete catecholamines in response to hypoxia due to membrane depolarisation (Inoue *et al.*, 1998).

The mechanism by which hypoxia acts to depolarise the chromaffin cell membrane has yet to be fully determined. In adult mammals hypoxia elicits a compensatory reflex increase in ventilation and sympathetic outflow due to the stimulation of small and highly vascularised organs located at the bifurcation of the carotid artery, known as the carotid bodies (Heymans & Neil, 1958; Little & Orberg, 1975). The chemoreceptive Type I glomus cells of the carotid body which are derived from neural crest cells and share a common embryological origin with the chromaffin cells of the adrenal medulla, also synthesise and secrete catecholamines, predominantly dopamine, in response to hypoxia (Doupe *et al.*, 1985; Fidone *et al.*, 1982; Gonzalez *et al.*, 1994; Rigual *et al.*, 1986). The pivotal role that Type 1 glomus cells play in oxygen homeostasis has led to the extensive investigation of the oxygen sensing mechanisms within these cells. Like chromaffin cells, there is an increase in intracellular calcium levels and membrane depolarisation associated with neurotransmitter release in response to hypoxia in Type I glomus cells (Biscoe & Duchen, 1990*b;* Buckler & Vaughan-Jones, 1994). The mechanism by which oxygen sensing occurs in the carotid body remains controversial but it appears multiple mechanisms may be involved (Acker & Xue, 1995). The most recent models of oxygen sensing in the carotid body put forward involve either the actions of a mitochondrial cytochrome (Lahiri, 1994) or oxygen-sensitive potassium channels (Lopez-Barneo *et al.*, 1993).

1.4.2.3.2.1 Mitochondrial oxygen sensing

The first model involves mitochondrial cytochrome aa_3 which has a relatively low affinity for oxygen (Acker & Xue, 1995). During hypoxia this cytochrome is unable to reduce oxygen leading to a diminished energy state and an efflux of calcium from mitochondrial stores, presumably through a mitochondrial Ca^{2+}-ATPase, with subsequent neurotransmitter release (Biscoe & Duchen, 1990*a;* Bunn & Poyton, 1996; Mulligan *et al.*, 1981). Spectral analysis of the carotid body during anoxia supports a functional role of the mitochondrial cytochrome aa_3 (Acker & Xue, 1995). Furthermore inhibition of mitochondrial respiration by the administration of cyanide or carbon monoxide are able to stimulate carotid body discharge (Biscoe & Duchen, 1990*a;* Lahiri, 1994; Wilson *et al.*, 1994). Photochemical action spectrum reveal that the cytochrome aa_3 is the key mitochondrial heme protein involved in the CO mediated increase in discharge (Lahiri, 1994; Wilson *et al.*, 1994). As mitochondrial PO_2 is extremely low and subject to metabolic fluctuations (Bunn & Poyton, 1996) it is unlikely that this mechanism is likely to function under physiological conditions but more so during anoxia or extreme hypoxia and also with cyanide and carbon monoxide poisoning. The release of catecholamines which has been reported to occur from bovine and guinea pig chromaffin cells independently of extracellular calcium during anoxia (Itoh *et al.*, 1994) and during metabolic inhibition with cyanide (Dry *et al.*, 1991; Inoue *et al.*, 1998) is also probably due to the release of calcium from mitochondrial stores.

1.4.2.3.2.2 Oxygen-sensitive potassium channels

Oxygen sensitivity may also be mediated by ion conductance (Lopez-Barneo, 1996). Oxygen-sensitive potassium channels have been identified in the O_2 sensing type I glomus cells of the carotid body (Lopez-Barneo *et al.*, 1988). These cells display voltage-sensitive, outward potassium currents which are reversibly inhibited by reductions in PO_2. (Lopez-Lopez *et al.*, 1989). Single channel analysis in excised membrane patches of Type I glomus cell membranes reveal a specific type of calcium-independent, potassium channel is responsible for the change in outward potassium current (Ganfornina & Lopez-Barneo, 1992). These channels which are voltage dependent, possess a slope conductance of 20 pS, have relatively fast activation and inactivation kinetics and are transient

voltage-dependent K^+ channels (Ganfornina & Lopez-Barneo, 1991). The O_2-sensitive K^+ channels of the Type 1 glomus cells display a reduced opening probability in response to hypoxia without any alteration in their conductance (Ganfornina & Lopez-Barneo, 1992). This effect due to alterations in the kinetic properties of the channel in response to reductions in oxygen tension therefore leads to a reduction in outward potassium flux, membrane depolarisation (Delpiano & Hescheler, 1989), enhanced calcium entry through voltage-sensitive calcium channels (Buckler & Vaughan-Jones, 1994), and finally neurotransmitter secretion (Fidone *et al.*, 1982; Rigual *et al.*, 1986). O_2-sensitive K^+ channels appear to have a relatively ubiquitous distribution (Lopez-Barneo, 1994) and have also been identified in a number of other tissues including pulmonary neuroepithelial bodies (Youngson *et al.*, 1993), pulmonary artery myocytes (Archer *et al.*, 1996; Post *et al.*, 1992), and central neurones (Jiang & Haddad, 1994).

The extremely small size of the carotid body ($\approx 1 \times 10^4$ cells) make the mechanisms of oxygen sensing difficult to study in this tissue (Beitner-Johnson *et al.*, 1997). The immortalised rat PC12 cell line which is derived from an adrenal medullary tumour shares morphological and phenotypical properties with Type I glomus cells with both cell types synthesising and secreting the neurotransmitter dopamine (Fidone & Gonzalez, 1986; Green & Tischler, 1987). Examination of this cell line as a potential model for oxygen sensing has revealed an oxygen dependent regulation of gene expression analogous to that observed in the glomus cells (see following sections for further detail) (Beitner-Johnson *et al.*, 1997; Czyzyk-Krzeska, 1997). Furthermore, it was subsequently established that PC12 cells also depolarise in response to hypoxia with a subsequent influx of calcium through voltage-sensitive calcium channels stimulating the secretion of dopamine (Beitner-Johnson *et al.*, 1997; Zhu *et al.*, 1996). Patch clamp studies also show that membrane depolarisation during hypoxia is due to the reduction of an oxygen sensitive, outward potassium current (Zhu *et al.*, 1996). The O_2-sensitive K^+ channels responsible for this current appear similar to those reported for the carotid body in that they are voltage dependent, calcium independent, and possess a unitary conductance of 20 pS (Conforti & Millhorn, 1997). The O_2-sensitive K^+ channels observed in PC12 cells however show somewhat slower inactivation kinetics than those of the type I glomus cells and are more

characteristic of delayed rectifiers (Conforti & Millhorn, 1997; Ganfornina & Lopez-Barneo, 1991).

Subsequent studies seeking to elaborate on the mechanisms of non-neurogenic catecholamine secretion from the adrenal medulla have also revealed the presence of O_2-sensitive K^+ currents in the chromaffin cells of the adult rat, neonatal rat, and the fetal sheep (Mochizuki-Oda *et al.*, 1997; Rychkov *et al.*, 1998; Thompson *et al.*, 1997). O_2-sensitive K^+ currents which display slow inactivation characteristic of delayed rectifier type potassium channels and are similar to those observed in PC12 cells were identified in both rat and fetal sheep adrenal chromaffin cells (Mochizuki-Oda *et al.*, 1997; Rychkov *et al.*, 1998; Thompson *et al.*, 1997). However, the slow inactivating outward potassium current observed in fetal sheep chromaffin cells was found to be a calcium-dependent (Rychkov *et al.*, 1998) whilst one study in the rat showed that it was calcium-independent (Mochizuki-Oda *et al.*, 1997) and the other failed to test the calcium dependency (Thompson *et al.*, 1997). A unique fast inactivating O_2-sensitive K^+ current which displays pharmacological and functional similarities to an I_A-type K^+ current was also identified in the chromaffin cells of the fetal sheep (Rychkov *et al.*, 1998). Interestingly the delayed rectifier type and the I_A type O_2-sensitive K^+ currents appeared separately in different chromaffin cell types (Rychkov *et al.*, 1998). The delayed rectifier type current was associated with small capacitance chromaffin cells (cf. 4.8 pF) whilst the I_A-type current was associated with large capacitance cells (cf. 12.3 pF) (Rychkov *et al.*, 1998). The functional significance of the two populations of chromaffin cells possessing two different types of O_2-sensitive K^+ current is not clear, although it is interesting to note that there is a specific distribution of the current types to large high capacitance and smaller low capacitance chromaffin cells which may reflect adrenaline storing and noradrenaline storing chromaffin cells, respectively (Rychkov *et al.*, 1998). The non neurogenic release of catecholamines in response to hypoxia though is characterised by a non-selective release of the catecholamines noradrenaline and adrenaline indicating a similar mechanism is operating in both cell types (Adams *et al.*, 1996; Slotkin & Seidler, 1988). It is also not clear whether the unique observation of the I_A-type current in fetal sheep adrenal chromaffin cells reflects a species difference or differences in cell

preparation and culture conditions which appear to influence the expression of this current (Rychkov *et al.*, 1998).

Interestingly the study of Thompson et al. (1997) reported that there was a loss of the O_2-sensitive K^+ current in the adrenomedullary chromaffin cells of the rat when taken after the onset of functional innervation of the gland. This was interpreted as a possible reason for the loss of the non-neurogenic response to hypoxia following innervation of the adrenal gland. Mochizuki-Oda (1997) and co-workers, however, reported the presence of the O_2-sensitive K^+ current in adult rats. O_2-sensitive K^+ currents also persist in isolated adrenal chromaffin cells from the adult sheep (G. Rychkov and M.L. Roberts, personal communication) as does non-neurogenic secretion in response to hypoxia (Cheung, 1989). As stated above the functional evidence suggests that it is a factor(s) transmitted via the splanchnic nerve which is responsible for the loss of non-neurogenic catecholamine secretion rather than a loss of potassium channels (Adams *et al.*, 1996).

The mechanism by which O_2-sensitive K^+ channels sense oxygen availability is uncertain, but appears to occur via membrane delimited mechanisms as reversible inhibition of potassium currents by hypoxia occurs in excised membrane patches of glomus cells and central neurones (Ganfornina & Lopez-Barneo, 1992; Ganfornina & Lopez-Barneo, 1991; Jiang & Haddad, 1994). There is evidence to suggest that the redox status of specific amino acid residues within the protein structure of potassium channels alters their conformation and activity (Lopez-Barneo, 1994). Specifically the formation of disulfide linkages between cystine residues in the reduced state has been found to reduce the activity of a cloned neuronal fast inactivating K^+ channel, RCK4 (Ruppersberg *et al.*, 1991). A putative membrane bound, multisubunit cytochrome *b*-like oxidase is speculated to function as the oxygen sensor, influencing protein redox status by the production of hydrogen peroxide (see below, Acker & Xue, 1995; Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997; Kummer & Acker, 1995). An alternative proposal is the formation of co-ordination complexes between oxygen and amino acids via metal containing sites in the main channel subunits, regulating channel conformation (Lopez-Barneo, 1996). The presence of oxygen sensitive potassium channels in adrenomedullary chromaffin cells could confer upon them the oxygen sensing capacity required for non-neurogenic catecholamine secretion.

1.4.2.4 Regulation of fetal adrenomedullary catecholamine biosynthesis

The regulation of catecholamine biosynthesis in fetal life is poorly understood. Major regulators of adrenal catecholamine biosynthetic enzyme activity in the adult, namely adrenal innervation and the HPA axis, do not reach functional maturity until relatively late in fetal development and in some cases, postnatally (Challis & Brooks, 1989; Comline & Silver, 1966; Seidler & Slotkin, 1986*b*).

As discussed in section 1.4.2.3, the adrenal medulla of the fetus possesses the unique capacity to secrete catecholamines in direct response to hypoxia (Cheung, 1990; Comline & Silver, 1961; Slotkin & Seidler, 1988). Whilst the effect of hypoxia on fetal catecholamine secretion from the adrenal medulla has been relatively well characterised, the direct actions of hypoxia on catecholamine biosynthesis in the fetal adrenal has not yet been examined to any great extent (DeCristofaro & LaGamma, 1994).

Activation of the fetal HPA axis is considered to be an important event in the maturation of the fetal adrenal medulla (Comline *et al.*, 1970). Surprisingly then, the impact of exogenous corticosteroid administration on adrenomedullary catecholamine synthesis in the fetus has not been fully examined. This may be highly significant in the clinical context in which corticosteroids are frequently administered to mothers in anticipation of premature delivery, in order to accelerate fetal lung maturation (Bishop, 1981; Schellenberg & Liggins, 1987).

1.4.2.4.1 Oxygen regulation of catecholamine biosynthesis

There is a relatively large body of literature on the regulation of tyrosine hydroxylase activity and synthesis in Type I glomus cells and PC12 cells (Beitner-Johnson *et al.*, 1997; Czyzyk-Krzeska, 1997). This is due to the key role that the catecholamine transmitter, dopamine, is speculated to play in oxygen sensing in the carotid body (Fidone *et al.*, 1990) and the information that its rate of synthesis is predominantly determined by tyrosine hydroxylase activity (Levitt *et al.*, 1965; Nagatsu *et al.*, 1964). Currently there is very little information available on the regulation of other catecholamine synthetic enzymes during hypoxia. In the absence of information on the regulation of catecholamine biosynthesis in the fetal adrenal in response to hypoxia the following discussion focusses on what is

currently known about this process in the post natal adrenal medulla and carotid body, as well as PC12 cells.

1.4.2.4.1.1 Hypoxia and catecholamine synthesis

Exposure to long term normobaric hypoxia or high altitude (hypobaric hypoxia) leads to morphological and biochemical changes within the carotid body (Soulier *et al.*, 1997). Long term hypoxia in the rat leads to a hypertrophy and hyperplasia of the Type I glomus cells (Dhillon *et al.*, 1984; Pequignot *et al.*, 1984). At the ultrastructural level these morphological changes are associated with increased mitochondrial volume density and enlarged dopamine-containing dense cored vesicles (Pequignot *et al.*, 1984). In association with the enlargement of dense cored vesicles there is a corresponding increase in the dopamine content of the carotid body (Hanbauer *et al.*, 1981; Schmitt *et al.*, 1992; Soulier *et al.*, 1997). Dopamine turnover is enhanced during chronic hypoxia, indicating that the increase in content is due to a marked increase in synthesis during hypoxia (Pequignot *et al.*, 1987; Schmitt *et al.*, 1992). The increase in dopamine synthesis is due to an increase in the activity (V_{max}) of TH during hypoxia, whilst the affinity for the enzyme for its pteridine co-factor (K_m) is unchanged (Hanbauer *et al.*, 1977). No significant changes in the activity of DBH have been reported to occur in the carotid body in response to chronic hypoxia (Hanbauer *et al.*, 1977; Starlinger *et al.*, 1983). The increase in TH V_{max} during chronic hypoxia is due to an elevation of the levels of the enzyme within the carotid body (Schmitt *et al.*, 1992; Soulier *et al.*, 1997). The morphological changes occur independently of neural and hormonal inputs implicating a direct affect of hypoxia on the carotid body (Mills & Nurse, 1993; Pequignot *et al.*, 1984). The increase in catecholamine turnover and synthesis during hypoxia, whilst not being totally reliant on carotid body innervation, are found to be modulated to a degree by neural input (Pequignot *et al.*, 1991).

The adrenal medulla displays hyperplasia in response to long term hypobaric hypoxia in the rat, although to a lesser extent than that which occurs in the carotid body (Gosney, 1985). An increase in catecholamine turnover within the adrenal medulla in response to both acute and chronic hypoxaemia has been observed using the tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine (Dalmaz *et al.*, 1994;

Johnson *et al.*, 1983; Lee *et al.*, 1987). Catecholamine stores within the adrenal medulla whilst initially being depleted in response to chronic hypoxia, display a subsequent increase in content (Dalmaz *et al.*, 1994; Steinsland *et al.*, 1970). Both acute and chronic hypoxia are established to stimulate an increase in TH activity (Dalmaz *et al.*, 1994; Hayashi *et al.*, 1990). The increase in TH V_{max} which occurs in response to acute hypoxia, independently of any change in K_m, may occur as a result of phosphorylation of the enzyme to a more active form (Hayashi *et al.*, 1990). Stimulation of TH activity during acute hypoxia requires functional adrenal innervation as it is abolished by prior transection of the splanchnic nerve (Hayashi *et al.*, 1990). In contrast, stimulation of TH activity during chronic hypoxia occurs independently of the carotid body reflex arc indicating that this effect may be mediated by direct, non-neurogenic mechanisms, or possibly by other peripheral chemoreceptor reflex pathways (Dalmaz *et al.*, 1994). Increased TH activity during chronic hypoxia is associated with a specific increase in tyrosine hydroxylase protein levels within the adrenal gland (Schmitt *et al.*, 1992; Soulier *et al.*, 1997).

1.4.2.4.1.2 Oxygen regulation of gene expression

Molecular oxygen plays a vital role in energy production in most eukaryotic organisms with oxygen delivery to organs and tissues in mammals being tightly regulated. A reduction in oxygen tension (hypoxia) stimulates a number of responses at the organismal level and equally importantly at the tissue and cellular level, which serve to increase oxygen delivery and preserve oxygen availability. There is a substantial body of evidence to show that many of the physiological adaptations which occur in response to hypoxia are in part mediated by alterations in oxygen-sensitive gene expression (Beitner-Johnson *et al.*, 1997; Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997).

The hormone erythropoietin (EPO), is released from the kidney and fetal liver to stimulate the production of oxygen-carrying red blood cells (Jelkmann, 1992). Hypoxia is able to induce a dramatic increase in EPO production which is primarily mediated through enhanced transcription of the EPO gene (Goldberg & Gaut, 1992; Jelkmann, 1992; Schuster *et al.*, 1989). Neovascularisation (angiogenesis) is stimulated by hypoxia in order to increase oxygen delivery to hypoxic tissues

(Shweiki *et al.*, 1992). Angiogenesis is driven by the activity of a number of cytokine growth factors whose expressions are found to be regulated by oxygen availability (Bunn & Poyton, 1996). One such cytokine growth factor is the ubiquitously expressed Vascular Endothelial Growth Factor (VEGF) (Claffey *et al.*, 1992; Minchenko *et al.*, 1994; Shweiki *et al.*, 1992). Nuclear run-off experiments find a marked increase in the transcription of the VEGF gene in response to hypoxia (Ikeda *et al.*, 1995).

A number of cell types are able to preferentially switch to non-oxidative glucose metabolism to generate energy by glycolysis when oxygen is limiting. The shift to anaerobic glycolysis (Embden-Meyerhof pathway) during hypoxia, which is known as the Pasteur effect, conserves oxygen but results in a less efficient production of ATP (18-fold lower) than the oxygen dependent Krebs cycle (Bunn & Poyton, 1996). To compensate for the decreased energy formation efficiency, hypoxia stimulates glycolytic enzyme activity and expression at the protein and mRNA level (Bunn & Poyton, 1996; Webster, 1987). Hypoxia also enhances glucose transport into cells by upregulation of insulin-independent glucose transporter 1 (GLUT-1) protein and mRNA levels (Bunn & Poyton, 1996).

1.4.2.4.1.3 Oxygen regulation of catecholamine biosynthetic enzyme genes

In terms of catecholamine biosynthesis, TH gene expression is well established to be regulated by hypoxia (Czyzyk-Krzeska *et al.*, 1992; Czyzyk-Krzeska *et al.*, 1994*a;* Czyzyk-Krzeska *et al.*, 1994*b;* Kroll & Czyzyk-Krzeska, 1998; Norris & Millhorn, 1995; Raymond & Millhorn, 1997). *In situ* hybridisation studies have revealed that adult rats exposed to mild hypoxia (10 % F_iO_2) for 1-48 h demonstrate marked increases in carotid body TH mRNA expression which are independent of hypercapnia and neural input (Czyzyk-Krzeska *et al.*, 1992). No significant increases in TH mRNA expression were evident in either the superior cervical ganglion (SCG) or adrenal medulla in response to the same stimulus, which led to the conclusion that TH mRNA expression is regulated by hypoxia in a tissue and cell specific manner (Czyzyk-Krzeska *et al.*, 1992). However, adrenomedullary TH mRNA expression was not examined independently of functional adrenal innervation which suppresses the direct stimulation of the adrenal medulla by hypoxia (Cheung, 1990; Slotkin & Seidler, 1988).

Furthermore, the modest level of hypoxia used in these studies may have been insufficient to result in a trans-synaptic activation of TH mRNA expression in the adrenal.

DeCristofaro and LaGamma (1994) examined the hypothesis that non-neurogenic stimulation of catecholamine secretion could stimulate catecholamine biosynthetic enzyme synthesis by acutely subjecting 4 day old rat pups to modest hypoxia (7-8 % F_iO_2 for 2 h) and measuring adrenal TH mRNA expression 24 h later (DeCristofaro & LaGamma, 1994). These investigators found that non-neurogenic stimulation of the adrenal medulla did not significantly increase TH mRNA expression following hypoxia (DeCristofaro & LaGamma, 1994). Examination of the timecourse of adrenal TH mRNA expression following the exposure of neonatal rats to hypoxia was not carried out raising the possibility that a change in TH mRNA expression following hypoxia may have been missed.

TH mRNA expression in the adrenal medulla of the adult and neonatal rat *in vivo* appears relatively unresponsive to modest hypoxia (Czyzyk-Krzeska *et al.*, 1992; DeCristofaro & LaGamma, 1994). It is interesting then that TH mRNA expression in the rat PC12 cell line exhibits an oxygen sensitivity which is analogous to that demonstrated in the type I glomus cells of the carotid body, with marked elevations of TH mRNA expression occurring in as little as 1 h in response to a 75% reduction in oxygen availability (Czyzyk-Krzeska *et al.*, 1994*a*). As outlined in the previous section the type I glomus cells of the carotid body, PC12 cells, and fetal adrenomedullary chromaffin cells posses functionally similar chemoreceptive mechanisms (Adams *et al.*, 1996; Buckler & Vaughan-Jones, 1994; Lopez-Barneo *et al.*, 1988; Mochizuki-Oda *et al.*, 1997; Rychkov *et al.*, 1998; Thompson *et al.*, 1997; Zhu *et al.*, 1996). The fetus lives at a much lower arterial PO_2 level than either the neonate or the adult and hence the modest levels of hypoxia used by previous investigators may have been insufficient to uncover any direct effect on hypoxia on adrenomedullary TH mRNA expression (Czyzyk-Krzeska *et al.*, 1992; DeCristofaro & LaGamma, 1994). It has yet to be established if hypoxia is able to directly regulate catecholamine synthetic enzyme mRNA expression in the fetus *in utero* or in adrenomedullary chromaffin cells *in vitro* which are not subject to neural influences.

The establishment of the PC12 cell line as a suitable *in vitro* model in which to study oxygen regulation of TH gene expression has greatly facilitated the elucidation of a number of potential mechanisms behind this process (Beitner-Johnson *et al.*, 1997). Nuclear run-off assays reveal that hypoxia increases transcription of the TH gene in PC12 cells (Czyzyk-Krzeska *et al.*, 1994*b*). Furthermore, studies using both transcriptional inhibitors and pulse chase techniques find that there is also an increase in the stability of TH mRNA in PC12 cells in response to hypoxia (Czyzyk-Krzeska *et al.*, 1994*b*). The two forms of mRNA expression regulation come into effect at different points during exposure to hypoxia with the increase in TH gene transcription prevailing early (< 5 h) during hypoxia. With longer exposure to hypoxia (< 10 h) there is an attenuation of gene transcription and stabilisation of the mRNA becomes the primary contributor to the overall increase in expression (Czyzyk-Krzeska, 1997; Czyzyk-Krzeska *et al.*, 1994*b*). Stabilisation of mRNA during hypoxia is advantageous as it provides greater energy efficiency, with cells utilising existing mRNA rather than transcribing new mRNA (Czyzyk-Krzeska, 1997).

1.4.2.4.1.4 Mechanisms of hypoxia induced TH gene transcription

The novel transcriptional factor, hypoxia-inducible factor 1 (HIF-1), interacts with specific *cis*-elements within the promoter and enhancer regions of numerous genes, including those for EPO (Semenza & Wang, 1992), VEGF (Forsythe *et al.*, 1996), GLUT-1 (Ebert *et al.*, 1995), and many glycolytic enzymes (Semenza *et al.*, 1994), to regulate transcription during hypoxia. HIF-1, which was first identified and characterised by Wang and Semenza, is induced by low cellular O_2 tension and consists of α and β subunits which must heterodimerise to bind to their consensus site and influence gene transcription (Jiang *et al.*, 1996; Semenza & Wang, 1992; Wang & Semenza, 1993*a;* Wang & Semenza, 1993*c;* Wang & Semenza, 1995; Wood *et al.*, 1996). HIF-1α and -1β belong to the basic helix-loop-helix family of transcription factors and whilst the Hif-1α subunit is a novel protein, the Hif-1β subunit has previously been identified as the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) functional in the transcriptional response to xenobiotics (Hoffman *et al.*, 1991; Wang *et al.*, 1995*a*).

Hypoxia induced upregulation of TH gene transcription in PC12 cells has been determined by TH-CAT reporter gene construct experiments to be dependent upon a short sequence of the 5'-proximal promoter region between bases -284 and -150 (Czyzyk-Krzeska *et al.*, 1994*b;* Norris & Millhorn, 1995). Analysis of this sequence reveals the presence of AP-1, AP-2, and HIF-1 regulatory *cis*-elements (Norris & Millhorn, 1995). Gel shift assays using a TH DNA fragment corresponding to bases -284 to -190 of the 5' promoter region show that there is increased PC12 cell nuclear protein binding to this fragment following prior exposure of these cells to hypoxia (Norris & Millhorn, 1995). Increased nuclear protein binding to the native TH DNA fragment following hypoxia can be partially competed for by the addition of excess TH HIF-1 element oligonucleotide (Norris & Millhorn, 1995). HIF-1 may, therefore, play a role in hypoxia induced regulation of TH gene transcription (Beitner-Johnson *et al.*, 1997; Czyzyk-Krzeska, 1997; Norris & Millhorn, 1995).

Aside from the HIF-1 regulatory element, the -284 to -190 base TH DNA fragment also contains the AP-1 regulatory element (Beitner-Johnson *et al.*, 1997; Norris & Millhorn, 1995). The importance of the AP-1 regulatory element in the TH gene transcriptional response to hypoxia is highlighted by a number of findings. First, the addition of unlabelled TH AP-1 element oligonucleotide attenuates the increase in hypoxia induced protein binding to the native TH -284 to -190 base DNA fragment (Norris & Millhorn, 1995). Secondly, the binding of PC12 cell nuclear protein extracts to double stranded oligonucleotides containing the TH AP-1 element sequence remains enhanced during prolonged hypoxia (Norris & Millhorn, 1995). Finally, mutation of the AP-1 element abolishes hypoxia induced TH (-284 to -150 base)-CAT reporter gene expression in PC12 cells (Norris & Millhorn, 1995).

UV cross linking experiments of PC12 nuclear protein extracts have found that the proteins which bind to the AP-1 site during hypoxia range in size from 20-85 kDa (Norris & Millhorn, 1995). The size range of these proteins fall within the range of several members of the *trans*-acting Fos and Jun family of proteins. Immunological gel shift assays revealed that antibodies against c-fos and jun-B produced supershifted bands resulting from their interaction with labelled TH AP-1 element / PC12 cell nuclear protein complexes (Norris & Millhorn, 1995). An

antibody against c-jun was unable to produce a supershifted band, indicating a lack of involvement of this protein in the hypoxia induced binding to the AP-1 element (Norris & Millhorn, 1995). The binding of both c-fos and jun-B to the AP-1 element were induced by hypoxia (Norris & Millhorn, 1995).

Interestingly, increases in intracellular calcium are required for hypoxia to stimulate an increase in TH mRNA expression in PC12 cells. The hypoxia induced increase in TH mRNA expression is abolished by the omission of calcium from the external medium or by the addition of calcium chelators (Raymond & Millhorn, 1997). The major signal transduction pathways that are activated by an increase in cytosolic Ca^{2+} levels are Ca^{2+} / calmodulin-dependent protein kinases (CamK), Ca^{2+} / calmodulin-dependent phosphatases and various isoforms of protein kinase C (PKC). Specific inhibitors of protein kinase C are able to block the hypoxia induced up-regulation of TH mRNA indicating that the Ca^{2+} / protein kinase C may play a critical role in hypoxia induced TH mRNA expression (Beitner-Johnson *et al.*, 1997; Raymond & Millhorn, 1997). Phosphorylation is a necessary requirement for the activation of a number of transcription factors, although HIF-1 phosphorylation occurs independently of PKC activation (Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997; Wang *et al.*, 1995*c*). The mechanisms by which hypoxia stimulates phosphorylation have yet to be determined but in the case of HIF-1 induction, pharmacological studies implicate the involvement of both serine / threonine kinases and phosphatases (Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997; Wang *et al.*, 1995*c*).

1.4.2.4.1.5 Mechanisms of hypoxia induced TH mRNA stabilisation

TH mRNA in PC12 cells has a half life of around 10 h and this is stabilised a further 2-4 fold during prolonged hypoxia (Czyzyk-Krzeska, 1997; Czyzyk-Krzeska *et al.*, 1994*b*). The increased stability of TH mRNA during hypoxia in PC12 cells appears to result from an increased binding of cytoplasmic protein to a pyrimidine-rich *cis*-acting sequence in the 3' untranslated region of the TH mRNA (Czyzyk-Krzeska *et al.*, 1994*a*). Gel retardation assays and RNase T1 mapping using fragments of rat TH mRNA incubated with cytoplasmic protein extract from PC12 cells, revealed that a sequence located between bases 1551-1579 in the 3' untranslated region exhibited increased protein binding following prior exposure of

the cells to hypoxia (Czyzyk-Krzeska & Beresh, 1996; Czyzyk-Krzeska *et al.*, 1994*a*). This sequence has been termed the Hypoxia-Inducible Protein Binding Sequence (HIPBS; Czyzyk-Krzeska, 1997). Mutation of this sequence abolishes protein binding, resulting in a decrease in the constitutive half life of TH mRNA as well as the abolition of the hypoxia induced increase in TH mRNA stability (Czyzyk-Krzeska & Beresh, 1996; Czyzyk-Krzeska *et al.*, 1997). Furthermore the mRNA of chloramphenicol acetyltransferase (CAT) reporter gene constructs containing the TH HIPBS sequence exhibit O_2 dependent regulation of stability which is not normally observed in the absence of this sequence (Czyzyk-Krzeska *et al.*, 1997). RNA affinity purification of hypoxia inducible proteins (HIP) which bind to the HIPBS yielded two protein factors with approximate molecular weights of 45 and 55-60 kD (Czyzyk-Krzeska *et al.*, 1997). Hypoxia inducible binding activity to HIPBS has also been identified in other catecholaminergic tissues such as the carotid body and the superior cervical ganglion (Czyzyk-Krzeska *et al.*, 1997). Post-transcriptional regulation of gene expression by the binding of proteins to stability elements within the 3'-untranslated region of the mRNA has also been described for other oxygen regulated genes including EPO and VEGF (Bunn & Poyton, 1996).

1.4.2.4.1.6 Oxygen sensing and TH gene transcription

The actions of molecular oxygen at the cellular level are in great part mediated by heme proteins. Heme proteins bind molecular O_2 via a central Fe^{2+} in the molecule which results in a conformational change from the deoxy to the oxy form. Conformational changes in the heme molecule affect their function and make them potential candidates for an "O_2 sensor" (for review see Bunn, 1996). There is evidence to suggest that heme proteins may be involved in the regulation of a number of oxygen sensitive genes. The transition metals cobalt and nickel which are able to substitute for iron in the heme moiety, locking it into the deoxy configuration due to their relatively low affinity for oxygen, are found to induce the expression of many oxygen regulated genes including EPO and VEGF, amongst others (Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997; Goldberg & Schneider, 1994; Ho & Bunn, 1996; Sinclair *et al.*, 1979; Sinclair *et al.*, 1982). Conversely, carbon monoxide which binds non-covalently to the ferrous heme group in a manner analogous to that of oxygen, locking it into the oxy-formation, antagonises

the hypoxia induced upregulation of EPO, VEGF, and other genes (Bunn & Poyton, 1996; Goldberg *et al.*, 1988; Goldberg & Schneider, 1994).

The oxygen sensor appears to be coupled to H_2O_2 production within the cell and hence is speculated to be a multisubunit oxidase (Bunn & Poyton, 1996). In PC12 cells which have been loaded with the H_2O_2-sensitive fluorescent dye 2',7'-dichlorofluorescein (DCF) and exposed to 5 % O_2, H_2O_2 levels are found to decrease as early as 1 h after exposure with the decrease reaching a maximum of 40 % after 6 h (Kroll & Czyzyk-Krzeska, 1998). Peroxide production is also found to be inhibited in a graded manner in EPO producing Hep 3B cells in response to decreasing oxygen tensions (Fandrey *et al.*, 1994). Therefore H_2O_2 production appears to be a plausible intracellular signal for oxygen regulated gene expression (Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997). In support of this theory, the hypoxia induced increase in TH mRNA expression in PC12 cells is found to be inversely related to intracellular H_2O_2 formation (Kroll & Czyzyk-Krzeska, 1998). Pretreatment of PC12 cells and Hep 3B cells with H_2O_2 prevents the induction of TH mRNA and EPO mRNA expression, respectively, in response to hypoxia (Fandrey *et al.*, 1994; Kroll & Czyzyk-Krzeska, 1998). Conversely, the administration of the H_2O_2 decomposing enzyme, catalase, or the reducing antioxidant *N*-(2-mercaptopropionyl)-glycine which decrease H_2O_2 formation are able to increase TH mRNA expression (Kroll & Czyzyk-Krzeska, 1998).

H_2O_2 is known as a reactive oxygen intermediate (ROI) and may mediate its effects on intracellular signal transduction pathways by regulation of intracellular redox status. Glutathione peroxidase decomposes H_2O_2 using reduced glutathione (GSH) as the hydrogen donor (Sies *et al.*, 1972). The reaction oxidises glutathione (GSSG), shifting the subcellular ratio of GSH:GSSG towards the oxidised form (Acker *et al.*, 1992). H_2O_2 can also generate more reactive oxygen compounds, such as hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen, via the Fenton reaction which is catalysed by intracellular iron (Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997; Isaacs & Binkley, 1977). These next-step ROIs interact with and oxidise glutathione and thiol groups (Czyzyk-Krzeska, 1997). Thus as a result of the decreased generation of H_2O_2 during hypoxia, decreased concentrations of ROIs and increased concentrations of reduced glutathione are expected (Acker, 1994; Acker & Xue, 1995; Czyzyk-Krzeska, 1997). Consequently the redox

equilibrium of protein thiols would shift towards the reduced form; affecting the nucleic acid-binding properties of regulatory proteins and influencing gene expression (Bunn & Poyton, 1996; Czyzyk-Krzeska, 1997). Reduction of next step ROI formation by the chelation of free intracellular iron using deferoxamine induces TH mRNA expression in PC12 cells (Kroll & Czyzyk-Krzeska, 1998), a phenomenon also observed in other cell lines for VEGF mRNA (Gleadle *et al.*, 1995) and EPO mRNA expression (Wang & Semenza, 1993*b*). Indeed AP-1 complexes require the reduction of specific cysteine residues in their DNA-binding domains for association with DNA *in vitro* (Abate *et al.*, 1990; Toledano & Leonard, 1991). HIF-1 binding to DNA is also redox sensitive and this is primarily due to redox dependent stabilisation of the HIF-1α subunit in the reduced form (Huang *et al.*, 1996; Wang *et al.*, 1995*b*). Furthermore HIBPs binding to TH mRNA is also dependent on these proteins being in the reduced form (Czyzyk-Krzeska, 1997).

1.4.2.4.2 Hormonal regulation of adrenomedullary development and catecholamine biosynthesis

As in the adult animal there is substantial evidence to suggest that glucocorticoids derived from the adrenal cortex play an important role in the regulation of catecholamine synthesis, particularly adrenaline synthesis, in the fetal adrenal gland. Aside from a regulatory role of glucocorticoids in fetal catecholamine biosynthesis, they also play an important role in development of the fetal adrenal medulla.

1.4.2.4.2.1 Glucocorticoids and adrenomedullary development

The neural crest is a transient embryonic structure consisting of precursor cells which migrate to specific locations and give rise to a diverse array of derivatives including sensory neurones, autonomic neurones, and glial cells (Anderson, 1993; Cochard *et al.*, 1978; Coulombre *et al.*, 1974; LeDouarin *et al.*, 1991; LeDouarin & Dupin, 1993). The fates of the multipotent neural precursor cells appear to be determined in large part by environmental factors encountered along the migratory route to their ultimate position (LeDouarin & Dupin, 1993; Stern *et al.*, 1991; Unsicker, 1985). One such neural crest derived cell lineage is the sympathoadrenal lineage which develops from sympathoadrenal progenitor cells

which migrate ventrolaterally from the apex of the neural tube to the dorsal aorta where they aggregate and differentiate to form sympathetic neurones or continue migration into the adrenal anlage where they differentiate to form chromaffin cells (Anderson, 1993; Patterson, 1990).

Previous studies have shown that when dissociated chromaffin cells from immature adrenals are cultured in the absence of glucocorticoids they exhibit poor long term survival in culture (Doupe *et al.*, 1985). Addition of nerve growth factor (NGF) to these cultures is able to rescue many of the chromaffin cells however they subsequently undergo a phenotypical change exhibiting extensive neurite outgrowth (Doupe *et al.*, 1985; Naujoks *et al.*, 1982; Unsicker *et al.*, 1978*a*). Glucocorticoid administration is able to block or delay NGF-induced neurite outgrowth (Doupe *et al.*, 1985; Unsicker *et al.*, 1978*a*). Long term culture of rat adrenal chromaffin cells in the presence of NGF and the absence of glucocorticoids results in a complete transition to a sympathetic neuron phenotype as assessed by a number of morphological and biochemical criteria (Doupe *et al.*, 1985). Furthermore *in vivo* studies show that injection of pregnant rats with NGF results in the replacement of fetal chromaffin cells with sympathetic ganglion cells (Aloe & Levi-Montalcini, 1979). Hence glucocorticoids appear to act as a survival factor for chromaffin cells and repress neuronal transdifferentiation of these cells (Doupe *et al.*, 1985; Unsicker *et al.*, 1978*a*). These data suggest that the sympathetic progenitor cells which migrate from the sympathetic ganglion primordium through the developing adrenal anlage develop into chromaffin cells in the adrenal medulla under the influence of the glucocorticoid rich environment.

More definitive proof of the role of glucocorticoids in the developmental regulation of the adrenal medulla arose with the development of antigenic markers which allowed for the identification of fetal sympathoadrenal (SA) progenitor cells (Anderson & Axel, 1986; Carnahan & Patterson, 1991*a;* Carnahan & Patterson, 1991*b*). Subsequently fetal SA progenitors were isolated and *in vitro* studies demonstrated that these cells are indeed bipotential and can develop into either sympathetic neurones or chromaffin cells depending on the hormonal milieu and culture conditions (Anderson & Axel, 1986; Carnahan & Patterson, 1991*b;* Michelsohn & Anderson, 1992). The development of a sympathetic neuron phenotype is initially dependent upon the presence of fibroblast growth factor and

depolarisation and subsequently upon the presence of NGF (Anderson, 1993; Anderson & Axel, 1986; Carnahan & Patterson, 1991*b;* DiCicco-Bloom & Black, 1989; Michelsohn & Anderson, 1992; Stemple *et al.*, 1988). Likewise the development of the chromaffin cell phenotype was found to be dependent upon the presence of glucocorticoids (Anderson, 1993; Anderson & Axel, 1986; Carnahan & Patterson, 1991*b;* Michelsohn & Anderson, 1992; Seidl & Unsicker, 1989).

In vivo individual sympathoadrenal progenitor cells which co-express neuron specific and chromaffin cell specific antigenic markers are observed in early (E12.5) sympathetic ganglion primordia in the fetal rat (Anderson *et al.*, 1991; Carnahan & Patterson, 1991*a*). The chromaffin specific markers expressed by these progenitors are lost in those cells which remain in the sympathetic ganglia whereas the neuron specific markers are extinguished in those cells which migrate through the adrenal primordium to form the adrenal medulla (Anderson, 1989; Anderson & Axel, 1986; Anderson *et al.*, 1991; Carnahan & Patterson, 1991*a;* Vogel & Weston, 1990).

The effect of glucocorticoids on the suppression of neural transdiffentiation of SA progenitor cells has been determined by pharmacological studies using specific receptor agonist and antagonists to be mediated by a high affinity type II glucocorticoid receptor (Michelsohn & Anderson, 1992). The levels of corticosterone present in the fetal rat adrenal gland at the time of invasion by the SA progenitor cells is more than sufficient to suppress neural transdifferentiation by these cells (Michelsohn & Anderson, 1992). Furthermore, studies in glucocorticoid receptor knockout mice, which die at birth due to respiratory failure, show that the adrenal glands of these mice essentially lack a central medulla despite markedly elevated glucocorticoid levels (Cole *et al.*, 1995). This would indicate that there is either a failure of SA progenitor cells to migrate or to survive and proliferate in the adrenal gland without functional glucocorticoid signalling. Interestingly, these glands do display small numbers of scattered noradrenaline chromaffin cells amongst the cortex (Cole *et al.*, 1995). Noradrenaline containing chromaffin cells generally account for a minor proportion of the adrenal medulla (ca ~ 15-20 % in the rat) whilst adrenaline containing cells account for the majority (Kent & Parker, 1993). Substantially higher levels of glucocorticoid receptor

immunoreactivity have been identified in the adrenaline-containing adrenomedullary chromaffin cells of the rat than is observed in the noradrenaline-containing cells (Ceccatelli *et al.*, 1989). This finding may imply one of two things; that different glucocorticoid-dependent and -independent SA progenitors may give rise to the adrenal medulla (Cole *et al.*, 1995), or that glucocorticoids do not induce the chromaffin phenotype *per se* but increase the probability of gaining chromaffin competency (Anderson, 1993).

1.4.2.4.2.2 Glucocorticoids and fetal catecholamine biosynthesis

Just as in the mature animal glucocorticoids appear to play an important role in the regulation of adrenaline synthesis in the developing adrenal by their actions on the enzyme, PNMT. In the immature adrenal of the neonatal rat, ACTH administration increases plasma corticosterone levels and also stimulates increases in both PNMT activity and adrenaline content (Kent and Parker, 1983; Banerji et al., 1986). Whilst hypophysectomy (HPX) in the fetal rat at E16.5 does not abolish the expression of adrenal PNMT at E21.5, it does dramatically reduce the developmental increase in PNMT expression (Bohn *et al.*, 1981).

The fact that PNMT expression could not be induced prematurely in fetal rat adrenals after both fetal and maternal corticosteroid and ACTH administration led Bohn and co-workers (1981) to conclude that glucocorticoids were not responsible for the initial induction of PNMT. Seidl and Unsicker (1989) reported that the induction of PNMT activity and adrenaline expression in the E17.3 rat adrenal coincided with a sharp increase in adrenal corticosterone levels and glucocorticoid receptor expression. These authors concluded that PNMT expression was induced in adrenergic chromaffin cells by glucocorticoids once functional receptors were present (Seidl & Unsicker, 1989). This contrasted with the findings of yet another study which found that PNMT mRNA and protein expression could be observed in the fetal rat adrenal gland at E15.5, prior to the corticosterone surge (Ehrlich *et al.*, 1989).

The conflicting results of the aforementioned studies were reconciled by some eloquent *in vitro* studies with freshly isolated E14.5 rat sympathoadrenal precursors (Michelsohn & Anderson, 1992). These studies revealed that glucocorticoids acting by specific type II glucocorticoid receptors were required for

the initial appearance of PNMT in the progenitor cells (Michelsohn & Anderson, 1992). Glucocorticoid exposure did not, however, control the timing of induction, with induction occurring along a similar time course to that observed *in vivo* (Michelsohn & Anderson, 1992). Thus the schedule of PNMT induction is a cell intrinsic timed process in chromaffin precursors which have previously committed to the chromaffin cell phenotype which as outlined above is also a glucocorticoid determined event (Michelsohn & Anderson, 1992). Measurements of fetal adrenal corticosteroid levels by these investigators also revealed that the levels present in the E15.5 adrenal are sufficient to account for the previous observation of PNMT positive cells within the adrenal at this age (Ehrlich *et al.*, 1989; Michelsohn & Anderson, 1992). The lack of sensitivity of the receptor binding assay used by Seidl and Unsicker (1989) may have contributed to the failure of these investigators to observe glucocorticoid receptors prior to E17.5 at which stage their expression increases dramatically (Seidl & Unsicker, 1989). Indeed a subsequent study was able to detect glucocorticoid receptor mRNA as early as E15.5 in rat adrenal chromaffin cells (Anderson & Michelson, 1989).

In the fetal sheep indirect evidence also suggests that glucocorticoids are necessary for the induction and maintenance of PNMT activity in the developing adrenal. Comline and Silver (1961) found that adrenaline content in the adrenal gland increases markedly over the 10-15 d preceding parturition which coincides with the prepartum surge in adrenal cortisol output (Challis & Brooks, 1989; Comline & Silver, 1961). In a subsequent study it was found that HPX in fetal sheep at 93-105 d gestation results in a failure of the adrenaline : noradrenaline ratio in the adrenal to exhibit the normal ontogenetic increase over late gestation (Comline *et al.*, 1970). This decrease in adrenaline production in the fetal sheep adrenal gland is also accompanied by adrenal atrophy, primarily due to lack of adrenocortical growth, and presumably decreased cortisol output (Comline *et al.*, 1970; Liggins *et al.*, 1973).

Despite the apparent stimulatory role adrenal glucocorticoids play on PNMT expression and activity in the fetal adrenal gland, recent *in vivo* studies in the sheep examining the effects of precocious, physiologically and clinically relevant increases in fetal plasma glucocorticoid levels, report a paradoxical suppressive effect on basal plasma adrenaline concentrations (Derks *et al.*, 1997; Stein *et al.*,

1995; Wood *et al.*, 1987). It has yet to be established if this glucocorticoid effect is manifested at the level of adrenaline synthesis, secretion, or metabolism. One possible explanation may be that the elevated peripheral glucocorticoids feedback at the pituitary to suppress ACTH secretion and hence reduce endogenous cortisol output from the adrenal gland (Challis & Brooks, 1989). The reduction in intra-adrenal cortisol levels could then result in a decrease of PNMT activity and adrenaline synthesis with a consequent reduction in adrenaline output.

1.5 AIMS AND HYPOTHESES

From the literature it can clearly be established that the adrenal medulla is functional and active in response to physiological stress *in utero* early in development, and hence is a key source of catecholamines in fetal life. Relatively little is known, however, about the regulation of fetal adrenal catecholamine synthesis in response to stimulation during both acute stress, and chronic stress in particular. The general aim of this thesis was therefore to examine the gene expression of the adrenal catecholamine synthetic enzymes, TH and PNMT, during development and in response to both acute and chronic physiological stress in the fetal sheep adrenal. Given the potential role that intra-adrenal opioid peptides play in modulating catecholamine secretion from the fetal adrenal medulla, the gene expression of the adrenal opioid peptide precursor molecule, PEnk A, will also be examined in a number of the studies in this thesis. The fetal sheep was employed as a model as it is a long gestation species in which many key aspects of adrenal development occur prenatally.

1.5.1 ONTOGENY OF TH, PNMT, AND PEnk A mRNA EXPRESSION IN THE FETAL SHEEP ADRENAL

It is well documented in the mature animal that factors associated with activation of the splanchnic nerve and glucocorticoids released from the adrenal cortex play an important role in the regulation of adrenal catecholamine biosynthesis. The developmental profiles of adrenal TH, PNMT, and PEnk A mRNA expression have hitherto not been examined over mid to late gestation in the fetal sheep. Hence the expression of TH, PNMT, and PEnk A mRNA will be examined in the adrenals of fetal sheep from mid to late gestation (80-146 d). This time frame coincides

with the development of functional splanchnic innervation and the prepartum surge in corticosteroid output from the cortex.

Hypothesis

That the developmental expression of adrenal TH, PNMT, and PEnk A are altered over mid to late gestation with the development of adrenal innervation and adrenocortical maturation.

1.5.2 IMPACT OF ACUTE HYPOXAEMIA ON THE EXPRESSION OF TH AND PNMT mRNA IN THE ADRENAL GLAND OF THE FETAL SHEEP PRIOR TO AND AFTER THE DEVELOPMENT OF FUNCTIONAL SPLANCHNIC INNERVATION

The fetal adrenal medulla is capable of secreting catecholamines in direct response to hypoxaemia prior to innervation. This is in contrast to the mature adrenal which requires reflex stimulation of the splanchnic nerve to stimulate catecholamine secretion. Neural stimulation of catecholamine release is associated with a compensatory increase in catecholamine synthesis known as stimulation-synthesis-coupling. It is not known whether the non-neurogenic release of catecholamines from the fetal adrenal gland during hypoxia is also associated with alterations in catecholamine synthesis. Hence the effect of acute fetal hypoxaemia on adrenal TH and PNMT mRNA expression before and after the development of functional splanchnic innervation will be examined in the fetal sheep.

Hypothesis

That acute hypoxia will act before the development of adrenal innervation to stimulate catecholamine synthetic enzyme mRNA expression. It is also hypothesised that the effect of hypoxia on catecholamine synthetic enzyme mRNA expression in the fetal adrenal will become dependent on reflex neural stimulation with the development of functional cholinergic innervation.

1.5.3 THE IMPACT OF A CHRONIC PHYSIOLOGICAL INCREASE OF PLASMA GLUCOCORTICOIDS ON THE EXPRESSION OF TH, PNMT, AND PEnk A mRNA IN THE ADRENAL GLAND OF THE FETAL SHEEP

Fetal plasma glucocorticoids are elevated in response to intrauterine stressors or as a consequence of therapeutic administration in anticipation of premature delivery. Despite well documented stimulatory effects of glucocorticoids on adult catecholamine synthesis and PNMT induction in sympathoadrenal progenitor cells, recent studies have found a fall in basal adrenaline levels in the late gestation sheep fetus in response to exogenous glucocorticoid administration. In an attempt to clarify whether the actions of exogenously administered glucocorticoids in the fetal sheep occur at the level of adrenal catecholamine synthesis, the impact of chronic intrafetal infusions of physiological levels of cortisol on adrenal TH, PNMT, and PEnk A mRNA expression will be determined.

Hypothesis

That exogenous cortisol administration to the fetal sheep acts via negative feedback on the HPA axis to cause a reduction in PNMT mRNA expression.

1.5.4 EFFECT OF PLACENTAL RESTRICTION ON TH, PNMT, AND PEnk A mRNA EXPRESSION IN THE ADRENAL GLAND OF THE LATE GESTATION SHEEP FETUS

Placental restriction is associated with chronic fetal hypoxaemia and hypoglycaemia. As a consequence basal plasma catecholamine levels are found to be elevated in the PR fetus. There is evidence to suggest that the capacity of the fetal and neonatal adrenal to secrete adrenaline in response to further stress is diminished with PR, however the impact of PR on catecholamine synthesis has yet to be determined. Adrenal expression of TH, PNMT, and PEnk A mRNA will be compared between normally grown and placentally restricted fetal sheep in late gestation.

Hypothesis

That the expression of TH mRNA will increase and that of PNMT mRNA will decrease in the chronically hypoxaemic, growth restricted sheep fetus.

2. ONTOGENY OF TYROSINE HYDROXYLASE, PHENYLETHANOLAMINE *N*-METHYLTRANSFERASE AND PROENKEPHALIN A mRNA EXPRESSION IN THE ADRENAL GLAND OF THE FETAL SHEEP

2.1 INTRODUCTION

Studies in a number of species have shown that the fetal adrenal medulla secretes noradrenaline and adrenaline in response to a range of perinatal stimuli including acute hypoxaemia, asphyxia, labour and vaginal delivery, and that these catecholamines play a key role in the initiation and coordination of cardiovascular and metabolic adaptations of the immature animal to these stimuli (Comline & Silver, 1961; Jones *et al.*, 1988; Lagercrantz & Bistoletti, 1973; Padbury *et al.*, 1982; Slotkin & Seidler, 1988). It is clear that noradrenaline, adrenaline, and Enk-containing peptides are present in the adrenal medulla and in the fetal circulation before birth, and that the adrenal medulla is the predominant source of circulating catecholamines during acute fetal hypoxaemia (Cohen *et al.*, 1991; Cohen *et al.*, 1984; Coulter *et al.*, 1990*a;* Coulter *et al.*, 1990*b;* Jones *et al.*, 1988). The regulation of the synthesis of catecholamines and enkephalins in the fetal adrenal before birth is, however, not well understood.

In the human and sheep, the adrenal medulla synthesises and secretes catecholamines throughout late gestation (Comline & Silver, 1961; Jones *et al.*, 1988; Padbury *et al.*, 1982; Wilburn *et al.*, 1986). In the adrenal of the fetal sheep, noradrenaline containing cells are predominantly localised in the centre of the adrenal medulla whereas adrenaline is present in peripheral columnar cells adjacent to, and interdigitating with, the inner zone of the adrenal cortex (McMillen *et al.*, 1988). Met-Enk containing peptides are also present in the fetal sheep adrenal (Coulter *et al.*, 1990*a;* McMillen *et al.*, 1988) from as early as 80 d gestation (term = 147 ± 3 d gestation) and it has been demonstrated that the pattern of localisation of PEnk A mRNA and Met-Enk containing peptides changes in the fetal adrenal during late gestation (McMillen *et al.*, 1988; Wan *et al.*, 1989*b*). Whilst there is only limited evidence for co-secretion of the enkephalin peptides with the catecholamines from the fetal adrenal (Coulter *et al.*, 1990*b;* Martinez *et al.*, 1990; Simonetta *et al.*, 1996*a*), there is evidence that intra-adrenal opioids may play an important role in modulating catecholamine secretion from the fetal

adrenal (Padbury *et al.*, 1987*b*). In the present study we have investigated the impact of increasing gestational age on the adrenal mRNA levels for TH, PNMT, and PEnk A in the fetal sheep.

2.2 METHODS

All procedures used in this study were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation

2.2.1 ONTOGENY STUDY

Eighteen dated pregnant Border Leicester x Merino ewes with gestational ages calculated from the date of mating, were electively killed by an intravenous overdose of sodium pentobarbitone (25 ml at 325 $mg.ml^{-1}$; Lethabarb, Virbac, Peakhurst, NSW, Australia) at 80-100 d (n = 6); 125 d (n = 6), and 140-146 d gestation (n = 6). Fetal sheep were delivered by laparotomy, weighed, and then killed by decapitation. Fetal adrenal glands were rapidly dissected, weighed, snap frozen in liquid N_2 and stored at -70°C prior to total RNA extraction.

2.2.2 RNA ISOLATION

Total RNA was extracted from fetal adrenal glands using modifications of the method previously described by Chirgwin *et al.* (1979). Individual fetal adrenal glands were homogenised using a Polytron PT3000 (Kinematica, Littau, Switzerland) at 30 000 rpm in 3 ml of guanidine isothiocyante (GIT) buffer, which consisted of : 4 *M* guanidine thiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5 % (w / v) sodium laurylsarcosine, and 0.3 % (v / v) Antifoam A (Sigma Chemicals, St. Louis, MO, USA). Caesium chloride (1.2 g) was added to the adrenal homogenate which was then carefully layered onto a 5.7 *M* CsCl cushion (1.2 ml) in an 11 x 60 mm polyallomer centrifuge tube (Beckman Instruments, Palo Alto, CA, USA). The samples were ultracentrifuged for 16 h at 130 000 *g* using a LE-80K Optima ultracentrifuge equipped with a SW60 rotor (Beckman Instruments). Following ultracentrifugation the supernatant was decanted and the insides of the tubes were wiped dry with a tissue with care taken not to disturb the pellets. The pellets were then briefly air dried by storing the tubes upside down at room

temperature (RT) for 5-10 min. The RNA pellets were subsequently resuspended in 100 µl of sterile water and transferred to sterile 1.5 ml eppendorf tubes. The RNA was further purified by the addition of 0.1 x volumes of 3 *M* sodium acetate and 2.5 x volumes of cold absolute ethanol, and reprecipitation on dry ice for 30 min. The RNA was pelleted by centrifuging the samples at 12 000 *g* for 30 min at 4°C. The supernatant was decanted from the pellet, which was air dried for 5 min and resuspended in a volume of sterile water estimated to give a final concentration of 4-5 µg total RNA.μl^{-1}. Maximum absorbance at 260 nm and 280 nm was used to quantify nucleic acid purity, yield, and concentration. RNA solutions were kept at -70°C until use.

2.2.3 OLIGONUCLEOTIDE PROBES

Oligonucleotide antisense probes complementary to mRNA nucleotide sequences for bovine PNMT (Baetge *et al.*, 1986; Wan *et al.*, 1989*b*, Figure 2.1A), bovine PEnk A (Noda *et al.*, 1982; Wan *et al.*, 1987, Figure 2.1B), and rat 18 S rRNA (Chan *et al.*, 1984, Figure 2.1C) were synthesised by a commercial manufacturer (Bresatec, Thebarton, SA, Australia) and supplied as desalted, lyophilised stocks. The oligonucleotide probes were resuspended in sterile water and further purified by elution from NICK columns containing Sephadex G-50 (Pharmacia, North Ryde, NSW, Australia) with Tris-EDTA buffer (10 m*M* Tris-HCl, 1 m*M* EDTA; pH 8.0).

2.2.4 TH cDNA PROBE

A 1.727 kb bovine TH cDNA (bTH) containing the full length coding sequence (D'Mello *et al.*, 1988) was used to detect TH mRNA. The TH cDNA which had been subcloned into the *Eco*RI site of pBluescript II SK- phagemid vector (Figure 2.2A) was a generous gift of Dr. B.B. Kaplan, University of Pittsburgh. There is extensive homology (~ 93 %) between bovine and ovine TH cDNA (Tillet *et al.*, 1997).

E. coli host strain DH5α (Department of Microbiology, The University of Adelaide) were made competent by the $CaCl_2$ protocol outlined by Sambrook *et al.* (1989). Plasmid DNA containing bTH (50 ng in 10 µl of sterile water) was added to 200 µl

of 0.1 *M* $CaCl_2$ containing competent DH5α *E. coli* and allowed to stand on ice for 30 min. The bacteria were subsequently heat shocked at 42°C for exactly 90 sec and then placed back on ice for a further 1-2 min. 800 µl of SOC medium (bacto-tryptone, 20 $g.l^{-1}$; bacto-yeast extract, 5 $g.l^{-1}$; NaCl, 0.5 $g.l^{-1}$; $MgCl_2$, 0.01 *M* ; Glucose, 20 m*M* ; pH 7.0) was added to the bacteria which were then incubated at 37°C for 45 min with gentle shaking (150 $cycles.min^{-1}$). The transformed bacteria were then plated out onto a LB agar plate (bacto-tryptone, 10 $g.l^{-1}$; bacto-yeast extract, 5 $g.l^{-1}$; NaCl, 10 $g.l^{-1}$; pH 7.0, with 1.5 % w / v bactoagar) containing ampicillin (60 $\mu g.ml^{-1}$; Boehringer Mannheim, Mannheim, Germany) to select bacteria expressing the ampicillin resistance marker, and incubated at 37°C overnight. A single bacterial colony was selected from the plate, inoculated into 10 ml of LB broth (bacto-tryptone, 10 $g.l^{-1}$; bacto-yeast extract, 5 $g.l^{-1}$; NaCl, 10 $g.l^{-1}$; pH 7.0) containing ampicillin (60 $\mu g.ml^{-1}$) and grown to late log phase (OD_{600} ~ 0.4-0.6) at 37°C with vigorous shaking (300 $cycles.min^{-1}$). Upon reaching late log phase 1 ml of the 10 ml starter culture was inoculated into a further 100 ml of ampicillin containing-LB broth and grown to saturation (12-16 h) at 37°C with vigorous shaking (300 $cycles.min^{-1}$). The bacteria were pelleted by centrifugation at 4000 *g* and the plasmid DNA was obtained and purified by alkaline lysis using a Maxiprep plasmid purification kit (Qiagen, Clifton Hill, VIC, Australia) as per manufacturer's instructions. The purified plasmid DNA was resuspended in Tris-EDTA buffer and the yield was quantitated by specrophotometric determination of OD_{260}.

The bTH cDNA insert was isolated from plasmid DNA by *Eco*R1 digestion. Plasmid DNA (20 µg) was incubated at 37°C for 16 h in a solution consisting of 1 x *Eco*R1 reaction buffer (MBI Fermentas, Vilnius, Lithuania), *Eco*R1 enzyme (30 U, MBI Fermentas) and sterile water to a total reaction volume of 100 µl. The cDNA was separated from the digested plasmid DNA by electrophoresis on a 1 % low melting point agarose gel (BDH, Poole, England) using 0.5 x TBE running buffer (5 x TBE: 0.045 *M* Tris-borate, 0.01 *M* EDTA, pH 8.0). The DNA was visualised by ethidium bromide staining of the gel and viewing under UV light. The cDNA insert was identified on the basis of size by comparison with λ DNA / *Eco*1301(*Sty* I) DNA size markers (MBI Fermentas) and the appropriate fragment

was excised from the gel using a sterile scalpel blade (Figure 2.2B). The cDNA was purified from the gel slice using a Bresa-Clean nucleic acid purification kit (Bresatec, Thebarton, SA, Australia) according to the manufacturer's instructions (Figure 2.2B).

2.2.5 PROBE LABELLING

The oligonucleotides were labelled using T4 polynucleotide kinase (7.9 $U.\mu l^{-1}$; Pharmacia, North Ryde, NSW, Australia) and γ-[^{32}P]ATP (4000 $Ci.mmol^{-1}$, Bresatec) according to the end labelling protocol for oligonucleotides outlined by Sambrook *et al.* (1989). Briefly, 3 µl of oligonucleotide (2 $pmoles.\mu l^{-1}$) was added to a sterile eppendorf tube, followed by 2 µl of 10 x One-Phor-All buffer (Pharmacia), 0.5 µl of sterile water, 13.5 µl of γ-[^{32}P]ATP, and 1 µl of T4 polynucleotide kinase (Pharmacia). The ingredients were mixed and allowed to incubate at 37°C for 1 h.

The cDNA probe was labelled by the random priming method with α-[^{32}P]dCTP (3000 $Ci.mmol^{-1}$, Bresatec) and Klenow fragment (6.4 $U.\mu l^{-1}$ Pharmacia) using an oligolabelling kit (Pharmacia) as per manufacturer's instructions. The labelling reaction was incubated for 60 min at 37°C.

For both the cDNA and oligoprobes, the labelled probes were separated from unincorporated radionucleotide by eluting them from NICK columns containing Sephadex G50 (Pharmacia) with Tris-EDTA buffer. The activities of the probes were determined by adding 2 µl of the labelled probe to 4 ml of aqueous counting scintillant (ACS, Amersham International, Buckinghamshire, UK) and counting the activity using a liquid scintillation β-counter (LS-3801, Beckman Instruments, Palo Alto, CA, USA).

2.2.6 NORTHERN BLOTTING

Total RNA samples (20 µg in 5.5 µl of sterile water) were denatured at 55°C for 10 min in 2.2 *M* formaldehyde, 50 % (v / v) formamide, and 1 x formaldehyde gel running buffer (5 x formaldehyde gel running buffer: 0.1 *M* 3-(*N*-morpholino)propane sulphonic acid, 40 m*M* sodium acetate, 5 m*M* EDTA, pH 7.0).

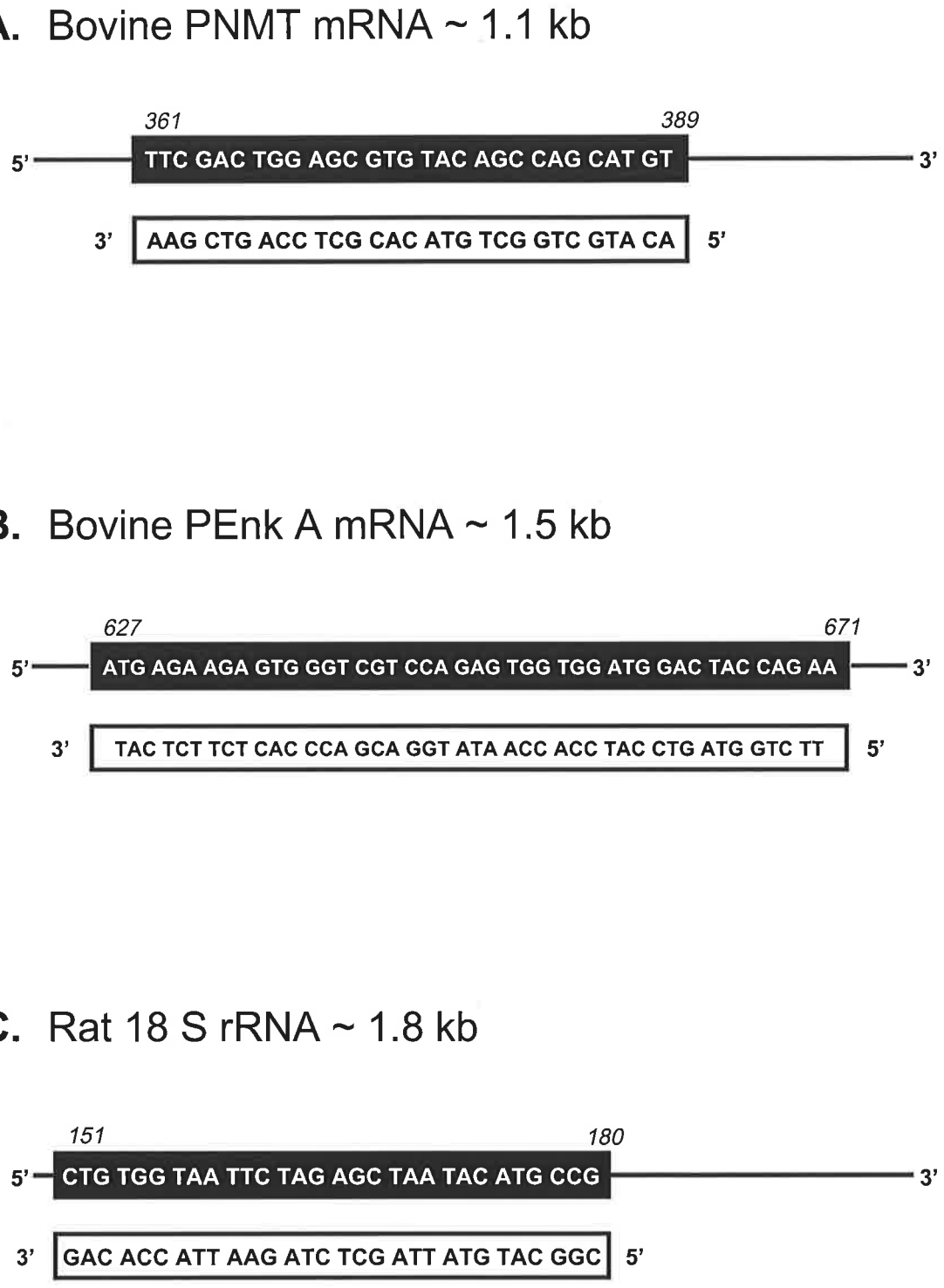
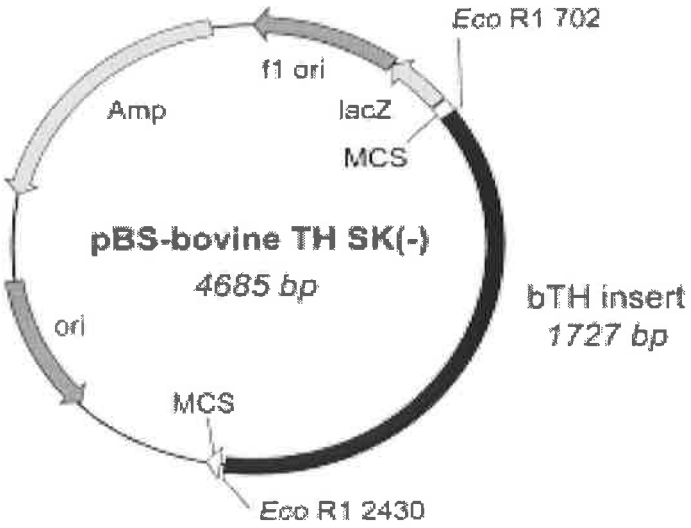


Figure 2.1 Target sequences of the bovine PNMT, PEnk A, and rat 18 S oligonucleotide probes

Schematic diagrams of the nucleotide sequences of the antisense oligonucleotides (open boxes) complementary for regions of bovine PNMT **(A)** and PEnk A mRNA **(B)** as well as rat 18 S rRNA **(C)** (solid boxes). Italicised numbers indicate the nucleotide number with respect to the start of the coding sequence.

A



B

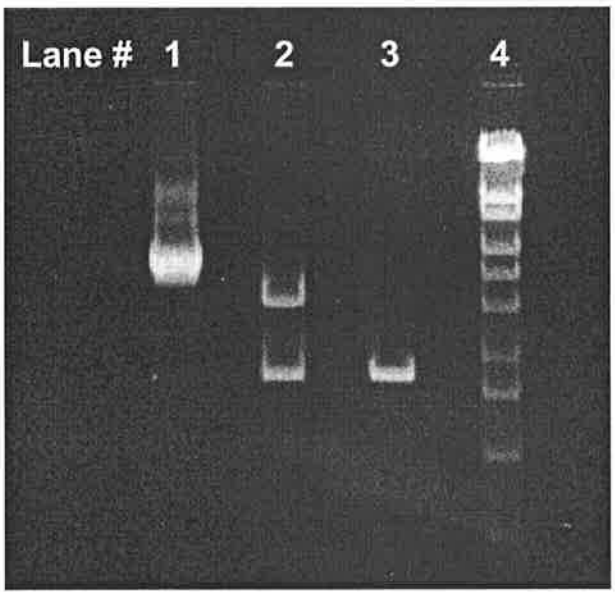


Figure 2.2 Isolation and purification of the TH cDNA probe from pBluescript II SK- phagemid vector

(A) Map of pBluescript II SK- phagemid vector with TH cDNA (solid black line) subcloned into the *Eco*RI region of the multiple cloning site (MCS).

(B) TH cDNA purification products following gel electrophoresis on a 1% Agarose TAE gel stained with ethidium bromide. Lane 1 : Purified plasmid vector DNA containing the TH cDNA subclone (4.68 kB) following extraction from DH5α *E. coli*. Lane 2: Separated pBluescript II SK- phagemid vector DNA (2.96 kB) and TH cDNA (1.73 kB) following *Eco*RI digestion. Lane 3: Purified TH cDNA (1.73 kB). Lane 4: λ DNA / *Eco*1301(*Sty* I) molecular weight markers.

After denaturation, 2 µl of loading buffer (50 % glycerol, 1 m*M* EDTA, 0.25 % bromophenol blue, and 0.25 % xylene cyanol) was added to the samples and total RNA was separated by electrophoresis on a denaturing gel containing 1 % agarose (BDH, Poole, England) and 2.2 *M* formaldehyde, using 1 x formaldehyde gel running buffer. Following gel electrophoresis, total RNA was transferred to Zetaprobe nylon membranes by capillary blotting (Biorad, Richmond, CA, USA) as described by Sambrook *et al.* (1989), using 10 x SSC (20 x SSC: 175.3 $g.l^{-1}$ NaCl, 88.2 $g.l^{-1}$ sodium citrate, pH 7.0) as the transfer buffer. Lanes containing RNA size markers (Millennium Markers; Ambion, Austin, TX, USA) were removed from the gels prior to total RNA transfer whereupon they were stained with ethidium bromide and photographed under UV light to allow for the verification of RNA transcript sizes. Membranes were washed in 10 x SSC for 10 min at RT before being baked for 1 h at 80°C and stored flat at -20°C until hybridisation.

2.2.7 MEMBRANE HYBRIDISATION

For oligonucleotide probes, membranes were prehybridised at 50°C for 18 h in a solution consisting of 5 x SSC, 20 m*M* NaH_2PO_4, pH 7.2, 7 % SDS, 5 x Denhardt's (50 x Denhardt's: 5 g Pharmacia Ficoll 400, 5 g polyvinylpyrollidone, 5 g bovine serum albumin) and 100 $\mu g.ml^{-1}$ of heat denatured salmon sperm DNA (Boheringer Mannheim). Membranes were hybridised with 1-2 x 10^6 $cpm.ml^{-1}$ of labelled oligonucleotide in 30 ml of fresh hybridisation solution for 20 h at 50°C. The same protocol was used for the TH cDNA probe, however the hybridisation solution consisted of 50 % (v / v) deionised formamide, 5 x SSPE (20 x SSPE: 175.3 $g.l^{-1}$ NaCl, 27.6 $g.l^{-1}$ $NaH_2PO_4.H_2O$, 7.4 $g.l^{-1}$ EDTA, pH 7.4), 7 % SDS, and 100 $\mu g.ml^{-1}$ of heat denatured salmon sperm DNA (Boehringer Mannheim), with hybridisation being carried out at 42°C. Following hybridisation the membranes were washed in 1 x SSC and 0.1 % SDS at their hybridisation temperatures (1 x 30 min). The membranes were then washed at their hybridisation temperature in 0.1 x SSC and 0.1 % SDS (2 x 30 min), briefly air dried, and exposed to a Fuji BAS-IIIs phosphorimager plate (Fuji Photo Film Co., Tokyo, Japan) for 24 - 48 h.

Autoradiographs were visualised using a Fuji BAS 1000 phosphorimager and quantitated using Fuji MacBAS software (Fuji Photo Film Co.).

2.2.8 DATA ANALYSIS

All data are presented as the mean ± standard error of the mean (SEM). The expressions of TH, PNMT, and PEnk A mRNA are presented as ratios of 18 S rRNA expression to correct for lane loading errors. Fetal growth, adrenal growth, total adrenal RNA yields and the ratios of TH, PNMT, and PEnk A mRNA : 18 S rRNA were compared amongst the different gestational age groups using one way ANOVA. A Fisher's post hoc test was subsequently used when significance was identified by ANOVA.

A probability of less than 5 % (ie. $P < 0.05$) was taken to be significant for all statistical analyses.

2.3 RESULTS

2.3.1 PROBE VALIDATION

Hybridisation of Northern blot membranes with the TH cDNA probe yielded a prominent band which migrated at around 1.8 kb (Figure 2.3A), consistent with the established size of bovine TH mRNA (D'Mello *et al.*, 1988). The PNMT and PEnk A antisense oligonucleotide probes hybridised to bands which migrated at approximately 1.1 and 1.5 kb respectively (Figures 2.3B & C), which is in agreement with the previously reported sizes for both bovine and ovine PNMT (Baetge *et al.*, 1986; Wan *et al.*, 1989*b*) and PEnk A mRNA transcripts (Jingami *et al.*, 1984; Wan *et al.*, 1987). The PNMT oligonucleotide probe also specifically labelled a second band of around 4.5 kb (not shown) which has also been reported by Wan and colleagues (Wan *et al.*, 1991*b*), and may represent a precursor RNA transcript for the mature PNMT mRNA. Only the signal intensity of the 1.1 kb band was measured in these studies to estimate PNMT mRNA expression. The 18 S antisense oligonucleotide probe hybridised to a single prominent band at 1.9 kb, which correlates well with the predicted size of rat 18 S rRNA (Chan *et al.*, 1984, Figure 2.3D). Autoradiographs of a Northern blot membrane onto which dilutions of fractionated total RNA from a 140 d fetal sheep adrenal had been transferred, demonstrated an increasing signal intensity for

each of the probes directly proportional to the amount of total RNA loaded (Figure 2.3).

2.3.2 FETAL OUTCOMES AND ADRENAL GROWTH WITH DEVELOPMENT

Fetal body weight significantly increased ($P < 0.0005$) with gestation in the age groupings studied (Table 2.1). Similarly, combined adrenal weight at 125 d was significantly greater ($P < 0.0005$) than at 80-100 d, and there was a further increase in adrenal weight at 140-146 d gestation (Table 2.1). The adrenal to body weight ratio however, was greatest at 80-100 d with a decline ($P < 0.0005$) in relative size occurring at 125 d, which was maintained at 140-146 d (Table 2.1). The average total RNA yield from the fetal adrenals was equivalent between the 80-100 d and 125 d gestational age groups but there was a significant increase ($P < 0.01$) in total adrenal RNA yield at 140-146 d gestation.

Table 2.1 Fetal weight, adrenal weight, and adrenal total RNA yield with development

Age Group	Fetal Weight (kg)	Combined Adrenal Weight (mg)	Adrenal Weight : Fetal Weight ($mg.kg^{-1}$)	Total Adrenal RNA Yield ($\mu g.mg^{-1}$)
80-100 d *(n = 6)*	0.64 ± 0.12^a	121 ± 10^a	201 ± 21^a	2.06 ± 0.12^a
125 d *(n = 6)*	2.73 ± 0.12^b	267 ± 19^b	98 ± 5^b	1.96 ± 0.12^a
140-146 d *(n = 6)*	5.01 ± 0.20^c	419 ± 27^c	84 ± 4^b	2.58 ± 0.12^b

Parameter values with different superscripts are significantly different ($P < 0.05$) from each other.

2.3.3 ONTOGENY OF ADRENAL TH, PNMT, AND PEnk A mRNA EXPRESSION

The ratio of adrenal TH mRNA : 18 S rRNA increased between 80-100 d (0.98 ± 0.13) and 125 d (1.40 ± 0.15, $P < 0.001$) (Figure 2.4A). After 140 d gestation, however, the adrenal TH mRNA : 18 S rRNA ratio was significantly lower (0.47 ± 0.05) than at either 80-100 d or at 125 d gestation (Figure 2.4A). The ontogenetic profile of adrenal PNMT mRNA differed from that of TH mRNA. The adrenal PNMT mRNA : 18 S rRNA ratio increased between 80-100 d (0.08 ± 0.01) and

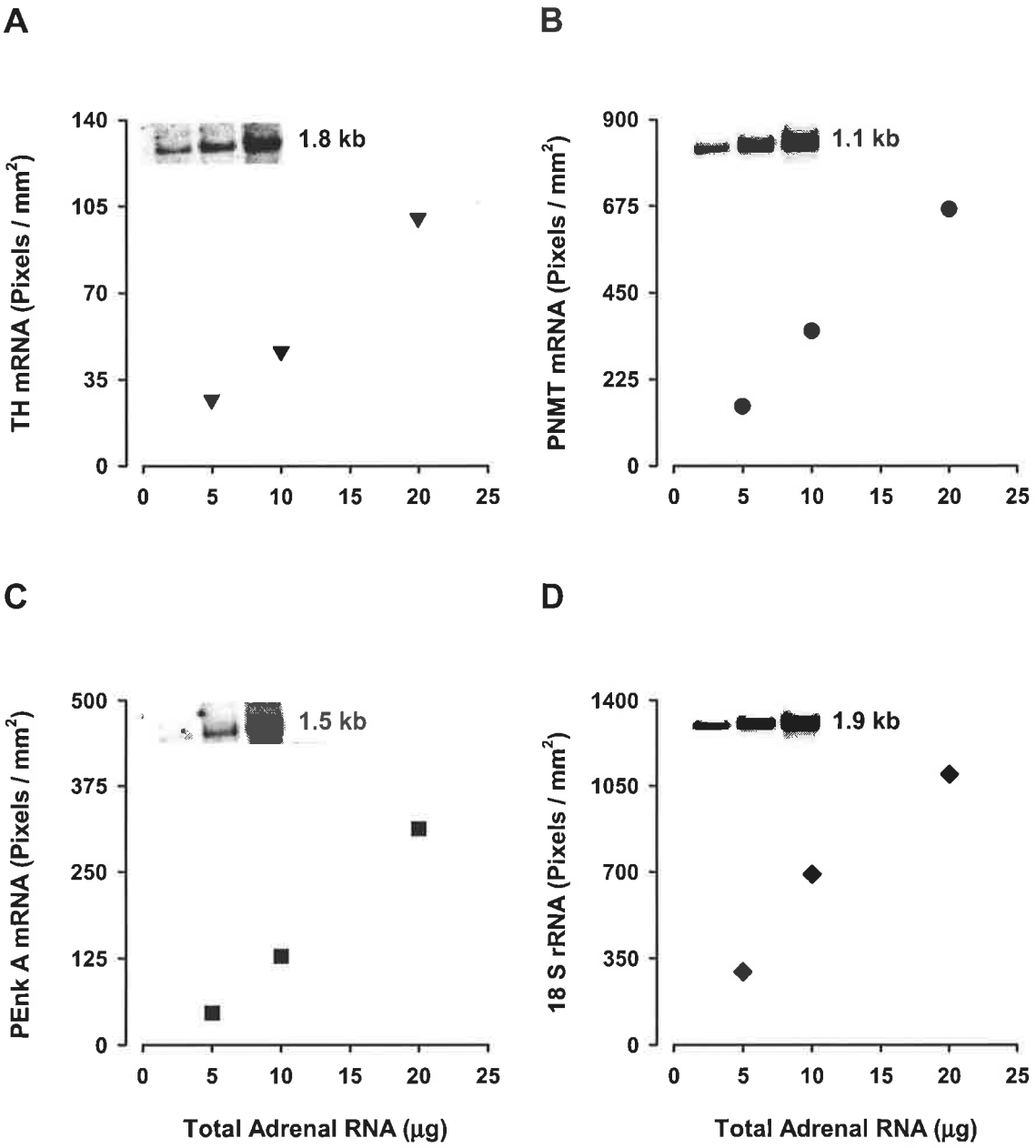


Figure 2.3 Validation of TH cDNA, PNMT, and PEnk A oligodeoxynucleotide probes

Autoradiographs (insets) and signal intensity values (Pixels / mm^2) from a Northern blot of total RNA obtained from the adrenal of a 140 d fetal sheep (5, 10, and 20 µg per lane respectively). The blots were hybridised with ^{32}P labelled (**A**) TH cDNA, (**B**) PNMT oligo, (**C**) PEnk A oligo, and (**D**) 18 S oligo probes. The size of the RNA transcripts are indicated alongside the autoradiographs.

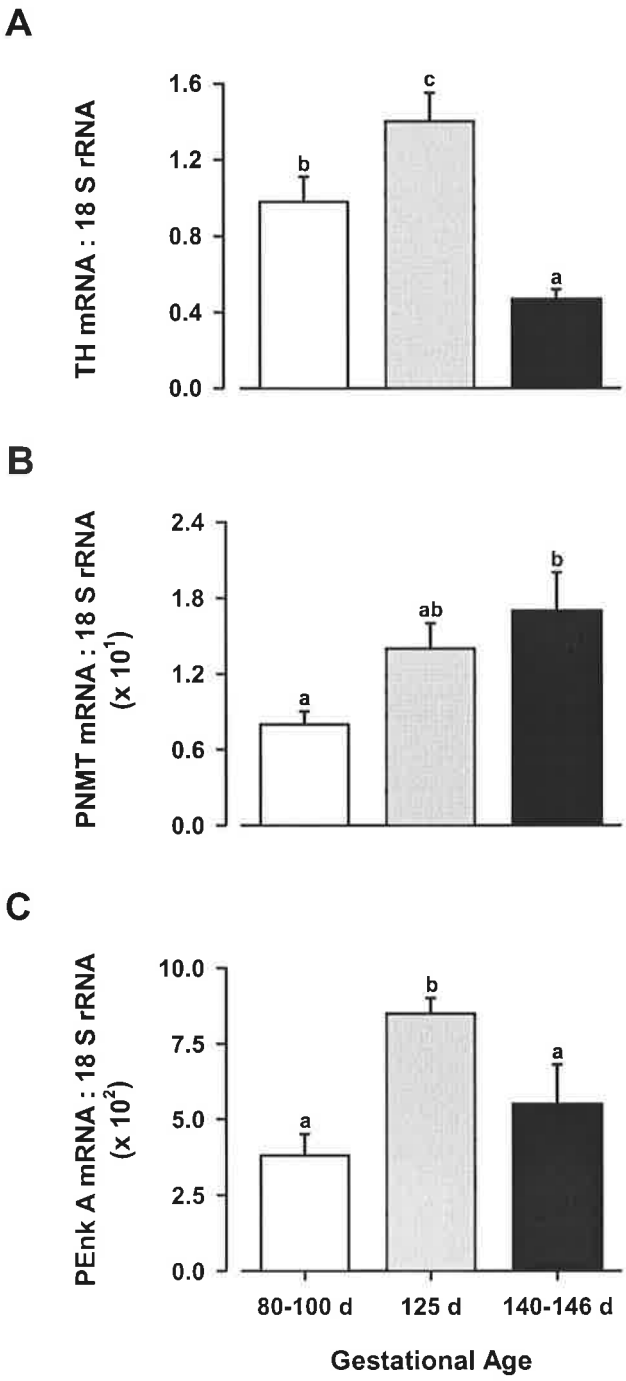


Figure 2.4 Ontogeny of TH, PNMT, and PEnk A mRNA expression in the fetal sheep adrenal
Gestational age comparison of adrenal TH mRNA (**A**), PNMT mRNA (**B**), and PEnk A mRNA (**C**) relative to 18 S rRNA (mean ± SEM) in fetal sheep aged 80-100 d (n = 6, white histograms), 125 d (n = 6, grey histograms), and 140-146 d (n = 6, black histograms). Significant differences [$P < 0.05$] between the mean ratios at different gestational ages are denoted by different superscript letters.

140-146 d gestation (0.17 ± 0.03, $P < 0.05$) (Figure 2.4B). The ratio of adrenal PEnk A mRNA : 18 S rRNA was higher ($P < 0.01$), however, at 125 d (0.085 ± 0.005) than at either 80-100 d (0.038 ± 0.007) or at 140-146 d gestation (0.055 ± 0.013) (Figure 2.4C).

2.4 DISCUSSION

The present study has found that there is a differential pattern of expression of catecholamine synthetic enzyme and PEnk A mRNA levels in the fetal sheep adrenal during late gestation. It was also established that the adrenal gland is proportionally larger in the 80-100 d gestation fetuses than it is in later gestation. This may reflect the importance of the fetal adrenal gland as a source of catecholamines when the peripheral sympathetic innervation is quite immature (Comline & Silver, 1966; Lebowitz *et al.*, 1972). Total adrenal RNA yield was significantly elevated in the 140-146 d gestation fetal sheep, coincident with the major phase of adrenocortical growth which is associated with both cellular hypertrophy and hyperplasia (Boshier & Holloway, 1989*b*).

In the present study adrenal TH mRNA levels increased between 100 and 125 d gestation and then significantly decreased after 140 d gestation. The increase in TH mRNA expression at 125 d is coincident with the onset of splanchnic innervation of the sheep adrenal. Ultrastructural studies have shown that synapses between splanchnic nerve terminals and adrenal chromaffin cells develop in the fetal sheep adrenal between 100 and 130 days gestation (Boshier *et al.*, 1989*a*) and there is evidence that functional splanchnic innervation of the adrenal develops around 120-130 days gestation (Cheung, 1990; Comline & Silver, 1961). It is possible that the onset of adrenal innervation is associated with a related stimulation of TH mRNA expression. There is evidence from studies in the adult rat and in bovine adrenomedullary cells that TH mRNA levels are increased after trans-synaptic or cholinergic stimulation and membrane depolarisation (Biguet *et al.*, 1986; Faucon Biguet *et al.*, 1991; Fossom *et al.*, 1991*b;* Hiremagalur *et al.*, 1993; Schalling *et al.*, 1988; Stachowiak *et al.*, 1994; Stachowiak *et al.*, 1990*b*). It is interesting that the increase in adrenomedullary TH mRNA expression at 125 d gestation is relatively transient in that there is a

subsequent decrease in adrenal TH mRNA levels in the week before delivery. We have previously shown that there is a decrease in the noradrenaline secretory response of the fetal sheep adrenal to sub-maximal doses of Acetylcholine (ACh) at 144 d compared with earlier in gestation and that this is not associated with a decrease in the size of the releasable pool of noradrenaline in the fetal adrenal (Butler *et al.*, 1995). One possible explanation for the parallel decrease in TH mRNA and adrenal sensitivity to ACh may be that the full establishment of functional innervation of the fetal adrenal in late gestation is associated with an absolute increase in the resting membrane potential, ie a relative hyperpolarisation of the noradrenaline containing cells.

Adrenal PEnk A mRNA levels also significantly increased at 125 d gestation, although there was no significant decline in the mRNA levels for the opioid peptide precursor after 140 d gestation. A range of studies in bovine adrenomedullary cells in culture have shown that PEnk A mRNA levels are increased in response to nicotinic stimulation and membrane depolarisation (Kley *et al.*, 1986; Kley *et al.*, 1987; Stachowiak *et al.*, 1990*c;* Wan *et al.*, 1991*b*) and it is therefore possible that the onset of splanchnic innervation stimulates a coordinated increase in PEnk A and TH mRNA levels. It is interesting that the pattern of PEnk A mRNA and Enk peptide localisation changes in the fetal sheep adrenal at around 125 d gestation, from localisation within both noradrenaline and adrenaline cells to the adult pattern of localisation of Enk-containing peptides predominantly within adrenaline cells (McMillen *et al.*, 1988; Wan *et al.*, 1989*b*). We have previously demonstrated that the change in Enk localisation in the sheep adrenal is dependent on an intact fetal pituitary-thyroid axis and have suggested that splanchnic innervation of the fetal adrenal may also be dependent on fetal thyroid status (Coulter *et al.*, 1989*a;* Simonetta *et al.*, 1996*c*). It appears that neural or hormonal events at around 125 d gestation produce both transient and long term changes in Enk gene expression and localisation within the sheep adrenal.

In contrast to TH mRNA, adrenal PNMT mRNA levels were highest after 140 days gestation than at any earlier gestational age studied. This gestational increase in PNMT mRNA levels is coincident with an increase in adrenal adrenaline content after 130 days gestation (Comline & Silver, 1961), an increase in the basal output

of adrenaline from the perfused adrenal preparation and in circulating adrenaline concentrations in the sheep fetus after 135 days gestation (Butler *et al.*, 1995; Simonetta *et al.*, 1993). It is likely that the prepartum increases in adrenal PNMT mRNA levels and adrenaline synthesis are directly related to the increase in adrenal corticosteroidogenesis which occurs in the last 10-15 d of gestation in the sheep (Challis & Brooks, 1989). It has previously been established in the fetal and neonatal rat that whilst the emergence of the adrenergic cell phenotype is not dependent on glucocorticoids (Bohn *et al.*, 1981) the subsequent maintenance of adrenaline synthesis is glucocorticoid dependent. Glucocorticoids have been shown to stimulate an increase in PNMT mRNA levels in bovine adrenomedullary cells in culture (Stachowiak *et al.*, 1990*d;* Wan & Livett, 1989), probably through an action at the glucocorticoid response element present in the 5' regulatory region of the PNMT gene (Ross *et al.*, 1990).

It is clear, that whatever the precise neural and hormonal factors involved in the control of catecholamine and opioid peptide biosynthesis, there is a differential regulation of the gene expression of TH, PNMT and PEnk A within the adrenal in late gestation in sheep.

3. IMPACT OF ACUTE HYPOXAEMIA ON THE EXPRESSION OF TH AND PNMT mRNA IN THE ADRENAL GLAND OF THE FETAL SHEEP

3.1 INTRODUCTION

In Chapter 2 it was found that the ontogenetic changes in adrenal TH and PNMT mRNA expression coincide with the development of functional splanchnic innervation and activation of the HPA axis. In the adult, both splanchnic innervation and the HPA axis play major roles in the regulation of catecholamine biosynthesis during stress (Fluharty *et al.*, 1985; Kvetnansky *et al.*, 1995; Ungar & Phillips, 1983). The secretion of catecholamines from the mature adrenal medulla in response to stress is predominantly stimulated by reflex activation of the splanchnic nerve (Biesold *et al.*, 1989; Lee *et al.*, 1987; Seidler & Slotkin, 1986*b*). It is well established that there is a functional coupling of catecholamine secretion and synthesis in the mature adrenal gland which aids in the maintenance of catecholamine stores in the face of increased secretory demands (Bygdeman & von Euler, 1958; Wakade *et al.*, 1988; Weiner, 1975). The stimulation-secretion-synthesis coupling of catecholamines within the chromaffin cells of the mature adrenal medulla is mediated by trans-synaptic events which occur in response to splanchnic nerve activation. Trans-synaptic stimulation of the adrenal medulla increases catecholamine synthetic enzyme levels, activity, and gene expression (Fluharty *et al.*, 1983; Fluharty *et al.*, 1985; Wong *et al.*, 1993; Wong & Wang, 1994).

Fully functional innervation of the fetal sheep adrenal gland by the preganglionic, cholinergic, splanchnic nerve does not develop until around 120 d gestation (Cheung, 1990; Comline & Silver, 1961). It is clear however, that the immature adrenal medulla of the sheep and a number of other species is capable of secreting catecholamines in response to hypoxia by a direct or non-neurogenic mechanism, prior to functional splanchnic innervation of the adrenal gland (Cheung, 1990; Comline & Silver, 1966; Seidler & Slotkin, 1986*b;* Slotkin & Seidler, 1988). It has yet to be determined if there is also an associated catecholamine synthesis-secretion coupling present in the immature fetal adrenal for non-neurogenic stimulation. The cellular mechanisms underlying the non-neurogenic secretion of catecholamines from the fetal and neonatal adrenal in

response to hypoxia have only recently been examined (Adams *et al.*, 1996; Rychkov *et al.*, 1998; Thompson *et al.*, 1997). Both neurogenic and non-neurogenic catecholamine secretion appear to share common mechanistic elements including membrane depolarisation and the influx of intracellular calcium into the cell via voltage sensitive Ca^{2+} channels (Adams *et al.*, 1996; Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997). In chromaffin cells obtained from mature bovine adrenal glands, cellular depolarisation and increased intracellular Ca^{2+} levels have each been demonstrated to stimulate catecholamine synthetic enzyme activity and the expression of TH and PNMT mRNA (Stachowiak *et al.*, 1994; Stachowiak *et al.*, 1990*c;* Wan *et al.*, 1991*b*).

Given the apparent similarities between the stimulation of catecholamine secretion by both neurogenic and non-neurogenic pathways it is hypothesised that there will also be changes in catecholamine synthesis associated with non-neurogenic catecholamine secretion. In order to examine this hypothesis the current study examined the effect of acute hypoxaemia upon the time course of expression of TH and PNMT mRNA in the adrenals of the fetal sheep both before and after the development of functional splanchnic innervation. In order to determine the relative contribution of the splanchnic nerve to catecholamine synthetic enzyme gene expression, adrenal TH and PNMT mRNA expression following acute hypoxia was also determined in fetal sheep in which cholinergic transmission was blocked by the nicotinic receptor antagonist, hexamethonium.

3.2 MATERIALS AND METHODS

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation.

3.2.1 ANIMALS AND SURGERY

Fifty two fetal sheep from timed pregnant Border Leicester x Merino ewes were used in this study. Surgery was performed on the ewes at either 88-92 d or 116-120 d gestation (calculated according to date of mating) under general anaesthesia induced by intravenous injection of sodium thiopentone (1.25 g; Pentothal, Rhone Merieux, Pinkenba, QLD, Australia) and maintained with halothane (3-4 % in oxygen; Fluothane, ICI, Melbourne, VIC, Australia). Ewes

were administered 2 ml of antibiotics (250 $mg.ml^{-1}$ procaine penicillin, 250 $mg.ml^{-1}$ dihydrostreptomycin sulphate, 20 $mg.ml^{-1}$ Procaine Hydrochloride; Penstrep Ilium, Troy Laboratories, Smithfield, NSW, Australia) intramuscularly, prior to surgery.

During surgery the uterus was exposed by a midline laparotomy incision and the fetal head was palpated. The fetal head and neck were then delivered through an incision in the uterine wall and the fetal membranes. Single lumen polyvinyl catheters (Critchley Electrical Products, Silverwater, NSW, Australia) were inserted into the fetal carotid artery and external jugular vein (outer diameter, 1.52 mm; inner diameter, 0.86 mm) and the amniotic sac (outer diameter, 2.70 mm; inner diameter, 1.50 mm) essentially as described by Dawes *et al.* (1972). The fetus was also administered 2 ml of antibiotics (Penstrep Ilium, Troy Laboratories) intramuscularly during surgery. The fetal head was then replaced and the uterus and fetal membranes were closed in a single layer using catgut (2 / 0; Ethicon, Johnson and Johnson, Sydney, NSW, Australia). A layer of inverting sutures (2 / 0 catgut, Ethicon, Johnson and Johnson) was subsequently placed in the myometrium. The maternal abdomen was closed in two layers, the peritoneum and rectus sheet followed by the skin and subcutaneous tissue, using catgut (3 / 0; Ethicon, Johnson and Johnson) and vetafil synthetic suture (0.3 mm; WDT, Garbsen, Germany) respectively. A catheter (outer diameter, 2.70 mm; inner diameter, 1.50 mm) was also inserted into the maternal external jugular vein. The catheters were flushed and filled with heparinised saline (500 $IU.ml^{-1}$; Multiparin, Fisons Pharmaceuticals, Sydney, NSW, Australia) and the fetal catheters were exteriorised through an incision in the ewe's flank.

3.2.2 POST OPERATIVE CARE

Ewes were housed in individual metabolic crates in animal holding rooms under a 12 h light-dark cycle and fed lucerne chaff once daily between 0900 and 1300 h with water ad libitum. Sodium ampicillin (500 mg, Austrapen, Commonwealth Serum Laboratories, Melbourne, VIC, Australia) was administered intra-amniotically to all fetal sheep for 4 consecutive days post operatively. A post operative recovery of at least a week was allowed before any experiment was conducted.

3.2.3 FETAL HYPOXIA

Separate groups of fetuses at 96-105 d (n = 15) and 129-144 d (n = 15) were subjected to 30 min of hypoxia. Fetal hypoxia was achieved in this study by altering the maternal F_iO_2. Following a 60 min baseline period a large clear polypropylene bag was tied loosely over the ewe's head and a mixture of air (39 %), N_2 (58 %), and CO_2 (3 %) was infused into the bag at a flow rate of 58 $l.min^{-1}$ (Figure 3.1). The gas mixture was titrated over the 30 min experimental period by adjusting the balance of air and nitrogen to maintain fetal S_aO_2 at approximately 50 % of baseline values. The normoxia controls for the hypoxia experiment consisted of 96-105 d (n = 5) and 129-144 d (n = 5) fetuses for which the pregnant ewes were administered a gas mixture which consisted of air (97 %) and CO_2 (3 %) at a flow rate of 58 $l.min^{-1}$ as outlined for the hypoxia experiment.

3.2.4 HEXAMETHONIUM

In a separate group of 129-144 d (n = 12) fetuses hexamethonium (hexamethonium bromide dihydrate; ICN Biomedicals, Cleveland, OH, USA) was administered by a 3 ml intravenous bolus (25 $mg.kg^{-1}$ in 0.9 % saline). The dose of hexamethonium used in this study has previously been demonstrated to produce a blockade of sympathetic function which persists for several hours in the fetal sheep (Brace & Brittingham, 1986). Hexamethonium was administered at -30 min relative to fetal exposure to either hypoxia (n = 6) or normoxia (n = 6).

3.2.5 BLOOD SAMPLING

Fetal arterial blood samples (0.5 ml) were collected once daily on the first four days following surgery and every second day thereafter for measurement of fetal blood gases and pH using an ABL 520 blood gas analyser (Radiometer, Copenhagen, Denmark) to assess fetal well being. During an experiment, 3.5 ml samples of fetal arterial blood were obtained at the following time points relative to the onset of maternal F_iO_2 regulation; -60, -35, -25, -5, 5, 10, 30, and 60 min. An equivalent volume of isotonic saline was infused into the fetus following the withdrawal of each sample. A fetal arterial blood sample (0.5 ml) was used for blood gas analysis as outlined above, whilst the remaining 3 ml was placed into

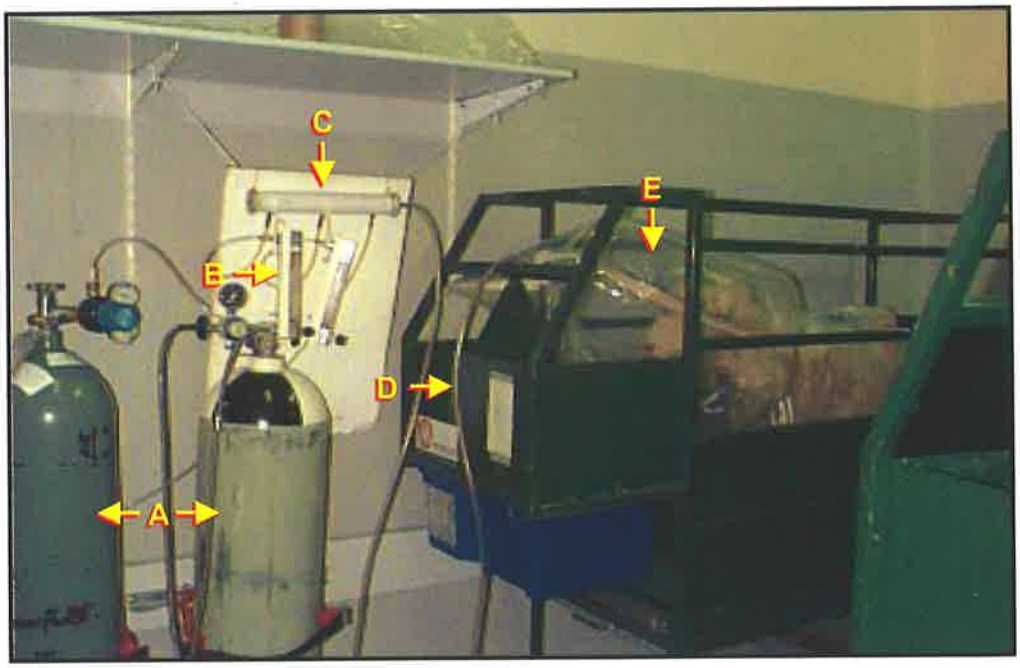


Figure 3.1 Experimental set up for the regulation maternal F_iO_2

Maternal F_iO_2 was regulated by the infusion of a gas mixture consisting of nitrogen, air, and carbon dioxide. Individual gases (A), the proportions of which could be controlled by flow regulators (B), entered a mixing chamber (C) and the mixture was infused via a hose (D) into a plastic bag (E) placed over the Ewe's head.

ice chilled heparinised tubes (125 U, Disposable Products, Adelaide, SA, Australia). The tubes were spun at 1800 *g* for 10 min at 4°C and the plasma was subsequently separated and transferred to polypropylene tubes whereupon they were snap frozen in liquid N_2. Plasma samples were stored at -20°C until assayed for catecholamine content.

3.2.6 POST MORTEM AND TISSUE COLLECTION

The ewes were sacrificed with an overdose of sodium pentobarbitone (8.125 g; Lethabarb, Virbac, Peakhurst, NSW, Australia) and the fetal sheep were delivered via laparotomy and killed by decapitation. The adrenal glands were dissected from each fetus, weighed, snap frozen in liquid N_2, and stored at -70°C until total RNA was extracted.

Adrenals were collected from fetuses sacrificed at 3-5 h (96-105 d, n = 5; 129-144 d, n = 5), 12 h (96-105 d, n = 5; 129-144 d, n = 5), and 20 h (96-105 d, n = 5; 129-144 d, n = 5) after the onset of hypoxia. Adrenals were collected from the fetal sheep in the control group (96-105 d, n = 5; 129-144 d, n = 5) following the onset of experimental normoxia over the range of time points described for the hypoxia group. For the hexamethonium treated fetuses adrenals were collected from fetuses 3-5 h after the onset of either experimental normoxia (n = 6) or hypoxia (n = 6).

3.2.7 PLASMA CATECHOLAMINE EXTRACTION

Prior to extraction, plasma samples collected at -25 and -5 min (basal) as well as the 5 and 10 min (hypoxia) samples were combined in equivalent portions for catecholamine measurement. The maximal rise in plasma catecholamines in the fetal sheep in response to acute hypoxia has been established within our laboratory to occur within the first 10 min of hypoxia (Simonetta *et al.*, 1996*a*). With the hexamethonium treated fetuses, the plasma samples collected at -60 and -35 min were also combined before extraction for the determination of basal catecholamine levels prior to the addition of hexamethonium.

Plasma catecholamines were partially purified by alumina absorption using a BAS plasma catecholamine kit (MF-9017; Bioanalytical Systems, West Lafayette, IN, USA) essentially as per the manufacturer's instructions with minor modifications.

Briefly 500 µl of fetal plasma was used and made up to a volume of 2 ml with distilled water and the sample pre treatment step was omitted. 3,4-dihydroxybenzylamine (DHBA; Bioanalytical Systems) was added to each sample (50 µl at 80 pmol.l^{-1} in 0.1 *M* perchloric acid) as an internal standard to monitor the recovery of catecholamines from plasma. The efficiency of catecholamine recovery averaged 67.4 ± 1.8 %.

3.2.8 CATECHOLAMINE MEASUREMENT

50 µl aliquots of the extracted samples were injected onto a BAS 200A HPLC system (Bioanalytical Systems) using a refrigerated microsampler (CMA / Microdialysis, Stockholm, Sweden) at 4°C. Separation of the catecholamines was achieved using a 3 µm ODS, 100 x 3.2 mm, Brownlee Velosep RP-18 cartridge column (Perkin Elmer, Norwalk, CT, USA) and MP-2 catecholamine mobile phase (Bioanalytical Systems). The column temperature was maintained at 40°C and the mobile phase flow rate was 1 ml.min^{-1}. The potential of the glassy carbon electrode was set at + 700 mV *vs.* a Ag / AgCl reference electrode whilst the gain was set to 0.5 nA. Catecholamines were automatically quantified by peak height integration using an IBM compatible personal computer equipped with Barspec Data System chromatographic software (Barspec, Rehovot, Israel). Peak heights referenced to standard curves generated for authentic catecholamine standards (norepinephrine bitartrate, epinephrine bitartrate, and DHBA hydrobromide; Bioanalytical Systems; 0.3125-20 nmol.l^{-1} in 0.1 *M* PCA; Figure 3.2) to determine the catecholamine content of the samples. The sensitivity of the assay was approximately 0.05 pmol.ml^{-1} and the intra- and inter-assay coefficients of variation (CVs) were less than 10 % for both noradrenaline and adrenaline.

3.2.9 TOTAL RNA EXTRACTION

Total adrenal RNA was extracted from each adrenal using Tri-reagent (Sigma Chemical Co., St. Louis, MO, USA), a proprietary version of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction (Chomczynski, 1993; Chomczynski & Sacchi, 1987). Briefly, adrenals were homogenised separately in 1 ml of Tri-reagent using a Polytron PT3000 homogeniser (Kinematica, Littau, Switzerland) at 30 000 rpm. The homogenate

was transferred to a sterile 1.5 ml eppendorf tube and bromo-3-chloropropane (0.1 ml) was added to the homogenate. The samples were then mixed and allowed to stand at room temperature (RT) for 5 min. The samples were centrifuged at 12 000 *g* for 10 min at 4°C and the upper aqueous phase was transferred to a separate sterile eppendorf tube. Total RNA was precipitated from the aqueous phase by adding isopropanol (0.5 ml) and allowing the samples to stand at RT. After 10 min, the samples were centrifuged (12 000 *g* for 10 min at 4°C) and the supernatant was subsequently removed. The RNA pellets were washed by adding 1 ml of 75 % ethanol and centrifuging at 12 000 *g* for 10 min at 4°C. Following the removal of the ethanol solution the RNA pellets were air dried and resuspended in an appropriate volume of sterile water. Nucleic acid concentration, yield, and purity were quantified by spectrophotometric measurement at 260 and 280 nm. Total RNA solutions were stored at -70°C prior to Northern blot analysis.

3.2.10 PROBES AND PROBE LABELLING

The probes utilised in this study and the radiolabelling methods for the probes are described in detail in chapter 2. To summarise, oligonucleotide antisense probes complementary to nucleotides 361-389 of the peptide coding region of bovine PNMT mRNA (29 mer, Wan *et al.*, 1989*b*), nucleotides 627-671 of the peptide coding region of bovine PEnk A mRNA (44 mer, Wan *et al.*, 1987), and nucleotides 151-180 of rat 18 S rRNA (30 mer, Chan *et al.*, 1984) sequence were synthesised (Bresatec, Thebarton, SA, Australia). A full length (1.73 kb) bovine TH cDNA (D'Mello *et al.*, 1988) was used to detect TH mRNA. The oligonucleotides were end labelled using T4 polynucleotide kinase 7.9 $U.\mu l^{-1}$, Pharmacia, North Ryde, NSW, Australia) and γ-[^{32}P]ATP (4000 $Ci.mmol^{-1}$, Bresatec) in accordance with the method described for the end labelling of oligonucleotides by Sambrook *et al.* (1989). The cDNA probe was labelled by the random priming method with α-[^{32}P]dCTP (3000 $Ci.mmol^{-1}$, Bresatec) and Klenow fragment (6.4 $U.\mu l^{-1}$) using an oligolabelling kit (Pharmacia) as per manufacturer's instructions.

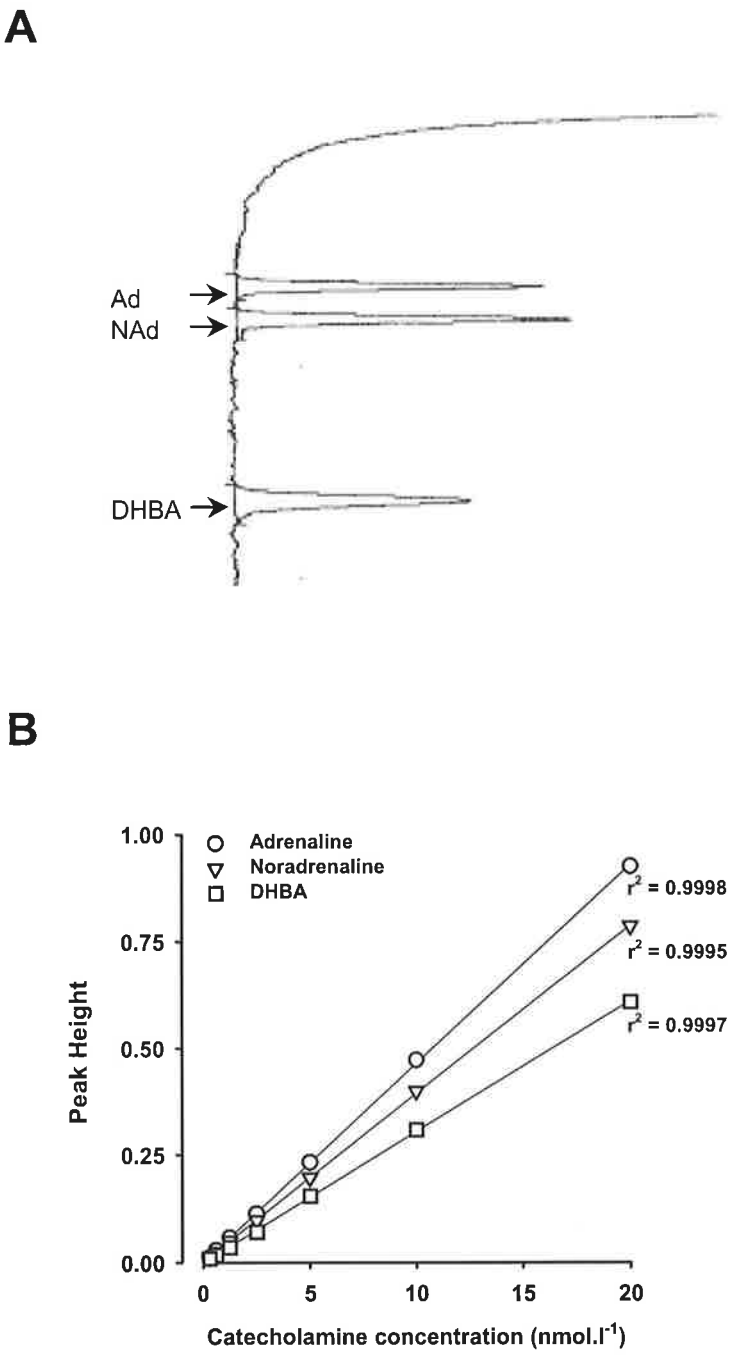


Figure 3.2 Example of catecholamine calibration curves generated using reverse phase HPLC coupled with ECD

(A) HPLC chromatogram of aqueous catecholamine standard solution containing adrenaline (Ad), noradrenaline (NAd), and DHBA (10 nmol.l^{-1}).

(B) Catecholamine standard curves generated for adrenaline (circles), noradrenaline (triangles), and DHBA (squares). 50 µl of aqueous catecholamine standard (0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 nmol.l^{-1}, in 0.1 M perchloric acid) was separated and analysed using reverse phase HPLC coupled with ECD as outlined in section 3.2.8. The peak height obtained was plotted against the concentration of catecholamine. The solid lines represent the lines of best fit derived by linear regression analysis with r^2 values indicated.

3.2.11 NORTHERN BLOT ANALYSIS

For each fetus, 20 μg of total adrenal RNA was denatured in 2.2 M formaldehyde and 50 % (v / v) formamide at 55°C for 10 min. Separation of the total adrenal RNA was achieved by electrophoresis on a 1 % agarose gel containing 2.2 *M* formaldehyde as described in detail in chapter 2. The separated total adrenal RNA was subsequently transferred to zetaprobe nylon membranes (Biorad, Richmond, CA, USA) via downward capillary transfer as described by Chomczynski & Mackey (1994) using 10 x SSC as the transfer buffer. Following transfer the membranes were washed in 10 x SSC for 10 min at room temperature and baked for 1 h at 80°C to fix the RNA to the membrane.

Prior to hybridisation with oligonucleotide probes the membranes were initially prehybridised at 50°C for 18 h in a solution consisting of 5 x SSC, 20 m*M* NaH_2PO_4, pH 7.2, 7% SDS, 5 x Denhardt's, and 100 $\mu g.ml^{-1}$ of heat denatured Salmon sperm DNA. Membranes were then hybridised for 20 h at 50 C in 15 ml of fresh hybridisation solution containing 1-2 x 10^6 $cpm.ml^{-1}$ of labelled oligonucleotide. For the TH cDNA probe equivalent prehybridisation and hybridisation times were used, however, the hybridisation solution consisted of 50 % (v / v) deionised formamide, 5 x SSPE, 7 % SDS, pH 7.2, and 100 $\mu g.ml^{-1}$ of heat denatured salmon sperm DNA, with hybridisation being carried out at 42°C in the presence of 1-2 x 10^6 $cpm.ml^{-1}$ of labelled probe.

Following hybridisation the membranes were washed in 1 x SSC and 0.1 % SDS (1 x 30 min) and then subsequently in 0.1 x SSC and 0.1 % SDS (1 x 30 min) at their hybridisation temperatures before being briefly air dried and exposed to a Fuji BAS-IIIs Phosphorimager Plate (Fuji Photo Co., Tokyo, Japan) for 24-48 h. Autoradiographs were obtained using a Fuji BAS 1000 Phosphorimager (Fuji Photo Co.) and quantitated using Fuji MacBAS software (Fuji Photo Co.)

3.2.12 STATISTICAL ANALYSIS

All data are presented as the mean ± SEM. Levene's tests for homogeneity of variance were conducted and logarithmic transformation of data carried out if appropriate. Blood gas variables over the time course of an experiment were examined within treatment groups using one way ANOVA with repeated

measures. Fisher's Post Hoc tests were used to identify significant differences between mean values. Unpaired one tailed Student's t-tests were used to compare the percentage changes of fetal plasma catecholamines from baseline levels in both the normoxia and hypoxia experiments. One way ANOVAs with Fisher's Post Hoc tests were used to compare the ratios of adrenal PNMT and TH mRNA : 18 S rRNA expression at different time points and in the hexamethonium experiments. Polynomial regression analyses were used to determine relationships of fetal adrenal PNMT mRNA and TH mRNA : 18 S rRNA expression with the experimentally induced changes in fetal arterial PO_2 during normoxia and hypoxia. Data from the hypoxia groups at the time points for which a significant change in adrenal mRNA expression first occurred were used for the regression analyses.

A probability of less than 5 % (ie. $P < 0.05$) was taken to be significant.

3.3 RESULTS

3.3.1 FETAL ARTERIAL BLOOD GASES AND EXPERIMENTAL HYPOXIA

3.3.1.1 Arterial PO_2

With experimental normoxia there were no significant changes in fetal arterial PO_2 from baseline values for either the 96-105 d (Figure 3.3A) or 129-144 d (Figure 3.3B) fetal sheep. With experimental hypoxia there were immediate and sustained reductions ($P < 0.0005$) in fetal arterial PO_2 from basal values for both the 96-105 d (41.9 ± 2.1 %, n = 15, Figure 3.3A) and 129-144 d (37.5 ± 1.8 %, n = 15, Figure 3.3B) fetal sheep. Fetal arterial PO_2 recovered to baseline values by 30 min after the cessation of hypoxia in both age groups (Figures 3.3A, B).

Hexamethonium administration at -30 min had no significant impact upon basal arterial PO_2 values in the 129-144 d fetuses (Figure 3.3B). There was no change in arterial PO_2 values with experimental normoxia in the hexamethonium treated fetuses (Figure 3.3B). The reduction in arterial PO_2 which occurred in response to experimental hypoxia was similar in the hexamethonium treated fetuses (41.5 ± 1.3 %, n = 6) to that observed in the absence of hexamethonium (Figure 3.3B).

There was also a recovery of arterial PO_2 levels by 30 min after the end of the hypoxia period in the hexamethonium treated fetuses (Figure 3.3B).

3.3.1.2 Arterial PCO_2

Fetal arterial PCO_2 remained constant with experimental normoxia in the 96-105 d fetuses (Figure 3.3C) in this age group there was a moderate but significant ($P <$ 0.0005) decline in fetal arterial PCO_2 from baseline levels (46.0 ± 0.7 mmHg) at 10 min (42.0 ± 1.1 mmHg) and 30 min (41.0 ± 1.0 mmHg) of hypoxia, which returned to baseline levels after 30 min of recovery (Figure 3.3C). In the 129-144 d fetuses there were no significant alterations in fetal arterial PCO_2 from baseline values with either experimental normoxia or hypoxia (Figure 3.3D).

Arterial PCO_2 levels in 129-144 d fetuses was unchanged by ganglionic blockade with hexamethonium at -30 min (Figure 3.3D). Furthermore, there were no significant effects of either experimental normoxia or hypoxia upon arterial PCO_2 levels in the hexamethonium treated fetuses (Figure 3.3D).

3.3.1.3 Arterial pH

For the 96-105 d fetuses, arterial pH values did not significantly change from their baseline levels during either experimental hypoxia or normoxia (Figure 3.3E). In the 129-144 d fetuses there was also no change in arterial pH values during experimental normoxia but with hypoxia there was a moderate but significant ($P <$ 0.0005) fall from basal levels (7.389 ± 0.006) at 30 min (7.369 ± 0.004) which persisted into the recovery period (60 min, 7.360 ± 0.004) (Figure 3.3F).

Basal arterial pH values were unchanged in the 129-144 d fetuses by the administration of hexamethonium. Hexamethonium treated fetuses displayed a small reduction ($P <$ 0.0005) in arterial pH over the duration of experimental normoxia (baseline, 7.393 ± 0.003; minimum, 5 min, 7.372 ± 0.006), which recovered after the experimental period. There was no significant effect of experimental hypoxia on arterial pH in hexamethonium treated fetuses (Figure 3.3F).

3.3.2 FETAL PLASMA CATECHOLAMINES

3.3.2.1 Basal plasma catecholamine levels

In the 96-105 d fetal sheep, plasma adrenaline concentrations frequently fell below the sensitivity of the assay. Basal (-25 and -5 min) noradrenaline levels and adrenaline levels, when they were detectable, in the 96-105 d fetal sheep (NAd, 0.32 ± 0.09 nmol.l^{-1}, n = 14; Ad, 0.13 ± 0.04 nmol.l^{-1}; n = 6) were significantly less ($P < 0.05$) than those observed in the 129-144 d fetal sheep (NAd, 0.78 ± 0.11 nmol.l^{-1}; Ad, 0.36 ± 0.04 nmol.l^{-1}; n = 19). The ratios of the basal concentrations of plasma noradrenaline to adrenaline were highly variable between individual fetuses and no significant differences were established between the 96-105 d (4.37 ± 2.49, n = 5) and 129-144 d (2.47 ± 0.38, n = 19) fetuses.

In the 129-144 d fetuses hexamethonium administration at -30 min appeared to lower basal plasma noradrenaline (-60 and -35 min, 1.24 ± 0.23 nmol.l^{-1}; -25 and -5 min, 0.86 ± 0.18 nmol.l^{-1}) and adrenaline (-60 and -35 min, 2.97 ± 2.38 nmol.l^{-1}; -25 and -5 min, 0.15 ± 0.02 nmol.l^{-1}) levels, although this effect was not significant.

3.3.2.2 Impact of experimental hypoxia on plasma catecholamine levels

For 96-105 d fetuses the net change in plasma noradrenaline levels from baseline (-25 and -5 min) during hypoxia (5 and 10 min) was significantly greater ($P < 0.05$) than that which occurred during normoxia (normoxia, 0.09 ± 0.05 nmol.l^{-1}, n =4; hypoxia, 0.43 ± 0.11 nmol^{-1}, n = 10). In those experiments in which plasma adrenaline was measurable, the net change in plasma adrenaline levels from baseline during normoxia was 0.01 nmol.l^{-1} (n = 1), and 0.09 ± 0.03 nmol.l^{-1} (n = 5) during hypoxia.

At 129-144 d gestation, hypoxia (5 and 10 min) stimulated increases in both plasma noradrenaline and adrenaline levels above baseline (-25 and -5 min) which were significantly greater ($P < 0.05$) than that observed during experimental normoxia (Figures 3.4, 3.5A). In the presence of hexamethonium the increase in plasma noradrenaline levels during hypoxia above those observed during normoxia was abolished, whilst a significant ($P < 0.05$) plasma adrenaline response to hypoxia was still maintained (Figure 3.5B).

3.3.3 HYPOXIA AND CATECHOLAMINE SYNTHETIC ENZYME GENE EXPRESSION

3.3.3.1 TH mRNA expression

In the 96-105 d fetal sheep, hypoxia resulted in a significant decline ($P < 0.05$) in adrenal TH mRNA : 18 S rRNA expression at 20 h following exposure to hypoxia (control, n = 5, 0.58 ± 0.12; 20 h, n = 4, 0.23 ± 0.06) (Figure 3.7A). For the 129-144 d fetuses there was also a significant decrease ($P < 0.005$) in TH mRNA expression following exposure to acute hypoxia however, this decrease in expression was evident as early as 3-5 h (control, n = 5, 0.42 ± 0.09; 3-5 h, n = 5, 0.13 ± 0.03) and maintained at 12 h (n = 5, 0.10 ± 0.01) with a recovery to control levels by 20 h following exposure (Figures 3.6, 3.7B).

3.3.3.2 PNMT mRNA expression

In the 96-105 d fetal sheep, adrenal PNMT mRNA expression increased significantly ($P < 0.01$) at 3-5 h (control, 0.12 ± 0.05, n = 5; 3-5 h, 0.24 ± 0.03, n = 5), and 12 h (0.24 ± 0.03, n =5) following exposure to hypoxia with a recovery to basal levels at 20 h (Figure 3.7C). At 129-144 d gestation adrenal PNMT mRNA expression also increased significantly ($P < 0.005$) 3-5 h following exposure to hypoxia (control, 0.23 ± 0.05, n = 5; 3-5 h, 0.53 ± 0.07, n = 5). PNMT mRNA expression in the older fetuses however, recovered to control values by 12 h (Figures 3.6, 3.7D).

3.3.3.3 Relationships of adrenal TH and PNMT mRNA expression with experimentally induced changes in fetal arterial PO_2

Adrenal TH mRNA expression for the 96-105 d fetuses in the control and 20 h post hypoxia groups displayed a significant, linear relationship with the experimentally induced changes in arterial PO_2 (ΔPO_2) from baseline values (TH mRNA = $3.10*\Delta PO_2 + 54.12$, $r^2 = 0.55$, $P < 0.05$) (Figure 3.8A). In the 129-144 d fetuses the relationship of adrenal TH mRNA expression with ΔPO_2 in the control and 3-5 h post hypoxia groups was more complex (Figure 3.8A), displaying a cubic relationship (TH mRNA = $-0.48*\Delta PO_2^3 - 4.10*\Delta PO_2^2 + 8.25*\Delta PO_2 + 117.92$, $r^2 = 0.82$, $P < 0.05$). TH mRNA expression in the 129-144 d fetuses appeared to

be maximal at baseline PO_2 levels and decreased until a ΔPO_2 of around -7 mmHg, (Figure 3.8A).

In contrast to adrenal TH mRNA, adrenal PNMT mRNA expression at 96-105 d displayed a significant inverse relationship (PNMT mRNA = $-18.98*\Delta PO_2 + 202.87$, $r^2 = 0.42$, $P < 0.05$) with experimentally induced changes in fetal arterial PO_2 following normoxia or at 3-5 h post hypoxia (Figure 3.8B). A similar relationship was also observed for PNMT mRNA expression with ΔPO_2 (PNMT mRNA = $-9.42*\Delta PO_2 + 75.62$, $r^2 = 0.69$, $P < 0.01$) in the 129-144 d fetuses following normoxia or at 3-5 h post hypoxia (Figure 3.8B)

3.3.4 EFFECT OF HEXAMETHONIUM ON CATECHOLAMINE SYNTHETIC ENZYME GENE EXPRESSION IN RESPONSE TO HYPOXIA

3.3.4.1 TH mRNA expression

The administration of hexamethonium to 129-144 d fetuses resulted in a significant reduction ($P < 0.0005$) in TH mRNA expression during normoxia to levels similar to those observed 3-5 h after hypoxia in the absence of hexamethonium (Figure 3.9A). With the induction of hypoxia following hexamethonium administration, there was no further reduction in TH mRNA expression 3-5 h afterwards (Figure 3.9A).

There was no relationship between adrenal TH mRNA expression and experimentally induced changes in fetal arterial PO_2 at 3-5 h following hypoxia in 129-144 d fetuses treated with hexamethonium (Figure 3.9B).

3.3.4.2 PNMT mRNA expression

The addition of hexamethonium to 129-144 d fetuses did not have any significant effect on PNMT mRNA expression during normoxia when compared to the values observed with saline administration (Figure 3.9C). Whilst there was a significant increase ($P < 0.05$) in PNMT expression above normoxia values at 3-5 h after hypoxia in the saline infused fetuses, the response was variable and non-significant in hexamethonium treated fetuses (Figure 3.9C).

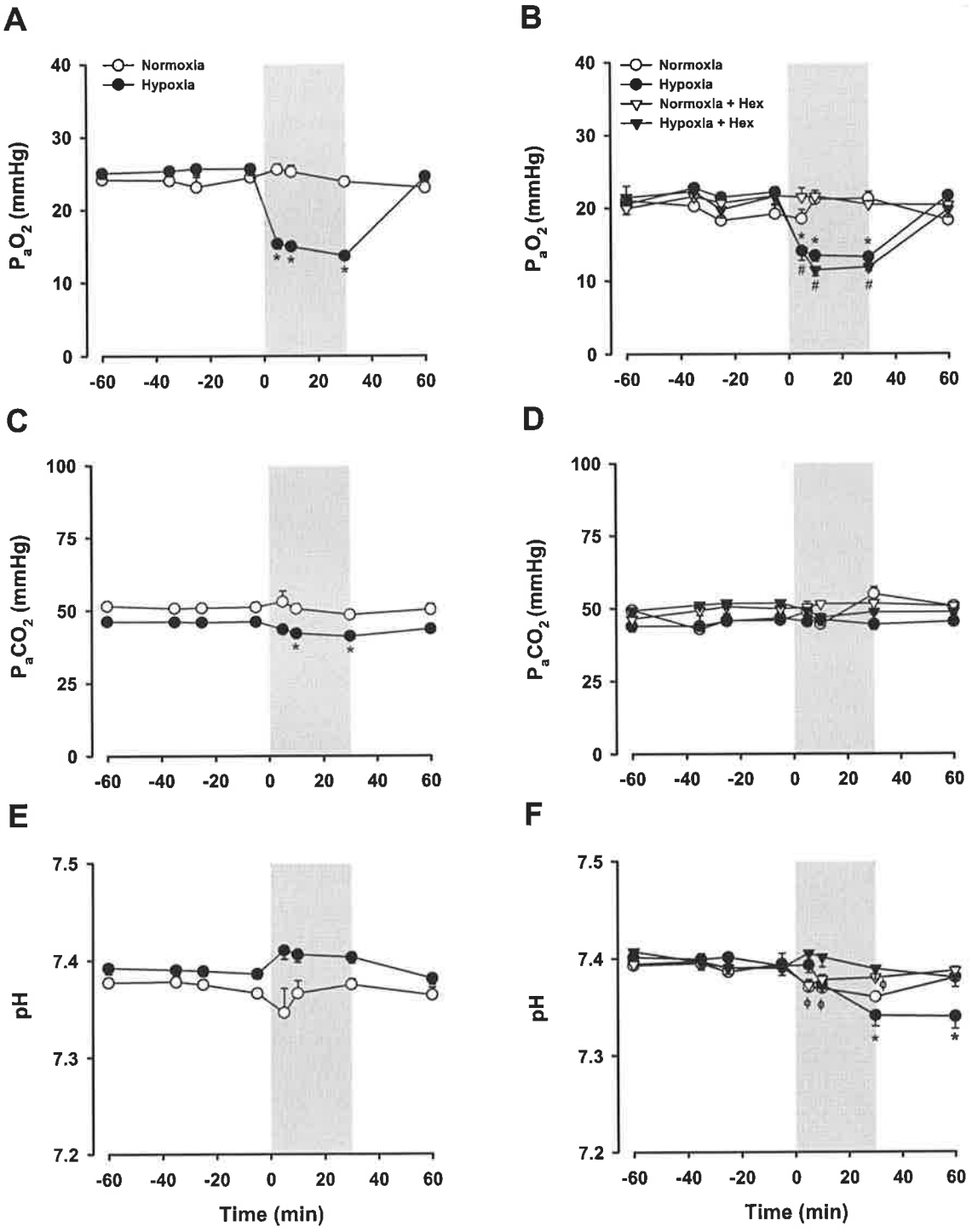


Figure 3.3 Effects of experimental hypoxia and normoxia upon fetal arterial blood gas variables

Average arterial PO_2 (**A**, **B**), PCO_2 (**C**, **D**), and pH (**E**, **F**) values for 96-105 d (left hand panel; **A**, **C**, **E**) and 129-144 d (right hand panel **B**, **D**, **F**) fetal sheep exposed to a 30 min period of experimental hypoxia (solid circles; 96-105 d, n = 15; 129-144 d, n = 15) or normoxia (open circles; 96-105 d, n = 5; 129-144 d, n = 4) as indicated by the shaded areas on the graphs. 129-144 d fetuses treated with hexamethonium (Hex, 25 $mg.kg^{-1}$) and exposed to either normoxia (open triangles, n = 6) or hypoxia (solid triangles, n = 6) are also shown. * $P < 0.05$ difference from basal values for hypoxia group, # $P < 0.05$ difference from basal values for hypoxia + Hex group, ϕ $P < 0.05$ difference from basal values for normoxia + Hex group.

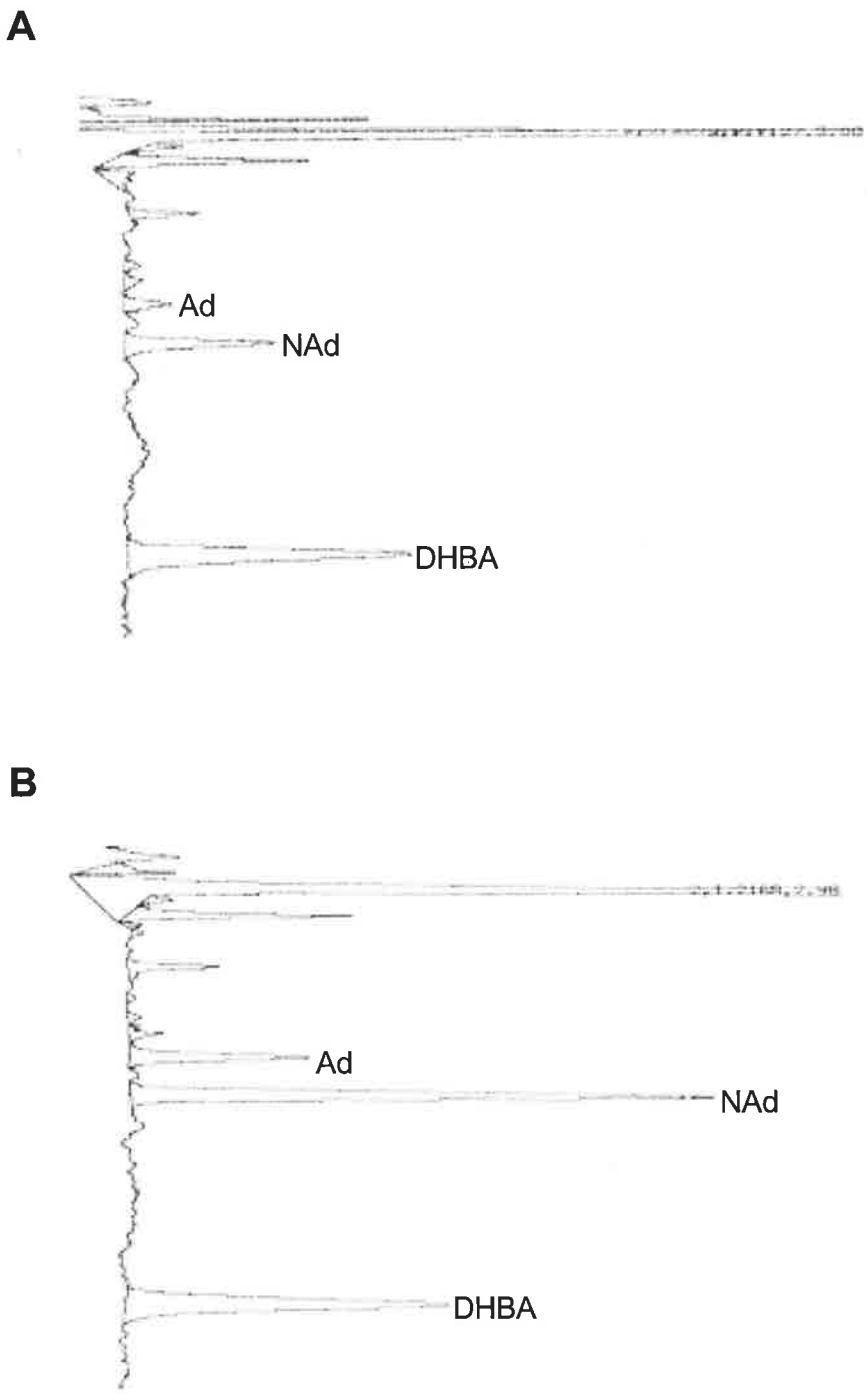


Figure 3.4 HPLC chromatograms of plasma catecholamine levels in a 141 d fetal sheep exposed to hypoxia

HPLC chromatograms of fetal plasma from a 141 d fetal sheep under basal conditions (A) and during hypoxia (B). Adrenaline (Ad), noradrenaline (NAd), and internal standard (DHBA) peaks are indicated. Note the increase in the peak height of adrenaline and noradrenaline in response to hypoxia.

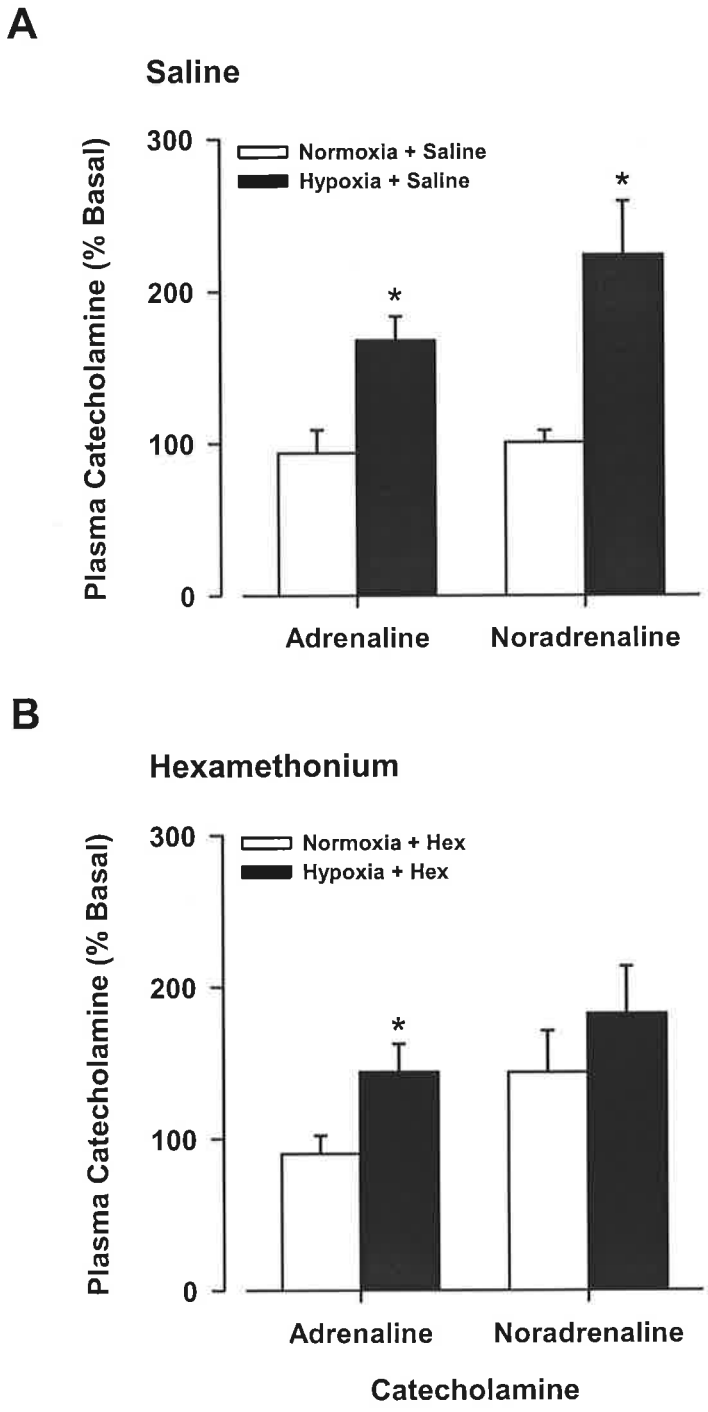


Figure 3.5 Impact of acute hypoxia on plasma catecholamine levels in the fetal sheep

Percentage change in fetal plasma catecholamine levels from average baseline values (-25, -5 min) in response to hypoxia (solid histograms; 5, 10 min) or normoxia (open histograms; 5, 10 min) for 129-144 d fetal sheep administered saline (**A**; normoxia, n = 4; hypoxia, n = 15), and 129-144 d fetal sheep treated with 25 $mg.kg^{-1}$ hexamethonium (**B**; normoxia, n = 6; hypoxia, n = 6). *$P < 0.05$ difference from normoxia value.

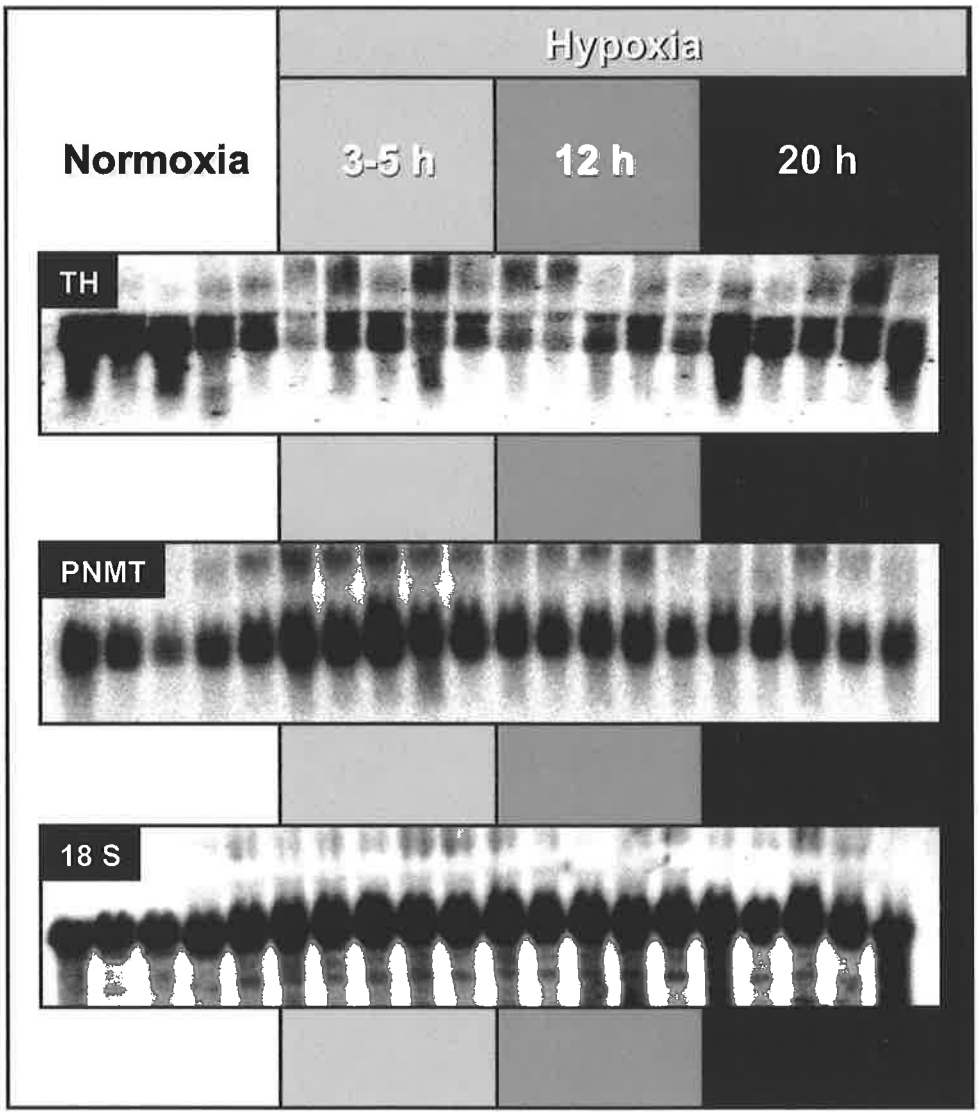


Figure 3.6 Autoradiographs of Northern blots of total adrenal RNA from fetal sheep exposed to acute hypoxia

Representative autoradiographs of Northern blots after hybridisation of radiolabelled TH cDNA, PNMT, and 18 S antisense oligonucleotide probes with total RNA (20 μg per lane) extracted from adrenals collected from fetal sheep at 129-144 d gestation after experimental normoxia (open panel, n = 5) or 3-5 h (light grey panel, n = 5), 12 h (dark grey panel, n = 5), and 20 h (black panel, n = 5) after 30 min of experimental hypoxaemia.

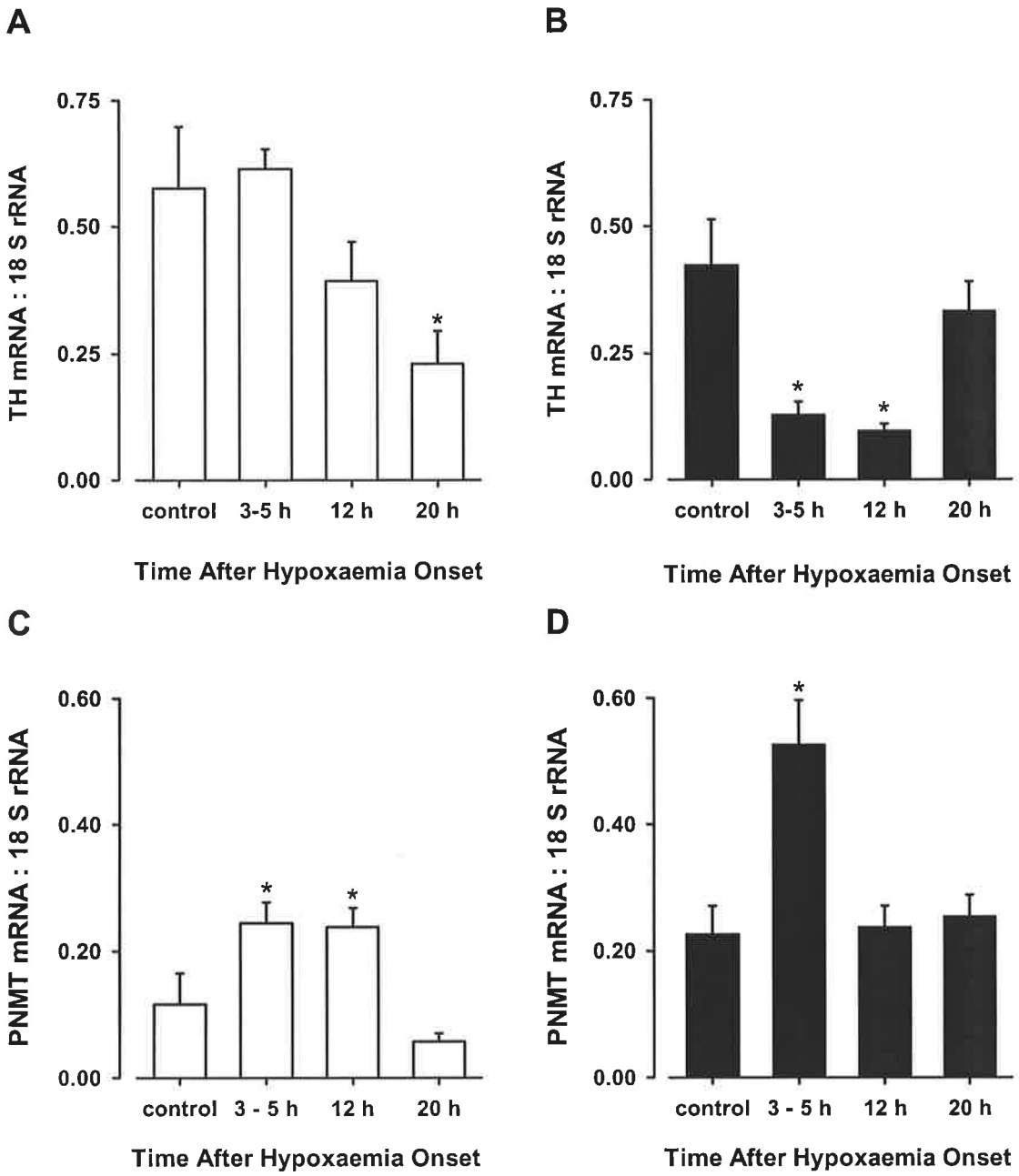


Figure 3.7 Time course of acute hypoxia induced changes in adrenal TH and PNMT mRNA expression in the fetal sheep

The ratios of adrenal TH mRNA : 18 S rRNA (**A**, **B**) and PNMT mRNA : 18 S rRNA (**C**, **D**) expression in 96-105 d (left hand panel; **A**, **C**) and 129-144 d (right hand panel; **B**, **D**) fetal sheep 3-5 h (96-105 d, n = 5; 129-144 d, n = 5), 12 h (96-105 d, n = 5; 129-144 d, n = 5) and 20 h (96-105 d, n = 4; 129-144 d, n = 5) after the onset of a 30 min period of experimental hypoxia or normoxia (control; 96-105 d, n = 4; 129-144 d, n = 5). * $P < 0.05$ difference from control value.

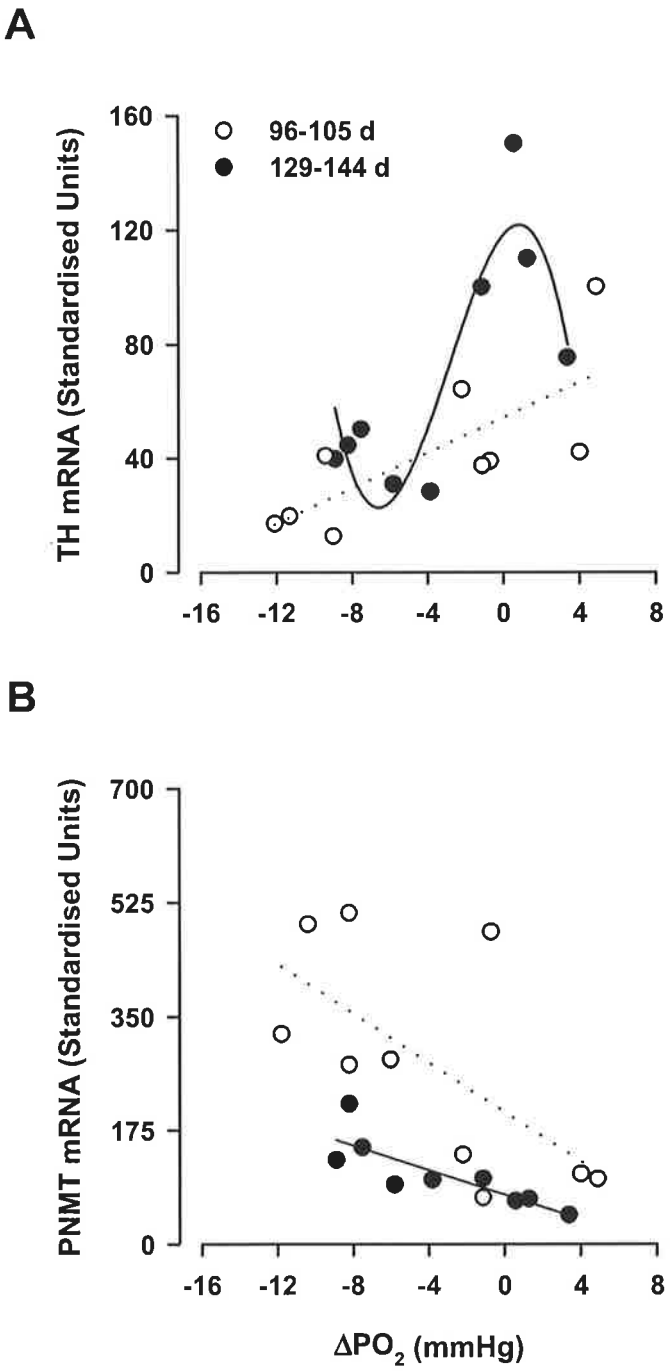


Figure 3.8 Relationships of adrenal TH and PNMT mRNA expression with experimentally induced changes of arterial PO_2 in fetal sheep

The expression of adrenal TH mRNA (**A**) and PNMT mRNA (**B**) plotted against the experimentally induced changes in arterial PO_2 (ΔPO_2) for 96-105 d (open circles; **A**, control and 20 h values: **B**; control and 3-5 h values) and 129-144 d fetal sheep (solid circles; **A**, control and 3-5 h values: **B**; control and 3-5 h values). Dotted and solid lines represent the lines of best fit derived by regression analysis for the 96-105 d and the 129-144 d groups, respectively.

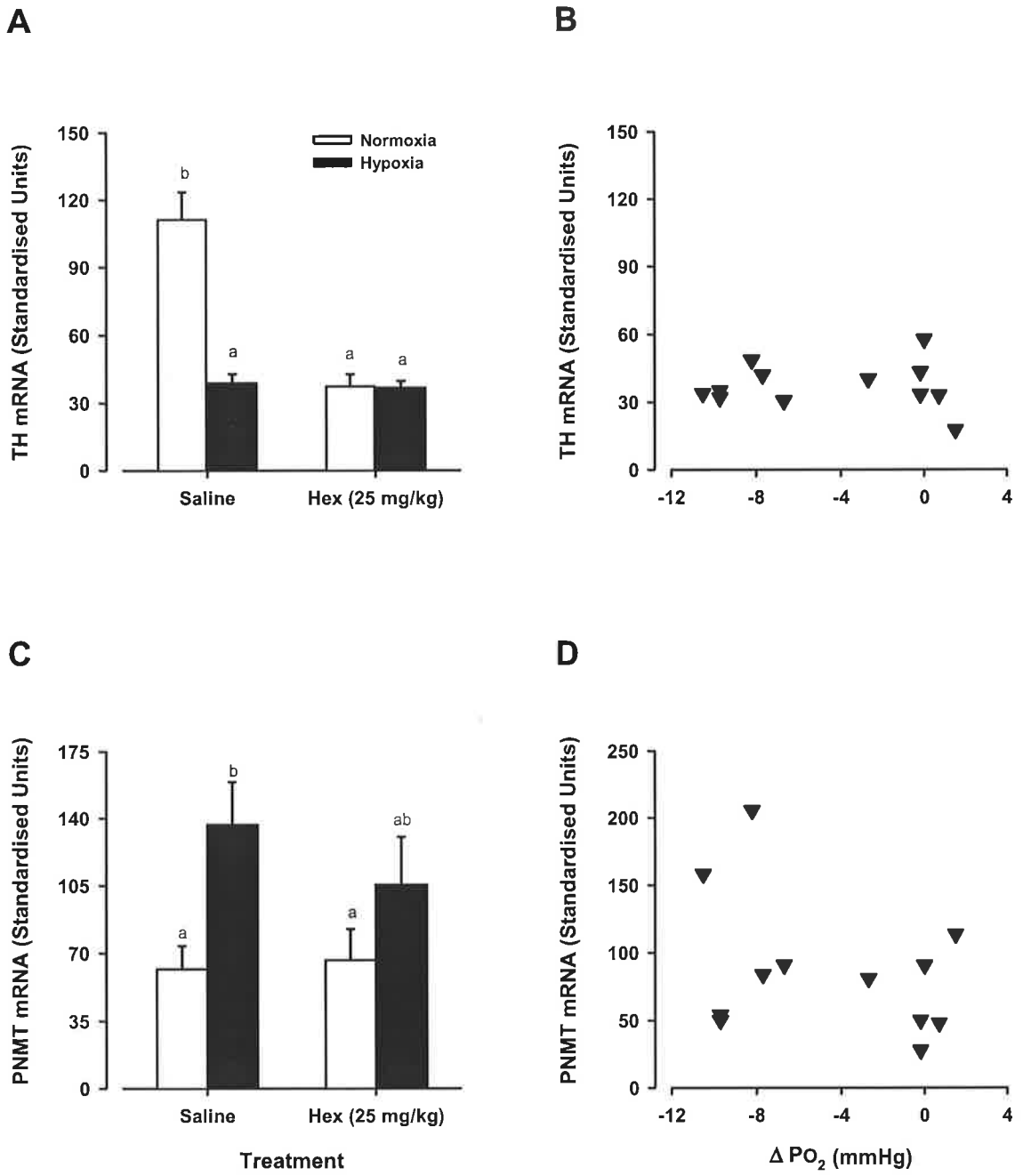


Figure 3.9 The effect of ganglionic blockade upon the hypoxia induced changes in adrenal TH and PNMT mRNA expression in the 129-144 d fetal sheep

Adrenal TH (**A**) and PNMT mRNA (**C**) expression in 129-144 d fetal sheep following exposure to experimental hypoxia (solid histograms, 3-5 h post hypoxia) or normoxia (open histograms) following the administration of either hexamethonium (Hex, 25 $mg.kg^{-1}$) or saline. Significant differences [$P < 0.05$] in TH and PNMT mRNA expression between groups are denoted by different superscript letters. The right hand panel shows the expression of adrenal TH mRNA (**B**) and PNMT mRNA (**D**) plotted against the experimentally induced changes in arterial PO_2 (ΔPO_2) for hexamethonium treated 129-144 d fetal sheep.

No relationship was observed between adrenal PNMT mRNA expression and experimentally induced changes in fetal arterial PO_2 in 129-144 d fetal sheep treated with hexamethonium (Figure 3.9D).

3.4 DISCUSSION

In the present study we found that basal circulating concentrations of noradrenaline and adrenaline increased with increasing gestational age in the fetal sheep. This agrees with the findings of Cheung (1990), who established that basal noradrenaline levels were positively correlated with gestational age and that adrenaline concentrations also tended to increase with gestational age. In the fetus, circulating noradrenaline is primarily derived from sympathetic neurones under basal conditions, as destruction of the peripheral sympathetic nerves after guanethidine administration markedly reduces plasma noradrenaline levels whilst they do not decrease after adrenal demedullation (Jones *et al.*, 1987). Hence the increase in circulating noradrenaline in late gestation may reflect maturational changes in the synthetic and secretory capacity the peripheral sympathetic nervous system (Simonetta *et al.*, 1993). In contrast plasma concentrations of adrenaline decrease significantly after adrenal demedullation (Jones *et al.*, 1987). There is also an increase in adrenaline output from the isolated perfused adrenal gland in late gestation (Butler *et al.*, 1995) which coincides with the increased expression of adrenal PNMT mRNA measured in the studies described in Chapter 2. It appears likely therefore that the increase in circulating adrenaline levels in the late gestation sheep fetus is derived in large part from an increased output from the fetal adrenal medulla.

A number of studies have previously established that hypoxia and asphyxia stimulate the secretion of catecholamines from the adrenal gland into the circulation in both anaesthetised fetal sheep during acute studies and in chronically catheterised conscious fetal sheep *in utero* (Cheung, 1990; Cohen *et al.*, 1991; Cohen *et al.*, 1984; Comline & Silver, 1966; Comline & Silver, 1961; Jones *et al.*, 1988; Widmark *et al.*, 1989). Plasma catecholamine levels and adrenal catecholamine output exhibit an inverse exponential relationship with the degree of fetal hypoxia (Cheung, 1990; Cohen *et al.*, 1984). Whilst a number of anatomical and functional studies in the fetal sheep have reported that

preganglionic cholinergic innervation of the adrenal gland does not occur until about 120 d gestation (Boshier *et al.*, 1989*a;* Comline & Silver, 1966; Comline & Silver, 1961), it is has been demonstrated that hypoxia can stimulate adrenomedullary catecholamine secretion before 120 d by a 'direct' or non-neurogenic mechanism (Adams *et al.*, 1996; Cheung, 1989; Cheung, 1990; Comline & Silver, 1966; Comline & Silver, 1961). In agreement with these previous findings the current study also found that acute hypoxia stimulated increases in plasma catecholamine levels in chronically catheterised fetal sheep *in utero* both before (96-105 d) and after (129-144 d) the development of functional splanchnic innervation.

The majority of nerve fibres projecting to the adrenal medulla via the splanchnic nerve are preganglionic, sympathetic, cholinergic nerves (Parker *et al.*, 1993; Robinson *et al.*, 1977*b*). Acetylcholine released from splanchnic nerve terminals acts at nicotinic and muscarinic receptors on chromaffin cells to stimulate catecholamine secretion from the adrenal medulla (Burgoyne, 1984; Ungar & Phillips, 1983). In the present study, administration of the nicotinic cholinoreceptor antagonist, hexamethonium, did not cause a decrease in the basal concentrations of plasma noradrenaline and adrenaline in fetal sheep between 129 and 144 days gestation. Hexamethonium administration did however, abolish the increase in plasma noradrenaline concentrations in response to acute hypoxaemia in these fetuses, supporting the previous observations of Cheung (1990) in chronically catheterised fetal sheep. In contrast, there was still a significant adrenaline secretory response to hypoxia in the older fetuses in the presence of hexamethonium. It appears therefore that after the development of splanchnic innervation, hypoxia stimulates noradrenaline secretion by reflex activation of nicotinic receptors. Adrenaline secretion, however did not appear to be completely dependent upon nicotinic stimulation.

It is possible that a component of the adrenaline secretory response to hypoxia in the older fetal sheep may be due to muscarinic stimulation. Previous studies in our laboratory using the retrogradely perfused adrenal gland preparation, demonstrated that acetylcholine evoked catecholamine secretion is not completely abolished by hexamethonium (Butler *et al.*, 1995). Furthermore Comline and Silver (1961) reported that hexamethonium treatment did not completely abolish

the catecholamine secretory response of the adrenal gland to electrical stimulation of the splanchnic nerve. Alternatively the rise in plasma adrenaline levels may be derived from sources other than the adrenal medulla and post ganglionic sympathetic nerves, such as the C1 neurones which project from the ventrolateral-medulla of the brainstem (Walker & Schuijers, 1989). This would appear to be unlikely as numerous studies which have examined the source of the plasma catecholamine rise in response to acute hypoxia have found that both the noradrenaline and adrenaline response to acute hypoxia are virtually abolished with adrenal demedullation or adrenalectomy (Jones *et al.*, 1988; Simonetta *et al.*, 1996*a*).

It has been demonstrated in a number of species that reflex neural stimulation of the adrenal medulla results in an increase in intracellular Ca^{2+} levels due to the activation of nicotinic and muscarinic receptors by acetylcholine (Cheek *et al.*, 1989; O'Sullivan *et al.*, 1989). Consequently a number of second messenger systems such as cAMP / PKA and PKC, which interact with transcription factors involved in the regulation of catecholamine synthetic enzyme gene expression, are also activated (Anderson *et al.*, 1992; Hwang *et al.*, 1997; Icard-Liepkalns *et al.*, 1992; Morita *et al.*, 1995*a;* Terbush *et al.*, 1988; Terbush & Holz, 1986). Thus there is a coupling of catecholamine secretion with catecholamine synthesis. The non-neurogenic catecholamine secretory response of fetal sheep and neonatal rat adrenomedullary chromaffin cells and PC12 cells to hypoxaemia is established to be mediated by oxygen-sensitive potassium channels (Rychkov *et al.*, 1998; Thompson *et al.*, 1997; Zhu *et al.*, 1996). The closing of these channels in response to hypoxia results in a depolarisation of the chromaffin cells and opening of voltage-sensitive calcium channels (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997). It has also been shown in studies using the retrogradely perfused fetal sheep adrenal gland, that hypoxia stimulates catecholamine secretion directly by a mechanism dependent on voltage-sensitive Ca^{2+} channels (Adams *et al.*, 1996). The subsequent influx of Ca^{2+} into the cell with non-neurogenic stimulation could therefore potentially activate a number of second messenger systems including isoforms of PKC, Ca^{2+} / calmodulin dependent protein kinases, and PKA via Ca^{2+} / calmodulin sensitive adenylate cyclase (Anderson *et al.*, 1992; Beitner-Johnson *et al.*, 1997). It is probable therefore that there is also a coupling of non-neurogenic stimulated secretion with catecholamine synthesis via the interaction of second

messenger systems with transcriptional factors which regulate catecholamine synthetic enzyme gene expression.

Surprisingly, acute hypoxaemia elicited a significant reduction in TH mRNA expression within the adrenal glands of fetal sheep both before and after the development of functional splanchnic innervation. Therefore acute hypoxia in the fetal sheep appears to be able to suppress TH mRNA expression by a non-neurogenic mechanism prior to functional splanchnic innervation of the adrenal gland. The non-neurogenic repression of TH mRNA expression in the fetal sheep adrenal was observed 20 h after the acute hypoxic and was directly related to the experimentally induced change in fetal P_aO_2. In contrast to the findings of the current study, Holgert and colleagues (1995) reported that adrenal TH mRNA expression increased in fetal rats at E21 following 48 h of maternal hypoxia. Therefore in the rat in which functional cholinergic innervation of the adrenal medulla does not develop until the first postnatal week, chronic hypoxia is able to stimulate TH gene expression by a direct non-neurogenic mechanism (Holgert *et al.*, 1995*b*). The fact that TH mRNA expression was decreased with fetal hypoxaemia in the current study may be attributable to species difference, degree of fetal hypoxaemia achieved, or the timing of the hypoxic insult, ie. acute *vs* chronic. It may also reflect that a rise in TH mRNA expression may have occurred earlier than 3-5 h after the hypoxic insult and was subsequently missed, although this would be highly unlikely as TH mRNA in most catecholaminergic tissues is relatively stable with a half life of around 10 h (Czyzyk-Krzeska, 1997; Czyzyk-Krzeska *et al.*, 1994*b*). Studies in the 1 d old neonatal rat and adult rat have found that moderate hypoxaemia does not significantly affect adrenal TH mRNA expression (Czyzyk-Krzeska *et al.*, 1992; DeCristofaro & LaGamma, 1994). Arterial PO_2, however, is much higher in the neonate and the adult than it is in the fetus and the lack of change in adrenal TH mRNA expression may indicate that the reductions in arterial PO_2 were insufficient to reach a response threshold (Czyzyk-Krzeska *et al.*, 1992; DeCristofaro & LaGamma, 1994).

With the development of functional adrenal innervation after 129 d gestation however, the suppression of adrenal TH mRNA expression was observed as early as 3-5 h after exposure to hypoxia. The relationship of TH mRNA expression with the change in arterial PO_2 was also more complex after 129 d gestation. There

was a tendency for TH mRNA expression to decrease with an increase in PO_2 relative to basal levels. This may reflect a reduction in basal sympathetic outflow and may suggest that there is a tonic activation of the sympathetic nervous system in the fetus during basal and normoxic conditions. As fetal arterial PO_2 decreased from baseline levels, TH mRNA expression also decreased reaching a minimum at a reduction of 7 mmHg. TH mRNA expression appeared to then rise with decreases in fetal arterial PO_2 below 7 mmHg and may imply that with marked decreases in PO_2 different secretion-synthesis coupling mechanisms operate which stimulate TH mRNA expression. After the administration of hexamethonium to the 129-144 d fetal sheep, there was a marked reduction in the basal expression of TH mRNA levels which implies that functional nicotinic stimulation is required for the maintenance of adrenal TH mRNA levels in fetal sheep after 129 days gestation. This is consistent with observations reported in Chapter 2, that there is a significant increase in adrenal TH mRNA expression at around 125 days gestation, ie coincident with the timing at which functional splanchnic innervation of the adrenal develops. In the present experiments there was no further reduction in TH mRNA levels with hypoxia in the presence of nicotinic blockade.

Studies of the regulation of TH gene expression and enzyme activity by hypoxia have been carried out in the type I glomus cells of the carotid body and PC12 cells, which synthesise and secrete dopamine and noradrenaline (Beitner-Johnson *et al.*, 1997; Czyzyk-Krzeska, 1997). Both these cell types are closely related to adrenal chromaffin cells but display a much greater sensitivity to TH gene regulation by hypoxia (Czyzyk-Krzeska *et al.*, 1992; Czyzyk-Krzeska *et al.*, 1994*b*). It has been established in these cell types that TH mRNA expression can be controlled by hypoxia at the level of transcription and translation (Czyzyk-Krzeska *et al.*, 1994*b*). Hypoxia stimulates TH gene transcription in PC12 cells by a mechanism which is calcium dependent and may involve the binding of HIF-1 and AP-1 transcription factors to regulatory elements on the TH gene (Beitner-Johnson *et al.*, 1997; Norris & Millhorn, 1995; Raymond & Millhorn, 1997). TH mRNA stability is also increased during hypoxia by the binding of hypoxia induced protein to stability elements in the 3' untranslated region of the TH mRNA (Czyzyk-Krzeska & Beresh, 1996; Czyzyk-Krzeska *et al.*, 1994*a;* Czyzyk-Krzeska *et al.*, 1997).

It is therefore likely that the reduction in fetal adrenal TH mRNA expression with hypoxia observed in both younger and older fetal sheep in the current study is due to decreased TH gene transcription. Basal TH mRNA expression in chromaffin cells has been shown to be dependent on a cAMP / PKA dependent pathway acting via a CRE on the TH gene promoter (Hwang *et al.*, 1997; Kim *et al.*, 1993*a*). Hypoxia (PO_2 ~ 18-66 mmHg) has been reported to directly stimulate cAMP levels within the carotid body (Cachero *et al.*, 1996; Delpiano & Acker, 1991; Wang *et al.*, 1991). Stimulation of cAMP synthesis in the adrenal gland and PC12 cells however can also induce the expression of an inducible cAMP early represser (ICER) which binds to the TH CRE to repress the activity of the TH gene promoter and inhibit induction by PKA (Molina *et al.*, 1993; Tinti *et al.*, 1996). It may be the case that the acute hypoxic stimulus utilised in this study preferentially induced ICER expression, consequently reducing adrenal TH mRNA expression in the fetal sheep adrenal. Alternatively, Fos family members of the AP-1 transcription factors in conjunction with c-jun have also been demonstrated to be capable of functioning as potent transrepressers of TH gene transcription by their interaction with the AP-1 site of the TH 'fat' specific element, which is an important regulator of basal activity (Gizang-Ginsberg & Ziff, 1994). As outlined in section 1.4.2.1.2, AP-1 transcription factors such as the Fos and Jun family of proteins are also involved in the stimulation of TH gene transcription and are induced by hypoxia (5 % O_2) in PC12 cells (Icard-Liepkalns *et al.*, 1992; Norris & Millhorn, 1995). The temporal relationship of the induction of the various AP-1 transcription factors appears to determine whether there is ultimately a positive or negative effect on TH gene expression (Gizang-Ginsberg & Ziff, 1994). The acute hypoxic stimulus in the current study may have induced the formation of an AP-1 complex with an inhibitory effect on TH gene transcription.

In these studies I have reported that there is a reduction in TH mRNA expression in response to moderate hypoxia ie within a range that may be clinically relevant. As TH is the rate limiting enzyme of catecholamine synthesis (Levitt *et al.*, 1965; Nagatsu *et al.*, 1964) there may be an impairment of adrenal catecholamine secretion to subsequent physiological challenges due to a reduced synthetic capacity. It must be borne in mind however, that alterations in TH mRNA expression may not always parallel changes in the biological activity of the enzyme (Kumer & Vrana, 1996). It has been noted in the rat that hypoxia can

stimulate an increase in the activity of the enzyme itself, possibly by phosphorylation to a more active form (Hayashi *et al.*, 1990). Clearly a determination of TH synthetic activity in the fetal sheep adrenal following exposure to acute hypoxia is required in future studies.

In the present study, adrenal PNMT mRNA expression increased after exposure to acute hypoxia both before and after the development of functional splanchnic innervation. Hence PNMT mRNA expression is regulated by non-neurogenic mechanisms in response to hypoxia before the development of splanchnic innervation. Adrenal PNMT mRNA expression increased as early as 3-5 h after hypoxia and exhibited an inverse linear relationship with the experimentally induced change in fetal P_aO_2, both before and after the development of adrenal innervation. After 129 d gestation the increase in PNMT mRNA expression in response to acute hypoxaemia was inhibited by hexamethonium, implicating the involvement of reflex nicotinic stimulation. There are relatively few studies on the effects of hypoxia on PNMT mRNA expression. This reflects the concentration of research on TH gene expression because of its role in the synthesis of dopamine, a major transmitter in the chemoreceptive type I glomus cells of the carotid body (Fidone *et al.*, 1990).

The factors which stimulate PNMT gene expression are not yet clearly defined. It is well established that glucocorticoids play an important role in the induction and maintenance of PNMT mRNA expression within adrenomedullary chromaffin cells (see section 1.3.4.2.2.1). Hypoxia stimulates an increase in plasma ACTH and cortisol concentrations in the late gestation fetal sheep (Akagi *et al.*, 1990; Braems *et al.*, 1996; Jones *et al.*, 1988). It is possible that an increase in adrenal cortisol output in response to hypoxia stimulates PNMT gene expression in the fetal sheep. However, the adrenal cortex of the fetal sheep at 96-105 d is relatively unresponsive to ACTH stimulation (Challis & Brooks, 1989), although maternally derived cortisol could contribute to an increase in fetal cortisol levels during hypoxaemia. This may suggest that the response to hypoxia both before and after 129 d gestation is associated with a pathway requiring membrane depolarisation, rather than via hormonal stimulation. The transcription factor Egr-1 is involved in neurally mediated increases in PNMT mRNA expression. Egr-1 expression is induced by neural stimuli whereupon it binds to its consensus element on the

PNMT gene promoter region to stimulate transcription (Ebert *et al.*, 1994; Ebert & Wong, 1995; Morita *et al.*, 1996). Protein kinase C activation stimulates PNMT mRNA expression, apparently through stimulation of Egr-1 binding to the PNMT gene promoter region (Morita *et al.*, 1995*a*). Interestingly, PKC has been implicated in the cellular adaptations of PC12 cells to hypoxia (Beitner-Johnson *et al.*, 1997; Raymond & Millhorn, 1997). Hence Egr-1 may play an important role in the induction of PNMT gene expression in the fetal adrenal medulla in response to acute hypoxia. It is interesting that hexamethonium abolishes the increase in PNMT mRNA expression in response to hypoxia after 129 d, which suggests that in the absence of nicotinic activation there is no residual non-neurogenic stimulation of PNMT mRNA expression at this age. It has previously been shown in the fetal sheep and neonatal rat that the development of functional splanchnic innervation appears to suppress the non-neurogenic stimulation of catecholamine secretion (Comline & Silver, 1961; Seidler & Slotkin, 1986*b*; Cheung, 1990).

The induction of PNMT mRNA expression in response to acute bouts of hypoxia may have physiological relevance in the fetal to neonatal transition whereby the fetus is frequently exposed to acute bouts of hypoxia during labour and delivery due to compression of the umbilical cord during uterine contractions (Giussani *et al.*, 1994). This may then stimulate adrenal PNMT mRNA expression and adrenaline synthesis. Increased adrenaline synthesis would serve to restore chromaffin cell adrenaline levels after the dramatic catecholamine surge which occurs in response to labour and delivery (Lagercrantz & Bistoletti, 1973; Padbury *et al.*, 1982). Adrenaline plays a key role in lung maturation and metabolism (Jones & Ritchie, 1978*b;* Padbury *et al.*, 1987*a;* Padbury *et al.*, 1984; Padbury *et al.*, 1987*c;* Sperling *et al.*, 1984) and it may be important that adrenaline levels are maintained by increased PNMT expression and adrenaline synthesis after the hypoxia associated with birth.

In summary this study has demonstrated that acute hypoxia which directly stimulates catecholamine secretion from the immature fetal adrenal medulla, also regulates TH and PNMT mRNA expression by a non-neurogenic mechanism. After the development of functional splanchnic innervation, it was found that the effect of acute hypoxia on adrenal TH and PNMT mRNA expression becomes dependent upon reflex nicotinic stimulation. We have also found that the

expression of adrenal TH and PNMT mRNA were differentially regulated by acute hypoxia. Further studies to determine the cellular and molecular mechanisms underlying the non-neurogenic and neurogenic regulation of the adrenal catecholamine synthetic enzymes during hypoxia in the fetus are required.

4. GLUCOCORTICOID REGULATION OF TH, PNMT AND PEnk A mRNA EXPRESSION IN THE FETAL SHEEP ADRENAL

4.1 INTRODUCTION

In the fetal sheep there are parallel increases in plasma concentrations of cortisol, adrenomedullary adrenaline content, and adrenal PNMT mRNA expression, in the 10-15 days preceding delivery (Chapter 2, Challis & Brooks, 1989; Comline *et al.*, 1970). Furthermore, fetal HPX prevents the late gestation rise in the ratio of adrenal adrenaline : noradrenaline content in the sheep (Comline *et al.*, 1970). It has therefore been concluded that the increased adrenal output of the glucocorticoid, cortisol, induces PNMT expression, with a consequent stimulation of adrenaline synthesis in the adrenal medulla during late gestation.

Whilst the evidence cited above supports a role for glucocorticoids in the stimulation of adrenaline synthesis in the fetal adrenal, a series of *in vivo* studies have found a paradoxical inhibitory effect of glucocorticoid infusion on plasma adrenaline concentrations. Wood and colleagues (1987) found that there was a significant fall in circulating catecholamine concentrations in fetal sheep after a 5 h cortisol infusion in late gestation. Stein and colleagues (1995) also found that a longer term cortisol infusion significantly reduced the plasma catecholamine surge which occurs after Caesarean delivery. Furthermore it has recently been demonstrated that intrafetal infusions of the synthetic glucocorticoids, betamethasone and dexamethasone, at doses used clinically to accelerate fetal lung development, suppress plasma concentrations of adrenaline and noradrenaline (Derks *et al.*, 1997).

It is possible that exogenous glucocorticoids may suppress the fetal hypothalamo-pituitary-adrenal axis via negative feedback, with consequent decreases in adrenal glucocorticoid output, PNMT expression, adrenaline synthesis, and adrenaline secretion. There have been no studies, however, on the effects of chronic physiological elevations of glucocorticoid concentrations on the expression of catecholamine biosynthetic enzymes in the adrenal medulla of a long gestation species before birth. The study reported in this chapter investigated the effect of a physiological elevation of fetal cortisol concentrations on the adrenal mRNA levels of TH and PNMT.

Intra-adrenal Enk-containing peptides co-localised in adrenaline containing chromaffin cells (McMillen *et al.*, 1988) may play an inhibitory role in regulating catecholamine release from the fetal adrenal (Padbury *et al.*, 1987*b*). Hence the effect of cortisol on the adrenal expression of PEnk A mRNA was also examined.

4.2 MATERIALS AND METHODS

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation.

4.2.1 ANIMALS AND SURGERY

Fifteen pregnant Border Leicester x Merino ewes were used in this study. Surgery was performed on the ewes at either 103 or 104 d gestation (calculated according to date of mating) under general anaesthesia induced by intravenous injection of sodium thiopentone (1.25 g; Pentothal, Rhone Merieux, Pinkenba, QLD, Australia) and maintained with halothane (3-4 % in oxygen; Fluothane, ICI, Melbourne, VIC, Australia). Ewes were administered 2 ml of antibiotics (250 $mg.ml^{-1}$ procaine penicillin, 250 $mg.ml^{-1}$ dihydrostreptomycin sulphate, 20 $mg.ml^{-1}$ Procaine HCl; Penstrep Ilium, Troy Laboratories, Smithfield, NSW, Australia) intramuscularly, prior to surgery. During surgery catheters were implanted into the fetal and maternal carotid artery and jugular vein as well as the amniotic sac, as described in detail in Chapter 3. The fetus was also administered 2 ml of antibiotics (Penstrep Ilium, Troy Laboratories) intramuscularly during surgery. The catheters were flushed and filled with heparinised saline (500 $IU.ml^{-1}$; Multiparin, Fisons Pharmaceuticals, Sydney, NSW, Australia) and the fetal catheters were exteriorised through an excision in the ewe's flank.

4.2.2 POST OPERATIVE CARE

Ewes were housed in individual metabolic crates in animal holding rooms under a 12 h light-dark cycle and fed lucerne chaff once daily between 0900 and 1300 h with water *ad libitum*. Sodium ampicillin (500 mg, Austrapen, Commonwealth Serum Laboratories, Melbourne, VIC, Australia) was administered intra-amniotically to all fetal sheep for 4 consecutive days post operatively. A

post operative recovery of 3-4 d was allowed before any blood sampling commenced.

4.2.3 BLOOD SAMPLING AND CORTISOL INFUSION

Fetal arterial blood samples (5 ml) were collected twice daily (10.00 and 17.00 h) from 107-116 d gestation. Aliquots of whole blood (2.5 ml) were immediately placed into ice chilled tubes for cortisol (heparinised tubes, 125 IU; Disposable Products, Adelaide, SA, Australia) and immunoreactive (ir) ACTH (tubes contained aprotinin, 1000 KIU.ml^{-1}, Sigma Chemicals, St Louis, MO, USA; and EDTA, 18.6 g.l^{-1} of whole blood) radioimmunoassay. Blood samples were centrifuged at 1800 *g* for 10 min at 4°C and the plasma was separated and stored at -20°C prior to assay.

Cortisol (2.5-3.0 mg.5 ml^{-1}.24 h^{-1}, n = 9; Solucortef, Upjohn Pharmaceutical's Ltd., Kalamazoo, MI, USA) or 0.9 % saline (n = 6) was infused intravenously into fetal sheep from 109 to 116 d gestation.

4.2.4 POST MORTEM AND TISSUE COLLECTION

At 116 d gestation the ewes were sacrificed with an overdose of sodium pentobarbitone (8.125 g; Lethabarb, Virbac, Peakhurst, NSW, Australia). Fetal sheep were delivered via laparotomy, weighed and killed by decapitation. The adrenal glands were dissected from each fetus and weighed. The left adrenal glands (n = 15) were snap frozen in liquid N_2 and stored at -70°C until total RNA was extracted. The right adrenal glands (n = 14) were cut into half, perpendicular to their poles, and placed into ice cold paraformaldehyde (4 %) fixative in sodium phosphate buffer (0.1 M, pH 7.0) for subsequent immunohistochemical analysis.

4.2.5 CORTISOL ASSAY

Total cortisol concentrations were measured in fetal sheep plasma by radioimmunoassay using an Orion Diagnostica kit (Orion Diagnostica, Turku, Finland). Prior to assay, cortisol was extracted from the plasma using dichloromethane (Bocking *et al.*, 1986). The efficiency of recovery of ^{125}I Cortisol from the fetal plasma using this procedure was 84 ± 1 %. The sensitivity of the assay was 0.39 nmol.l^{-1}. The rabbit cortisol antibody had a cross reactivity of

< 1 % with cortisone and 17-hydroxyprogesterone and < 0.01 % with pregnenolone, aldosterone, progesterone and estradiol (product information supplied by Orion Diagnostica). The inter- and intra-assay coefficients of variation (CV) were always less than 10 %.

4.2.6 ACTH ASSAY

The concentrations of plasma irACTH were measured by radioimmunoassay using an ICN Biomedicals kit (ICN Biomedicals, Seven Hills, NSW, Australia). The sensitivity of the assay was 7 $pg.ml^{-1}$ and the rabbit anti-human ACTH (1-39) had a cross reactivity of < 0.01 % with β-endorphin, α-MSH, α-Lipotrophin, and β-Lipotrophin (product information supplied by ICN Biomedicals). The inter-assay CV was 14.6 % and the intra-assay CV was < 10 %.

4.2.7 PLASMA GLUCOSE AND LACTATE DETERMINATION

Plasma concentrations of glucose and lactate were determined by enzymatic analysis using a COBAS MIRA automated analysing system (Cobas, Roche Diagnostics, Basle, Switzerland). The enzymes hexokinase and glucose 6-phosphate dehydrogenase were used for glucose determination whilst lactate dehydrogenase and glutamate-pyruvate transaminase were used for lactate determination. The photometric determination of NADH formation was used to calculate the amounts of glucose and lactate present in the sample. The intra-assay CVs were 1.4 % and 1.3 % for glucose and lactate, respectively.

4.2.8 TOTAL RNA EXTRACTION

Total adrenal RNA was extracted according to the method of Chirgwin *et al.* (1979), as outlined in Chapter 2. Briefly, adrenals from cortisol infused (n = 9) and saline (n = 6) fetuses were homogenised in 4 *M* guanidine thiocyanate buffer and ultracentrifuged at 130 000 *g* through a 5.7 *M* CsCl cushion. The supernatant was removed with the pellets being resuspended in sterile water and subsequently reprecipitated using 0.3 *M* sodium acetate and absolute ethanol on dry ice. The supernatant was again removed before the pellets were washed with 70 % ethanol and then resuspended in sterile water. Nucleic acid concentration, yield, and purity were quantified by spectrophotometric measurement at 260 and 280 nm. Total RNA solutions were stored at -70°C prior to Northern blot analysis.

4.2.9 PROBES AND PROBE LABELLING

The probes utilised in this study and the radiolabelling methods for the probes are described in detail in Chapter 2. To summarise, oligonucleotide antisense probes complementary to nucleotides 361-389 of the peptide coding region of bovine PNMT mRNA (29 mer, Wan *et al.*, 1989), nucleotides 627-671 of the peptide coding region of bovine PEnk A mRNA (44 mer, Wan *et al.*, 1987), and nucleotides 151-180 of rat 18 S rRNA (30 mer, Chan *et al.*, 1984) sequence were synthesised (Bresatec, Thebarton, SA, Australia). A 1.7 kb bovine TH cDNA (D'Mello *et al.*, 1988) was used to detect TH mRNA. The oligonucleotides were end labelled using T4 polynucleotide kinase (7.9 $U.\mu l^{-1}$, Pharmacia, North Ryde, NSW, Australia) and γ-[^{32}P]ATP (4000 $Ci.mmol^{-1}$,Bresatec) in accordance with the method described for the end labelling of oligonucleotides by Sambrook *et al.* (1989). The cDNA probe was labelled by the random priming method with α-[^{32}P]dCTP (3000 $Ci.mmol^{-1}$, Bresatec) and Klenow fragment (6.4 $U.\mu l^{-1}$, Pharmacia) using an oligolabelling kit (Pharmacia) as per manufacturer's instructions.

4.2.10 NORTHERN BLOT ANALYSIS

For each sample 20 µg of total adrenal RNA was denatured in 2.2 *M* formaldehyde and 50 % (v / v) formamide at 55°C for 10 min. Separation of the total adrenal RNA was achieved by electrophoresis on a 1 % agarose gel containing 2.2 *M* formaldehyde. The separated total adrenal RNA was subsequently transferred to Zetaprobe nylon membranes (Biorad, Richmond, CA, USA) via capillary blotting. Membranes were washed in 10 × SSC for 10 min at room temperature and then baked for 1 h at 80°C to fix the RNA to the membrane. Prior to hybridisation with oligonucleotide probes the membranes were initially prehybridised at 50°C for 18 h in a solution consisting of 5 × SSC, 20 m*M* NaH_2PO_4, pH 7.2, 7 % SDS, 5 × Denhardt's, and 100 $\mu g.ml^{-1}$ of heat denatured Salmon sperm DNA. Membranes were then hybridised for 20 h at 50°C in 30 ml of fresh hybridisation solution containing 1-2 × 10^6 $cpm.ml^{-1}$ of labelled oligonucleotide. For the TH cDNA probe equivalent prehybridisation and hybridisation times were used, however, the hybridisation solution consisted of 50 % (v / v) deionised formamide, 5 × SSPE, 7 % SDS, pH 7.2, and 100 $\mu g.ml^{-1}$ of

heat denatured salmon sperm DNA, with hybridisation being carried out at 42°C in the presence of 1-2 x 10^6 cpm.ml^{-1} of labelled probe. Following hybridisation the membranes were washed in 1 × SSC and 0.1 % SDS (1 × 30 min) and then subsequently in 0.1 × SSC and 0.1 % SDS (1 × 30 min) at their hybridisation temperatures before being briefly air dried and exposed to a Fuji BAS-IIIs phosphorimager plate (Fuji Photo Co., Tokyo, Japan) for 24-48 h. Autoradiographs were obtained using a Fuji BAS 1000 phosphorimager (Fuji Photo Co.) and quantitated using Fuji MacBAS software (Fuji Photo Co.).

4.2.11 ANTIBODIES

Rabbit anti-bovine PNMT was generously supplied by Professor PRC Howe (Department of Biomedical Science, The University of Wollongong, NSW, Australia) and rabbit anti-rat TH was purchased (Chemicon International, Temecula, CA, USA). The rabbit anti-PNMT and anti-TH primary antibodies were used at working dilutions of 1:1500 and 1:1000 respectively. The specificity of these antibodies has been previously demonstrated (McMillen *et al.*, 1988; Varndell *et al.*, 1982).

4.2.12 IMMUNOHISTOCHEMISTRY

Following fixation in paraformaldehyde (4 %) for 24 h adrenals were washed in phosphate buffered saline (0.9 % saline, 0.1 *M* phosphate buffer, pH = 7.0) at 4°C (2 × 24 h) and then stored in ethanol at 4°C before being embedded in paraffin wax. Transverse adrenal sections of 5 μm were cut and mounted onto poly-L-lysine coated slides.

Semi adjacent sections were either processed for TH and PNMT immunoreactivity using a Vectastain avidin-biotin peroxidase complex kit (PK-4001, Vector Laboratories, Burlingame, CA, USA) or stained with haemotoxylin (Harris' Haematoxylin, Sigma Diagnostics, St Louis, MO, USA) and eosin (0.5 % in 90 % Ethanol). The sections were dewaxed by immersion (2 × 5 min) in Histoclear (National Diagnostics, Somerville, NJ, USA) and subsequently rehydrated by immersion in a graded ethanol series (100 %, 2 × 5 min; 90 %, 1 × 2 min; 70 %, 1 × 2 min; distilled H_2O, 1 × 2 min). Sections were washed in 0.01 *M* phosphate buffered saline (PBS, pH 7.0; Sigma Chemicals, St. Lois, MO, USA) for 20 min,

before being incubated with blocking serum (3 % Normal Goat Serum in PBS) for 30 min at room temperature. Excess blocking serum was blotted away and the sections were covered with the primary antibody diluted in PBS containing 1 % BSA. After overnight incubation at 4°C in a humidified container, the sections were washed in (PBS, 3 × 5 min) and goat anti-rabbit biotinylated secondary antibody was applied to the sections for 60 min at room temperature. Following washing (PBS, 3 × 5 min) the sections were then incubated with avidin-biotin-peroxidase complex (Vector Laboratories) at room temperature for 60 min before being washed again (PBS, 3 × 5 min). The reaction was visualised by the addition of 3,3-diaminobenzadine tetrahydrochloride (DAB, 0.5 $mg.ml^{-1}$ in PBS buffer and 0.02 % H_2O_2) at room temperature for 10 min. Sections were then washed (PBS, 3 × 5 min) and dehydrated through a graded ethanol series (distilled H_2O, 1 x 2 min; 70 % ethanol, 1 x 2 min; 90 %, 1 x 2 min; 100 %, 2 x 5 min; Histoclear, 2 x 5 min) before coverslips were mounted with DPX (BDH Laboratory Supplies, Poole, England).

4.2.13 ADRENAL MORPHOMETRY

Adrenal section images were captured and digitised using a CCD black and white video camera (SSC-M370CE, Sony Corp., Tokyo, Japan) mounted on a dissecting microscope (SZ-40, Olympus Optical Co., Tokyo, Japan) via an SZ-CTV photomount tube (Olympus Optical Co.). The image was digitised as a grey scale image and positively stained areas were measured using a Power Mac 8500 / 120 (Apple, Cupertino, CA, USA) equipped with NIH Image V1.61 software. Total adrenal area was measured as the area of the section stained with Haematoxylin and Eosin. The area of the adrenal medulla was defined as the area of the gland which was positively stained with anti-TH. The areas of the sections which were positively stained with anti-PNMT were also calculated. The relative areas of the adrenal gland occupied by the medulla was calculated by dividing the area stained by anti-TH by the total adrenal area. The relative area of the adrenal medulla occupied by PNMT positive cells was obtained by dividing the area stained with anti-PNMT by the area stained with anti-TH. In all cases dual measurements of area on serial sections were made and the measurements were averaged.

4.2.14 STATISTICAL ANALYSIS

All data are presented as the mean ± standard error of the mean (SEM). The ratios of TH, PNMT, and PEnk A mRNA : 18 S rRNA expression were compared between the saline and cortisol infused fetuses using two-tailed, unpaired Student's t-tests. Similarly two tailed, unpaired, Student's t-tests were also used to compare fetal body weights, adrenal weights, adrenal total RNA yields and adrenal morphometry data. One way ANOVA was used to compare the plasma cortisol and irACTH concentrations under basal conditions and during infusion with either cortisol or saline. Where significant differences were identified by ANOVA a Fisher's post hoc test was used. The relationship between fetal adrenal PNMT protein expression and mRNA expression was determined by regression analysis.

A probability of less than 5% (ie. $P < 0.05$) was taken to be significant.

4.3 RESULTS

4.3.1 FETAL OUTCOMES

There was no significant effect of cortisol infusion on fetal body weight, combined adrenal weight, nor the total RNA yield from fetal adrenals, when compared with saline infused animals (Table 4.1).

Table 4.1 Fetal outcomes for saline and cortisol infused fetuses at 116 d gestation

Treatment	Fetal Weight (kg)	Adrenal Weight (g)	Total Adrenal RNA Yield ($\mu g.mg^{-1}$)
Saline *(n = 6)*	2.32 ± 0.26	0.26 ± 0.03	1.67 ± 0.12
Cortisol *(n = 9)*	2.17 ± 0.08	0.23 ± 0.01	1.62 ± 0.07

4.3.2 FETAL ENDOCRINE AND METABOLIC STATUS

Cortisol infusion produced a highly significant 30-fold increase ($P < 0.0005$) in fetal plasma cortisol concentrations, whereas cortisol levels remained unchanged from baseline in the saline infused controls (Figure 4.1A). Plasma irACTH concentrations were unchanged from baseline values following infusion with either

cortisol or saline (Figure 4.1B). Furthermore no significant differences in average plasma ACTH levels were identified between saline and cortisol infused fetuses during both baseline and infusion conditions (Figure 4.1B).

Cortisol infusion resulted in a moderate but significant increase ($P < 0.05$) in fetal plasma glucose levels (1.57 ± 0.07 mmol.l^{-1}, n = 9) when compared with saline infused fetuses (1.32 ± 0.09 mmol.l^{-1}, n = 6). Fetal plasma lactate concentrations were not significantly different, however, between the cortisol (0.91 ± 0.11 mmol.l^{-1}, n = 9) and saline infused fetuses (0.66 ± 0.06 mmol.l^{-1}, n = 6).

4.3.3 CORTISOL INFUSION AND ADRENAL TH, PNMT AND PEnk A mRNA EXPRESSION

There were no significant differences in the ratios of either adrenal TH mRNA : 18 S rRNA expression (saline, 2.1 ± 0.1; cortisol, 2.6 ± 0.2) or PEnk A mRNA : 18 S rRNA expression (saline, 0.089 ± 0.020; cortisol, 0.055 ± 0.009) between treatment groups (Figure 4.2, Figures 4.3A & C). In contrast, the ratio of adrenal PNMT mRNA : 18 S rRNA was significantly reduced [$P < 0.0005$] by approximately 75 % in the cortisol infused fetuses (0.085 ± 0.015) when compared with the saline infused group (0.376 ± 0.067) (Figure 4.2, Figure 4.3B).

4.3.4 CORTISOL INFUSION AND ADRENAL MORPHOMETRY

There were no significant differences observed between the saline and cortisol infused animals in either the total adrenal area or relative area of the gland occupied by the medulla (Table 4.2). Staining with anti-TH was localised throughout the adrenal medulla in all sections with no positive staining observed in the cortex (Figures 4.4A & C). Positive staining with anti-PNMT was present in the peripheral columnar cells of the adrenal medulla which interdigitated with the adrenal cortex and in some medullary cells adjacent to the central adrenal vein and sinusoids (Figures 4.4B & D). The proportion of the adrenal medulla which stained positively with anti-PNMT was significantly reduced ($P < 0.007$) by around 40 % in the fetal sheep adrenals from the cortisol infused group when compared with the control group (Table 4.2). There was also a significant correlation between the ratio of PNMT mRNA : 18 S rRNA expression and the relative area of the adrenal gland which was PNMT positive [Proportion of adrenal medulla

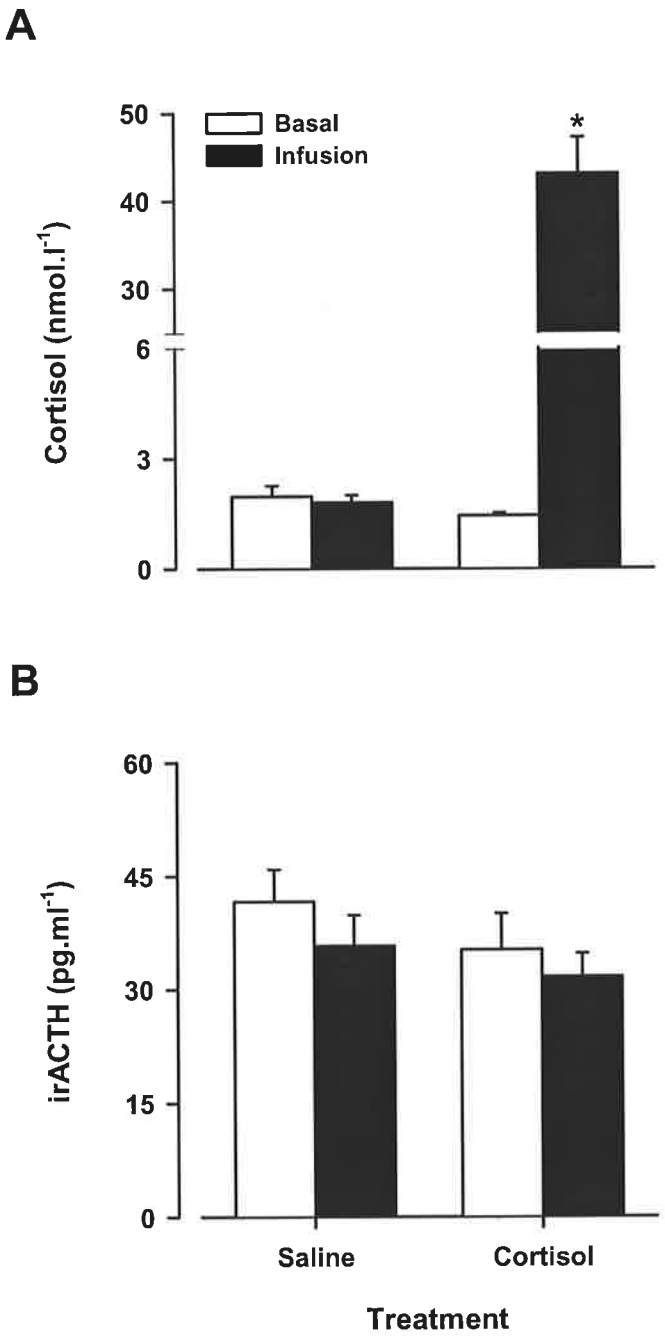


Figure 4.1 Effect of cortisol infusion on plasma cortisol and irACTH concentrations in the fetal sheep

Plasma cortisol (A) and irACTH (B) concentrations in fetal sheep infused with either cortisol (2.5-3.0 mg.5 ml^{-1}. 24 h^{-1}, n = 9) or saline (0.9%, n = 6) between 109 d and 116 d gestation. Basal values (open histograms) represent the average values of samples taken prior to infusion (107-109 d gestation) whilst infusion values (solid histograms) represent the average values over the duration of the infusion (110-116 d gestation). * $P < 0.05$ difference from respective basal value.

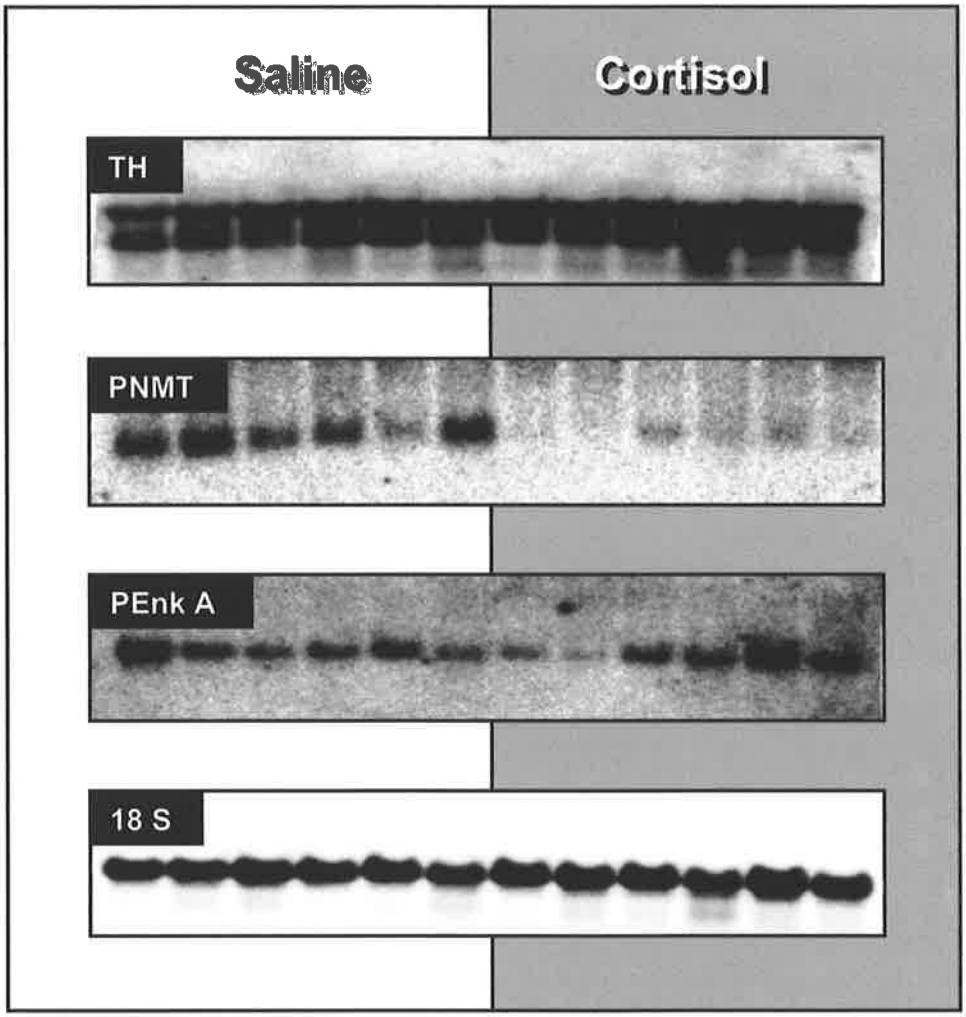


Figure 4.2 Representative autoradiographs of Northern blots of total adrenal RNA from cortisol and saline infused fetal sheep hybridised with TH cDNA, PNMT, PEnk A, and 18 S rRNA oligodeoxynucleotide probes

Representative autoradiographs of Northern blots after hybridisation of radiolabelled TH cDNA, PNMT, PEnk A, and 18 S antisense oligonucleotide probes with total RNA (20 µg per lane) extracted from adrenals collected from fetal sheep at 116 d gestation after infusion of either saline (open panel, n = 6) or cortisol (2.5-3.0 mg.5 ml^{-1}.24 h^{-1}, shaded panel, n = 6)

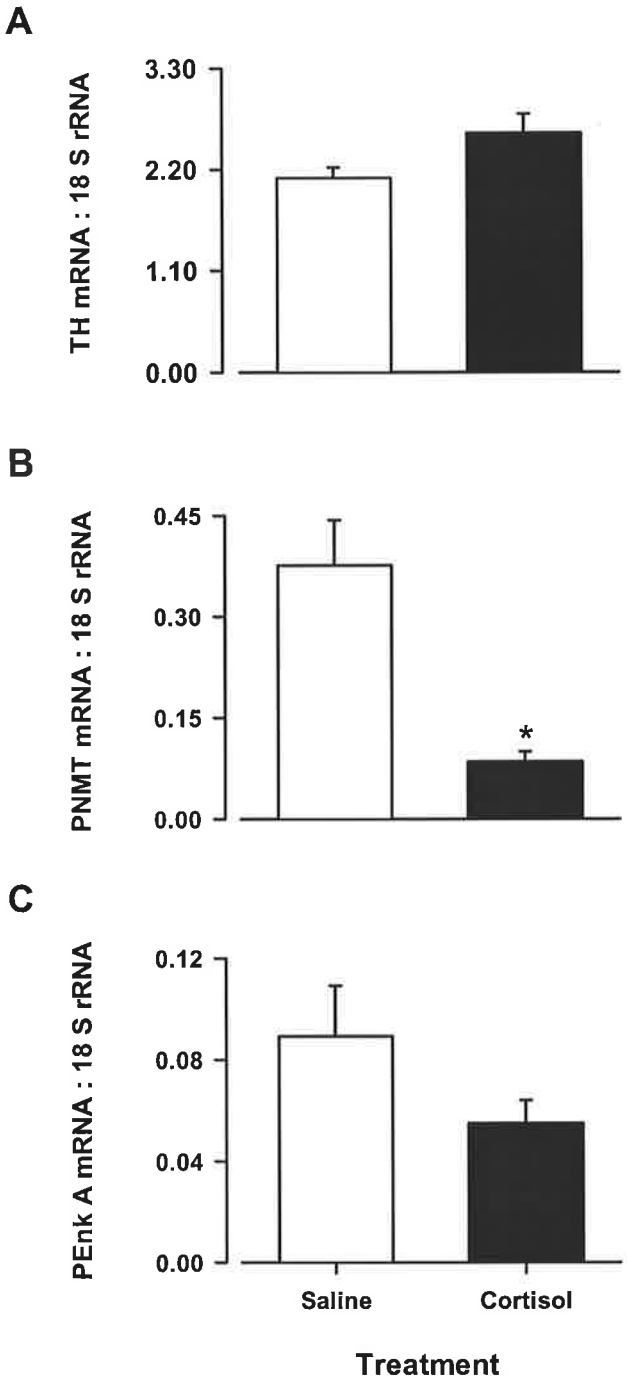


Figure 4.3 Impact of cortisol infusion on adrenal catecholamine synthetic enzyme and PEnk A mRNA expression in the fetal sheep

TH mRNA (A), PNMT mRNA (B), and PEnk A mRNA (C) expression relative to 18 S rRNA expression (mean ± SEM) in total adrenal RNA obtained from fetal sheep at 116 d gestation after infusion with either saline (open histograms, n=6) or cortisol (solid histograms, 2.5-3.0 mg.5 ml^{-1}.24 h^{-1}, n = 9). The mean PNMT mRNA : 18 S rRNA ratio was significantly [$P < 0.0005$] lower in the cortisol infused group when compared to the saline infused group as denoted by the asterisk.

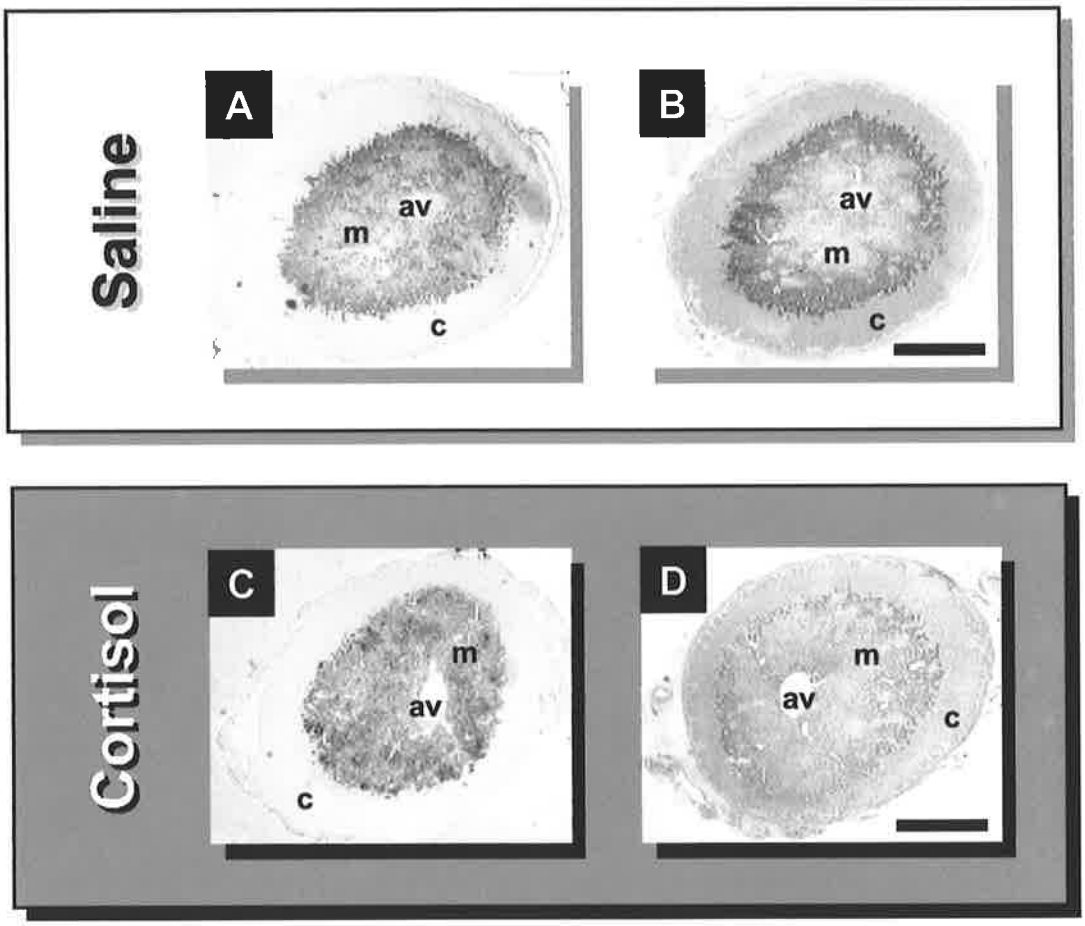


Figure 4.4 Immunohistochemical staining of adrenal sections from saline and cortisol infused fetal sheep with antibodies to TH and PNMT

Immunohistochemical staining of sagittal adrenal sections with anti-TH (A, C) and anti-PNMT (B, D) from fetal sheep infused with saline (A, B; open panel) or cortisol (C, D; shaded panel). Anti-TH stained sections (A, C) displayed immunoreactivity throughout the adrenal medulla for both saline and cortisol infused fetuses. Note that in the anti-PNMT stained sections (B, D), PNMT immunoreactivity, visible as the darkly stained areas, is primarily located in the peripheral regions of the medulla (m) which interdigitate with the cortex (c) and occasionally around the central adrenal vein (av) and sinusoids. There was a significant ($P < 0.007$) reduction in the relative area of adrenal medulla which was PNMT positive in the cortisol infused group when compared with the saline infused group. Scale bar represents 500 μm.

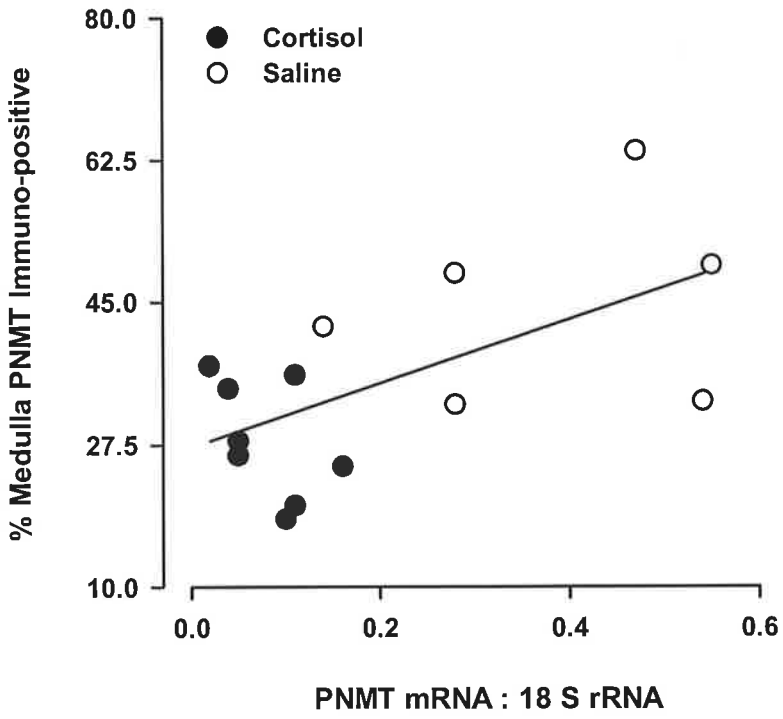


Figure 4.5 Relationship between adrenal PNMT mRNA expression and PNMT immunoreactivity within the adrenal medulla in saline infused and cortisol infused fetal sheep

The ratio of adrenal PNMT mRNA : 18 S rRNA expression plotted against the area of the adrenal medulla immuno-positive for PNMT in saline infused (open circles) and cortisol infused (solid circles, 2.5-3.0 mg. 5 ml^{-1}. 24 h^{-1}) 116 d fetal sheep. The solid line represents the line of best fit derived by regression analysis. A significant positive relationship [y = 43.47x + 26.28; r = 0.652; $P < 0.02$] was established.

stained with anti-PNMT = 43.47*(PNMT mRNA:18 S rRNA) + 26.28; r = 0.65; $P < 0.02$] (Figure 4.5).

Table 4.2 Adrenal morphometry data for saline and cortisol infused fetuses at 116 d gestation

Treatment	Total Adrenal Area (mm^2)	Adrenal Medulla (% of total gland area)	PNMT Staining (% of total medullary area)
Saline *(n = 6)*	6.7 ± 0.26	44.9 ± 4.3	44.8 ± 4.8
Cortisol *(n = 8)*	6.9 ± 0.08	52.3 ± 1.7	28.1 ± 2.5*

*$P < 0.05$ difference between groups.

4.4 DISCUSSION

A number of recent studies in the sheep have found that intrafetal infusion of clinically relevant doses of both endogenous and synthetic glucocorticoids leads to a decrease in plasma adrenaline and noradrenaline levels (Derks *et al.*, 1997; Stein *et al.*, 1995; Wood *et al.*, 1987). The data obtained in this study indicate that the glucocorticoid induced fall in plasma adrenaline levels may be a consequence of the dramatic decrease in PNMT mRNA and protein expression in the fetal adrenal. The effect of cortisol infusion was specific for adrenal PNMT as TH and PEnk A mRNA levels were not significantly altered. Interestingly, there was also a significant increase in fetal glucose levels in the cortisol infused fetuses which may relate to the inductive effect of cortisol on hepatic gluconeogenic enzymes (Fowden, 1995). The plasma cortisol concentrations achieved during infusion in this study were similar to those measured after 140 days gestation or after exposure to acute stress, such as hypoxaemia in late gestation (Braems *et al.*, 1996; Challis & Brooks, 1989).

A large body of evidence exists which demonstrates that glucocorticoids are necessary for the induction and maintenance of PNMT activity in the adrenal. Dexamethasone administration in the rat reverses the fall in PNMT activity which occurs following hypophysectomy (HPX) (Jiang *et al.*, 1989; Wurtman & Axelrod, 1966) an effect which is dependent on increased PNMT mRNA expression and protein synthesis (Evinger *et al.*, 1992; Jiang *et al.*, 1989; Wurtman & Axelrod, 1966). Furthermore, translation of total adrenal RNAs from HPX rats *in vitro*

produces higher levels of immunoprecipitable PNMT in rats treated with dexamethasone (Evinger *et al.*, 1992). Finally, glucocorticoids also act to reduce PNMT degradation by stabilising the enzyme co-factor *S*-adenosylmethionine (Ciaranello, 1978; Wong *et al.*, 1992).

In rats with an intact and functional hypothalamo-pituitary adrenocortical (HPA) axis, glucocorticoids are also able to stimulate adrenal PNMT mRNA expression and enzyme activity although at supraphysiological doses (Wong *et al.*, 1995). The magnitude of the increase in peripheral glucocorticoid levels required to stimulate PNMT mRNA expression and enzyme activity in intact animals probably reflects the portal nature of the adrenal gland, in which the medulla is exposed to corticosterone concentrations estimated to be as high as 10^{-5} M (Jones *et al.*, 1977). In primary cultures of isolated bovine chromaffin cells which have been removed from any cortical influence, dexamethasone is a powerful inducer of PNMT activity with an EC_{50} for both mRNA expression induction and activity induction of 1-10 nM (Kelner & Pollard, 1985; Wan & Livett, 1989). Bovine chromaffin cells have high affinity glucocorticoid binding receptors with a K_d of around 1 nM (Kelner & Pollard, 1985) and the specific glucocorticoid type II receptor antagonist, RU 38486, blocks the induction of PNMT activity by glucocorticoids (Betito *et al.*, 1992).

In the present study, however, adrenal PNMT mRNA and protein levels clearly decreased following exposure to excess glucocorticoids. Interestingly, low dose glucocorticoid treatment has been found to reduce PNMT activity in adult rats (Wurtman *et al.*, 1967), and to attenuate the immobilisation stress induction of PNMT mRNA expression after a 1 d infusion of cortisol (Kvetnansky *et al.*, 1995). These authors concluded that the inhibitory effect of glucocorticoids on PNMT activity and mRNA expression were due to negative feedback on the HPA axis suppressing endogenous adrenal glucocorticoid output and intra-adrenal glucocorticoid levels (Kvetnansky *et al.*, 1995; Wurtman *et al.*, 1967).

Cortisol infusion was found in the current study to have had no significant effect on circulating irACTH levels, adrenal weights, or adrenomedullary and adrenocortical proportions in the fetal sheep. Furthermore, cortisol infusion did not result in a decrease in the mRNA expression of the adrenal steroidogenic

enzymes 3β-hydroxysteroid dehydrogenase / Δ^5, Δ^4-isomerase, P-$450_{17\alpha}$, or P-450_{SCC} in these studies (Ross *et al.*, personal communication). This concurs with the findings that fetal adrenal corticosteroid production and output is minimal before 120 days gestation due to a low pituitary corticotrophic drive (Hennessy *et al.*, 1982; Wintour, 1984), and that the HPA axis is relatively insensitive to glucocorticoid negative feedback in fetal sheep at this gestational age range under basal conditions (Challis & Brooks, 1989; Ozolins *et al.*, 1990). Whilst our findings do not support an indirect action of cortisol via negative feedback effect on pituitary ACTH secretion to reduce PNMT expression, it is possible that cortisol acts at the fetal pituitary to alter the post-translational processing of the ACTH precursor molecule, proopiomelancortin (POMC) to generate POMC derived peptides which are less bioactive than ACTH (Ozolins *et al.*, 1991*a;* Zehnder *et al.*, 1998).

Another possible explanation for the observed decrease in the expression of adrenal PNMT mRNA may be a down-regulation of adrenal type II glucocorticoid receptors in the cortisol infused group. Binding of these receptors by glucocorticoids results in homo-dimerisation and interaction of the complex with glucocorticoid response element motifs on the PNMT gene to stimulate transcription (Wright *et al.*, 1993). Studies have shown that there is a loss of type II glucocorticoid receptors present in chromaffin cells after prolonged exposure to glucocorticoids (Betito *et al.*, 1993).

Analysis of the transcription regulatory regions upstream of the coding region of the PNMT gene in the human (Kaneda *et al.*, 1988), mouse (Morita *et al.*, 1992), and cow (Batter *et al.*, 1988) reveal the presence of a number of glucocorticoid response element-like motifs. Fusion gene construct experiments using a CAT reporter gene have revealed the GRE which is 513 bp upstream of the coding region of the bovine PNMT gene, stimulates transcription when exposed to glucocorticoids (Ross *et al.*, 1990). It may be that other GREs on the gene may regulate transcription in an inhibitory way such that there may be a biphasic response to glucocorticoids depending on dose and / or duration of exposure to excess glucocorticoids.

Adrenal TH mRNA expression was not affected by cortisol infusion and this is in agreement with previous studies which show that adrenal TH mRNA expression and enzyme activity are predominantly regulated by neural factors rather than glucocorticoids (Faucon Biguet *et al.*, 1991; Muller & Unsicker, 1986; Naranjo *et al.*, 1986; Stachowiak *et al.*, 1990*b*). In the fetal sheep, basal plasma noradrenaline levels are predominantly determined by the degree of spill over from sympathetic nerve terminals as destruction of the adrenal medulla does not significantly alter plasma noradrenaline levels (Jones *et al.*, 1988). Hence it appears that the effect of excess glucocorticoids on plasma noradrenaline levels in the late gestation fetal sheep (Derks *et al.*, 1997; Stein *et al.*, 1995; Wood *et al.*, 1987) may be due to a decrease in noradrenaline overspill from peripheral sympathetic nerves. In the mature animal glucocorticoids appear to have an inhibitory influence on many aspects of sympathoadrenal activity particularly at the sympathoneural level (Kvetnansky *et al.*, 1995). It has previously been reported that the elevated plasma levels of noradrenaline measured in the adrenalectomised rat are reversed after glucocorticoid administration (Brown & Fisher, 1986). Exogenous glucocorticoid administration to humans also inhibits sympathetic nerve outflow and consequently reduces circulating noradrenaline concentrations (Lenders *et al.*, 1995).

Glucocorticoid receptor binding sequences have been identified on the rat preproenkephalin gene (LaGamma & Alder, 1987) and glucocorticoids stimulate both PEnk A mRNA expression and Enk-containing peptide levels in cultures of bovine and rat adrenal chromaffin cells (Henion, 1992; LaGamma & Alder, 1987; Naranjo *et al.*, 1986; Stachowiak *et al.*, 1990*b;* Stachowiak *et al.*, 1988*b;* Yoburn *et al.*, 1987). The maximal induction of PEnk A mRNA in cultured chromaffin cells by glucocorticoids occurs at very low concentrations (Naranjo *et al.*, 1986) and in the current study no significant change in adrenal PEnk A mRNA expression was observed by increasing circulating cortisol levels in fetal sheep. Fraser *et al.* (1997) reported that a 96 h infusion of cortisol to 124-129 d fetal sheep actually suppressed adrenal PEnk A mRNA expression. Furthermore, it has previously been shown that post-translational processing of Enk-containing peptides in the fetal sheep adrenal is glucocorticoid independent (Coulter *et al.*, 1992). Hence it is unlikely that cortisol infusion in the fetal sheep acts to suppress plasma catecholamine levels by increasing adrenal opioid peptide synthesis.

Our data suggest that there is an effect of excess glucocorticoids on adrenal adrenaline biosynthesis which may, in part, explain the reported decrease in plasma adrenaline after intrafetal administration of clinically relevant doses of glucocorticoids (Derks *et al.*, 1997; Stein *et al.*, 1995; Wood *et al.*, 1987). These findings are significant in the context of current clinical practice in which exogenous synthetic glucocorticoids are administered to the pregnant woman to mature fetal lung development in anticipation of premature delivery (Bishop, 1981; Schellenberg & Liggins, 1987). It is likely that a decrease in adrenaline biosynthesis would limit the adrenal responses to the stress of vaginal or Caesarean delivery and to a range of perinatal stressors in the immediate newborn period. Prenatal dexamethasone treatment within therapeutic dose ranges has been reported to produce a dose-dependent increase in mortality rates in neonatal rats exposed to physiological levels of hypoxia (Kauffman *et al.*, 1994). The deleterious effects of dexamethasone on neonatal survival during hypoxia are associated with specific decreases in both adrenal adrenaline secretion and content as well as a premature loss of specialised cardiac α_2-adrenergic receptors which preserve fetal and neonatal cardiac function (Kauffman *et al.*, 1994).

In summary, we have found that a chronic, physiological elevation of plasma cortisol levels in the fetal sheep from 109-116 d gestation specifically reduced adrenal PNMT mRNA expression with a consequent decrease in PNMT protein expression. This decline in PNMT expression does not appear to be mediated by negative feedback of the exogenously administered cortisol on the fetal HPA axis and may represent a direct or indirect novel glucocorticoid regulatory effect on PNMT synthesis. The decline in PNMT synthesis is consistent with previous studies which have reported a fall in circulating adrenaline levels following glucocorticoid infusion in fetal sheep. The consequent impairment of adrenal adrenaline synthesis by physiologically and clinically relevant doses of glucocorticoids could result in altered adrenomedullary responses to perinatal stressors in the fetus and neonate.

5. EFFECT OF PLACENTAL RESTRICTION ON TH, PNMT, AND PEnk A mRNA EXPRESSION IN THE ADRENAL GLAND OF THE LATE GESTATION SHEEP FETUS

5.1 INTRODUCTION

The fetus responds to acute episodes of hypoxia with an increase in adrenal catecholamine ouput (Cheung, 1990; Cohen *et al.*, 1984; Jones *et al.*, 1988) and in Chapter 3 it was reported that there are also associated changes in adrenal TH and PNMT gene expression. It is not yet established if exposure of the fetus to chronic stress *in utero* evokes long term changes in catecholamine synthetic enzyme and proenkephalin A (PEnk A) gene expression which could potentially impact upon the synthesis and secretion of adrenomedullary catecholamines.

An inability of the placenta to supply the developing fetus with adequate oxygen and nutrients has been implicated in the pathogenesis of intrauterine growth restriction (see Robinson *et al.*, 1985 and Robinson *et al.*, 1995 for reviews). Cordocentesis studies in the human demonstrate that small for gestational age fetuses are frequently hypoxaemic and hypoglycaemic (Economides *et al.*, 1991). Catecholamines and their metabolites are also elevated in the amniotic fluid and umbilical cord blood in growth retarded human fetuses (Divers *et al.*, 1981; Lagercrantz *et al.*, 1980; Puolakka *et al.*, 1984; Weiner & Robillard, 1988). Experimental models of IUGR in which the fetus is chronically hypoxaemic there are also sustained increases in the fetal plasma concentrations of catecholamines (Gagnon *et al.*, 1994; Hooper *et al.*, 1990; Simonetta *et al.*, 1997). Despite increased plasma catecholamines under basal conditions with experimental IUGR, studies both in the rat (Shaul *et al.*, 1989) and lamb (Jones & Robinson, 1983) reveal that the catecholamine response, particularly adrenaline, is impaired in response to an additional acute hypoxic challenge.

Intrauterine growth restriction and chronic fetal hypoxaemia are therefore associated with long term changes in basal circulating catecholamine levels and adrenomedullary responsiveness to subsequent acute stressors. Such changes may reflect alterations in fetal catecholamine secretion, synthesis, and metabolism. It is unknown to what degree any of these determinants of circulating catecholamine levels are altered in the fetal adrenal gland in response to chronic

stress. The aim of this study was therefore to compare the adrenal expression of TH, PNMT, and PEnk A mRNA in growth restricted and normally grown fetal sheep in late gestation. For this study a well characterised experimental model of IUGR was utilised which involved the surgical restriction of placental implantation in the sheep thus restricting placental growth and function (Alexander, 1964; Owens *et al.*, 1989).

5.2 METHODS

All procedures were approved by the University of Adelaide Standing Committee on Ethics in Animal Experimentation.

5.2.1 ANIMALS AND SURGERY

Twenty one Border Leicester x Merino ewes were used in this study. In ten ewes, the majority of visible endometrial caruncles, were removed from the uterus prior to conception as previously described (Robinson *et al.*, 1979*a*). This procedure limits the number of placental cotyledons formed following mating and hence restricts placental and fetal growth. Caruncle ctomy was performed under anaesthesia induced by intravenous injection of sodium thiopentone (1.25 g; Pentothal, Rhone Merieux, Pinkenba, QLD, Australia) and maintained with halothane (3-4 % in oxygen; Fluothane, ICI, Melbourne, VIC, Australia). Ewes were administered 2 ml of antibiotics (250 $mg.ml^{-1}$ procaine penicillin, 250 $mg.ml^{-1}$ dihydrostreptomycin sulphate, 20 $mg.ml^{-1}$ Procaine HCl; Penstrep Ilium, Troy Laboratories, Smithfield, NSW, Australia) intramuscularly prior to surgery. The uterus was exteriorised through a low abdominal midline incision and subsequently opened along the antimesometrial border from the level of the cervix to close to the uterotubule junction in both horns. Most of the visible endometrial caruncles were then excised and the incisions in the uterus were sutured in a single layer using 2 / 0 cat gut (Ethicon, Johnson and Johnson, Sydney, NSW, Australia) through the myometrium and serosa. Once sutured the uterus was replaced and the abdomen closed in two layers, the peritoneum and rectus sheet, followed by the skin and sub cutaneous tissue, using 4 / 0 coated Vicryl (Ethicon). Ewes were kept under observation for 4-7 d post surgery and following a minimum of 10 weeks recovery entered a mating program with the date of mounting taken

as day 0 of pregnancy. Pregnancies were confirmed by ultrasound at approximately 60 d gestation.

In the 10 carunclectomised ewes (Placental Restriction group) and 11 control ewes a second surgery was carried out between 102 and 112 d gestation under general anaesthesia as described above. Catheters were inserted into the fetal and maternal carotid artery and jugular vein as well as the amniotic sac as described in Chapter 3. Catheters were flushed and filled with heparinised saline and the fetal catheters were exteriorised through an excision in the ewes flank.

5.2.2 POST OPERATIVE CARE

Following surgery the ewes were housed in individual metabolic crates in animal holding rooms under a 12 h light-dark cycle and fed lucerne chaff once daily between 0900 and 1300 h with water *ad libitum*. Sodium ampicillin (500 mg, Austrapen, Commonwealth Serum Laboratories, Melbourne, VIC, Australia) was administered intra-amniotically to all fetal sheep for 4 consecutive days post operatively.

5.2.3 POST MORTEM AND TISSUE COLLECTION

The ewes were electively killed between 140 and 141 d gestation with an overdose of sodium pentobarbitone (8.125 g; Lethabarb, Virbac, Peakhust, NSW, Australia) and both the uterus and fetus were removed by hysterotomy. The fetuses were weighed and then decapitated and the fetal adrenal glands were quickly dissected, weighed, and either snap frozen in liquid N_2 or placed into ice-cold paraformaldehyde fixative. Placental cotyledons were individually dissected from the uteri of carunclectomised (n = 7) and control ewes (n = 3) and weighed.

5.2.4 BLOOD SAMPLING PROTOCOL

Fetal (3.5 ml) and maternal (5 ml) arterial blood samples were collected every second day between 104 and 140 d gestation. An additional 0.5 ml of whole arterial blood was also collected in order to determine arterial PO_2, pH, PCO_2, O_2 saturation (SO_2) and haemoglobin (Hb) content using an ABL 330 acid base analyser and an OSM2 Hemoximeter (Radiometer, Copenhagen, Denmark). Blood samples were collected into heparinised tubes on ice and centrifuged at

4°C at 1500 *g* for 10 min. The plasma was separated and a 1 ml aliquot was placed into a polypropylene tube (12 x 75 mm) containing antioxidant (50 µl of 2 m*M* ascorbic acid) for subsequent catecholamine analysis. All plasma aliquots were subsequently snap frozen in liquid nitrogen and stored at -70°C until analysed for catecholamines and glucose.

5.2.5 PLASMA GLUCOSE DETERMINATION

Plasma concentrations of glucose were measured across gestation in placentally restricted (PR, n = 9) and control fetuses (n = 9) using a COBAS MIRA automated Analysing System (Cobas, Roche Diagnostics, Basle, Switzerland). The system determined plasma glucose concentrations by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase and measuring the formation of NADH spectrophotometrically at 340 nm. The intra-assay coefficient of variation was 1.4%.

5.2.6 PLASMA CATECHOLAMINE EXTRACTION

Catecholamines were extracted by alumina absorption from gestational plasma samples obtained from PR (n = 7) and control (n = 6) fetuses and measured in accordance with the methods of (Schuijers *et al.*, 1986). Thawed 1 ml aliquots of plasma were transferred to borosilicate glass culture tubes (12 x 75 mm) containing 10 mg of acid washed alumina (Hart Analytical, Melbourne, Australia) and 30 µl of 10 m*M* sodium metabisulphite. An additional 1 ml of distilled water was added to the plasma, as well as 100 µl of DHBA (20 pmol / 100 µl 0.01*M* sodium metabisulphite) which was used as an internal standard to monitor catecholamine recovery. Tris-EDTA buffer (400 µl, 1*M* Tris base, 2 % EDTA, pH 8.6) was added to the tubes which were then vigorously agitated at 4°C for 40 min on a multi-tube vortexer (Baxter Healthcare Corp., Miami, FL, USA). The samples were subsequently centrifuged at 1800 *g* for 10 min at 4°C and the supernatant was discarded. The alumina was then washed 4 times with distilled deionised water (0.5 ml) and following the last wash the samples were centrifuged at 1800 *g* for 10 min at 4°C, with the supernatant again being discarded. The catecholamines were desorbed from the alumina by the addition of perchloric acid (100 µl, 0.1 *M* in 0.01 *M* sodium metabisulphite) prior to the samples being

vortexed for a further 10 min at 4°C. Following centrifugation at 1800 *g* for 10 min at 4°C the supernatant was aspirated and filtered through 0.45 μm syringe filters (Filter type HV; Nihon Millipore Kogyo K.K., Yonezawa, Japan) to remove any remaining alumina particles. Samples were stored at -70°C after extraction until they were assayed using reverse phase HPLC combined with electrochemical detection. The average recovery of catecholamines with this extraction procedure was 59.2 ± 0.9 %.

5.2.7 CATECHOLAMINE MEASUREMENT

30 μl aliquots of the extracted samples were injected onto a BAS 200A HPLC system (Bioanalytical Systems Inc., West Lafayette, IN, USA) using a CMA 200 refrigerated microsampler (CMA / Microdialysis AB, Stockholm, Sweden) at 4°C. Separation of catecholamines was achieved using a 3 μm ODS, 100 x 3.2 mm, phase II cartridge column (Bioanalytical Systems Inc.) and an aqueous mobile phase which consisted of (m*M*): sodium acetate, 50; citric acid, 20; octanesulphonic acid, 3.75; EDTA, 2; and methanol 5% v / v, at a pH of 4.3. The mobile phase flow rate was 0.7 $ml.min^{-1}$ and the potential of the glassy carbon detector electrode was set at + 750 mV *vs.* a Ag / AgCl reference electrode. Catecholamines were automatically quantified by peak area integration using an IBM compatible personal computer equipped with Barspec Data System chromatographic software (Barspec Ltd., Rehovot, Israel). Peak areas were compared with to those obtained for catecholamine standards of known concentration (Norepinephrine Bitartrate, Epinephrine Bitartrate, and DHBA Hydrobromide; 20 pmol / 100 μl 0.4 *M* PCA; Bioanalytical Systems Inc.) to determine the noradrenaline and adrenaline content of the samples. The sensitivity of the catecholamine assay was 0.07 $pmol.ml^{-1}$ for both noradrenaline and adrenaline and the intra- and inter-assay coefficients of variation were less than 10 %.

5.2.8 RNA ISOLATION AND ANALYSIS

Total adrenal RNA was extracted according to the method of Chirgwin *et al.* 1979, as outlined in Chapter 2. Briefly, adrenals from 6 PR fetuses and 6 control fetuses were homogenised in 4 *M* guanidine thiocyanate solution and ultracentrifuged at

130 000 *g* through a 5.7 *M* CsCl cushion. The RNA pellet was then resuspended in water and purified by re-precipitation with 0.3 *M* sodium acetate and absolute ethanol. The RNA pellet was washed in 70 % ethanol and again resuspended in water. Maximum absorbance was measured spectrophotometrically at 260 nm and 280 nm and used to quantify nucleic acid purity, yield, and concentration. RNA solutions were kept at -70° C until use.

5.2.9 PROBES AND PROBE LABELLING

The probes utilised in this study and the radiolabelling methods for the probes are described in detail in Chapter 2. To summarise, oligonucleotide antisense probes complementary to nucleotides 361-389 of the peptide coding region of bovine PNMT mRNA (29 mer, Wan *et al.*, 1988), nucleotides 627-671 of the peptide coding region of bovine PEnk A mRNA (44 mer, Wan *et al.*, 1988), and nucleotides 151-180 of the rat 18 S rRNA (30 mer, Chan *et al.*, 1984) sequence were synthesised (Bresatec, Thebarton, SA, Australia). A 1.7 kb bovine TH cDNA (D'Mello *et al.*, 1988) was used to detect TH mRNA. The oligonucleotides were end labelled using T4 polynucleotide kinase (7.9 $U.\mu l^{-1}$, Pharmacia, North Ryde, NSW, Australia) and γ-[^{32}P]ATP (4000 $Ci.mmol^{-1}$,Bresatec) in accordance with the method described for the end labelling of oligonucleotides by Sambrook *et al.* (1989). The cDNA probe was labelled by the random priming method with α-[^{32}P]dCTP (3000 $Ci.mmol^{-1}$, Bresatec) and Klenow fragment (6.4 $U.\mu l^{-1}$, Pharmacia) using an oligolabelling kit (Pharmacia) as per manufacturer's instructions.

5.2.10 NORTHERN BLOT ANALYSIS

Northern blotting was performed as described by Sambrook *et al.* (1989) and outlined in detail in Chapter 2. Total RNA samples (20 µg) were denatured in 2.2 *M* formaldehyde and 50 % (v / v) formamide at 55°C for 10 min. The denatured total RNA was subsequently separated using gel electrophoresis with 1 % agarose containing 2.2 *M* formaldehyde and then transferred by capillary blotting to Zetaprobe nylon membranes (Biorad, Richmond, CA, USA). Membranes were washed in 10 x SSC for 10 min at room temperature before being baked for 1 h at 80°C and stored flat at -20°C until hybridisation.

For oligonucleotide probes the membranes were prehybridised at 50°C for 18 h in a solution consisting of 5 x SSC, 20 m*M* NaH_2PO_4, pH 7.2, 7 % SDS, 5 x Denhardt's, and 100 $\mu g.ml^{-1}$ of heat denatured Salmon sperm DNA. Membranes were hybridised with 1-2 x 10^6 $cpm.ml^{-1}$ of labelled oligonucleotide in 30 ml of fresh hybridisation solution for 20 h at 50°C. The same protocol was used for the TH cDNA probe, however the hybridisation solution consisted of 50 % (v / v) deionised formamide, 5 x SSPE, 7 % SDS, pH 7.2, and 100 $\mu g.ml^{-1}$ of heat denatured salmon sperm DNA, with hybridisation being carried out at 42°C. Following hybridisation the membranes were washed in 1 x SSC and 0.1 % SDS at their hybridisation temperatures (1 x 30 min). The membranes were then washed at their hybridisation temperature in 0.1 x SSC and 0.1 % SDS (2 x 30 min), briefly air dried, and exposed to a Fuji BAS-IIIs phosphorimager plate (Fuji Photo Co., Tokyo, Japan) for 24-48 h. Autoradiographs were visualised using a Fuji BAS 1000 phosphorimager (Fuji Photo Co.) and quantitated using Fuji MacBAS software (Fuji Photo Co.).

5.2.11 IMMUNOHISTOCHEMISTRY

Adrenals from PR (n = 6) and control (n = 6) fetuses were dissected into halves perpendicular to their poles at postmortem and immersed in ice-cold paraformaldehyde fixative (4 % in sodium phosphate buffer, 0.1 *M*, pH 6.4) for 24 h. Following fixation the adrenals were washed in ice cold sodium phosphate buffer (2 x 24 h). After washing, adrenals were stored at 4°C in 70 % ethanol before being embedded in paraffin wax.

Transverse adrenal sections (5 µm) were cut on a microtome and mounted onto glass slides coated with poly-L-lysine. Semi adjacent sections were either processed for TH and PNMT immunoreactivity using a Vectastain avidin-biotin peroxidase complex kit (PK-4001, Vector Laboratories, Burlingame, CA, USA) or stained with haematoxylin and eosin. The antibodies and immunohistochemical procedures used in this study have been described in detail in Chapter 4. Briefly, the sections were deparaffinised by immersion in Histoclear (National Diagnostics, Somerville, NJ, USA) and then rehydrated by immersion through a graded ethanol series and rinsed in distilled water. Endogenous peroxidase activity was quenched by a 30 min incubation with 0.5 % H_2O_2 in methanol. Sections were

washed (1 x 20 min, 0.01 *M* PBS, pH 7.0) and then pre-incubated with blocking solution (3 % NGS in 0.01 *M* PBS containing 1 % BSA) for 30 min. Following pre-incubation the sections were covered with primary antibody at the appropriate dilution (rabbit anti-bovine PNMT, 1:1500; rabbit anti-rat TH, 1:1000) in PBS containing 1 % BSA. Sections were incubated overnight in an air tight humidified container at 4°C. Following incubation with the primary antibody sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vectastain kit PK-4001) for 60 min at room temperature and then with ABC (Vectastain kit PK-4001) for a further 60 min. The sections were washed with PBS (0.01 *M*, 3 x 5 min) following each incubation. The sections were subsequently covered with DAB (0.5 $mg.ml^{-1}$ in PBS buffer and 0.02 % H_2O_2) at room temperature for 10 min. Sections were finally washed in PBS (3 x 5 min), dehydrated and coverslipped.

5.2.12 ADRENAL MORPHOMETRY

The transverse areas of a number of adrenal compartments were determined from mid glandular sections. Images of adrenal sections were captured using a CCD black and white video camera (SSC-M370CE, Sony Corp., Tokyo, Japan) mounted on a dissecting microscope via an SZ-CTV photomount tube (Olympus Optical Co., Tokyo, Japan). The image was digitised as a grey scale image using a Power Mac 8500 / 120 (Apple, Cupertino, CA, USA) equipped with NIH Image V1.61 software. Total adrenal area was obtained by defining and measuring the boundary around the adrenal section image. Relative vascular area was calculated by subtracting the area of the section positively stained with Haematoxylin and Eosin from the total area of the section. The area of the medulla was defined as the area of the gland which stained positively with anti-TH. The areas of the sections which were positively stained for PNMT was also calculated.

5.2.13 DATA ANALYSIS

All data are presented as the mean ± standard error of the mean (SEM). A mean gestational value for each of the blood gas variables and plasma glucose concentrations were calculated for each animal in the PR and control groups and the group mean values were compared using one-tailed Student's t-tests. Adrenal TH, PNMT, and PEnk A mRNA : 18 rRNA ratios in the PR and control groups

were compared using two-tailed Student's t-tests. The relationships between mean gestational arterial PO_2 or plasma glucose and adrenal PNMT mRNA expression in PR and control fetal sheep were determined using regression analysis.

The areas of the adrenal medulla and cortex, and the relative areas of the vascular, TH, and PNMT containing regions of the adrenal sections in the PR and control groups were compared using two-tailed Student's t-tests.

A probability of less than 5% (ie. $P < 0.05$) was taken to be significant for all statistical analyses.

5.3 RESULTS

5.3.1 PLACENTAL RESTRICTION AND FETAL OUTCOMES

There was a decrease in fetal and placental weight with PR (Table 5.1). The combined adrenal weight was not significantly different between placentally restricted and control fetuses (Table 5.1). There was an increase, however, in adrenal weight when expressed relative to fetal body weight in the PR group (Table 5.1). The yield of total adrenal RNA was equivalent in the PR (1.30 ± 0.07 $\mu g.mg^{-1}$, n = 6) and control (1.27 ± 0.07 $\mu g.mg^{-1}$, n = 6) fetuses.

Table 5.1 Fetal, placental, and adrenal weights for control and placentally restricted fetal sheep at 140 d gestation

Treatment	Fetal Weight (kg)	Placental Weight (g)	Adrenal Weight (mg)	Adrenal : Fetal Weight ($mg.kg^{-1}$)
Control	4.64 ± 0.23 *(n =11)*	573.3 ± 64.9 *(n = 3)*	372.0 ± 11.7 *(n = 11)*	81.7 ± 3.8 *(n = 11)*
PR	2.94 ± 0.24* *(n = 10)*	187.6 ± 30.8* *(n = 7)*	350.6 ± 26.1 *(n = 9)*	114.8 ± 17.0* *(n = 9)*

* $P < 0.05$ compared to control

The number of animals used for each observation is indicated in parenthesis.

5.3.2 PLACENTAL RESTRICTION AND FETAL BLOOD GAS, METABOLIC AND ENDOCRINE STATUS

The mean gestational (104-140 d) values for arterial PO_2 and O_2 saturation were significantly lower whereas mean arterial haemoglobin (Hb) content and PCO_2

were elevated in the PR group when compared to controls (Table 5.2). Despite the apparent chronic hypoxaemia and hypercapnia in PR fetuses there was no associated acidaemia (Table 5.2).

Mean gestational plasma glucose levels were significantly lower in PR fetuses when compared to their control counterparts (Table 5.2).

Mean plasma noradrenaline concentrations were approximately 2.1-fold higher in fetuses which were placentally restricted (Figure 5.1A). Similarly, plasma adrenaline levels were 2.5 times higher across late gestation in placentally restricted fetuses when compared to their respective controls (Figure 5.1B).

Table 5.2 Mean gestational values for arterial blood gas variables and plasma glucose in control and placentally restricted fetal sheep

Treatment	P_aO_2 (mmHg)	P_aCO_2 (mmHg)	Hb (g / dl)	S_aO_2 (%)	pH	Glucose ($mmol.l^{-1}$)
Control	23.6±0.9 *(n = 11)*	45.6±0.8 *(n = 11)*	10.2±0.3 *(n = 11)*	68.1±2.0 *(n = 11)*	7.363±0.009 *(n = 11)*	1.42±0.07 *(n = 9)*
PR	15.1±0.8* *(n = 10)*	49.5±1.0* *(n = 10)*	11.5±0.6* *(n = 10)*	44.3±3.7* *(n = 10)*	7.355±0.012 *(n = 10)*	1.13±0.09* *(n = 9)*

* $P < 0.05$ compared to control

The number of animals used for each observation is indicated in parenthesis

5.3.3 PLACENTAL RESTRICTION AND ADRENAL TH, PNMT, AND PEnk A mRNA EXPRESSION

There was no significant difference in the ratios of either adrenal TH mRNA : 18 S rRNA or PEnk A mRNA : 18 S rRNA between the PR and control groups (Figures 5.2, 5.3A & C). In contrast, the ratio of adrenal PNMT mRNA : 18 S rRNA was significantly reduced in the PR fetal sheep (0.003 ± 0.002) when compared with control animals (0.011 ± 0.002) (Figures 5.2 and 5.3B). There was also a significant correlation between the ratio of adrenal PNMT mRNA : 18 S rRNA and the mean arterial PO_2 in the fetal sheep in the PR and control groups (PNMT mRNA : 18 S rRNA = $0.113 * PO_2 - 1.444$, r = 0.88, $P < 0.001$) (Figure 5.4A). There was no significant correlation, however, between the ratio of adrenal PNMT mRNA : 18 S rRNA and the mean gestational plasma glucose concentrations (Figure 5.4B).

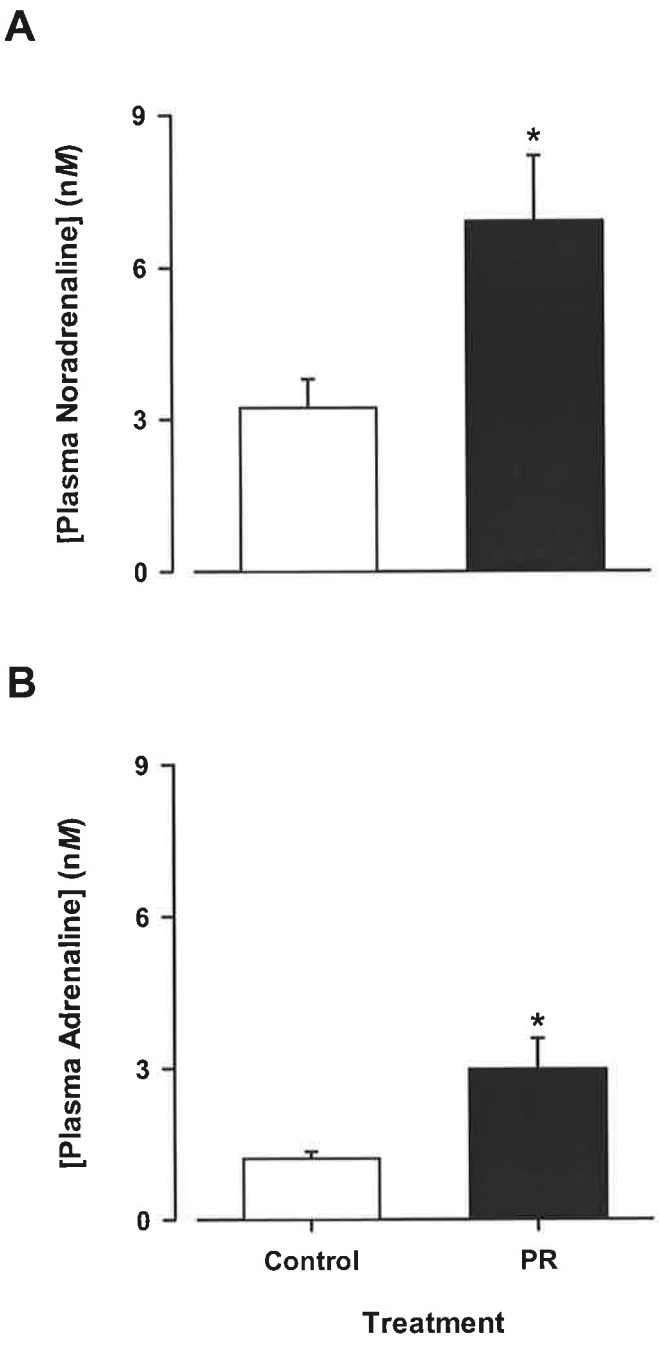


Figure 5.1 The effect of placental restriction on basal plasma catecholamine concentrations in the fetal sheep

Mean (± SEM) gestational concentrations of noradrenaline (**A**) and adrenaline (**B**) in the plasma of control (n = 6, open histograms) and PR (n = 7, solid histograms) fetal sheep from 104-140 d gestation. Plasma noradrenaline and adrenaline concentrations were significantly [$P < 0.05$] higher in the PR fetuses when compared to their respective controls as denoted by the asterisk.

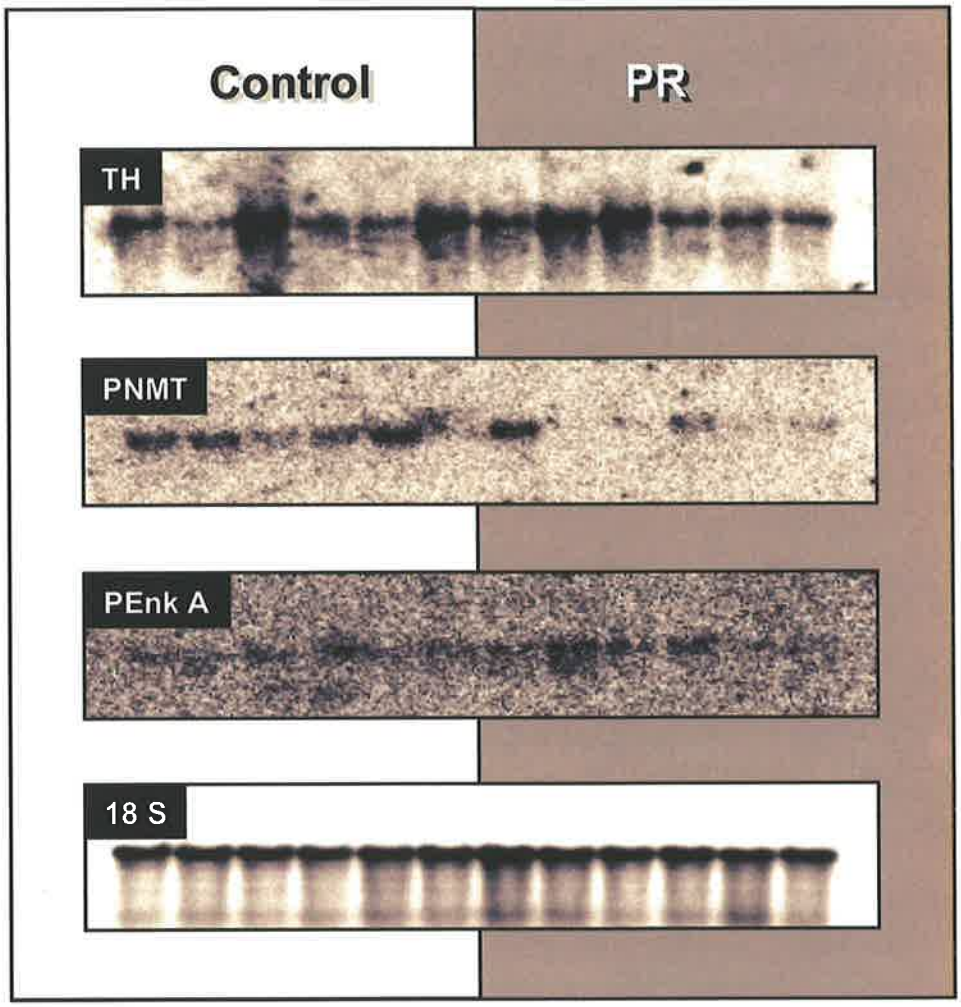


Figure 5.2 Representative autoradiographs of Northern blots of total adrenal RNA from control and placentally restricted fetuses, hybridised with TH cDNA, PNMT, PEnk A, and 18 S oligodeoxynucleotide probes

Representative autoradiographs of Northern blots after hybridisation of radiolabelled TH cDNA, PNMT, PEnk A, and 18 S antisense oligonucleotide probes with total RNA (20 µg per lane) extracted from adrenals collected from control (open panel, n = 6) or placentally restricted (shaded panel, n = 6) fetal sheep at 140 d gestation.

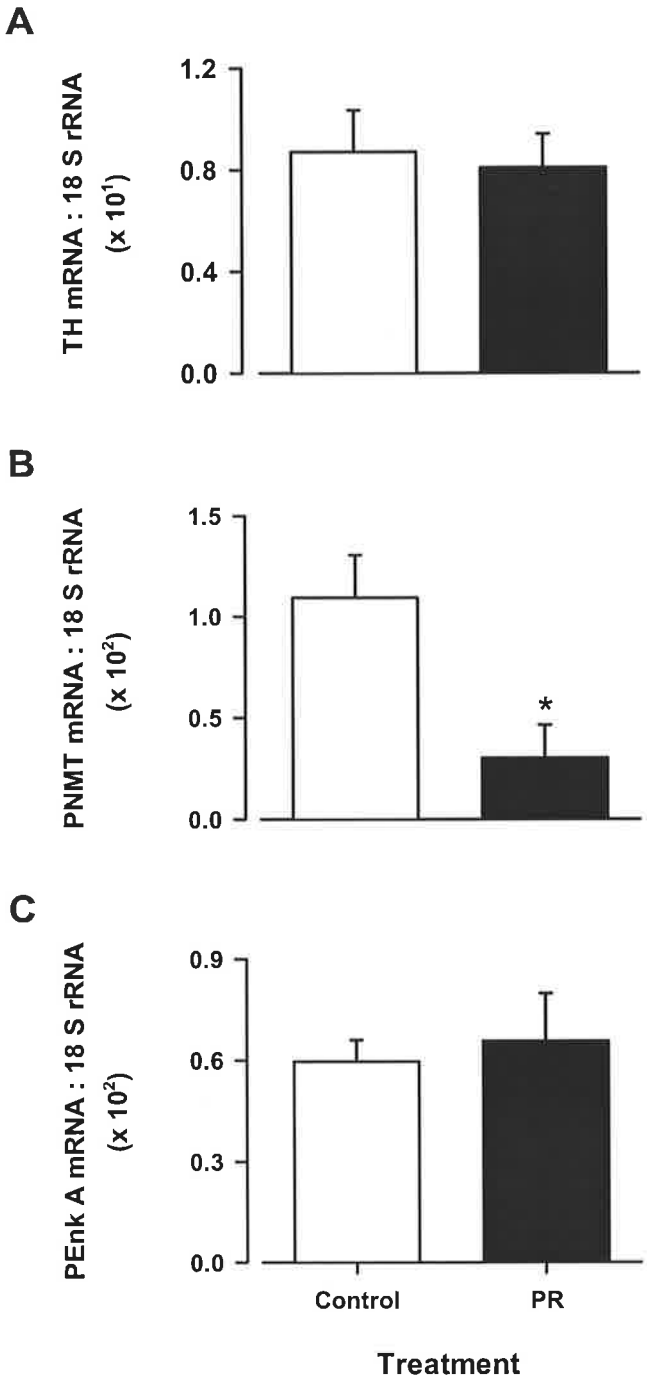


Figure 5.3 The effect of placental restriction on adrenal TH, PNMT, and PEnk A mRNA expression in the fetal sheep

TH mRNA (**A**), PNMT mRNA (**B**), and PEnk A mRNA (**C**) relative to 18 S rRNA (mean ± SEM) in the adrenals of 140 d fetal sheep from the control (n = 6, open histograms) and PR (n = 6, solid histograms) groups. The mean PNMT mRNA : 18 S rRNA ratio was significantly [$P < 0.05$] lower in the PR group when compared to the control group as denoted by the asterisk.

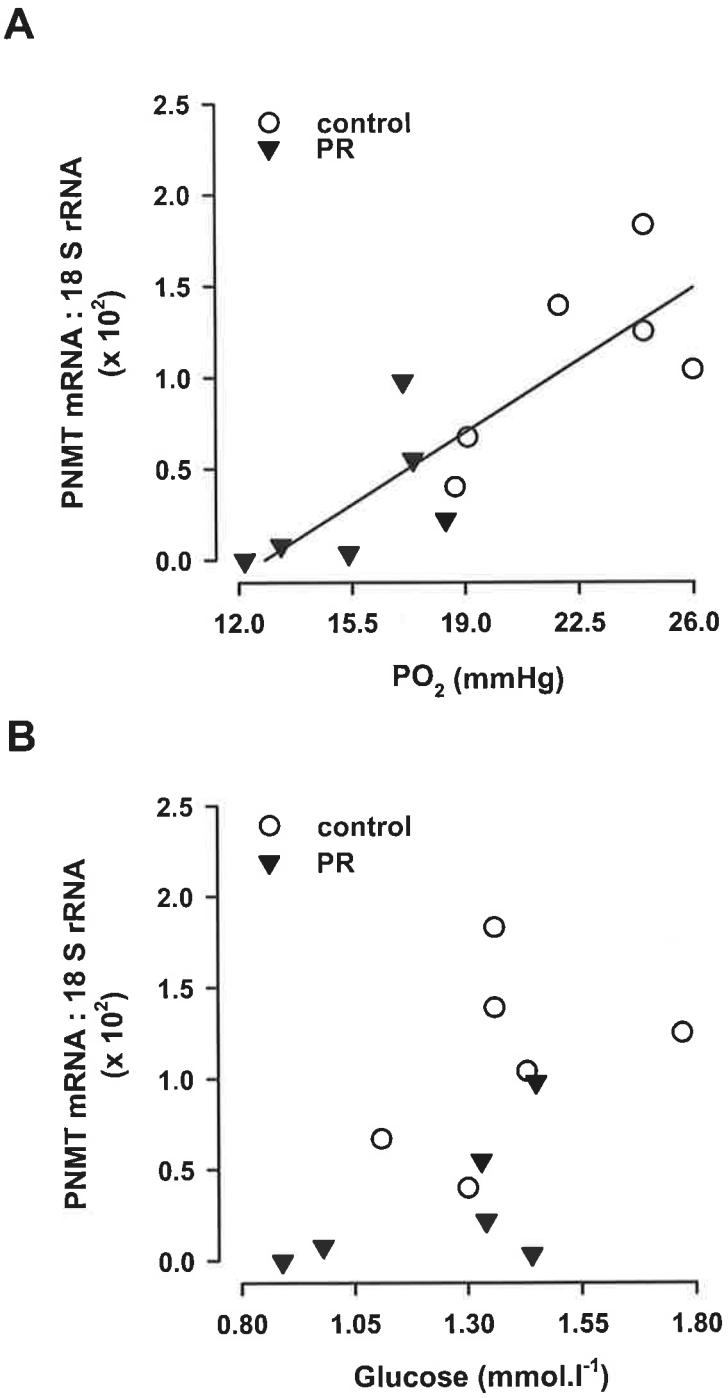


Figure 5.4 The relationship of adrenal PNMT mRNA expression with mean gestational arterial PO_2 and plasma glucose concentration in the fetal sheep

The relationship of adrenal PNMT mRNA : 18 S rRNA expression with mean gestational arterial PO_2 (**A**) and mean gestational plasma glucose concentrations (**B**) in both control (open circles) and PR (closed triangles) fetal sheep. A significant relationship [r^2 = 0.70, P < 0.001] was found between adrenal PNMT mRNA : 18 S rRNA expression and mean arterial PO_2, the solid line represents the line of best fit derived by linear regression analysis.

5.3.4 PLACENTAL RESTRICTION AND ADRENAL CATECHOLAMINE SYNTHETIC ENZYME LOCALISATION

Whilst there were also no differences between the PR and control groups in total adrenal area, the relative vascular area was found to be significantly greater in the PR group than in the controls (Table 5.3).

Neither TH nor PNMT immunoreactivity were observed in cortical cells in any adrenal sections. In fetal adrenals from both the control and placentally restricted groups TH immunoreactivity was observed throughout the adrenal medulla. The area of the adrenal medulla as determined by the presence of TH immunoreactivity was not significantly altered by placental restriction (Table 5.3). PNMT immunoreactivity on the other hand was observed in control fetal adrenals in the peripheral region of the medulla, in the rim of columnar cells which interdigitate with the cortex. Positive staining with anti-PNMT was also present in some medullary cells adjacent to the central adrenal vein and sinusoids (Figure 5.5A). In adrenals collected from PR fetuses, staining for PNMT was also predominantly confined to the peripheral region of the medulla (Figure 5.5B). The proportion of the adrenal medulla which was positively stained with anti-PNMT, however, was significantly lower in the PR fetal sheep compared to control animals (Table 5.3). There were no significant differences between the groups in the intensities of immunohistochemical staining (mean grey scale value in arbitrary units) for TH (control, n = 6, 102.4 ± 1.0; PR, n = 6, 122.5 ± 15.7), and PNMT (control, n = 6, 209.5 ± 1.0; PR, n = 6, 211.8 ± 0.7).

Table 5.3 Adrenal morphometry data for control and placentally restricted fetal sheep at 140 d gestation

Treatment	Total Adrenal Area (mm^2)	Relative Vascular Area (%)	Relative Medullary Area (%)	Medullary Area PNMT Immunopositive (%)
Control (*n* = 6)	12.9 ± 0.9	2.8 ± 0.8	38.6 ± 3.1	44.6 ± 4.0
PR (*n* = 6)	11.9 ± 1.0	5.2 ± 0.4*	40.8 ± 1.6	21.4 ± 7.4*

* $P < 0.05$ compared to control

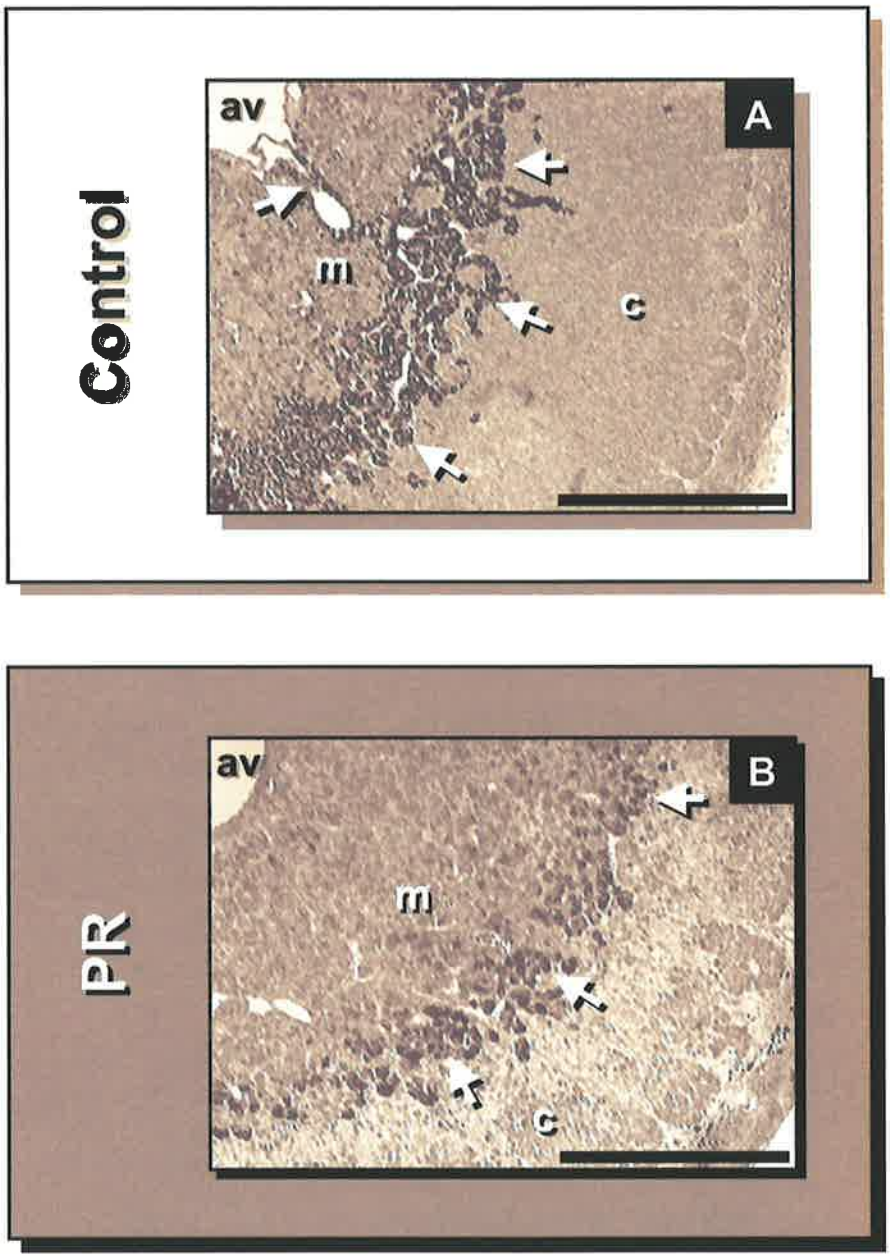


Figure 5.5 Immunohistochemical staining of adrenal sections from placentally restricted and control fetal sheep with PNMT antibody

Immunohistochemical staining of sagittal adrenal sections with anti-PNMT. Adrenal sections were obtained from both control (A; open panel) and PR (B; shaded panel) fetal sheep at 140 d. Note that PNMT positive cells which are darkly stained (indicated by arrows) are located in the peripheral region of the medulla (m) which interdigitates with the cortex (c). Some positive staining can also be seen around the central adrenal vein (av) and adrenal sinusoids. In the PR group adrenals (B) anti-PNMT staining appears patchy and is less dense compared to that observed in the controls (A). Scale bar = 500 μm.

5.4 DISCUSSION

Placental restriction was achieved in this study by the surgical removal of endometrial caruncles from the uterus of the ewe prior to mating and pregnancy. This resulted in the expected limitation of placental growth. The impairment of placental growth and hence function resulted in chronic fetal hypoxaemia with a lower mean gestational arterial PO_2, SO_2, and elevated haemoglobin levels in placentally restricted fetuses. Impaired placental function was further implicated by fetal hypercapnia and hypoglycaemia over late gestation. These changes in blood gas and metabolic variables are consistent with the reports of previous studies which utilised the carunclectomy model of intrauterine growth retardation (Robinson *et al.*, 1979*a;* Simonetta *et al.*, 1997).

As a consequence of decreased oxygen and nutrient supply there was a compensatory decrease in fetal growth. The growth of the adrenal gland however, was maintained and in relative terms increased in placentally restricted fetuses. The sparing of adrenal gland growth despite fetal IUGR has previously been demonstrated in a number of experimental models, including carunclectomy, and may relate to a redistribution of blood flow to the gland (Block *et al.*, 1984; Creasy *et al.*, 1972; Koritnik *et al.*, 1981; Oyama *et al.*, 1992; Robinson *et al.*, 1979*a*). Interestingly placental restriction was associated with an increase in adrenal vascular area which would be consistent with enhanced proportional blood flow to the gland with IUGR (Block *et al.*, 1984; Creasy *et al.*, 1972). The relative increase in fetal adrenal gland size which is found to occur with placental restriction is not found to be due to a selective increase in either of the cortical or medullary components.

Despite maintained adrenal growth, IUGR was specifically associated with decreased PNMT mRNA expression and immunoreactivity in the adrenal gland of the late gestation fetal sheep. Furthermore, there was a strong, direct relationship between mean gestational arterial PO_2 and adrenal PNMT mRNA expression. This relationship appeared to be specific for arterial PO_2 as there was no correlation between adrenal PNMT mRNA expression and mean gestational plasma glucose levels. A direct relationship between PNMT mRNA expression and arterial PO_2 raises the possibility that chronic low arterial PO_2 may act by

either neurogenic or non neurogenic mechanisms to suppress PNMT synthesis. Suppression of adrenal PNMT immunoreactivity in the fetal sheep in response to placental restriction has recently been reported to occur as early as 90 d gestation (Coulter *et al.*, 1998). It is therefore highly likely that hypoxia acts directly by a non-neurogenic mechanism to decrease adrenal PNMT gene expression, as functional innervation of the fetal sheep adrenal has yet to become established at this age (Boshier *et al.*, 1989*a;* Cheung, 1990; Comline & Silver, 1961). It is noteworthy that the fetal adrenal response to chronic hypoxaemia / placental restriction differed from that which occurred in response to acute hypoxia (Chapter 3) where PNMT mRNA expression increased in a manner which related to the degree of hypoxia. Hence there appears to be a temporal aspect to the regulation of PNMT gene expression by hypoxia in the fetal adrenal gland.

Whilst glucocorticoids clearly play an important role in the development of the adrenaline synthesising cells within the adrenal medulla (Cole *et al.*, 1995; Michelsohn & Anderson, 1992), it was demonstrated in Chapter 4 that inappropriate exposure of the fetal adrenal to high plasma glucocorticoid levels reduces PNMT mRNA expression. Increased plasma glucocorticoid levels have been reported for both clinical and experimental fetal growth restriction (Economides *et al.*, 1991; Economides *et al.*, 1988; Gagnon *et al.*, 1994; Sug Tang *et al.*, 1992). Elevated plasma cortisol levels in late gestation have also been observed in the placental restriction model of growth restriction utilised in the current study (Phillips *et al.*, 1996). It is unlikely however, that a premature increase in fetal glucocorticoid levels is responsible for the suppression of fetal adrenal PNMT levels as this rise occurs after 125 d gestation (Phillips *et al.*, 1996), subsequent to the reduction in PNMT immunoreactivity at 90 d gestation (Coulter *et al.*, 1998).

No effect of placental restriction was found on TH mRNA expression or TH immunoreactivity within the adrenal gland of the fetal sheep, despite elevated plasma noradrenaline levels. Basal noradrenaline levels in normally developed fetal sheep are predominantly determined by overspill from sympathetic neurones (Jones *et al.*, 1987). The indirectly acting sympathomimetic, tyramine, which selectively displaces catecholamines from sympathetic nerve terminals produces an increased plasma noradrenaline response in placentally restricted fetuses

(Simonetta *et al.*, 1997). Therefore the increased noradrenaline levels in placentally restricted fetuses may be attributed to increased overspill from sympathetic terminals rather than increased medullary output (Simonetta *et al.*, 1997). Holgert and colleagues (1995) using *in situ* hybridisation techniques reported that exposure of E18 fetal rats to 48 h of moderate hypoxaemia led to an increase in adrenal TH mRNA expression. Unlike the sheep, the rat does not develop functional adrenal innervation until postnatal life (Lau *et al.*, 1988; Seidler & Slotkin, 1986*b*) and hence the increase in TH mRNA expression observed by Holgert et al. (1995), must have been non-neurogenic in origin. In adult rats for which functional splanchnic innervation of the adrenal is established, exposure to 2 h of hypoxia leads to a neurally dependent increase in TH enzyme activity (Hayashi *et al.*, 1990) whilst no effect is observed for TH mRNA expression after 6 h of hypoxia (Czyzyk-Krzeska *et al.*, 1992). Long term hypoxia (3-28 d) in the adult rat also leads to an increase in TH activity although an increase in TH protein levels has been reported to accompany this response (Dalmaz *et al.*, 1994; Schmitt *et al.*, 1992). Chronic hypoxia associated with placental restriction in the fetal sheep may alter TH activity without affecting its synthesis.

Insulin induced hypoglycaemia is able to stimulate an increase in adrenal TH mRNA in adult rats, but not in neonatal pups which lack adrenal innervation (DeCristofaro & LaGamma, 1994). Despite the fact that the PR fetuses in this study tended to be hypoglycaemic, TH mRNA expression was apparently unaffected. This may be because the degree of hypoglycaemia achieved in PR is below the threshold for adrenal stimulation or alternatively placental restriction may be associated with a disruption of adrenal innervation.

PEnk A mRNA expression was not found in this study to be altered by placental restriction. In contrast immunological and biochemical techniques reveal that the expression of Enk-containing peptides is reduced in the adrenal gland of the 90 d fetal sheep with placental restriction (Coulter *et al.*, 1998). Whilst the total amount of Enk-containing peptides is reduced in placentally restricted fetuses at 90 d gestation, post-translational processing of these peptides is unchanged (Coulter *et al.*, 1998). Adrenal Enk-containing peptide levels were not determined in the current study therefore it cannot be determined if the lack of difference in PEnk A mRNA expression between control and placentally restricted fetuses in late

gestation reflects a relative recovery of Enk-containing peptide levels in the PR fetuses. Moderate fetal hypoxaemia over 48 h in late gestation has been demonstrated to increase adrenal PEnk A mRNA expression in the sheep (Fraser *et al.*, 1997). Reflex activation of the splanchnic nerve by insulin-induced hypoglycaemia also stimulates PEnk A mRNA expression in the rat (Kanamatsu *et al.*, 1986). *In vitro* and *in vivo* studies reveal that glucocorticoids and ACTH stimulate adrenal PEnk A mRNA expression and Enk-containing peptide levels in the rat and fetal sheep in a dose and developmentally dependent manner (Chapter 4, Coulter *et al.*, 1992; Fraser *et al.*, 1997; Naranjo *et al.*, 1986; Stachowiak *et al.*, 1988*b*). Glucocorticoids also act to potentiate the effects of trans-synaptic activation on PEnk A mRNA expression (Naranjo *et al.*, 1986). It is possible that functional splanchnic innervation, hypoxaemia, hypoglycaemia, and elevated glucocorticoid levels could stimulate a recovery of adrenal PEnk A mRNA expression and Enk-containing peptide levels in the placentally restricted fetus in late gestation.

The data presented in this study suggest for the first time that PR has a major impact on the capacity of the adrenal to synthesise adrenaline via effects on PNMT gene expression. This study and a number of other studies have clearly shown that there is an increase in the basal circulating concentrations of noradrenaline and paradoxically of adrenaline in the growth restricted sheep fetus (Jones & Robinson, 1983; Simonetta *et al.*, 1995). Plasma noradrenaline levels are found to be inversely related to arterial PO_2 under basal conditions in both normally grown and placentally restricted fetuses (Jones & Robinson, 1983; Simonetta *et al.*, 1997). Under basal conditions, plasma adrenaline concentrations are inversely related to arterial PO_2 in normally grown fetuses whereas they are be positively correlated with PO_2 in placentally restricted fetuses (Simonetta *et al.*, 1997). Therefore despite the higher basal adrenaline levels in placentally restricted fetuses there is a tendency for these levels to decrease as arterial PO_2 decreases which would be consistent with a limitation of adrenaline synthesis and secretion in these animals.

The impact of intrauterine hypoxaemia on adrenaline synthesis may have significant physiological consequences before, during, and immediately after birth. Superimposition of an acute bout of fetal hypoxia results in a substantially blunted

adrenaline secretory response in placentally restricted fetuses whilst a large noradrenaline response occurs which is similar in relative magnitude to that which is observed in control fetuses (Jones & Robinson, 1983). The mobilisation of glucose and the suppression of insulin secretion in response to acute hypoxic challenge are also impaired in placentally restricted fetuses which would be consistent with a reduced adrenaline secretory response (Jones & Ritchie, 1978*b;* Jones & Robinson, 1983; Padbury *et al.*, 1987*c*; Robinson *et al.*, 1983*a*). Growth restriction of fetal sheep by single umbilical artery ligation results in an inability of the fetus to maintain the adrenaline response to delivery (Oyama *et al.*, 1992). In addition to the failure to maintain adrenaline secretion in response to delivery, there are associated disruptions in blood pressure as well as glucose and free fatty acid mobilisation in growth retarded fetuses (Oyama *et al.*, 1992). The effects of intrauterine growth retardation may persist postnatally as 1 d old rat pups, growth retarded by chronic ligation of the maternal uterine artery, display impaired adrenaline secretory responses to acute hypoxia (Shaul *et al.*, 1989). Interestingly, chronic prenatal hypoxia in the rat is also associated with long term reductions of PNMT protein levels within respiratory neurones of the medulla oblongata which may compromise responses to acute hypoxia in early postnatal life (White & Lawson, 1997).

In summary, placental restriction and chronic hypoxaemia resulted in a specific suppression of PNMT mRNA levels and a concomitant decrease in the PNMT containing region of the fetal adrenal. As placental restriction was not associated with any changes in adrenal TH and PEnk A mRNA levels, or in the amount and intensity of immunochemical staining for TH, it appears that adrenaline, noradrenaline, and Enk-containing peptide synthesis are differentially regulated in the adrenal of the growth restricted fetus in late gestation. The potential limitation of adrenaline synthesis and secretion may have serious consequences for the homeostatic responses of the fetus to subsequent acute physiological challenges and the transition to postnatal life.

6. SUMMARY AND CONCLUSIONS

The fetal adrenal medulla is a key source of circulating catecholamines during intrauterine stress, such as the imposition of hypoxaemia (Cohen *et al.*, 1984; Comline & Silver, 1961; Jones *et al.*, 1988). There have been relatively few studies, however, which have investigated the impact of physiological stress upon catecholamine synthesis in the fetal adrenal medulla in a long gestation species such as the sheep. Given that the catecholamine synthetic capacity of the fetal adrenal medulla will determine its capacity to meet increased secretory demands (Bygdeman & von Euler, 1958; Wakade *et al.*, 1988) this is an important area for investigation. The studies presented in this thesis have examined the gene expression of the catecholamine synthetic enzymes, TH and PNMT, in the adrenal gland of the fetal sheep during development and in response to acute and chronic stress. Adrenal PEnk A mRNA expression was also examined as intra-adrenal opioid peptides have been implicated in the regulation of catecholamine secretion during stress in the fetus (Padbury *et al.*, 1987*b*). This final chapter will summarise the main findings and implications of this thesis and discuss directions for future research.

In Chapter 2 the expression of adrenal TH, PNMT, and PEnk A mRNA was determined in the fetal sheep adrenal between 80 and 146 d gestation. This developmental window encompasses a number of key aspects of adrenal development in the fetal sheep, including the establishment of functional splanchnic innervation (Boshier *et al.*, 1989*a;* Comline & Silver, 1961) and the maturation and activation of the fetal HPA axis (Challis & Brooks, 1989). Whilst catecholamines and enkephalin-containing peptides are co-stored within the adrenal chromaffin cells of the fetal sheep (McMillen *et al.*, 1988; Wan *et al.*, 1989*b*), there was a differential regulation of TH, PNMT, and PEnk A mRNA expression across mid to late gestation. Adrenal TH and PEnk A mRNA expression peaked at 125 d gestation coincident with the onset of establishment of functional cholinergic innervation of the adrenal gland by the splanchnic nerve. TH and PEnk A mRNA expression then decreased after 140 d gestation. PNMT mRNA expression on the other hand increased in late gestation and peaked at 140-146 d in parallel with the activation of fetal HPA axis and the prepartum increase in cortisol output from the fetal adrenal cortex. In Chapter 2 I have

demonstrated that blockade of nicotinic receptors suppressed adrenal TH mRNA levels during basal or normoxic conditions. This indicates that adrenal TH mRNA expression is maintained by splanchnic nerve activation in the late gestation sheep fetus. It is case however, that basal adrenal PNMT mRNA expression was not suppressed by the addition of hexamethonium to the late gestation sheep fetus. It appears likely therefore that the expression of these two catecholamine synthetic enzyme genes are differentially regulated in the adrenal during late gestation. It would be of interest to determine the effects of disruption of splanchnic innervation to the adrenal, by nerve sectioning or fetal athyroidism, upon the ontogenetic expression of adrenal TH, PNMT, and PEnk A mRNA.

The interaction of the HPA axis in the regulation of catecholamine synthetic enzymes and enkephalin peptides has been well studied in the adult rat and in cell culture. Surprisingly, there is relatively little information on the regulation of catecholamine synthetic enzymes in the fetus by the HPA axis, particularly in a long gestation species. Hypophysectomy in the fetal sheep results in the failure of the adrenaline content of the fetal adrenal to increase in late gestation (Comline *et al.*, 1970), and also has a number of consequences for the localisation, synthesis, and processing of the enkephalin containing peptides (Coulter *et al.*, 1989*a;* Coulter *et al.*, 1991; Coulter *et al.*, 1992). Further understanding of the role of the HPA axis in the developmental regulation of adrenal TH, PNMT, and PEnk A mRNA expression could be gained in studies which prevent the prepartum activation of the HPA axis by either hypothalamic-pituitary disconnection or the use of 11β hydroxylase inhibitors, such as metyrapone which inhibit cortisol synthesis.

Acute fetal hypoxaemia can potentially arise from a number of situations (Giussani *et al.*, 1994). Catecholamines secreted from the fetal adrenal gland in response to hypoxia elicit a number of cardiovascular and metabolic adaptations (Jones *et al.*, 1988). In the functionally innervated adrenal gland, hypoxia stimulates catecholamine secretion by reflex neural stimulation (Biesold *et al.*, 1989; Cheung, 1990; Jensen & Hanson, 1995; Seidler & Slotkin, 1985). Factors associated with neural stimulation are well established to regulate catecholamine synthetic enzyme gene expression in the adult adrenal medulla. Prior to adrenal innervation however, hypoxia stimulates catecholamine secretion from the adrenal

gland by a 'direct' non-neurogenic mechanism (Cheung, 1990; Comline & Silver, 1966; Comline & Silver, 1961; Seidler & Slotkin, 1985). In Chapter 3 it was found that acute exposure to moderate hypoxaemia reduced TH mRNA and increased PNMT expression in the fetal adrenal gland in a manner which related to the degree of change in arterial PO_2. This effect of hypoxaemia was evident before the development of functional splanchnic innervation to the adrenal, implicating a non-neurogenic regulation of TH and PNMT gene expression. After the development of functional splanchnic innervation however, the regulation of TH and PNMT mRNA expression in response to acute hypoxia was dependent upon reflex nicotinic stimulation.

The non-neurogenic regulation of TH mRNA and PNMT mRNA expression in adrenomedullary chromaffin cells in responses to changes in oxygen tension has yet to be examined. Future studies would be required to focus upon the determination of the second messenger systems and transcription factors activated at the cellular level in adrenomedullary chromaffin cells in response to hypoxia. This would then provide further insight into the potential mechanisms whereby hypoxia could directly influence gene expression independently of neural stimulation in adrenomedullary chromaffin cells.

Cortisol is also secreted from the fetal adrenal cortex in response to a number of prenatal stressors (Akagi & Challis, 1990; Ozolins *et al.*, 1991*b;* Rose *et al.*, 1978). It has been established that glucocorticoids play an important role in the induction and maintenance of adrenal PNMT expression and adrenaline synthesis in chromaffin cells in culture and in the developing and mature rat adrenal *in vivo*. There is also indirect evidence to suggest that in the fetal sheep glucocorticoids are important for the regulation of adrenaline synthesis (Comline *et al.*, 1970). Paradoxically, however, physiologically and clinically relevant doses of glucocorticoids act to decrease plasma noradrenaline and adrenaline levels in the fetal sheep (Derks *et al.*, 1997; Stein *et al.*, 1995; Wood *et al.*, 1987). In Chapter 4 the effect of a physiological increase in plasma cortisol levels between 109 and 116 d gestation was examined and it was found that there was a specific decrease in adrenal PNMT mRNA and protein expression whilst TH and PEnk A mRNA expression were not substantially altered. The specific decrease in PNMT mRNA expression with increased glucocorticoid levels implies that the reduction in

adrenaline levels observed in previous studies maybe attributable to a decrease in adrenal adrenaline synthesis. We have made the novel observation that a precocious increase in plasma cortisol levels in the fetal sheep actually has a suppressive effect on adrenal PNMT expression.

The mechanisms by which increased peripheral cortisol levels act to suppress adrenal PNMT mRNA expression in the fetal sheep clearly needs to be addressed given the clinical significance of prenatal glucocorticoid treatment (Schellenberg & Liggins, 1987). It is unlikely that the exogenous cortisol is acting to feedback negatively on the fetal HPA axis to reduce intra-adrenal cortisol levels but adrenal venous effluent could be collected during intrafetal cortisol infusion to ensure that there is no reduction in adrenal cortisol output. A possible mechanism for the glucocorticoid induced suppression of PNMT mRNA expression is that cortisol may act to downregulate of type II glucocorticoid receptors (GR) on the adrenomedullary chromaffin cells (Betito *et al.*, 1993). GR are involved in the stimulation of PNMT gene expression (Betito *et al.*, 1992; Wan & Livett, 1989) and a reduction in GR may negatively impact upon PNMT mRNA expression. Molecular and immunological techniques could be used to study the expression of GR mRNA and protein in the adrenals of cortisol infused fetal sheep and their relationship to PNMT mRNA expression. Glucocorticoids have also been implicated in the long term programming of fetal cardiovascular function (Dodic *et al.*, 1999*a;* Dodic *et al.*, 1999*b*) and it would be interesting to determine if the impact of prenatal cortisol infusion on PNMT mRNA expression also continues into postnatal life. In the rat, prenatal administration of dexamethasone at E17-E19 decreases adrenal adrenaline content at postnatal day 1 and led to an impaired adrenaline secretory response of these neonatal rat pups in response to hypoxia (Kauffman *et al.*, 1994).

It can be speculated that the reduction in plasma noradrenaline levels found in this study after cortisol infusion is not due to a reduction in adrenal TH gene expression. Glucocorticoids have been reported to reduce sympathetic outflow (Lenders *et al.*, 1995) hence a reduction in sympathetic outflow in response to glucocorticoid treatment may be responsible for the reduction in basal plasma noradrenaline levels observed in the fetal sheep. PEnk A mRNA levels were not significantly altered in response to glucocorticoid infusion and hence it is unlikely

that an increase in enkephalin-containing peptide synthesis at the pre-translational level is responsible for the inhibitory effects of glucocorticoids upon basal catecholamine output.

Intrauterine growth retardation is a relatively common pathological condition frequently associated with chronic fetal hypoxaemia and hypoglycaemia (Economides *et al.*, 1991; Robinson *et al.*, 1985). Plasma catecholamine levels are increased in both clinical IUGR and experimental paradigms (Lagercrantz *et al.*, 1980; Simonetta *et al.*, 1997). In Chapter 4 adrenal TH, PNMT, and PEnk A mRNA expression were examined at 140 d following experimental IUGR in fetal sheep by placental restriction. Despite elevated plasma catecholamine concentrations in PR fetal sheep, adrenal PNMT mRNA expression and immunoreactivity were specifically decreased at 140 d gestation. Curiously, PNMT mRNA expression exhibited a strong positive relationship with mean gestational levels of arterial PO_2. This is in contrast to acute hypoxia where adrenal PNMT mRNA increases with decreasing PO_2 (Chapter 3), therefore there appears to be a temporal aspect to the regulation of PNMT gene expression in the fetal sheep by oxygen. There was no significant effect of PR on adrenal TH and PEnk A mRNA expression at 140 d gestation.

It appears reasonable to conclude that the increased basal output of adrenaline and noradrenaline with PR are not attributable to increased synthesis within the fetal adrenal gland, or reduced levels of intra-adrenal opioid peptides. Recent isotope dilution studies in the fetal sheep indicate that the increased circulating catecholamine levels in IUGR fetuses are due to enhanced spill-over from the sympathoadrenal system (Smolich, 1999). It has previously been demonstrated in our laboratory that tyramine, which selectively displaces catecholamines from sympathetic nerve terminals, elicits significantly higher plasma noradrenaline and adrenaline levels in PR sheep (Simonetta *et al.*, 1997). Therefore the increase in basal catecholamine levels in PR fetal sheep appears to be due to increased overspill from developing sympathetic neurones. This possibility could be studied by measuring plasma catecholamine concentrations in PR fetal sheep following adrenal demedullation or chemical destruction of the peripheral sympathetic nervous system with guanethidine or 6-hydroxydopamine.

The adrenaline secretory capacity of the fetal sheep adrenal is paradoxically reduced with PR despite an increase in basal plasma adrenaline levels. This may therefore affect the reserve of the fetal adrenal gland to respond to further acute or chronic stress. There is evidence to suggest that this is indeed the case as PR fetal sheep have been reported to have a reduced adrenaline secretory response to superimposed acute hypoxia (Jones & Robinson, 1983). A number of metabolic responses attributable to increased adrenaline levels in response to hypoxaemia are also blunted in PR fetal sheep (Jones & Robinson, 1983; Robinson *et al.*, 1983*a*). The impaired adrenaline secretory response to hypoxia persists in neonatal rats which have been subjected to the Wigglesworth model of IUGR (Shaul *et al.*, 1989). Based on strong epidemiological evidence, IUGR has been proposed to be a risk factor in the development of cardiovascular and metabolic disturbances in adult life (Barker, 1999). It would be of interest to determine if the effect of IUGR on the adrenaline synthetic capacity of the adrenal gland in the sheep persists into post natal life.

In summary the studies presented in this thesis demonstrate that there is a differential regulation of adrenal catecholamine synthetic enzyme and PEnk A gene expression in the fetal sheep with development. There is also differential regulation of adrenal catecholamine synthetic enzyme gene expression during acute hypoxaemia in the fetal sheep, before and after functional splanchnic innervation. Cortisol, which is also released from the adrenal during fetal hypoxaemia, was demonstrated to have a novel suppressive effect on adrenal PNMT expression in the fetal sheep. Experimental IUGR with chronic hypoxaemia was established to result in a specific reduction in adrenal PNMT mRNA expression and immunoreactivity which was closely related to arterial oxygenation over gestation in the fetal sheep. Clearly intrauterine stress impacts upon catecholamine synthetic enzyme gene expression in the fetal sheep. Long term alterations in adrenal catecholamine synthetic enzyme gene expression will influence the catecholamine synthetic capacity of the fetal and neonatal gland and hence may result in altered secretory responses to homeostatic challenges. Future functional and molecular studies will be required to determine the full array of non-neurogenic and neural factors which regulate catecholamine synthetic enzyme gene expression during intrauterine stress.

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