A study of C-type natriuretic peptide in cerebrospinal fluid and related tissues of sheep, and its regulation by dexamethasone

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Michele Odile Wilson

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Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

A study of C-type natriuretic peptide in cerebrospinal fluid and related tissues of sheep, and its regulation by dexamethasone

By Michele Odile Wilson

C-type natriuretic peptide (CNP) has high abundance in cerebrospinal fluid (CSF) and central tissues, and has been implicated in the regulation of important processes in the central nervous system (CNS) including the stimulation of neuronal branching, regulation of blood-brain barrier permeability and neuroprotection. Despite the many important putative roles that have been attributed to CNP during development and in adult life through *in vitro* studies, little is known about the regulation of CNP *in vivo* — and its usefulness in clinical settings of pathophysiological CNS disorders remains untapped. The aim of this PhD project was to explore the regulation of CSF concentrations of CNP and the amino-terminal fragment of proCNP (NTproCNP) in sheep, and relate this to central sources.

To determine whether CSF concentrations of CNP peptides are affected by altered levels in plasma, concurrent CSF and plasma samples were collected from 15 pregnant sheep throughout the gestation period (days 4, 87 and 116) — a time when plasma concentrations become markedly increased. Compared with non pregnant sheep (n = 15), plasma concentrations of CNP peptides were elevated by 30-fold at days 87 and 116 in pregnant sheep, yet CSF concentrations of CNP and NTproCNP did not differ between the groups — which indicated that central levels of CNP peptides were independently regulated from those in plasma in sheep. As there were no known compounds or physiological setting capable of acutely affecting CSF concentrations of CNP peptides, a series of pilot studies was carried out to screen a number of candidate drugs in sheep, and to examine the effect of changes in live weight and appetite in red deer stags. Despite the dramatic changes in appetite and live weight that occurred throughout the breeding season in red deer stags (> 30 kg live weight gain), CSF concentrations of CNP peptides remained stable. Similarly, CNP and NTproCNP concentration in CSF of sheep remained unchanged following administration with all compounds (including anaesthetics, morphine, a pyrogen and l-deprenyl) except for one; a single

i.v. dose of dexamethasone (0.25 mg/live weight) was shown to reliably induce a marked increase in CNP and NTproCNP concentration in CSF within 8 h.

Following the successful identification of a secretagogue for CNP, subsequent studies were carried out to examine the increases in CNP peptide concentration in CSF and plasma following different doses of dexamethasone (0, 0.025, 0.063, 0.125, 0.25 mg/kg live weight), and to associate dexamethasone-stimulated increases in peptide levels in CSF with changes in peptide concentration and gene expression of *NPPC*, *NPRB* and *NPRC* in tissues sampled from the brain and anterior and posterior pituitary glands. Whereas plasma concentrations of CNP peptides were increased following all doses of dexamethasone, concentrations in CSF were elevated only at the two highest doses. Compared with saline-treated sheep, CNP and NTproCNP content in nervous tissue of dexamethasone-treated sheep (0.25 mg/kg, i.v.) was significantly higher throughout the brain in 6 and 11 of the 14 tissues considered, respectively.

Gene expression of *NPPC* was upregulated in several tissues (anterior pituitary gland, posterior pituitary gland, hypothalamus, hippocampus and pons), which is supportive of increased synthesis of CNP following dexamethasone. The pituitary gland (anterior and posterior), which is known for its enriched levels of CNP content — had a similar concentration of NTproCNP when compared with most tissues sampled from the brain. Furthermore, the NTproCNP:CNP concentration ratio in pituitary gland (1:1) differed from brain (5:1 to 10:1), suggesting that little CNP degradation occurs in the pituitary gland — however the expression of *NPRC* did not differ between pituitary and brain tissue. Gene expression of *NPPC* was upregulated in both the anterior and posterior pituitary gland following dexamethasone, despite no significant increase in CNP or NTproCNP concentration in these tissues.

Analysis of the molecular forms present in various fluids and tissues using size-exclusion high performance liquid chromatography revealed similar profiles for anterior pituitary gland and plasma extracts, whereby proCNP was present, CNP-53 was the prevalent form and CNP-22 was virtually absent. Together, these findings suggest that CNP processing differs between brain, anterior and posterior pituitary glands and supports the possibility that CNP is secreted directly from the anterior pituitary gland into the circulation. The widespread increase of CNP secretion in multiple tissues across the brain in response to dexamethasone implicates CNP in glucocorticoid actions — possibly related to inflammation or local fluid dynamics mediated by glial cells. These findings pave the way for future studies designed to establish the role of CNP in the CNS in normal

health *in vivo*, and to establish a clinical application for this peptide in central settings of pathophysiological disorders in the CNS.

Keywords: C-type natriuretic peptide, CNP, NTproCNP, cerebrospinal fluid, CSF, sheep, central nervous system, CNS, dexamethasone, glucocorticoids, brain, pituitary

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Statement

The studies presented in this thesis were carried out as part of an ongoing collaboration between Lincoln University (Christchurch, NZ) and the Department of Medicine, University of Otago, (Christchurch, NZ). I was involved in all aspects of the studies including experimental design, coordination of animal trials, sample collection and analysis, writing of the manuscript, and my specific input is outlined at the beginning of each chapter. Statistical analysis was conducted following consultation with a biometrician. It must be acknowledged that this work benefitted significantly from input by my PhD supervisors and co-authors. All animal procedures were carried out in accordance with the Animal Welfare Act 1999 (NZ), and were approved by the Lincoln University Animal Ethics Committee.

Publications arising from thesis

Journal articles

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List of Abbreviations

AI	Artificial insemination
ANP	Atrial natriuretic peptide
AQP4	Water channel aquaporin-4
BBB	Blood-brain barrier
BNP	Brain natriuretic peptide
cGMP	Cyclic guanosine monophosphate
CIDR	Controlled internal drug release (device)
CNS	Central nervous system
CSF	Cerebrospinal fluid
DRG	Dorsal root ganglia
EDTA	Ethylenediaminetetraacetic acid
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
IDE	Insulin degrading enzyme
i.m.	Intramuscular
i.v.	Intravenous
ir	Immunoreactive
LH	Luteinising hormone
LPS	Lipopolysaccharide
MR	Mineralocorticoid receptor
NO	Nitric oxide
NPPC	C-type natriuretic peptide preprohormone gene
NPR	Natriuretic peptide receptor
NPR2	Natriuretic peptide receptor B gene
NPR3	Natriuretic peptide clearance receptor gene
NPR-A	Natriuretic peptide receptor A
NPR-B	Natriuretic peptide receptor B
NPR-C	Natriuretic peptide clearance receptor
NPs	Natriuretic peptides
NTproCNP	Amino-terminal pro C-type natriuretic peptide
RIA	Radioimmunoassay
s.c.	Subcutaneous
s.e.	Standard error of the mean
SE-HPLC	Size-exclusion high performance liquid chromatography
TFA	Trifluoroacetic acid

Chapter 1. General Introduction

C-type natriuretic peptide (CNP) is a member of the natriuretic peptide family, a group of structurally related but functionally distinct peptides. While the heart is the main site of production for the cardiac natriuretic peptides (atrial natriuretic peptide: ANP, and brain natriuretic peptide: BNP), sources of CNP are widespread. Unlike the other natriuretic peptides, CNP circulates at very low concentrations in the blood, leading to the conclusion that CNP acts locally in a paracrine/autocrine manner. Indeed, CNP has been shown to be a local regulator of bone growth and vasodilation.

Interest in the role of CNP in the central nervous system (CNS) has been growing since CNP was discovered in the porcine brain (Sudoh *et al.* 1990). The widespread distribution of CNP in the rat and human brain (Komatsu *et al.* 1991) and high concentration in cerebrospinal fluid (CSF), which exceeds that of other natriuretic peptides and its own concentration in the plasma (Kaneko *et al.* 1993), are suggestive of a homeostatic role in the brain. Indeed, *in vitro* studies have shown a role for CNP in several neuroregulatory processes, including nervous system development (Kishimoto *et al.* 2008) and neuroprotection (Ma *et al.* 2010). In 2011, Schouten *et al.* reported concurrent measurements of CNP and the stable amino-terminal fragment of proCNP (NTproCNP), where it was reported that CSF and plasma concentrations of the two peptides were independently regulated – suggestive that central and peripheral sources of CNP were separate.

Little is known about the regulation of CNP synthesis and secretion *in vivo* and its actions in adult life are poorly understood. Understanding CNP physiology may be useful in settings of central pathological disorders, however study of CNP in CSF is limited in human studies given the unlikelihood of obtaining healthy human CSF samples. Consequently, this PhD project was initiated to advance our knowledge about central dynamics, sources and regulation of CNP, using a large animal model where CSF samples could be obtained from healthy individuals.

To facilitate repeated CSF sampling from healthy sheep, a method was developed for cannulation of the cisterna magna (Chapter 3). To test the hypothesis that CNP peptides are independently regulated in CSF and plasma in sheep – as in humans – CNP peptides were measured in a unique ruminant setting where circulating CNP peptide concentrations are markedly elevated, i.e. throughout gestation (Chapter 4). In order to identify central site(s) of CNP synthesis, pilot studies were conducted to identify a stimulus or suppressant capable of altering CNP concentrations in

CSF (5.1). Following reports of a possible central role of CNP in energy balance and appetite – and in the context of identifying factors capable of influencing central levels of CNP peptides – temporal changes in CNP peptides were characterised in CSF and plasma of red deer stags, which undergo major seasonal changes in live weight and appetite (5.2). Having identified a potential candidate stimulus – dexamethasone – changes in CNP peptide concentration were formally characterised by use of a dose-response study (Chapter 6). Hypothesising that dexamethasone-induced increases in CNP concentration in CSF were due to increased peptide release from central tissue to CSF, the concentration of both peptides was measured in dexamethasone-stimulated and non-stimulated brain and pituitary tissues. Gene expression of the CNP precursor, receptor, and clearance receptor was also measured in a selection of tissues to provide further insight into the possible synthesis, action and clearance of CNP in specific tissues after dexamethasone stimulation. Specific molecular forms of CNP peptides were characterised in CSF, plasma, hypothalamus, anterior and posterior pituitary glands and results are presented in Chapter 7. The novel findings and main contributions of this work are discussed in Chapter 8, as well as potential avenues for further study.

Chapter 2. Literature Review

2.1 Introduction to CNP

2.1.1 The natriuretic peptide family

The natriuretic peptide (NP) family consists predominantly of the three hormones; atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Due to their crucial role in blood pressure and plasma volume homeostasis (Nishikimi *et al.* 2006), and consequential diagnostic value in the identification of chronic heart failure (Burnett *et al.* 1986, Mukoyama *et al.* 1991, Falcão *et al.* 2004, Bettencourt 2005), ANP and BNP are arguably the best known and most widely studied of these NPs (returning close to 20,000 and 15,000 PubMed search returns, respectively, compared with approximately 1600 for CNP — March 2017, using text in full e.g. 'atrial natriuretic peptide').

ANP and BNP are referred to as the 'cardiac NPs' (Rademaker & Richards 2005) and are released into the circulation in response to hypervolaemia-induced stretch of the atria and ventricles, and act in an endocrine fashion to regulate blood volume by antagonising the renin-angiotensin-aldosterone system and by enhancing natriuresis, diuresis and vasodilation (Richards 1996, Ito *et al.* 2003, Nishikimi *et al.* 2006). Other NPs have been identified, including dendroapsis natriuretic peptide in snake venom, human plasma and atrial myocardium (Schweitz *et al.* 1992, Shirger *et al.* 1999) and ventricular natriuretic peptide in cardiac tissue in eels (Takei *et al.* 1994), however in mammals, ANP, BNP and CNP are considered to be the major members of this family. In the literature, the acronym 'CNP' is used to refer to the originally discovered 22-residue form (CNP-22, Figure 2.1) as well as CNP-53, the peptide from which the former is putatively cleaved from (discussed below).

NPs contain an obligatory C-terminal 17-residue disulphide ring structure (Potter 2011a), of which 11 residues are conserved across the family (Figure 2.1). These peptides are expressed in a broad range of tissues (Minamino *et al.* 1988, Sudoh *et al.* 1990, Ruskoaho 1992, Chrisman *et al.* 1993, Chen & Burnett 1998, Stepan *et al.* 1999, Woodard *et al.* 2002, Kalra *et al.* 2003, Horio *et al.* 2003, Kalra *et al.* 2004, Prickett *et al.* 2005, Zhao & Ma 2009, Del Ry *et al.* 2011), although the main sites of NP production are the atria and ventricles for ANP and BNP, and vasculature and central nervous system for CNP.

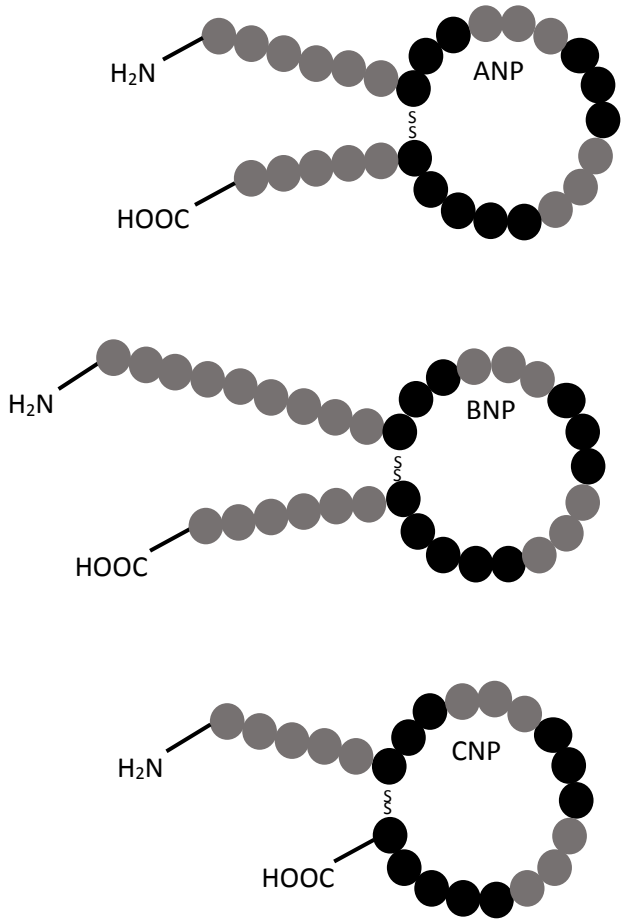

Figure 2.1 Primary structure of human natriuretic peptides.
Black circles represent amino acids that are conserved across the natriuretic peptide family and grey circles represent amino acids that differ. (ANP: atrial natriuretic peptide, BNP: brain natriuretic peptide, CNP: C-type natriuretic peptide, figure adapted from Potter 2011b).

The process of mature peptide formation is similar for each of the NPs, as all are derived from a preprohormone which is converted to a prohormone by removal of a signal peptide (Wu *et al.* 2003). The prohormone is then cleaved by various enzymes (Table 2.1) to produce two fragments which are either stored or immediately released: the respective natriuretic peptides ('mature forms'), as well as an amino-terminal fragment which is presumed to be bio-inactive. Of the prohormones, only proBNP has been identified in the circulation (Seferian *et al.* 2007).

ANP is stored in granules in the atria, where it is primarily expressed, and its release is stimulated by atrial wall stretch which results from increased blood volume (de Bold *et al.* 1986, Edwards *et al.* 1988). BNP is not stored in granules in the ventricles where it is primarily expressed – although

it is stored in atrial granules with ANP (Potter *et al.* 2006) – and is synthesised and released in proportion to the degree of left ventricular dysfunction, and following acute myocardial infarction and in chronic heart failure (Omland *et al.* 2002, Richards *et al.* 2003). CNP has been identified in small vesicles in pinealocytes described as being similar to ANP-containing atrial granules (Middendorf *et al.* 1996) and in human aortic endothelial cells staining with granularity (Stingo *et al.* 1992) – the latter of which was consistent with CNP-53. However, the extent to which CNP is stored and released, as opposed to synthesised *de novo* is largely unknown for many tissues, including those of the central nervous system.

Differing features of NPs include the location of genes which encode the preprohormone, cleavage enzymes, and size/sequence of preprohormones, prohormones, mature forms and amino-terminal fragments. Despite close structural similarities, NPs differ largely in their biological function (reviewed extensively by Potter *et al.* 2006, 2009). A comparison of general characteristics of NP biology is discussed below and summarised in Table 2.1.

Table 2.1 A comparison of general characteristics of the main natriuretic peptides.

	ANP	BNP	CNP	References
Location of gene which encodes for precursor	Chromosome 1 (human), chromosome 4 (mouse)	Chromosome 1 (human), chromosome 4 (mouse)	Chromosome 2 (human, mouse, sheep)	Lima *et al.* 2008, Tamura *et al.* 1996, NCBI database
Gene name	*NPPA*	*NPPB*	*NPPC*	
Number of residues in preprohormone	151	134	126	Itoh *et al.* 1988, Seilhamer *et al.* 1989, Tawaragi *et al.* 1991
Number of residues in prohormone	126	108	103	Itoh *et al.* 1988, Seilhamer *et al.* 1989
Number of residues in mature protein	28	Varies across and within species as several truncated forms are known to circulate, e.g.: 32 (human, pig, dog) 45 (rat, mouse)	22, 53 (two forms)	Potter *et al.* 2006, Seilhamer *et al.* 1989, Sudoh *et al.* 1988, Ogawa *et al.* 1994, Kojima *et al.* 1990, Yandle & Richards 2015
Enzyme responsible for cleavage of prohormone	Corin	Furin or corin	Furin	Sawada *et al.* 1997, Yan et al. 2000

Mode of action	Endocrine, paracrine and autocrine	Endocrine, paracrine and autocrine	Presumed to be paracrine and autocrine	Nishikimi *et al.* 2006
Receptor responsible for mediating effects	Guanylyl cyclase A (GC-A), also known as natriuretic peptide receptor A (NPR-A)	Guanylyl cyclase A (GC-A), also known as natriuretic peptide receptor A (NPR-A)	Guanylyl cyclase B (GC-B), also known as natriuretic peptide receptor B (NPR-B or NPR2)	Potter *et al.* 2006
Half-life	2 minutes (humans), 0.5 – 4 minutes in mice, rats, rabbits, dogs and monkeys	Varies for different components, from 3.9 – 20.7 minutes	2.6 minutes (humans), 1.6 minutes (sheep)	Nakao *et al.* 1986, Ruskoaho 1992, Mukoyama *et al.* 1991, Yandle *et al.* 1986, Hunt *et al.* 1994, Charles *et al.* 1995
Primary roles	Reduction of blood volume and pressure	Reduction of blood volume and pressure, prevention of cardiac fibrosis	Stimulation of endochondral bone growth, reduction of cardiac fibrosis, dilation of blood vessels	Tamura *et al.* 2000, Horio *et al.* 2003, Nishikimi *et al.* 2006
Current diagnostic/ therapeutic uses	Indicator of heart failure	Detection of ventricular dysfunction and likely heart failure	Clinical trial underway using CNP analogue to treat achondroplasia	Falcão *et al.* 2004, Bettencourt 2005, Lorget *et al.* 2012

2.1.2 CNP and NTproCNP secretion

First discovered in the porcine brain in 1990, CNP (as it is most commonly known) is composed of 22 amino acid residues (Sudoh *et al.* 1990, Figure 2.1) and differs from ANP and BNP in that it terminates in the second cysteine residue, lacking a further C-terminal extension (Sudoh *et al.* 1990). The CNP precursor peptide gene (*NPPC*) is located on chromosome 2 in humans, mice (Ogawa *et al.* 1994) and sheep (NCBI database) and contains two exons separated by an intron, which encode prepro-CNP, a 126 residue CNP precursor (Ogawa *et al.* 1994).

The precursor peptide is converted to a 103-amino acid prohormone that is processed intracellularly by furin to yield NTproCNP (amino acids 1-50) and CNP-53 (amino acids 51-103) (Wu *et al.* 2003). Both of these forms should occur in equal proportions intracellularly and must be secreted from cells in equimolar amounts, assuming the theoretical models of CNP processing described by Prickett *et al.* (2001) and Wu *et al.* (2003) are true. NTproCNP is not considered to have any biological function but it is a stable product of CNP gene expression that is simply co-

secreted with CNP (Prickett *et al.* 2001). Aitken *et al.* (1999) reported that the ovine amino acid sequence of NTproCNP differs only slightly from that of the human and pig (92 and 98 % homology, respectively).

CNP-53 is further cleaved to produce CNP-22 (Figure 2.2), the latter of which has been described as the mature and most biologically active form of the CNP fragments (Baxter 2004), since the 22-amino acid sequence was found to be carried in CNP-53 in porcine brain extracts (Minamino *et al.* 1990). However, labelling CNP-22 as the 'mature form' is misleading as it may imply that CNP-53 is 'immature' and potentially inactive. Furthermore, there are no data to suggest that CNP-22 is more potent than CNP-53, in fact *in vitro* data demonstrates equi-potency between the two forms (Yeung *et al.* 1996a).

In fact, Yasoda *et al.* (2009) suggested that CNP-53 may be more potent than CNP-22, as a higher plasma CNP concentration from systemically administered synthetic CNP-22 was required to rescue a mouse model of achondroplasia (fibroblast growth factor receptor (3) mutation which results in the inhibition of chondrocyte hypertrophy), than the same model of achondroplasia crossed with a transgenic mice with liver-specific CNP overexpression (that raised plasma concentrations of CNP – presumably CNP-53 and CNP-22). However, it is likely that this difference is largely explained by the fact that increased levels of circulating CNP in transgenic mice were apparent just after birth, whereas peripheral infusions of CNP began three weeks after birth.

The mechanism for the cleavage of CNP-53 to CNP-22 is unknown, although it is likely to take place extracellularly and after secretion, given the predominance of CNP-53 in endothelial cells (Stingo *et al.* 1992), the absence of CNP-22 in the ovine pituitary gland (Pemberton *et al.* 2002) and the predominance of CNP-22 in the circulation (Stingo *et al.* 1992). In contrast, Yandle *et al.* (1993) showed that similar amounts of CNP-53 and CNP-22 existed in the ovine hypothalamus, indicating that processing of CNP may differ between tissues.

There have been conflicting reports about which CNP form is dominant in CSF; Togashi *et al.* (1992) reported CNP-22 as the main form (and that CNP-53 was the dominant form in brain tissue), whereas Schouten *et al.* (2011) reported the main form to be CNP-53. In the former study, it was also noted that CNP-53 was present, and, consistent with models of intracellular processing of proCNP 1-103 (Prickett *et al.* 2001, Wu *et al.* 2003), there was no evidence of proCNP 1-103 in CSF.

Despite the minor differences in exon sequences between species, CNP-22 has a high degree of homology across species – it is identical in sheep, humans, pigs and rats and is the most highly conserved natriuretic peptide in vertebrates (Aitken *et al.* 1999), having been identified in fish (Inoue *et al.* 2003), snake brain (Hayashi *et al.* 2003) and snake venom (Soares *et al.* 2005). In contrast, human CNP-53 differs slightly from the pig and rat forms, as it has two amino acid substitutions (Tawagari *et al.* 1991). It has not been sequenced in the sheep. However, Aitken *et al.* (1999) reported that the translated region of exon 1 (encodes the signal peptide and the first 7 amino acids of NTproCNP) and exon 2 (encodes the remainder of NTproCNP and the entire mature hormone) in sheep showed a strong sequence identity (93-98 %) with the human and pig sequences.

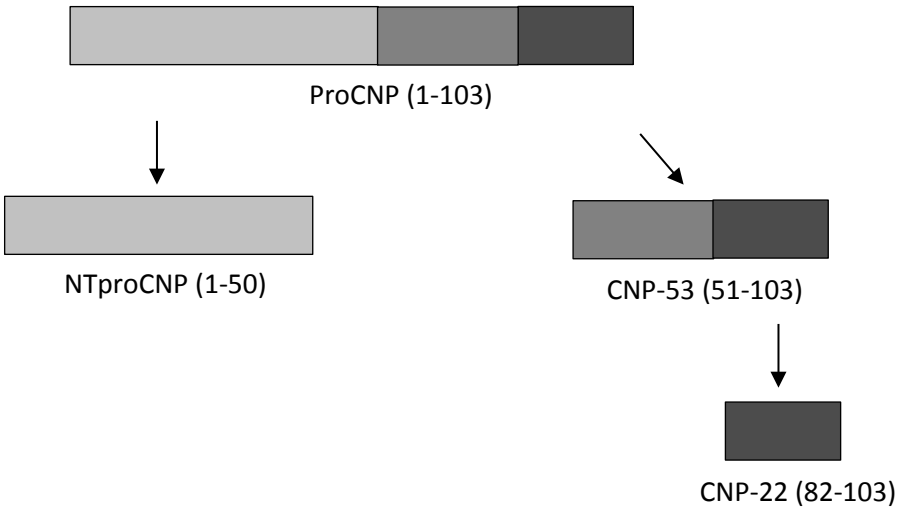

Figure 2.2 Schematic diagram of the processing of the CNP prohormone to smaller fragments. ProCNP (1-103) is cleaved to form NTproCNP (1-50) and CNP-53 (51-103), which contains the CNP-22 fragment (82-103).

2.1.3 CNP receptors and its clearance

So far three distinct natriuretic peptide receptors have been identified: natriuretic peptide receptors A and B (NPR-A and NPR-B, also termed GC-A and GC-B, respectively), which are approximately 1030-amino acid transmembrane guanylyl cyclases, and NPR-C, a 496-amino acid transmembrane protein with a short 37-amino acid cytoplasmic tail (Koller *et al.* 1991) which is also a guanylyl cyclase (GC-C). ANP and BNP are ligands for NPR-A (Potter 2011a), whereas NPR-B (which is homologous to NPR-A) is potently and selectively activated by CNP which has a binding affinity for NPR-B 50- or 500-fold higher than ANP or BNP, respectively, in humans (Koller *et al.* 1991).

Guanylyl cyclases exist in two forms – particulate (membrane bound) and soluble – and both forms catalyse the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), a secondary intracellular messenger that is presumed to be responsible for almost all effects of particulate guanylyl cyclases. The resulting increase in cGMP concentration triggers a signalling cascade which involves activation of protein kinases, regulation of cGMP-gated cation channels and cGMP-regulated phosphodiesterase – thereby mediating a range of downstream effects, including ion channel conductance, smooth muscle relaxation, and neurotransmission (Denninger & Marletta 1999).

NPR-A, NPR-B and NPR-C are particulate guanylyl cyclases which are involved in a range of physiological processes, including the regulation of gut ion/water transport, phototransduction, olfaction and thermosensation (Kuhn 2016). Soluble guanylyl cyclases were traditionally considered to belong exclusively to the cytoplasm, however it has been reported that they may translocate to the plasma membrane after ligand binding, potentially with the assistance of molecular chaperones (Agullo *et al.* 2005). Regardless, soluble guanylyl cyclases are likely to be mostly cytoplasmic, and are receptors for gaseous ligands, which are primarily nitric oxide (NO) and carbon monoxide (Evgenov *et al.* 2006). Soluble guanylyl cyclases contain a haem moiety which is essential for NO-binding (Martin *et al.* 2005). It is known that NPs and NO elicit cGMP synthesis and activate common downstream pathways, however many aspects of the interaction between CNP, NO and cGMP as regulators of endochondral ossification remain to be explored (reviewed by Teixeira *et al.* 2008). There is evidence to suggest that cGMP-independent functions are mediated by some specific protein-protein interactions (Kuhn 2016), although none have been reported for NPR-B or NPR-C.

In keeping with the autocrine/paracrine action of CNP, *NPRB* expression closely matches that of *NPPC*, with major sites of expression in endothelial cells and cardiomyocytes (Del Ry *et al.* 2011), osteoblasts (Hiroyuki *et al.* 2008), kidney (Canaankuhl *et al.* 1992) and in neural tissues such as the dorsal root spinal and cranial ganglia during embryonic development (DiCicco-Bloom *et al.* 2004). NPR-B has been identified in many brain regions, including throughout the brainstem of non-human primates (Abdelalim & Tooyama 2011), as well as in the rat and mouse pituitary gland (McArdle *et al.* 1994).

NPR-C (also known as NPR3) comprises ~95 % of the NPR population (Maack 1992) and has a major functional role as the clearance receptor for the natriuretic peptide family as it is able to

bind, internalise and target the peptides for lysosomal ligand hydrolysis (Maack *et al.* 1987, Potter 2011b) and the ligand-free receptor is recycled back to the plasma membrane (Potter 2011b). Although most of the physiological effects of natriuretic peptides are mediated by NPR-A and NPR-B, there is evidence which supports the hypothesis that NPR-C has functional roles — other than clearance. The unique intracellular tail of NPR-C contains a pertussis toxin-sensitive G-binding domain (Anand-Srivastava *et al.* 1996, Murthy & Makhlouf 1999). Consequently, NPR-C may have a signalling role through G-protein coupling, such as the inhibition of adenylyl cyclase activity (Pagano & Anand-Srivastava 2001) and stimulation of phosphoinositide hydrolysis (Murthy *et al.* 2000). Furthermore, NPR-C activation is linked to the smooth muscle hyperpolarisation induced by CNP in resistance arteries where CNP acts as an endothelium-derived hyperpolarising factor (Chauhan *et al.* 2003). Recently, Egom *et al.* (2015) studied the effects of NPR-C ablation on aspects of cardiac functioning using a knockout mouse model. Mice lacking in NPR-C were more susceptible to atrial fibrillation and had slower electrical conduction in the sinoatrial node and atrium (and also exhibited long bone growth). Atrial fibrillation was associated with enhanced collagen expression and deposition in the atria, suggesting a protective role for NPR-C in the heart.

Unlike NPR-B, NPR-C has a similar affinity for all the natriuretic peptides and is the most widely and abundantly expressed natriuretic peptide receptor. It is highly expressed in many tissues that express guanylyl cyclase receptors (Matsukawa *et al.* 1999); for example, kidney glomeruli (Maack *et al.* 1987). NPR-C distribution includes most of the major endocrine glands, lungs, kidneys and the vascular wall where it is the most represented NPR in endothelial cells (Leitman *et al.* 1986). The expression of *NPRC* is also widespread throughout the brain in rats (Herman *et al.* 1996a) with its mRNA shown to be present in deep layers of the neocortex and limbic cortex, posterior cortical amygdala, ventral subiculum, amygdalohippocampal area, and dentate gyrus.

In addition to specific receptor-mediated degradation, natriuretic peptides are also degraded by extracellular proteases (Potter 2011b), neprilysin (Kenny *et al.* 1993) and insulin degrading enzyme (IDE, Ralat *et al.* 2011). Enzymatic degradation may be as important as NPR-C clearance and it is responsible for the steady state level of CNP in endothelial cells (Komatsu *et al.* 1996). Neprilysin is a zinc-containing, membrane-bound, ectoenzyme (Kerr & Kenny 1974) that binds and degrades CNP as it does with other similar peptide hormones such as angiotensin II (Stephenson & Kenny 1987). Although neprilysin cleaves CNP at multiple sites, the primary site of hydrolysis occurs between cysteine^{6} and phenylalanine^{7} residues (Watanabe *et al.* 1997). IDE also

cleaves CNP (and ANP, but not BNP) at multiple sites, and inactivates the ability of CNP to raise intracellular cGMP (Ralat *et al.* 2011). Conversely, reduced IDE expression enhanced the stimulation of NPR-B by CNP which is consistent with IDE-dependent degradation and inactivation of CNP (Ralat *et al.* 2011).

2.1.4 NTproCNP: a marker of CNP production

Due to its rapid receptor-mediated and enzymatic clearance, CNP has the shortest circulating half-life (2.6 min) of all the natriuretic peptides in humans (Hunt *et al.* 1994) and a similarly short half-life (1.6 min) in sheep (Charles *et al.* 1995). The short half-life of CNP has made it a particularly difficult peptide to study, as circulating levels may not reflect tissue concentrations near the site of production (Kalra *et al.* 2003). Therefore, NTproCNP may be considered as a long-lived surrogate for CNP, so its levels in body fluids can provide an index of CNP secretion/activity (Prickett *et al.* 2001). NTproCNP has been established as a stable product of CNP gene expression and its concentration in plasma is highly correlated with CNP concentration (Prickett *et al.* 2001). It is unlikely that NTproCNP is hydrolysed by neprilysin or cleared from the circulation by NPR-C (Charles *et al.* 2006, Schouten *et al.* 2011), therefore its prolonged half-life makes it a more reliable indicator of CNP activity *in vivo* than measurement of CNP itself (Prickett *et al.* 2001).

Different clearance rates explain why the plasma concentration ratio of the two peptides (NTproCNP:CNP) is consistently high across different species; 31:1 in adult humans (Schouten *et al.* 2011), 26:1 in 4-week-old lambs (Prickett *et al.* 2009), and 28:1 in adult ewes (Prickett *et al.* 2010). This ratio is even higher for CSF concentrations; i.e. 144:1 in human CSF (Schouten *et al.* 2011). This may be due to the abundance of neprilysin (Facchinetti *et al.* 2003) and the widespread distribution of NPR-C in the brain and spinal cord tissues (Herman *et al.* 1996a) which depletes the concentration of CNP relative to NTproCNP (Schouten *et al.* 2011). There is one study which reported that the NTproCNP:CNP ratio in human CSF increased with age (Schouten *et al.* 2011).

2.2 Function and regulation of CNP in central and peripheral tissues

2.2.1 Peripheral roles for CNP

Although the focus of these studies are of CNP in the central nervous system, CNP is involved in the regulation of many important peripheral roles which are discussed below. Given the potent natriuretic effects of the cardiac natriuretic peptides, similar roles for CNP have been investigated, however it has been established that CNP is significantly less potent for inducing natriuretic (Hunt *et al.* 1994), diuretic or hypotensive effects *in vivo* than the cardiac natriuretic peptides (ANP and BNP, Sudoh *et al.* 1990). Although CNP appears to have some involvement in the regulation of blood pressure – it is produced by the vascular endothelium and causes vasodilation *in vitro* (Wei *et al.* 1993) – this is through local regulation of vascular tone (Chauhan *et al.* 2003) in an autocrine or paracrine manner. Also, CNP circulates at much lower concentrations than ANP so it appears to have little function in common with the classic natriuretic peptides. However, it is becoming clear that CNP is involved in the regulation of a much wider range of physiological mechanisms, which is reflected by the many peripheral sites which express *NPPC*, including the vascular endothelium (Chen & Burnett 1998), vascular smooth muscle cells (Woodard *et al.* 2002), myocardial tissue (Kalra *et al.* 2003, Del Ry *et al.* 2011), cardiac fibroblasts (Horio *et al.* 2003), reproductive tissues (Chrisman *et al.* 1993), bone (Prickett *et al.* 2005), kidney (Kalra *et al.* 2004) and heart (Vollmar *et al.* 1993).

Trans-organ sampling in anaesthetised sheep revealed that multiple organs contribute to circulating levels of CNP and NTproCNP (heart, liver, kidney, hind limb) in ewes, as demonstrated by a positive arterio-venous gradient (Charles *et al.* 2006). Positive arterio-venous gradients of CNP forms across the head and neck in trans-organ regional sampling studies have raised the possibility that cerebral tissues could be an important source of CNP in the systemic circulation in humans (Palmer *et al.* 2009) and sheep (Prickett *et al.* 2009). Unlike ANP and BNP, no significant net extraction of CNP across any tissue bed has been measured. However, a negative veno-arterial gradient between both the inferior vena cava and carotid artery, and the jugular vein and carotid artery, may implicate the lung in extraction of CNP from the circulation (Charles *et al.* 2006).

CNP is known to perform major functions in these tissues through the binding of NPR-B or by signalling through NPR-C, and stimulates growth of bone explants in tissue culture by acting on proliferative and hypertrophic zones (Yasoda *et al.* 1998). CNP transcripts and NPR-B are expressed in chondrocytes of growth plates (Hagiwara *et al.* 1994) and there are many reports

which demonstrate that the CNP signalling pathway is crucial for normal bone growth *in vivo* — in particularly, the regulation of endochondral ossification (Yasoda *et al.* 2004a, Yasoda *et al.* 2004b). *NPPC*-knockout mice have a dwarfed phenotype and experience early death (Chusho *et al.* 2001), and a loss-of-function mutation in NPR-B in humans also results in acromesomelic dysplasia (Marateaux type) and as a result, also have a dwarfed phenotype (Bartels *et al.* 2004). Similarly, mice with targeted disruption of cGMP-dependent kinase II, a downstream mediator of cGMP, also have compromised endochondral ossification and show a dwarfed phenotype (Pfeifer *et al.* 1996). Individuals with mutations in the *NPRC* gene display skeletal overgrowth/gigantism, as has been shown in mice (Jaubert *et al.* 1999, Matsukawa *et al.* 1999). Skeletal overgrowth results from *NPPC* overexpression in humans (Ko *et al.* 2015) and rodents (Fujii *et al.* 2010). It is clear that proper regulation of natriuretic peptide levels is crucial for normal development – and that improving our understanding of NP regulation may lead to promising clinical applications (reviewed by Peake *et al.* 2014).

CNP has been shown to rescue a mouse model of achondroplasia, whereby CNP inhibited proliferation and induced hypertrophy of chondrocytes when overexpressed in the liver, or infused into the circulation (Yasoda *et al.* 2004b). The use of a CNP analogue in treatment of achondroplasia in children is under investigation following reports that BMN 111, a 39-amino acid CNP analogue, was capable of inhibiting fibroblast-growth-factor-mediated MAPK activation (mitogen-activated protein kinases), and improving aspects of skeletal development (Lorget *et al.* 2012).

Several studies have investigated the role of CNP in appetite and energy expenditure, following observations that *NPPC*-knockout mice are typically leaner (Chusho *et al.* 2001, Tamura *et al.* 2004). It was unclear whether this phenotype was due to CNP playing a role in energy balance, or an indirect result of the *NPPC*-knockout mice featuring a misalignment of their teeth and jaws from skull and cervical spine deformities – causing mice to have difficulty with eating (Tamura *et al.* 2004). Inuzuka *et al.* (2010) addressed this dilemma by breeding *NPPC*-knockout mice with chondrocyte-targeted expression (CNP-Tg/*NPPC*$^{-/-}$) – i.e. rescuing the skeletal deformities and growth retardation, while maintaining an absence of CNP elsewhere. CNP-Tg/*NPPC*$^{-/-}$ mice had reduced body fat accumulation and better insulin sensitivity, suggesting that CNP may be a positive regulator of energy balance – either via reducing energy expenditure in brown adipose tissue and/or increasing food intake (Inuzuka *et al.* 2010). However, there is also evidence to support an opposite role for CNP; Yamada-Goto *et al.* (2013) reported that

intracerebroventricular administration of CNP suppresses food intake in mice via activation of the melanocortin system (Yamada-Goto *et al.* 2013), suggesting CNP as a negative regulator of energy balance.

The involvement of CNP in regulating aspects of reproduction is also unclear, however several lines of evidence from a range of species imply that it has a many-faceted role (Walther & Stepan 2004) and the expression of *NPPC* is altered in the rat ovary and uterus during the oestrous cycle (Dos Reis *et al.* 1995, Huang *et al.* 1996). In addition, CNP is involved in the regulation of erectile tissue and testicular function (Middendorff *et al.* 2000, Küthe *et al.* 2003), male mouse fertility (Sogawa *et al.* 2014) and is produced by trophoblast binucleate cells in the ruminant placenta during pregnancy – resulting in plasma CNP concentrations that are approximately 30-fold higher than non pregnant values (McNeill *et al.* 2011). CNP maintains oocyte meiotic arrest in early antral follicles of many species including mice (Zhang *et al.* 2010, Tsuji *et al.* 2012), cats (Zhong *et al.* 2016), pigs (Hiradate *et al.* 2014) and goats (Zhang *et al.* 2015) and this inhibitory effect is relieved by the luteinising hormone (LH) surge *in vivo* (Zhang *et al.* 2015). This action of CNP may be beneficial to infertility treatments, i.e. in *in vitro* maturation of oocytes whereby the synchronisation of nuclear and cytoplasmic maturation can be influenced by CNP to improve fertility outcomes (Wei *et al.* 2017).

CNP exerts a myriad of effects on local vasculature which extend beyond simply promoting vasodilation and controlling blood flow in the resistance vessels (Chauhan *et al.* 2003). Other effects include inhibition of vascular smooth muscle cell proliferation and migration (Morishige *et al.* 2000), and the stimulation of re-endothelialisation (Doi *et al.* 2001, Ohno *et al.* 2002). Scotland *et al.* (2005a) demonstrated that CNP dose-dependently reduces leukocyte rolling and platelet activity, during basal conditions and also under acute inflammatory conditions (induced by interleukin1-β and histamine). It was found that these effects were dependent on NPR-C activity, specifically via suppression of the expression of P-selectin, a surface adhesion molecule found on endothelial cells and platelets. Although the mechanisms for effects of CNP on the vasculature are not all understood, such as its ability to protect against against myocardial ischemia/ reperfusion injury (Hobbs *et al.* 2004, Soeki *et al.* 2005) and prevention of cardiac hypertrophy (Wang *et al.* 2007, Tokudome *et al.* 2004), there is certainly enough evidence to warrant further research into these mechanisms as potential therapeutic targets for cardiovascular diseases (reviewed by Scotland *et al.* 2005b and Lumsden *et al.* 2010).

2.2.2 CNP in cerebrospinal fluid

It has been established that the concentration of CNP in human CSF is the highest of any natriuretic peptide, and that the concentration of CNP peptides in CSF is substantially higher than in plasma (Togashi *et al.* 1992, Kaneko *et al.* 1993, Ikeda *et al.* 2001, Schouten *et al.* 2011). The concurrent measurement of CNP in CSF and plasma in healthy patients revealed a concentration of 2.13 pmol/L in CSF, and a CNP concentration that was too low to be detected in plasma (assay sensitivity < 0.2 fmol/mL, Kaneko *et al.* 1993). Ikeda *et al.* (2001) measured CNP concentration as 6.10 pmol/L in CSF, which was about 2.6 times higher than the concomitant plasma CNP concentration (2.37 pmol/L).

Schouten *et al.* (2011) reported the concentration of CNP peptides in CSF of human patients undergoing spinal anaesthesia (age 51-90). Consistent with previous reports, levels in CSF were markedly higher than in plasma (nearly 10-fold). In addition, this publication provided the first report of NTproCNP concentration in CSF, whereby NTproCNP concentration in CSF was 1045 ± 359 pmol/L which is 67 times higher than the value for plasma (Schouten *et al.* 2011). Further, the NTproCNP:CNP concentration ratio was greater in CSF (145:1) compared with plasma (31:1); indicating that degradation of CNP is more rapid in the CNS than in peripheral tissues. This also highlights the diagnostic importance of NTproCNP measurement in CSF, as an indicator of CNP release which would have been grossly underestimated by measurement of CNP alone.

2.2.3 Sources, targets and roles of CNP in the central nervous system

Several studies of neuroendocrine functions of natriuretic peptides (Doczi *et al.* 1987, Samson 1987) were carried out prior to the discovery of CNP in the porcine brain (Sudoh *et al.* 1990). However, it has since been established that CNP is the most abundant natriuretic peptide in the CNS and is widely distributed in the brain, e.g. of the rat (Minamino *et al.* 1991), sheep (Pemberton *et al.* 2002) and human (Ueda *et al.* 1991, Totsune *et al.* 1994). Furthermore, the expression of *NPPC* in the CNS has been described for the mouse (Stepan *et al.* 1999, Mirczuk *et al.* 2014) and rat (Minamino *et al.* 1991, Stepan *et al.* 1999). Komatsu *et al.* (1991) published the first report of markedly high levels of CNP in the rat pituitary gland, compared with other cranial structures. This finding was confirmed in sheep (Pemberton *et al.* 2002) and was shown to be unique to CNP; ANP and BNP were present in the pituitary gland at levels similar to those in brain tissue. Also, CNP is found in higher concentrations in the rat anterior pituitary gland than in the posterior pituitary gland (Komatsu *et al.* 1991) indicating some differentiation of its roles in glandular versus neural tissue. Collectively, these studies have paved the way for investigations of potential roles of CNP in the central nervous system.

There is evidence to suggest that both neuronal and glial cells are sources and targets of CNP (reviewed by Prado *et al.* 2010). In the dorsal root entry zone of the spinal cord, CNP-induced cGMP production via NPR-B was shown to be essential for sensory axon bifurcation (Schmidt *et al.* 2009). In agreement with this, Abdelalim *et al.* (2013) reported that NPR-B is expressed in neuronal cell bodies and processes in dorsal root ganglia (DRG) of the rat. Glial cells of the retina (Müller cells) showed a strong presence of CNP, particularly in their endfeet which closely enwrap blood vessels, suggesting involvement in the regulation of blood flow and intraocular pressure (Fernández-Durango *et al.* 1999). This is consistent with prior findings from Mori *et al.* (1997) who confirmed the vasodilator effects of CNP on cerebral arterioles in rats. *NPPC* expression has been reported in mouse astrocytes and rat glioma cells (Yeung *et al.* 1996b), and the ability of CNP to stimulate cGMP has been demonstrated in cultures of rat (Kobayashi *et al.* 1993) and mouse (Yeung *et al.* 1992) glial cells. *NPRB* is expressed in rat microglia (Moriyama *et al.* 2006), although the level of expression was lower than was found for NPR-A. Consistent with widespread NPR-B distribution, CNP-stimulated cGMP accumulation has been established in astrocytes (Deschepper & Picard 1994) and neurons (Xia *et al.* 2013). In summary, there is evidence for CNP-related physiology in both neurons and glial cells, including microglia and astrocytes.

Samson *et al.* (1993) demonstrated that central administration of CNP to conscious rats resulted in a decrease in circulating plasma LH concentration, which was mediated via NPR-B. This was a follow-up from observations whereby ANP had produced the same effect, however it was established that this ANP effect was due to competitive binding and displacement of CNP from NPR-C; increased ANP concentration decreased NPR-C availability, which in turn accentuated the reduction of LH by CNP.

Given that the presence of NPR-B has been confirmed in the rat anterior pituitary gland, it is possible that CNP is an autocrine/paracrine regulator of gonadotrope function. CNP has been co-localised with immunoreactive LH/FSH (follicle-stimulating hormone) cells; McArdle *et al.* (1994) reported that the majority of CNP-containing cells in the rat anterior pituitary gland were gonadotropes. Furthermore, when cultures of rat anterior pituitary cells were exposed to a toxin targeted to GnRH (gonadotropin-releasing hormone) receptor-expressing cells, there was a significant reduction in CNP-stimulated cGMP production and GnRH-stimulated LH release (McArdle *et al.* 1994).

The widespread distribution of CNP gene expression in the central nervous system suggests that CNP has a multifunctional role in neuroendocrine regulation (Stepan *et al.* 1999). For example, the high expression in the rodent tegmentum (brainstem) indicates an involvement of CNP in autonomic control and its expression in the rat hippocampus suggests a role in learning and memory (Stepan *et al.* 1999). CNP affects bidirectional plasticity in the hippocampus (Decker *et al.* 2010), by influencing hippocampal network oscillations in adult rats (Decker *et al.* 2009) — which raises the question as to whether CNP is involved with learning and memory. In support of this, central administration of CNP in rats has been shown to facilitate learning and consolidation of passive avoidance learning (Telegdy *et al.* 1999).

Aside from memory and learning, CNP is involved with other neuroregulatory processes such as nervous system development (Kishimoto *et al.* 2008). CNP stimulates axonal branch formation of DRG neurons in the spinal cord, and provides a cue that is necessary for bifurcation of central sensory afferents (Zhao & Ma 2009). In cultures of embryonic DRG neurons, CNP stimulates branch formation, induces axonal growth, and attracts growth cones (Zhao & Ma 2009). In addition, NPR-B is the most highly expressed natriuretic peptide receptor in the developing rat brain, and its expression is highest around the first postnatal day of life, which coincides with an elevated expression of nestin — a marker protein for stem/progenitor cells — which indicates a role for NPR-B in perinatal neurogenesis (Müller *et al.* 2009). Other suggested roles include neuroprotection (Ma *et al.* 2010), regulation of blood-brain barrier permeability (Bohara *et al.* 2014), involvement in pathways underlying pain hypersensitivity (Shepherd *et al.* 2014), and regulation of cocaine-induced changes in gene expression responsible for neuronal plasticity (Jouvert *et al.* 2004).

Since its discovery in 1990, it has become clear that the effects of CNP in the central nervous system are extremely diverse. However, many of the studies have been conducted *in vitro,* so there is a need to establish how far the crucial roles for CNP determined from these studies extend to the *in vivo* situation. Measurement of CNP concentrations under various neurological conditions will be essential for adding to the 'bigger picture', as has been demonstrated by analysing CSF from patients with Parkinson's disease (Espiner *et al.* 2014).

2.2.4 Regulation of CSF concentrations of CNP

To date, there are no published data on CNP peptide concentrations in sheep CSF. However, pilot studies conducted with sheep (G Barrell, Lincoln University, 2009-2010, unpublished) revealed much higher concentrations of CNP and NTproCNP in CSF (1.5 – 6.1 and 58-332 pmol/L, respectively) than in peripheral plasma (0.6 – 1.7 and 16 – 33 pmol/L, respectively). In addition, there was some indication that CNP peptide concentrations were higher in CSF from lumbar than from cervical samples (3/5), and that concentrations tended to be lower in the first sample that was collected when sheep were still anaesthetised, suggesting that the anaesthetic drugs used for this work (diazepam and ketamine) suppressed CNP peptide secretion into the CSF. Following this, CNP peptide concentrations in CSF and plasma of adult sheep that were studied over a 24-hour period revealed a complete absence of any diurnal pattern of secretion (Wilson *et al.* 2015). Further studies showed that both cortical activation via transcranial magnetic stimulation, and activation of the sympathetic nervous system via an intravenous bolus of caffeine, had no effect on CNP peptide concentrations in CSF or plasma (Wilson *et al.* 2011, unpublished). CNP and NTproCNP were present in CSF at high concentrations at all times in the sheep with no apparent effect of these two potentially stimulatory treatments on central or peripheral concentrations of the peptides.

The CSF of human subjects undergoing spinal anaesthesia for arranged orthopaedic procedures has been analysed for concentrations of CNP peptides (Schouten *et al.* 2011). Some of the patients had been on long-term medication for a variety of issues. Although the number of patients receiving any one class of drug limits conclusions from these studies, the only drug that had any effect on CNP levels in CSF was furosemide. Patients receiving long term furosemide treatment, which is known to potently reduce CSF formation (Johanson *et al.* 2008) and therefore CSF reabsorption, had a significantly increased NTproCNP:CNP ratio in CSF, compared with those not receiving the drug (Schouten *et al.* 2011). Furosemide did not affect the NTproCNP:CNP concentration ratio in plasma. The other drug classes used in these procedures which were without effect included a statin, angiotensin inhibitor, beta blocker, calcium channel blocker, anti-depressant, proton pump inhibitor, and a glucocorticoid.

2.3 Rationale for thesis

CNP has a high abundance in brain tissues relative to other natriuretic peptides, and is unique in that its concentration in CSF far exceeds circulating levels. *In vitro* studies show that CNP influences a diverse range of central processes, including stimulating neural growth and connectivity (Zhao & Ma 2009), neuroplasticity in hippocampal tissues (Decker *et al.* 2010), and effects on blood-brain barrier permeability (Bohara *et al.* 2014), however the functional role of CNP *in vivo* is unclear. At the time of commencing this thesis, there was only one report of CNP and NTproCNP concentrations in concurrent samples of CSF and blood (Schouten *et al.* 2011) which indicated that central and peripheral concentrations of CNP peptides are independently regulated, at least in healthy humans.

It was hypothesised that these high concentrations of CNP peptides in CSF reflected secretion from nervous tissues, however there were no reports linking nervous tissue concentrations of CNP to levels in the CSF. Animal models are necessary for progress in this area — given the unlikelihood of obtaining healthy samples of human CSF — and large animal models are advantageous as repeated samples of CSF can be obtained to allow for studies of temporal changes in hormone concentrations. Given the potential clinical applications of elucidating the function(s) of a novel neuropeptide involved in the regulation of many important central processes, there is first a need to establish fundamental aspects of CNP physiology in the CSF, i.e. identify central sources and investigate factors capable of influencing central levels of CNP *in vivo*.

Chapter 3. General Methods

This chapter describes methods which are common to several or all studies in this thesis. Other techniques or assays specific to individual studies are described in their respective chapters.

3.1 Measurement of live weight and animal handling

Live weight of sheep was measured by placing individual animals in a holding crush fitted with electronic scales (to the nearest 0.5 kg). Sheep were brought indoors and individually housed at Johnstone Memorial Laboratory (Lincoln University) throughout all trials, except for the study of CNP in pregnant sheep described in Chapter 4 where sheep were kept at Ashley Dene farm for the last 2 months of gestation. Jugular blood samples and CSF (from previously inserted cannulae) were collected by having an assistant lightly restrain the sheep in their individual pens.

3.2 Measurement of natriuretic peptides

3.2.1 Extraction of peptides from CSF and plasma

NPs were extracted from plasma and CSF samples using solid-phase extraction columns (Sep-Pak C_{18}, Walters, Milford, Massachusetts, USA). The columns were prepared by the addition of 5 mL of 100 % methanol followed by 5 mL of 0.1 % trifluoroacetic acid (TFA). Plasma (2 mL) and CSF (1 mL) samples were then passed through the columns, followed by a further 5 mL of 0.1 % TFA. Peptides were eluted using 2 mL of 80 % isopropanol in 0.1 % TFA into polystyrene collection tubes containing 10 µL of 0.1 % Triton X-100 (addition of Triton to the collection tubes prevents binding of NPs to the tube). The eluant was collected, dried at 37 °C under an air stream and frozen at -20 °C. Dried down plasma extracts (from 2 mL plasma) were reconstituted in 500 µL of RIA assay buffer to ensure they were above the detection limit. Dried down CSF extracts (from 1 mL CSF) were reconstituted in 1 mL of RIA assay buffer.

3.2.2 Radioimmunoassay of natriuretic peptides

Samples were assayed in duplicate, and all samples from individual sheep were included in the same assay to reduce the contribution of inter-assay variation. The detection limit, and between- and within-assay coefficients of variation reported in subsequent chapters reflect calculations based over a one year period of the respective study. These parameters were also calculated for the several years spanning the time period of this thesis and are reported below.

CNP

CNP was assayed as previously described (Yandle *et al.* 1993, Prickett *et al.* 2007), with minor changes: 50 µL standard (human proCNP (82-103) or sample extract was pre-incubated with 50 µL

of a commercial primary rabbit antiserum raised against proCNP (82-103) (Phoenix Pharmaceuticals Inc., Belbont, California, USA, catalogue number G-012-03) and diluted to 1:2000 in RIA assay buffer, then incubated for 22-24 hours prior to the addition of 50 µL of tracer (^{125}I labelled Tyr^{0}-proCNP(82-103)) containing 3000 cpm. The antisera raised against proCNP (82-103) shows 100 % cross-reactivity with CNP-22 and hCNP-53 (Phoenix Pharmaceuticals data sheet). Within- and between-assay coefficients of variation were 5.1 % and 9.8 % respectively, at 9 pmol/L, and the detection limit was 0.6 pmol/L.

NTproCNP

NTproCNP was measured as previously described (Prickett *et al.* 2001, Prickett *et al.* 2007) with minor changes (see appendix for a comparison of parallelism of ovine and human samples): 100 µL standard or sample extract was pre-incubated with 50 µL primary rabbit antiserum raised against human NTproCNP (1-15) (J39, diluted 1:6000 in RIA assay buffer) then incubated for 22-24 hours prior to addition of 50 µL of tracer(^{125}I labelled proCNP(1-15)Tyr^{16}) containing 3000 cpm. Following a further 22-24 h incubation period, bound and free labelled antigen were separated by addition of 500 µL of solid phase secondary antibody (5 % v/v donkey anti-rabbit Sac-cell (IDS Ltd, UK) diluted in potassium phosphate assay buffer). Peptide standards were made from synthetic ovine proCNP (1-19) (Mimotopes Pty Ltd, Clayton, Victoria, Australia). Within- and between-assay coefficients of variation were 7.8 % and 12.6 % respectively, at 64 pmol/L, and the detection limit was 2.7 pmol/L.

3.3 Methods of cannulation for repeated CSF sampling

The following section (3.3.1) describes the method that was employed on commencing this thesis and was used to collect CSF in pilot studies (5.1.2) and in 2 of 8 sheep in the dose-response study in Chapter 6. CSF was collected on most occasions, however the success rate of cannulation was poor, and each cannula would typically only last for approximately 6 days before CSF could no longer be drawn. Therefore, a new method with a longer patency period was sought and is described in 3.3.2. This new method was used for the remaining 6 sheep in the dose-response study, as well as the collection of CSF for size-exclusion high performance liquid chromatography described in Chapter 7.

3.3.1 Cannulation of the cervical subarachnoid space

Animals were fasted and had no access to water for 12 h before the cannulation procedure. Wool was clipped from one side of the neck and the skin was sprayed with 70 % v/v ethyl alcohol. Sheep were individually anaesthetised by a single intravenous injection (into the jugular vein)

with an equal volume mixture of diazepam (Ilium diazepam, 5 mg/mL, Troy Laboratories NZ Pty Ltd, Auckland, NZ, 0.5 mg/kg live weight) and ketamine hydrochloride (Phoenix Ketamine injection, 100 mg/mL, Phoenix Pharm Distributors Ltd, Auckland, NZ, 10 mg/kg live weight) which provided 20-30 minutes of sedation and analgesia.

Once sedated, each animal was placed into sternal recumbency, with the forelimbs folded underneath with the hips flexed and the pelvic limbs under the body or extended alongside the abdomen. A handler gently straddled the sheep and held the sheep's head steady while a 50 cm^2 area over the midline occipital region of the skull was clipped free of wool. The injection site was surgically prepared with a disinfectant scrub (1.6 % w/v available iodine (19.7 mg/mL crude iodine): Vetadine - Iodine Animal Wash, Bomac Laboratories Ltd, Wiri Station Road, Manakau City, Auckland, NZ) followed by 70 % v/v ethyl alcohol.

Ventral-flexion of the head and neck of the sheep was maintained throughout the procedure in order to enlarge the opening of the atlanto-occipital space and allow for easy entry of the Tuohy needle. Care was taken not to obstruct the sheep breathing, which was regularly checked whilst held in this position. In most sheep, a palpable depression down the dorsal midline could be identified near the top of the triangle which joins the caudal aspect of the occipital protuberance and the wings of the first cervical vertebra of the spine. The insertion site of the cannula was targeted at this palpable depression (Plate 3.1).

Plate 3.1 The site for CSF collection in anaethetised sheep.

A Tuohy needle (16G x 3.23 inch, i.e. 1.7 x 80 mm, Braun Perican®, Melsungen, Hesse, Germany) was inserted in a caudal direction at an angle of approximately 15° from the vertical plane (Plate 3.1). The tissues penetrated, in order, were: the skin and sub subcutaneous tissue, nuchal ligament,

dura mater and the arachnoid mater to reach the subarachnoid space of the cervical region. The nuchal ligament presents resistance to the passage of the needle. Correct placement of the cannula was identified by a distinct 'pop' and was confirmed when CSF could be slowly withdrawn from the needle hub by gentle aspiration. A polyamide catheter (0.6 x 1.05 x 1000 mm, Braun Perifix®ONE, Bethlehem, Pennsylvania, USA) was introduced through it, and fastened in place with cyanoacrylate adhesive (Superglue) plus adhesive tape.

3.3.2 Cannulation of the cisterna magna

The following describes the modification of a previously described method (Falconer *et al.* 1985) which allows for repeated collection of CSF from the cisterna magna, and was published in *Laboratory Animals*, 2015.

Modification of a method for cannulation of the cisterna magna in sheep to enable chronic collection of cerebrospinal fluid

Wilson, M.O., Barrell, G.K. (2015). *Laboratory Animals* 49: 85-87

3.3.2.1 Abstract

A method is described for chronic cannulation of the cisterna magna to enable repeated sampling of cerebrospinal fluid from conscious, ambulatory sheep by means of a flexible vinyl tube. Ease of sampling and duration of cannula patency are similar to those obtained with rigid, metal cannulae, but this modified method minimizes the degree of surgical intervention, and possible trauma, occurring during placement of the cannula.

Keywords
cannulation, cerebrospinal fluid, sheep, cisterna magna

3.3.2.2 Introduction

A variety of methods for cannulation of the cisterna magna and other cerebroventricular sites in sheep and goats have been described. However we consider the use of rigid, metal cannulae, with the attendant need to excavate a large section of the parietal bone and place securing attachments into the skull, to be unnecessarily invasive. To enable investigations of central roles of C-type natriuretic peptide,[1] we have sought to establish a method that enables repeated collection of cerebrospinal fluid (CSF) from the cisterna magna of sheep. CSF can be collected from sheep at cervical and lumbar sites,[2] although use of sedation is needed for this procedure

and it is not amenable to repeated and/or chronic sampling. We describe a simple modification of a reported method,[3] which allows for repeated CSF collection with minimal surgical intervention.

3.3.2.3 Materials and Methods

Six yearling Coopworth ewes (live weight between 30 and 51 kg) were fasted for 24 h prior to surgery and water was withheld overnight. Each animal was given an intramuscular injection of analgesic (1 ml of 324 µg/ml buprenorphine hydrochloride, Temgesic injection – Reckitt Benckiser Healthcare (UK) Ltd., Slough, UK). Anaesthesia was induced with an intravenous injection of a combination of ketamine (10 mg/kg ketamine hydrochloride, Phoenix ketamine injection, Phoenix Pharm Distributors Ltd, Auckland, NZ) and diazepam (0.5 mg/kg, Pamlin injection, Parnell Laboratories New Zealand Ltd, Auckland, NZ). After endotracheal intubation, anaesthesia was maintained with 2 % v/v isoflurane in oxygen. Post surgery, each sheep received analgesic (as above) every 8 h for 2 days and rectal temperature was monitored daily. These procedures were approved by the Lincoln University Animal Ethics Committee.

To maintain the sheep's head in a stable position during surgery, its mandible was placed on a horizontal bar attached to an external steel frame on the operating table and a tapered bar, clamped one on each side of the same frame, was secured into each auditory meatus (Plate 3.2).

Plate 3.2 Position of the sheep for cannulation of the cisterna magna.
The anaesthetized animal is placed in a prone position with its mandible resting on a steel rod and the head secured by tapered steel bars which extend into each auditory meatus.

The surgical site was clipped free of wool, cleaned with povidone-iodine solution and disinfected with 70 % v/v isopropanol. Using aseptic procedures a 6 cm sagittal skin incision was centred over the depression on the parietal bone and underlying tissues were revealed with use of a retractor (e.g. Weitlaner) and further dissection. The periosteum was scraped from the underlying parietal bone to clear the entry site for drilling, the midline about 3 mm rostral to the nuchal ligament aponeurosis. A 2.5 mm hole was drilled by hand through the bone at 15° rostral to the vertical (90°), until the bit passed through the bone. The hole was enlarged to 5 mm diameter and bone chips were removed with cotton swabs and by flushing with saline solution. Each cannula was made from a 30 cm length of clear polyvinyl chloride tubing (Dural Plastics & Engineering, Auburn, New South Wales, Australia) with an outside diameter (o.d.) of 2.5 mm (internal diameter 1.5 mm). At one end, the tip was sliced at an angle (45º) and 2 holes (2 mm diameter) were cut through the wall of the cannula on opposite sides, within 15 mm of the tip. Access to the distal end was provided by insertion of a sawn-off 1.65 mm o.d. (16 G) hypodermic needle with Luer hub. The dura mater and arachnoid membranes were carefully pierced in a caudal direction with a 10 G (3.4 mm outside diameter x 76.2 mm length) steel needle (Industrial Strength Llc., Wallingford, Connecticut, USA), through which the tip of the cannula was introduced and advanced caudally towards the cisterna magna (about 45 mm) until CSF flowed freely in the tubing – see Plate 3.3. Alternatively, the cannula could sometimes be introduced directly into the cisterna magna after the meningeal membranes were pierced. Appearance of CSF around the needle during its insertion indicated successful penetration of the meninges. The cannula was secured by placing an absorbable suture on to subcutaneous tissue near the bone entry site and tying it to the cannula with Chinese finger trap knotting. The needle was placed subcutaneously within the incision site and directed outwards so that it exited through the skin 5 cm lateral to the incision and was used to exteriorise the distal end of the cannula. After removal of the needle and attachment of the Luer hub to the cannula, a sampling port (BD Q-Syte^{TM} 0.10 ml, Becton Dickinson Infusion Therapy Systems Inc., Sandy, Utah, USA) was fitted to the hub. Subcutaneous tissues were repaired above the bone entry site with continuous suturing (absorbable) and the skin was closed with interrupted sutures (non-absorbable). Plastic surgical adhesive tape (5 cm length) was placed on the cannula 10 cm from the skin entry site and secured to the skin with cyanoacrylate adhesive (‘instant glue’), and the skin entry site was sealed with a generous bleb of antiseptic ointment (Betadine povidine-iodine antiseptic-germicide ointment, The Purdue Frederick Company, Norwalk, Connecticut, USA). The exterior section of the cannula and the surgical area were covered with sterile cotton swabs that were held in place with flexible synthetic netting. The cannula port was disinfected with 70 % v/v isopropanol prior to sample

collection, after which 0.5 ml of sterile saline solution (0.9 % w/v) was injected into the cannula to occupy the dead space, antiseptic ointment was reapplied and cotton swabs were replaced.

Plate 3.3 Mid-sagittal view of the head of a sheep showing the cerebrospinal fluid (CSF) cannula *in situ*.

The cannula is introduced through a hole drilled in the parietal bone about 3 mm rostral to the nuchal ligament aponeurosis and inserted in a caudal direction so that the tip is located in the cisterna magna.

3.3.2.4 Results

All six sheep were successfully cannulated, and recovered completely to provide CSF samples. The cannulae remained patent for 9 to 19 days (mean 13.5 days) and 25 to 46 samples of CSF (1.0 to 1.2 ml), were collected from each sheep. In most cases at 1 to 2 days before cannula patency failed, CSF flow into the collection syringe became noticeably reduced, which could be alleviated sometimes by prior administration of a small volume (< 0.5 ml) of saline solution through the cannula. After CSF ceased to flow, cannulae were removed and all sheep were returned to the farm in good health.

3.3.2.5 Discussion

This method enables repeated sampling of CSF from sheep with minimal restraint, without need for sedation post surgery and without ill effects. Sampling could be repeated on numerous occasions in a day for more than 2 weeks, which is a similar sampling performance to that described previously.[3] However, we consider that the present technique is a refinement because it is less invasive due to the use of flexible tubing, rather than the rigid metal needle used previously,[3] and requires less removal of the parietal bone. This contrasts with the relatively

major excavation of bone that is required for placement of a rigid metal needle and thus our method reduces the possibility of trauma to the dorso-caudal surfaces of the cerebellum. The utility of this technique for repeated collection of CSF from sheep will likely reduce the number of animals required to be subjected to the procedure.

In situ cannulae in the cerebral ventricles and cisternae of goats and sheep invariably become coated with a fibrous membrane, so that the functionality of such cannulae has a limited time-span.[4] Because the ability to collect CSF repeatedly from sheep does not extend beyond two to three weeks, it is desirable to have a cannulation technique that has minimal impact on welfare of the animals but provides ready access for sampling. The method described meets these criteria and makes some progress on two of the three Rs of humane animal experimentation.

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Declaration of Conflicting Interests
None Declared

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3.3.2.6 References

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Chapter 4. Sustained increases in plasma C-type natriuretic peptides fail to increase concentrations in cerebrospinal fluid: evidence from pregnant sheep

Statement

All aspects of this chapter have been published in *Peptides* (Wilson *et al.* 2015). I was involved in the study design, animal procedures, statistical analysis and writing of the manuscript. All natriuretic peptide assays were carried out by me, except those of the diurnal variation study which were executed by Sengodi Madhavan. The published manuscript which forms this chapter benefitted significantly from contributions from all authors, and from comments from anonymous reviewers.

Sustained increases in plasma C-type natriuretic peptides fail to increase concentrations in cerebrospinal fluid: evidence from pregnant sheep

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4.1 Abstract

C-type natriuretic peptide (CNP) is a paracrine growth factor with high abundance in CNS tissues and cerebrospinal fluid (CSF). Consistent with findings of CNP transcripts in the cerebral microvasculature and hypothalamus, CNP increases the permeability of the blood-brain barrier and reduces food intake when administered intracerebroventricularly in rodents. Whether high concentrations of CNP in plasma can affect CSF levels is unknown. Accordingly we have studied changes (days 4, 87 and 116) in concurrent plasma and CSF concentrations of CNP peptides in pregnant sheep – a physiologically unique setting in which plasma CNP is elevated for prolonged periods. Preliminary studies in non pregnant sheep showed stable CNP levels in CSF during repetitive sampling. Compared with values in non pregnant controls, plasma concentrations of CNP peptides were markedly raised (30-fold) at days 87 and 116 in pregnant sheep, yet CSF levels in the two groups did not differ. CNP peptides in CSF decreased from day 4 to day 87 in pregnant sheep, possibly reflecting an adaptive response of the cerebral vasculature to increased

hemodynamic load. We conclude that sustained high concentrations of CNP – far exceeding levels encountered in human pathophysiology – fail to affect CNP peptide levels in CSF.

Keywords: blood-brain barrier; C-type natriuretic peptide; cerebrospinal fluid, NTproCNP, pregnancy

4.2 Introduction

C-type natriuretic peptide (CNP), a paracrine growth factor regulating cell proliferation and maturation in many tissues,[1] has a high abundance in tissues of the central nervous system (CNS)[2,3] including cerebrospinal fluid (CSF).[4,5] CNP transcripts are prominent in the cerebral microvasculature[6] and specific CNP receptors (NPR-B) are expressed in the choroid plexus and in the hypothalamic neuroendocrine circuits.[7] CNP promotes neurogenesis[8] and connectivity within CNS structures[9] and intracerebroventricular administration of CNP has been shown to reduce food intake in rodents[10] and lower arterial blood pressure in sheep.[11] Recently, studies in rodents[12] show that CNP also increases the permeability of the blood-brain barrier (BBB). Taken together these findings raise the possibility that, as found for other systemic peptides affecting vegetative functions[13] and/or neural development,[14] CNP may also access the brain parenchyma and CSF.

In health, CSF concentrations of CNP (i.e. proCNP 82-103; 2.2 kDa) and the amino-terminal (bio-inactive) fragment of its propeptide (amino-terminal proCNP 1-50, i.e. NTproCNP; 5 kDa) far exceed concentrations in plasma in humans.[5] These findings suggest that CNP is independently regulated in central and systemic circulations, and that transfer from plasma to CSF is minimal. Plasma CNP concentrations in adult humans are close to detection limits (approximately 1 pmol/L) in normal health but may achieve levels as high as 10-20 pmol/L in subjects with septic shock.[15] Whether such high concentrations of CNP can affect CSF concentrations has not been studied but is important to assess for the above reasons, especially now that treatment using systemic administration of CNP is undergoing trials in children with disorders of skeletal growth (ClinicalTrials.gov.NCT02055157).

Hypothesising that CSF concentrations of CNP will not be changed by sustained elevations in the systemic circulation, we have now measured CNP forms in time-matched samples of CSF and venous plasma obtained from pregnant sheep – a physiologically unique setting in which the circulating concentrations of CNP forms in blood are markedly increased for prolonged periods.[16] Concomitant samples of cervical atlanto-occipital CSF and venous plasma were collected from 15

twin-bearing ewes throughout gestation and peptide concentrations were compared with values in 15 non pregnant control ewes similarly sampled over the same time period. To assess possible changes in BBB permeability in late gestation, plasma-CSF gradients in small molecules were also measured.

4.3 Materials and methods

Animal procedures

All procedures involving animals were carried out in accordance with the Animal Welfare Act 1999 (NZ) and were approved by the Lincoln University Animal Ethics Committee. Animals were adult crossbred Coopworth-based female sheep (15 non pregnant ewes and 15 pregnant ewes with twins), live weight 63.2 ± 1.6 kg (mean ± s.e.) that grazed pasture during the study. Pregnancy and day of conception were ascertained by collecting ewes that had been naturally mated in a single 48 h period. Ultrasound scanning 9 weeks later was used to select those with twin fetuses. Unmated ewes from the same flock were randomly selected for the non pregnant (control) group. All sheep were brought indoors briefly on 3 occasions throughout the gestation period; at 4, 87 and 116 days post conception (gestation length 145 ± 6 days, mean ± s.e.) for collection of samples of blood and CSF. On these occasions the ewes were weighed one day prior to sampling and had no access to feed for 24 h after which 10 mL of blood was collected via jugular venepuncture into an evacuated tube containing 18 mg of potassium ethylenediamine tetra acetate (BD Vacutainer®, Becton Dickinson, Franklin Lakes, NJ, USA), placed on ice then centrifuged at 2500 rpm for 10 minutes at 4 ºC. The time from blood collection until centrifugation did not exceed 2 h. Plasma was stored in polycarbonate tubes at -20 °C until assayed.

Ewes were individually anaesthetised with 25 mg diazepam and 500 mg ketamine hydrochloride delivered in combination by i.v. injection into an external jugular vein. Wool was clipped from the CSF sampling site and the skin cleaned with povidone iodine solution and sprayed with 70 % (v/v) ethanol. While an assistant held the head of the animal in a nose-down flexed position, a 30 mm x 0.70 mm (22 G) hypodermic needle was inserted into the atlanto-occipital space as described by Scott[17] to obtain CSF. The landmark for needle incision into the atlanto-occipital space was the centre of an imaginary line connecting the caudal ear margins and the occipital protuberance. A palpable depression down the cisternal dorsal midline could be identified at the sampling site. 1.5 mL of CSF was drawn through the needle into a 3 mL syringe then transferred to a polycarbonate tube and placed on ice, before being transferred to a freezer (-20 °C) within 2 h. On occasions CSF could not be obtained but at least 13 animals per group (pregnant, non pregnant) were successfully sampled on each occasion.

To determine stability of CSF levels of CNP peptides and the effects of anaesthetics used in sample collection, preliminary studies were performed. Seven non pregnant Coopworth ewes aged 3-5 years (average live weight 50 kg) were housed indoors, exposed to natural lighting, and fed 0.9 kg lucerne chaff once daily at 0900 with free access to water for 7 days prior to study. These conditions were maintained during the intervention, except ewes were fed immediately after 0900 h samples were collected. Using an intravenous catheter (BD Angiocath™ IV Catheter, Franklin Lakes, NJ, USA) temporarily emplaced in the cervical subarachnoid space[17] as described above, samples of CSF (1.0 – 1.2 ml) were withdrawn at two-hourly intervals for 24h. Jugular blood samples (10ml) were collected at the same times, and processed as described above. After the study period had finished, cannulae were removed and all sheep were returned to the farm in good health. The effects of anaesthetic agents were separately studied in 6 non pregnant female Coopworth ewes aged 3-7 years, mean live weight 70.2 ± 2.7 kg. Animals were housed as above, except they were fed lucerne pellets (SealesWinslow performance nutrition, Ashburton, NZ). Animals were similarly sampled before and 10 minutes after receiving diazepam (0.5 mg/kg Ilium diazepam, Troy Laboratories NZ Pty Ltd, Auckland, NZ) and ketamine hydrochloride (10 mg/kg, Phoenix Ketamine injection, Phoenix Pharm Distributors Ltd, Auckland, NZ) in combination.

Hormone assays

Plasma was stored in polycarbonate tubes at -20 °C until assayed. Hormones in CSF and plasma were measured by radioimmunoassay after extraction using Sep-Pak cartridges (Walters Corp., Milford, MA, USA). All samples from an individual animal were processed in duplicate in a single assay. CNP was assayed as previously described[18] using commercial CNP-22 antiserum (catalogue number G-012-03, Phoenix Pharmaceuticals, Belmont, CA, USA). Within- and between-assay coefficients of variation were 5.9 and 10.4 % respectively. NTproCNP was measured in plasma and CSF by radioimmunoassay as described previously[5,19] except that synthetic ovine proCNP (1-19) (Mimotopes Pty Ltd, Clayton, Victoria, Australia) was used as standard. Rabbit antiserum J39, raised against human proCNP (1-15), was used as the primary antibody. Since the sequence of ovine and human proCNP (1-15) differ at amino acid residue 14, concentrations obtained using the new ovine standard also differ from those previously reported.[16] The following formula describes the numeric relationship for sheep plasma concentration read from human vs sheep NTproCNP standard curves: [log sheep NTproCNP] = 1.4 [log human NTproCNP] - 0.30. Within- and between-assay coefficients of variation were 6.7 and 7.3 %, respectively. The ratio of NTproCNP to CNP (NTproCNP:CNP) was calculated from molar concentrations of the respective peptides in each sample analysed.

Plasma and CSF gradients of small metabolites

Glucose and β-hydroxybutyrate were measured in CSF and plasma samples collected from 8 non pregnant and 8 pregnant sheep on day 116. Analytes were measured enzymatically on an automated analyser (Hitachi Modular P, Hitachi High-Technologies, Corporation, Tokyo, Japan) at a commercial analytical laboratory (Gribbles Veterinary Pathology, Christchurch, NZ) using proprietary kits (glucose – hexose kinase, Roche Diagnostic Corporation, Indianapolis, IN, USA and β-hydroxybutyrate, Randox Laboratories Limited, Crumlin, County Antrim, UK).

Statistical analysis

Statistical analyses were performed using Genstat (16th edition, VSN International Ltd., Hemel Hempstead, UK). Repeated-measures ANOVA was used for each hormone in each fluid to assess whether a diurnal rhythm was present, to determine a difference in concentration over time for pregnant sheep, and to compare hormone concentrations and NTproCNP:CNP ratios between pregnant and non pregnant sheep at different time points. A Bonferroni *post hoc* analysis was carried out to determine changes within the pregnant group. A two-tailed *t*-test was used to compare values before and after the anaesthetic regimen, and to compare glucose and β-hydroxybutyrate concentrations and ratios in CSF and plasma from pregnant and non pregnant sheep. Data are presented as mean ± s.e.

4.4 Results

Stability studies and effect of anaesthesia

As shown in non pregnant sheep (Figure 4.1), repetitive sampling of plasma and CSF show relatively stable concentrations of both CNP peptides throughout the 24 h period of study (P > 0.05). There was no evidence of any diurnal rhythm.

Compared with concentrations in CSF before anaesthesia (CNP 1.85 ± 0.08, NTproCNP 730 ± 48 pmol/L), values did not differ significantly 10 minutes after drug administration (1.69 ± 0.08 and 682 ± 39 pmol/L, respectively). Likewise, plasma concentrations of CNP (0.84 ± 0.04 before, 0.78 ± 0.04 pmol/L after) and NTproCNP (35.0 ± 2.6 before; 36.1 ± 1.8 pmol/L after) did not differ.

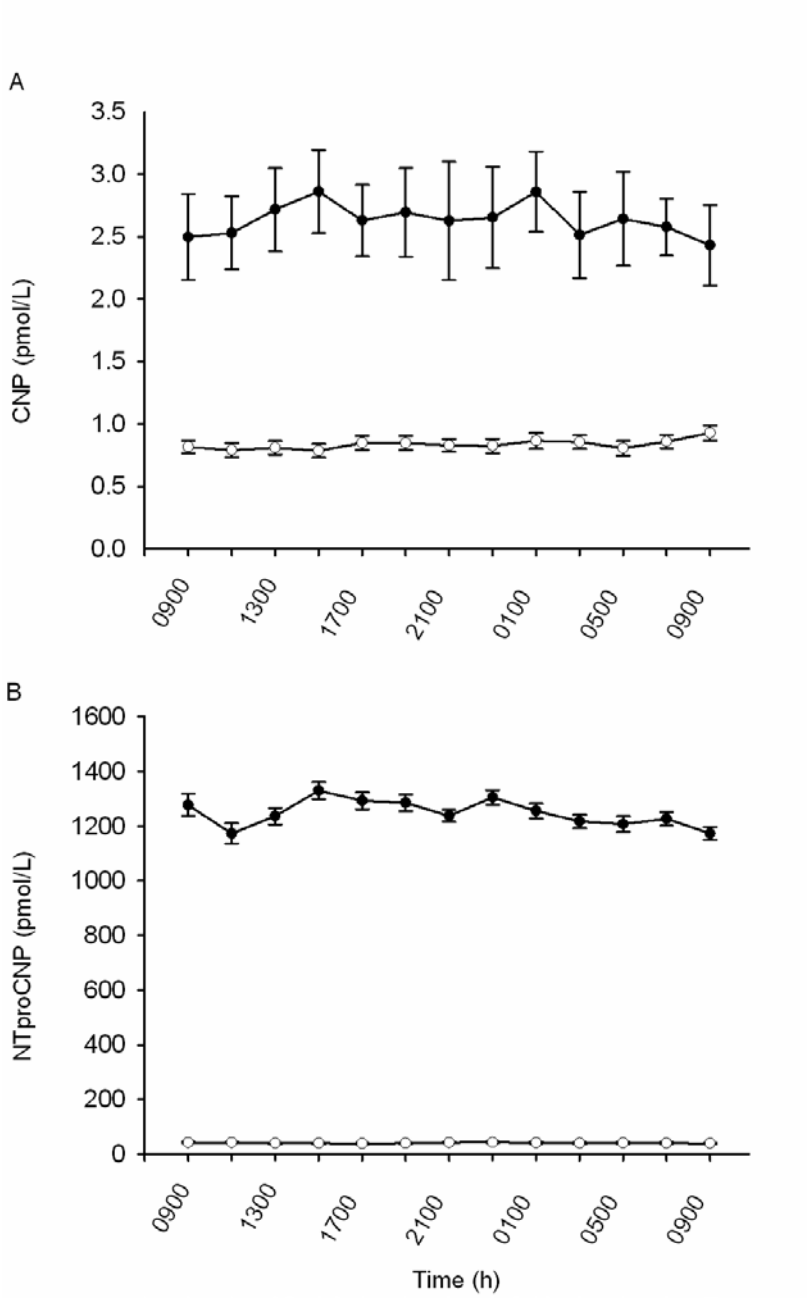

Figure 4.1 Diurnal variation of CSF and plasma concentrations of CNP peptides.
CNP (A) and NTproCNP (B) concentrations in CSF (closed circles) and plasma (open circles) in 7 non pregnant ewes sampled at two-hourly intervals for 24 h. Data are mean ± s.e., pmol/L.

Effect of pregnancy on plasma and CSF peptides and metabolites

Thirteen of the 15 pregnant sheep remained well during gestation and delivered healthy twin lambs without incident. However, one sheep died from causes unrelated to the study after the last sampling occasion prior to parturition, and one delivered a malformed lamb that died several days after birth. Mean gain in live weight from day 4 to day 116 was 7.6 ± 1.0 kg in pregnant sheep. Non pregnant ewes (controls) also remained in good health with minimal change in live

weight (mean loss 0.6 ± 1.1 kg) over the same time period. None of the animals exhibited signs of meningeal infection in any of the studies being reported.

Changes in CNP peptides in plasma and CSF are shown in Figure 4.2. Plasma CNP concentrations in non pregnant ewes (Figure 4.2 A) were 0.81 ± 0.03, 0.81 ± 0.05 and 0.97 ± 0.05 pmol/L on day 4, 87 and 116 respectively. In strong contrast, while similar on day 4 (0.91 ± 0.04 pmol/L), plasma CNP in pregnant ewes had risen markedly by day 87 (24 ± 1.5 pmol/L), increasing further by day 116 (31.9 ± 2.0 pmol/L) as shown in Figure 4.2 A. Compared with day 4 values, these increases were highly significant ($P < 0.001$ by ANOVA). Concentrations of CNP in CSF in non pregnant ewes (Figure 4.2 C) were higher than concurrent plasma concentrations; 3.22 ± 0.3, 3.04 ± 0.25 and 3.60 ± 0.28 pmol/L on day 4, 87 and 116 respectively. Despite the > 25-fold increase in plasma CNP concentration during gestation (Figure 4.2 A), concurrent levels of CNP in CSF in these pregnant sheep (3.15 ± 0.21, 2.43 ± 0.17, and 4.08 ± 0.33 on day 4, 87 and 116 respectively) did not change ($P = 0.068$), and did not differ from those in non pregnant ewes ($P = 0.15$, by repeated-measures ANOVA, Figure 4.2 C).

As described above for plasma CNP, a similar pattern of change was observed in NTproCNP concentrations in plasma of pregnant sheep (Figure 4.2 B). Plasma NTproCNP concentrations in non pregnant ewes were 47 ± 3.0, 49 ± 2.0 and 55 ± 2.7 pmol/L at day 4, 87 and 116 respectively whereas values were much higher during gestation after day 4 (54 ± 2.0, 1423 ± 106 and 1904 ± 276 pmol/L respectively; $P < 0.001$ by ANOVA). As noted with CNP, CSF concentrations of NTproCNP in non pregnant ewes (Figure 4.2 D) were much higher than concurrent levels in plasma. Again, in the face of greatly increased time-matched NTproCNP concentrations in plasma during gestation, concentrations of NTproCNP in CSF in these pregnant sheep did not differ from non pregnant values.

Comparing values in pregnant and non pregnant sheep at individual time points, with the exception of the highly significant differences observed for both CNP and NTproCNP in plasma at 87 and 116 days, values of CSF CNP and NTproCNP concentration did not differ between the groups at any time point. CSF CNP (Figure 4.2 C) and NTproCNP (Figure 4.2 D) concentrations both decreased significantly from day 4 to day 87 in pregnant sheep ($P < 0.05$, *post hoc* Bonferroni) despite the elevation in plasma concentrations of both peptides. Values did not differ significantly between 4 and 116 days in pregnant sheep.

The overall NTproCNP:CNP concentration ratio in plasma was 62 ± 2.6 and did not differ between pregnant and non pregnant sheep (P = 0.997, ANOVA), nor over time (P = 0.999, ANOVA). Similarly, values for the NTproCNP:CNP ratio in CSF (overall mean 299 ± 19) did not differ between pregnant and non pregnant sheep (P = 0.228, ANOVA).

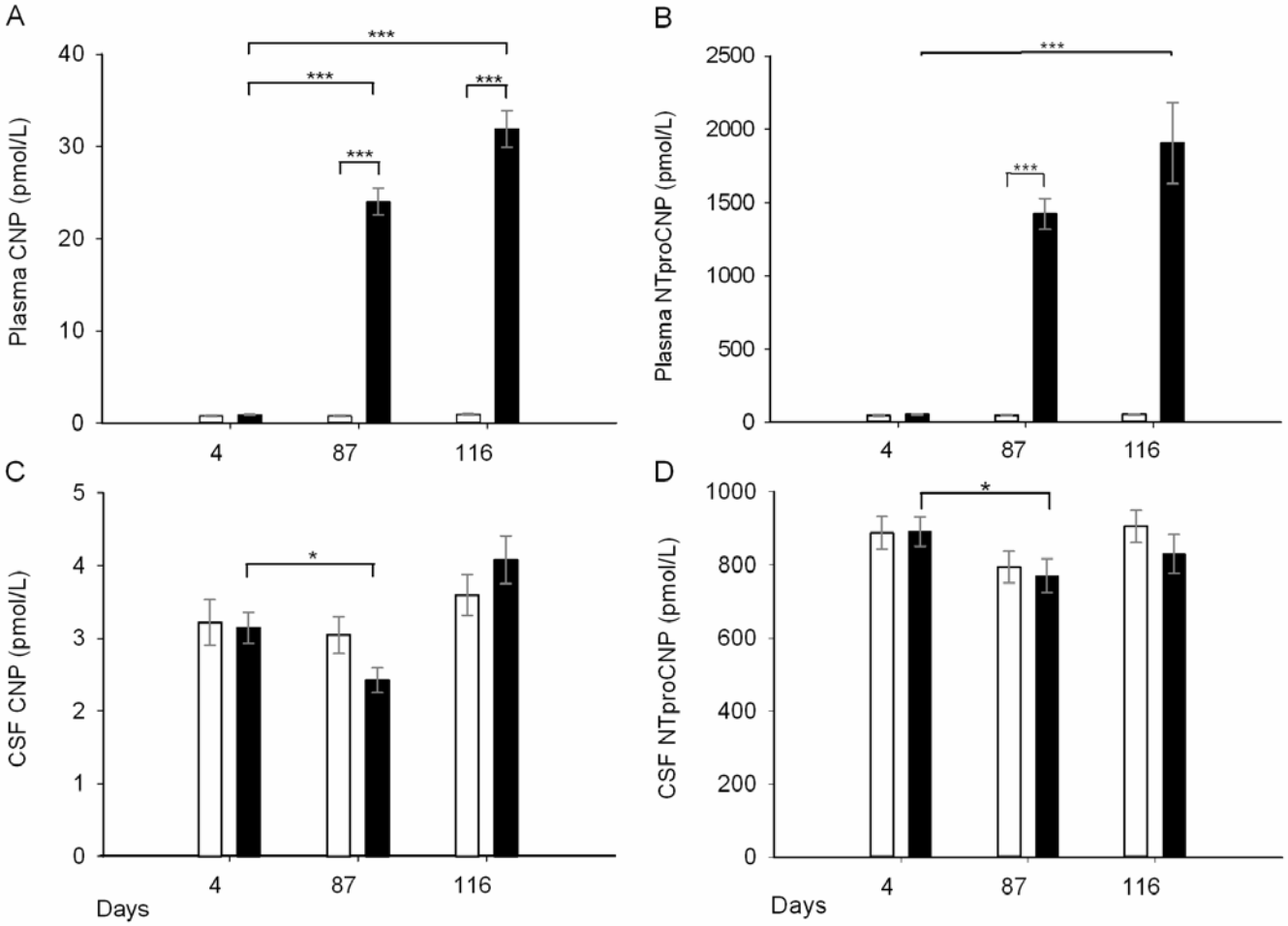

Figure 4.2 Effect of pregnancy on CNP peptides in plasma and CSF.
Concentration of CNP and NTproCNP in plasma (A, B) and CSF (C, D) of non pregnant (open bars) and twin-bearing (solid bars) ewes throughout the gestation period. Data are mean ± s.e., pmol/L, n = 15 per group; * P < 0.05, *** P < 0.001.

As shown in Table 4.1, both glucose and β-hydroxybutyrate concentrations were higher in plasma than in CSF irrespective of status on day 116. Whereas plasma β-hydroxybutyrate concentration increased significantly in pregnancy, plasma glucose concentration fell. However, the plasma:CSF concentration ratio of β-hydroxybutyrate did not differ between pregnant (8.5 ± 0.51) and non pregnant sheep (8.2 ± 0.77, P = 0.68) despite significant differences in concentration in CSF (Table

4.1). Similarly, there was no difference in the plasma:CSF concentration ratio of glucose between pregnant (1.72 ± 0.25) and non pregnant sheep (1.74 ± 0.16, *P* = 0.96) despite significant changes in glucose concentration during pregnancy.

Table 4.1 Concentration of small metabolites in CSF and plasma.

Concentration of β-hydryoxybutyrate and glucose in CSF and plasma in pregnant and non pregnant sheep on day 116. Data are mean ± s.e. (mmol/L), n = 8 per group.

	CSF pregnant	CSF non pregnant	*P*	Plasma pregnant	Plasma non pregnant	*P*
β-hydroxybutyrate	0.11 ± 0.01	0.05 ± 0.003	<0.001	0.93 ± 0.12	0.38 ± 0.02	0.002
Glucose	1.59 ± 0.21	2.23 ± 0.15	0.027	2.55 ± 0.31	3.73 ± 0.16	0.005

4.5 Discussion

Findings that CNP increases the permeability of the cerebrovascular endothelium[12] raise the possibility that systemic concentrations of the peptide may access the central circulation and contribute to concentrations of CNP in CSF. This has not been studied previously but is an important issue as intraventricular administration of CNP affects food intake and energy regulation in rodents[10] and lowers systemic blood pressure in sheep.[11] In healthy adults, plasma CNP concentrations are low (< 1-2 pmol/L),[20] less than 30 % of CSF levels,[5] making it unlikely that systemic CNP contributes significantly to CSF levels of CNP. However at much higher concentrations – as may be achieved during septic shock[15] or by exogenous treatments in children in order to promote skeletal growth – conditions of access may be more favourable. The present study – capitalising on a unique finding of sustained increases in plasma CNP during ovine pregnancy[16] – shows that CSF levels are unaffected by these high levels in the peripheral circulation which also fail to affect the permeability of the BBB to small molecules. In addition we report significant reduction in CSF concentrations of both CNP forms at mid gestation – possibly mediated by hemodynamic changes in the systemic circulation associated with pregnancy. Although concentrations of both CNP and NTproCNP in CSF are much higher than plasma levels in humans,[5] contributions from specific brain nuclei, spinal cord or CNS vascular tissues to levels in CSF are not known. In preliminary studies in non pregnant sheep, here we show that, as in humans, concentrations of both peptides were much higher in CSF compared with concurrent plasma levels. CSF concentrations of both CNP peptides were relatively stable during a prolonged

period of repetitive sampling and showed no diurnal changes. Further, levels in CSF and plasma were unaffected by anaesthesia induced by the drugs used in the study. Consistent with this evidence of stability is the close agreement in peptide levels within the two study groups (pregnant and non pregnant) on day 4. Changes in plasma CNP in ovine pregnancy (approximately 25-fold increase), induced by greatly increased placental secretion[16] and achieving levels of 30-40 pmol/L, clearly fail to increase concurrent levels in CSF. It is possible, that failure to detect evidence of access to CSF is a consequence of some adaptive or compensatory change associated with the pregnant state. For example, changes in protein binding or other protein-protein interactions, or alterations in clearance or degradation of CNP could be responsible. For several reasons this is unlikely. Our previous studies of CNP in ovine pregnancy[21] show no evidence of protein binding of CNP. More importantly, the ratio of NTproCNP to CNP in CSF does not differ between pregnant and non- pregnant groups. Conceivably, pregnancy associated changes in CNP clearance (by NPR-C) or altered degradative pathway activity could affect CNP but such changes would not be expected to affect NTproCNP.[1,5] For both peptides to be changed similarly (in both direction and degree) such that the ratio was unchanged we consider implausible but cannot be completely excluded.

Whether our findings extend outside pregnancy also requires consideration. Pregnancy represents a major hemodynamic challenge[22] and results in significant changes in cardiac output, redistribution of regional blood flow and remodelling in cerebral blood vessels, where parenchymal arterial lumen diameter is increased and the vascular wall becomes thinner[23] – all of which could affect BBB integrity. Although the threshold affecting transfer of solutes and small molecules at different levels of arterial pressure may change, several lines of evidence indicate that the BBB remains intact and unaltered in normal gestation.[23] Our observation that small molecule plasma:CSF gradients, as measured on day 116, do not change in pregnancy are consistent with this view. It is therefore reasonable to conclude that our findings will have general application – including non pregnant states. However it is relevant to note that while plasma CNP values in the current study greatly exceed those likely to be observed in pathophysiological states in humans,[20,24,25] they are several orders of magnitude lower than those (1-100 nmol/L) shown to increase BBB permeability in tissue preparations – or achieved after intravenous doses (10 nmol/kg) that increase permeability in mice.[12] Whether equivalent (e.g. 20 µg/kg) or higher doses of CNP administered to humans alter BBB permeability therefore remains unknown.

Significant reduction in both CSF CNP and NTproCNP concentrations at mid gestation in pregnant sheep was an unexpected finding. As already discussed, hemodynamic changes associated with pregnancy also impact on the cerebral vasculature. Of note, natriuretic peptides of brain origin are reported to participate in the regulation of CSF formation and volume.[26, 27] In a previous study of concurrent CSF and plasma CNP forms in humans, a significant inverse correlation was found between plasma and CSF concentrations of CNP.[5] Since there was also a significant association of plasma CNP with arterial blood pressure it was suggested that these systemic factors may reduce 'vascular CNP' sourced within the BBB[28,6] – and constitute a servo mechanism contributing to the autoregulation of cerebral blood flow and the maintenance of intracranial fluid volume.[26] In pregnancy, although no change occurs in cerebral blood flow or pressure, there is extensive remodelling within brain parenchymal arterioles, with consequential increase in wall tension and wall stress.[23] This, along with increased capillary density and vascular volume in pregnancy could reflect the afferent limb in the servo mechanism previously postulated. In diminishing CNP production within the brain's vasculature, such changes could be important in the homeostatic regulation of intracranial fluid volume.[26,27] Hemodynamic stress is likely to be near maximum by mid gestation - at least in humans[22] and could account for the temporal changes we observe in CSF CNP. In future studies it will be instructive to examine temporal changes in cerebral blood flow, BBB integrity and changes in CNP during pregnancy, and the effects of specific CNP antagonists once they become available.

In summary, sustained high concentrations of CNP forms in the systemic circulation – greatly exceeding concentrations encountered in human pathophysiology – do not increase CNP levels in CSF. Reduced CSF CNP forms at mid gestation may reflect an adaptive response of the cerebral vasculature to increased circulating blood volume.

Disclosure/conflict of interest

EAE is a consultant for BioMarin Pharmaceutical. The authors have no conflicts of interest to disclose.

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We thank Colin Pettigrew and Hélène de Batz for their assistance with management of sheep, and Martin Wellby and Sengodi Madhavan for technical assistance.

Author contributions statement

M.O.W. and G.K.B. ran the animal trial and collected CSF and plasma samples, and M.O.W. conducted CNP and NTproCNP assays. T.C.R.P. and M.O.W. carried out the statistical analysis. Results were discussed and the manuscript was written with contributions from all authors, with significant input into the discussion from E.A.E.

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4.7 Additional information, excluded from the manuscript

Pregnant and non pregnant sheep were obtained using the following procedure: after progesterone controlled internal drug release devices (CIDRs) were inserted and withdrawn, a crayon-harnessed vasectomised ram was introduced in order to identify ewes that were cycling. Ewes that were marked with the crayon by the vasectomised ram were deemed non pregnant — 15 of which were randomly selected to comprise the non pregnant group of sheep for the current study — and the rest were subject to artificial insemination (as part of a separate study). 12 days following AI, an entire crayon-harnessed ram was introduced to the mob for mating with the ewes that did not conceive to the AI. Only 15 pregnant sheep were required for this study, however as it was too early in the gestation period to confirm pregnancy status via ultrasound scanning, samples of CSF and plasma were collected from 20 of these sheep. Once pregnancy status was confirmed after the first sampling period, 15 sheep were randomly selected to remain in the trial.

Chapter 5. Investigation of candidate stimuli to evoke changes in CSF concentration of CNP peptides

Statement

I was involved with the study design and animal procedures, and all assays were carried out by me except for the testosterone assay, which was done by Canterbury Health Laboratories (Christchurch, NZ). I received significant technical assistance with animal procedures, and the writing of this chapter benefitted from comments from my PhD supervisors.

5.1 Investigation of candidate stimuli to evoke changes in CSF concentration of CNP peptides in sheep

5.1.1 Introduction

As discussed in Chapter 2, there are many regions of the CNS that express CNP and its receptors, any of which could be candidates for sources of CNP. At the onset of these studies, no physiological event or drug treatment had been reported to be capable of altering CNP peptide concentration in CSF. Preliminary studies on sheep, carried out as an Honours degree project, revealed the absence of a diurnal rhythm (Wilson *et al.* 2015), and a lack of response to cerebral cortex activation (in the form of transcranial magnetic stimulation) and general sympathetic activation (peripheral administration of caffeine; unpublished, Wilson *et al.* 2011). To overcome this lack of a known stimulus/suppressant that will acutely elevate or reduce the CSF concentrations of CNP peptides, a series of pilot studies was undertaken in search of a candidate for this purpose. Certainly, identification of a treatment stimulus or suppressant that is able to alter CNP peptide concentrations in CSF would be invaluable in future studies to determine the source of CNP peptides in CSF. Compounds were selected that targeted a range of major brain pathways, and/or showed potential in the literature to play a role in the regulation of CNP secretion in the brain (5.1.2). The studies described in this section were pilot studies which served the sole purpose of identifying a central stimulus or suppressant of central CNP. Therefore, they involved small group sizes, no statistical analysis and sometimes no measure of drug efficacy (e.g. l-deprenyl and progesterone). As part of the same general objective aimed at identifying central regulators of CNP peptides, studies were undertaken in a second animal model — the red deer stag — to investigate whether the profound seasonal changes in appetite and reproductive activity exhibited by this species affected CSF levels of the CNP peptides (5.2).

5.1.2 Rationale for selection of candidate stimuli to effect changes in CSF concentration of CNP peptides

Anaesthetics/sedatives

In earlier pilot studies designed to develop a method for repeated collection of CSF samples in sheep, it was observed that CNP peptide concentration was lower in the initial samples which were obtained — whilst the animals were under the influence of ketamine/diazepam — than in subsequent samples (n = 5, Lincoln University 2009–2010, unpublished). This suggested that brain regions sensitive to the depressive effects of anaesthetics and sedatives were the possible sources of CNP peptides appearing in CSF. Consequently, samples of CSF were collected before, during and after anaesthesia to map any changes in CSF concentrations of CNP peptides which could be linked to the decrease and subsequent increase in arousal level that accompanies anaesthesia and recovery. The compounds investigated were ketamine (on its own or in conjunction with the sedative, diazepam) and isoflurane. The sedative, diazepam, was also tested on its own.

Lipopolysaccharide

There are many studies linking CNP to inflammation described in the literature, however the nature of CNP's involvement is unclear. Whereas higher plasma concentrations of CNP are associated with severe inflammation in adults (Koch *et al.* 2011), acutely ill children were shown to have lower plasma concentrations of the CNP peptides (Prickett *et al.* 2013). Evidence for an anti-inflammatory effect of CNP was published by Qian *et al.* (2002) where it was shown that overexpression of CNP reduced macrophage infiltration in injured arteries, and suppressed the expression of adhesion molecules that were stimulated by the injury *in vitro*. Elevated plasma concentrations of CNP have been observed in patients diagnosed with Crimean-Congo fever (Turkdogan *et al.* 2012) although it is unlikely that this is related to CNP having an active role in this setting — instead CNP may be released from endothelial cells which are damaged by the virus (Ergonul *et al.* 2004).

Suga *et al.* (1993) reported that lipopolysaccharide (LPS), a pyrogenic endotoxin, induces CNP secretion from bovine endothelial cells *in vitro*. Furthermore, Osterbur *et al.* (2013) found that LPS, tumour necrosis factor-α and interleukin-1β stimulated NTproCNP secretion from canine aortic vascular endothelium *in vitro*. Interleukin 1-β, which can act as an endogenous pyrogen in the rat brain (Dascombe *et al.* 1989), was the strongest stimulant of NTproCNP secretion of all the stimulants tested (Osterbur *et al.* 2013). CNP may also be involved in the central control of

thermoregulation, because it was shown that intracerebroventricular administration of CNP acutely elevated colon temperature in rats (Pataki *et al.* 1999).

LPS, when administered to sheep at a dose of 700 ng/kg live weight, reliably produces a fever in our laboratory. It was used here to provide a model inflammatory response to determine the possible role of this stimulus on the concentration of CNP peptides in CSF.

Progesterone

There are several studies linking sex steroids to the regulation of CNP synthesis. Exogenous oestrogen has been shown to increase plasma concentrations of CNP peptides in pre-pubertal ewes (Prickett *et al.* 2008). Testosterone treatment in children with growth hormone deficiency and idiopathic short stature is accompanied by markedly increased plasma concentrations of NTproCNP (Olney *et al.* 2007). However, evidence against a direct effect of testosterone on CNP synthesis was provided in later studies by Prickett *et al.* (2008), where it was shown that exogenous testosterone had no effect on plasma concentrations of CNP peptides in pre-pubertal lambs. In contrast to testosterone and oestrogens, the effects of progesterone on CNP peptides have not been studied. Controlled internal drug release devices (CIDRs) are silicon coated devices containing progesterone which are inserted intra-vaginally to large animals such as sheep and cattle (Wheaton *et al.* 1993). After a period of time whereby the progesterone is slowly released, the CIDRs are removed, allowing oestrus to be synchronised throughout a herd of animals. CIDRs were used here as a convenient progesterone delivery source to investigate its possible role in physiology of the CNP peptides in the CNS.

Morphine

Opioid receptors are widely distributed in the brain and are bound by endogenous ligands including β-endorphin and various forms of encephalin; their actions possibly providing analgesic effects under normal physiological conditions (Wolozin & Pasternak 1981). Morphine is a selective μ opioid receptor agonist which is commonly prescribed to relieve acute and chronic pain (reviewed by Mellon & Bayer 1998) and targets a specific set of brain pathways. Furthermore, Babarczy *et al.* (1995) reported that central administration of CNP prior to a morphine injection depressed the antinociceptive properties of the opiate in mice. The effect of morphine on the affective state, locomotor activity and other behavioural aspects have been well characterised in sheep (Verbeek *et al.* 2012). A dose was selected that has been shown to be safe

but sufficient to induce behavioural changes and affect arousal status, as measured by an increase in locomotor activity and number of vocalisations (Verbeek *et al.* 2012).

L-deprenyl

Espiner *et al.* (2014) showed that CSF concentrations of NTproCNP were reduced in Parkinson's disease patients — compared with CSF from individuals without a neurological disorder — and that the decline seen with disease progression was prevented in patients who received L-deprenyl. L-deprenyl (Selegiline) was developed in the 1960s for possible use as an anti-depressant, but was approved by the FDA (Food and Drug Administration, USA) in 1989 for treatment of Parkinson's disease because it is a potent and selective inhibitor of monoamine oxidase B enzyme (Knoll 1983). Monoamine oxidase B enzyme degrades dopamine, and loss of dopamine (due to neurodegeneration in the substantia nigra) is one of the hallmarks of Parkinson's disease (Grosch *et al.* 2016). Whether changes in dopamine levels would affect CSF concentrations of CNP peptides is unknown, however several studies have reported the ability of CNP to influence dopamine pathways. Thiriet *et al.* (2001) reported that microinjections of CNP into the rat brain prevented cocaine-induced dopamine release and altered transcription factors that coordinated changes in gene expression underlying neuronal plasticity. Jouvert *et al.* (2004) later confirmed the site of action of CNP and showed that these changes were mediated via cGMP-dependent protein kinase I. The most common therapeutic dose that is prescribed for Parkinson's disease patients is 10 mg l-deprenyl, taken daily through the form of two 5 mg tablets. Hypothesising that changes in dopamine levels may alter CSF concentrations of CNP peptides, the effect of a single dose of 10 mg l-deprenyl was investigated here.

Dexamethasone

Several studies have shown that glucocorticoids have the potential to regulate CNP peptide pathways, however whether they act at the level of the CNS is not known. Prickett *et al.* (2009) reported that plasma concentrations of CNP and NTproCNP in growing lambs were lower 24 and 48 h after repeated (125 µg/kg live weight for 2 days) administration of dexamethasone, a synthetic glucocorticoid. In contrast, it has been shown that dexamethasone increases expression of *NPPC* in chondrocytes *in vivo* (Agoston *et al.* 2002). On this background of contradictory results, the present study investigated the effect of a single peripherally-administered bolus of dexamethasone on central concentrations of CNP peptides.

5.1.3 Methods

Sheep were brought indoors one week prior to each trial and fed a maintenance level diet of lucerne chaff and pellets (SealesWinslow performance nutrition, Ashburton, NZ) with water available *ad libitum*. Food and water was withheld for the night prior to the cannulation procedure, whereby individual animals were cannulated at the cervical epidural space for repeated collection of CSF samples as described in 3.3.1. Prior to the cannulation procedure, sheep were given a single s.c. injection of an antibiotic (2.5 mL Depocillin, 300 mg/mL procaine penicillin, MSD Animal Health, Upper Hutt, NZ). CSF sampling commenced 2 days after the cannulation procedure. When catheter patency allowed, different studies were conducted with individual sheep, with a minimum of 2 days between treatments. As the objective of this series of studies was to identify a compound capable of acutely altering CSF concentrations of CNP peptides, multiple samples were collected within several hours of treatment in most cases and concurrent blood samples (for plasma) were collected on the same occasions. CSF and plasma samples were assayed for CNP and NTproCNP as previously described in 3.2.2. Assay detection limits were 0.61 and 5.4 pmol/L, respectively.

Ketamine and diazepam in combination

CSF and plasma samples were collected immediately prior to a single i.v. bolus of a combination of diazepam and ketamine (0.5 mg/kg live weight, Ilium diazepam, Troy Laboratories NZ Pty Ltd, Auckland, NZ and 10 mg/kg live weight, Phoenix Ketamine injection, Phoenix Pharm Distributors Ltd, Auckland, NZ, respectively, n = 4) and at 10, 30 and 120 minutes post injection.

Diazepam

CSF and plasma samples were collected immediately prior to a single i.v. bolus of diazepam (1.0 mg/kg live weight, Ilium diazepam, Troy Laboratories NZ Pty Ltd, Auckland, NZ, n = 4) and at 10, 30 and 120 minutes post injection.

Ketamine

CSF and plasma samples were collected immediately prior to a single i.v. bolus of ketamine (20 mg/kg live weight, Phoenix Ketamine injection, Phoenix Pharm Distributors Ltd, Auckland, NZ, n = 4) and at 10, 30 and 120 minutes post injection.

Isoflurane

CSF and plasma samples were collected immediately before anaesthesia was induced with isoflurane by mask (in oxygen, 5 % v/v) and maintained with an endotracheal tube at 2-3 % for 30

minutes (Isoflurane, registered to Piramal Healthcare Limited, India. Marketed and distributed by: Merial New Zealand Ltd, Manukau City, NZ, n = 4). CSF and plasma samples were collected 10, 30 and 120 minutes after induction of anaesthesia.

Lipopolysaccharide

An estimate of core temperature was obtained by using a digital rectal thermometer (Geratherm Medical AG, Geschwenda, Germany) prior to and after a single i.v. bolus of LPS (Sigma-Aldrich, St Louis, Missiouri, USA, Lot 30 K4114, Serotype 055:B5, 700 ng/kg, n = 4) dissolved in 0.9 % w/v saline solution at a rate of 20 µg/mL. Rectal temperature was regularly recorded and CSF and plasma samples were collected immediately prior to treatment and at various time points after as core temperature increased between 150 and 210 minutes post injection.

Progesterone

CSF and plasma samples were collected immediately prior to the intra-vaginal insertion of a progesterone CIDR (Eazi-breed CIDR, Interag, Hamilton, 0.3 g progesterone/insert. Distributed by Pfizer, Auckland, n = 4), which was administered using a plastic applicator and removed after 3 h. Samples were collected at 3 and 6 h after CIDR insertion.

Morphine

CSF and plasma samples were collected immediately prior to the i.v. administration of a single bolus of morphine (0.84 mg/kg live weight, morphine hydrochloride BP oral solution 2 mg/mL, Pharmacia Cooporation, New Jersey, USA, n = 4) and at 40 minutes and 2, 3, and 24 h after treatment.

L-deprenyl

CSF and plasma samples were collected immediately prior to a single i.v. bolus of l-deprenyl (10 mg/sheep, Selegiline hydrochloride, Sigma-Aldrich, St Louis, Missouri, USA, dissolved in 0.9 % w/v saline solution at a rate of 2 mg/mL, n = 3) and at 30, 100 and 150 minutes after treatment. In a subsequent trial, the same protocol was followed with a different group of sheep (n = 3).

Dexamethasone

CSF and plasma samples were collected immediately prior to a single i.m. bolus of dexamethasone (Dexa 0.2, PhoenixPharm Distributors Ltd, Auckland, NZ, 0.25 mg/kg live weight), and 24 h following treatment (n = 4). In a subsequent trial, the same protocol was followed

except the route of administration was i.v. and samples were collected at 2, 4, 8, 24 and 48 h post treatment (n = 3).

5.1.4 Results

Most samples were collected as intended, however occasionally a CSF sample could not be obtained due to a loss of patency of the catheter. Mean catheter patency was 6 days. In cases of premature failure of a cannula, a replacement animal was introduced to the trial.

CSF concentrations of NTproCNP were elevated 24 h after dexamethasone administration in all 4 sheep (Figure 5.9), and CSF concentrations of both peptides were elevated in 1 of 3 sheep administered with l-deprenyl (Figure 5.8). Therefore, the effect of the respective compounds on CSF concentrations of CNP peptides was investigated further with 3 different sheep. This 'follow–up' study revealed a marked increase in CSF concentrations of NTproCNP 8 h after dexamethasone in all 3 sheep. CSF concentrations of CNP were elevated 8 h after dexamethasone in 2 sheep. In plasma, CNP and NTproCNP concentration was markedly elevated 2, 4 and 8 h after dexamethasone, but had returned to baseline concentrations 24 h post treatment. In contrast, NTproCNP concentrations in CSF remained elevated in all 3 sheep (Figure 5.11).

Whereas a marked increase in CNP peptide concentration was observed after dexamethasone, there was no consistent change in CNP or NTproCNP concentration in CSF or plasma after l-deprenyl (Figure 5.10). There were no consistent changes in CSF concentrations of CNP and/or NTproCNP at any time point following adminstration of ketamine, diazepam, diazepam and ketamine in combination, isoflurane, LPS, progesterone and morphine (Figure 5.1 to Figure 5.7). Behavioural changes were observed in all sheep that received anaesthetics; and there was a noticable reduction in locomotor activity after 10 minutes, i.e. all sheep were lying down, following administration with diazepam. Most sheep were standing again 30 minutes after injection and activity levels were close to normal 2 h post treatment. After ketamine, several behaviours were displayed that were indicative of its dissociative effect, including an increase in vocalisations, increase in eye and head movement shown 10 and 30 minutes post injection, with most sheep returning to their normal (calmer) state by 2 h. Following diazepam and ketamine (in combination), sheep were anaethetised within several minutes and remained in this state for 20-30 minutes. Similarly, isoflurane induced then maintained anaesthesia for 30 minutes.

Behavioural changes were observed for the first 30 minutes after morphine; three sheep began grinding their teeth whilst maintaining a fixed gaze within minutes of treatment, and the fourth

sheep showed an increase in alertness and interest in its surroundings. A mild elevation of temperature was induced by LPS. Mean rectal temperature was 39.1°C prior to LPS administration and 39.3 and 39.7 °C at 2.5 h and 3 h 20 minutes later, respectively.

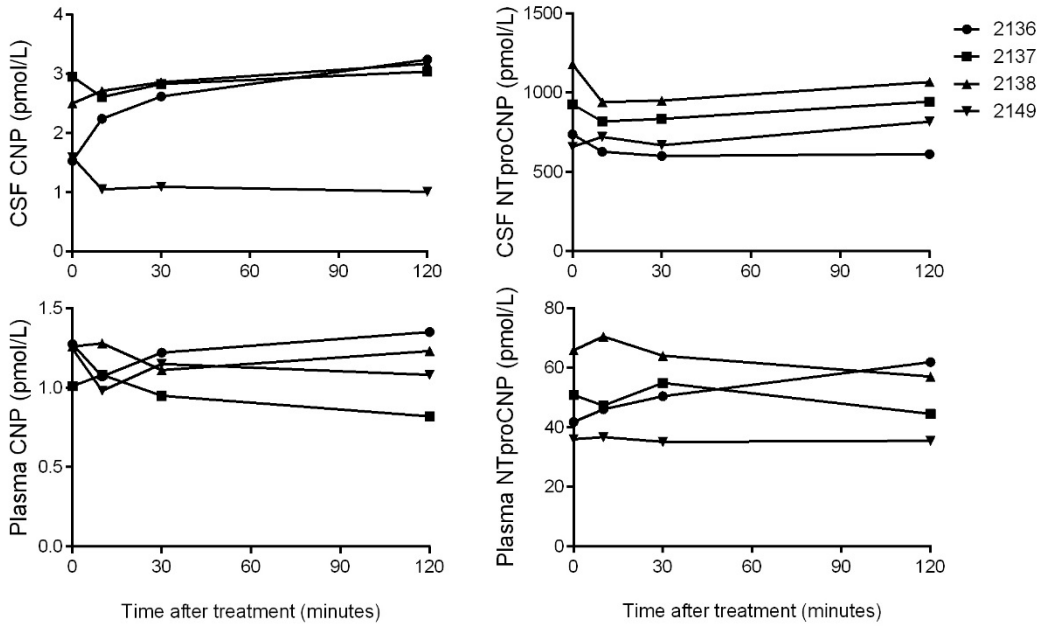

Figure 5.1 Effect of diazepam.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following administration with a single bolus of diazepam (1.0 mg/kg live weight, i.v.).

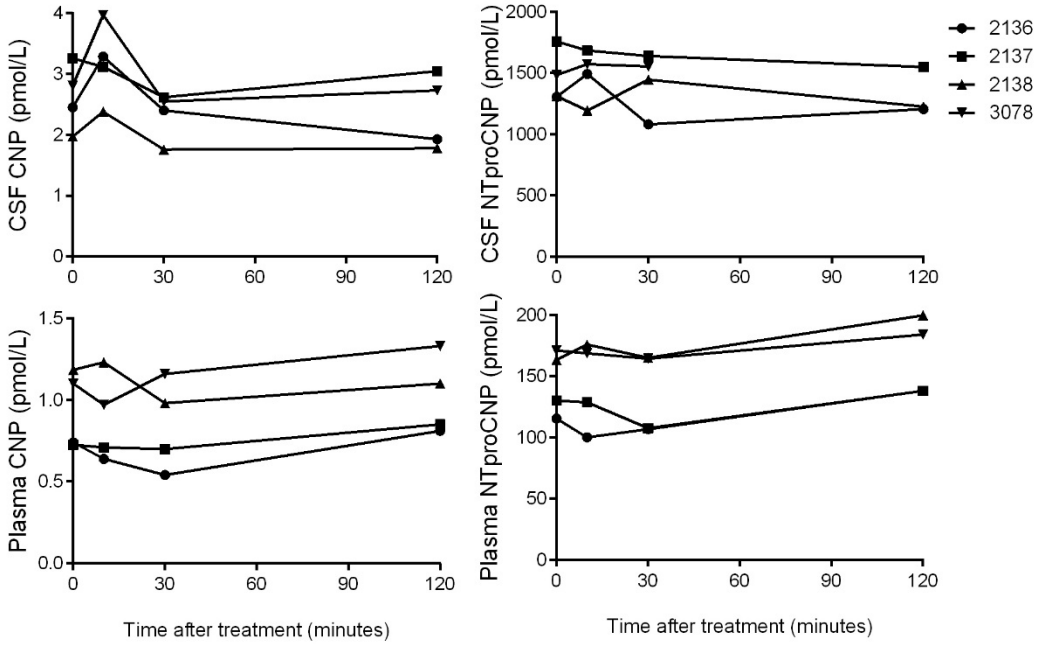

Figure 5.2 Effect of ketamine.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following administration with a single bolus of ketamine (20 mg/kg live weight, i.v.).

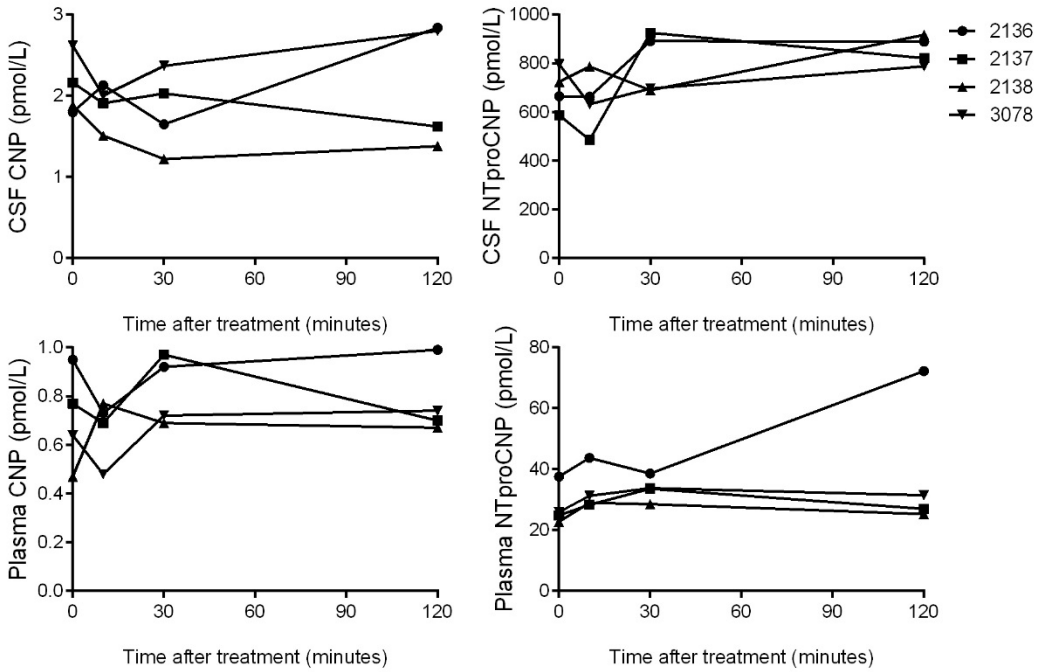

Figure 5.3 Effect of diazepam and ketamine in combination.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following administration with a single bolus of diazepam and ketamine in combination (0.5 and 10 mg/kg live weight, respectively, i.v.).

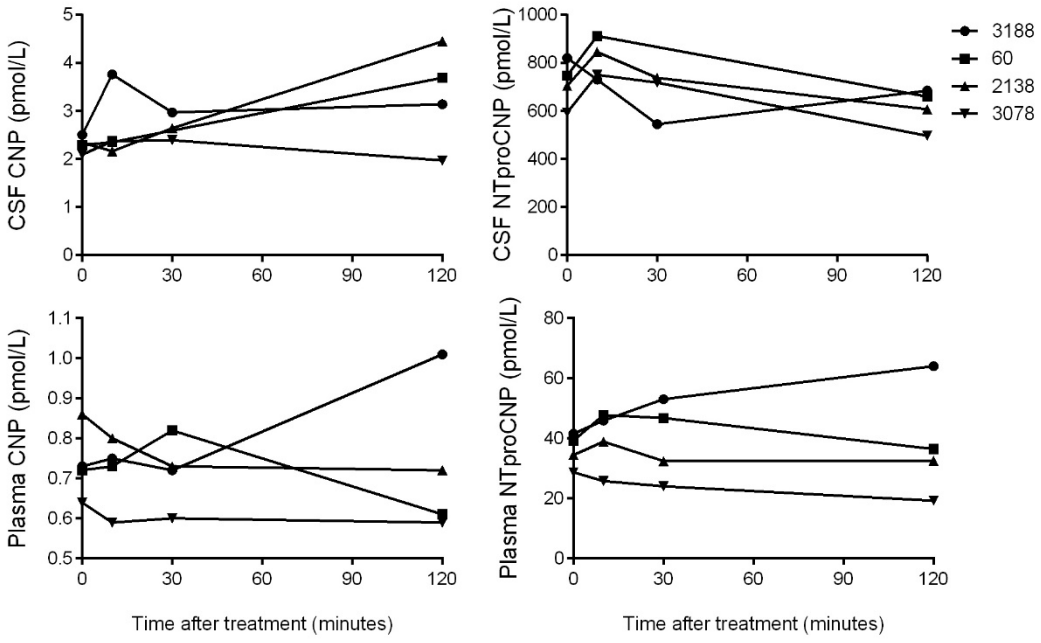

Figure 5.4 Effect of isoflurane.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) anaethetised with isoflurane (induced at 5 % and held at 2-3 % for 30 minutes).

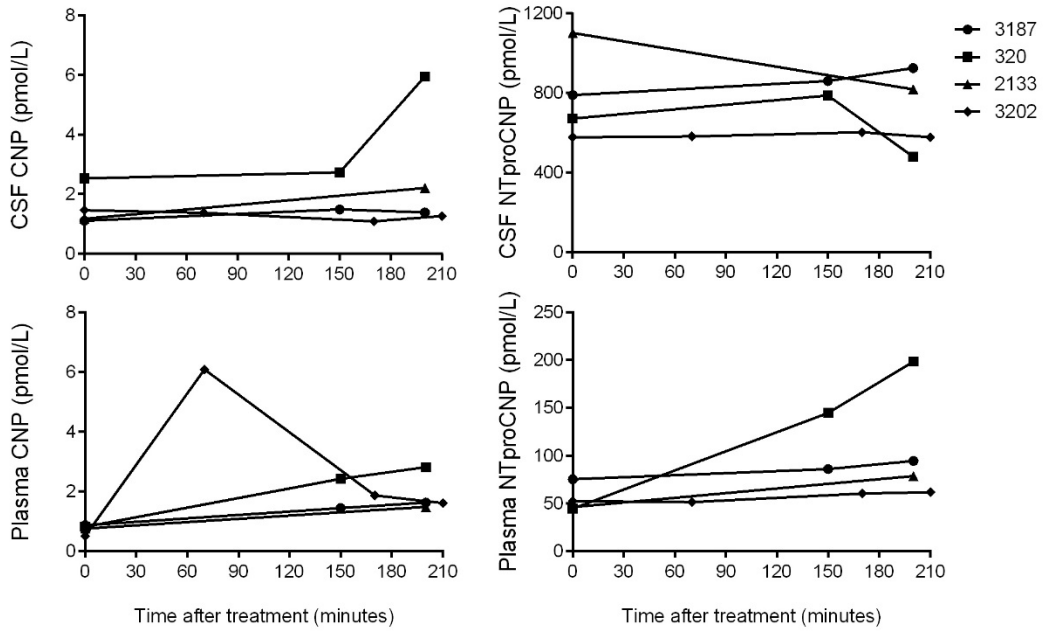

Figure 5.5 Effect of LPS.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following administration of a single bolus of LPS (0.7 µg/kg live weight, i.v.).

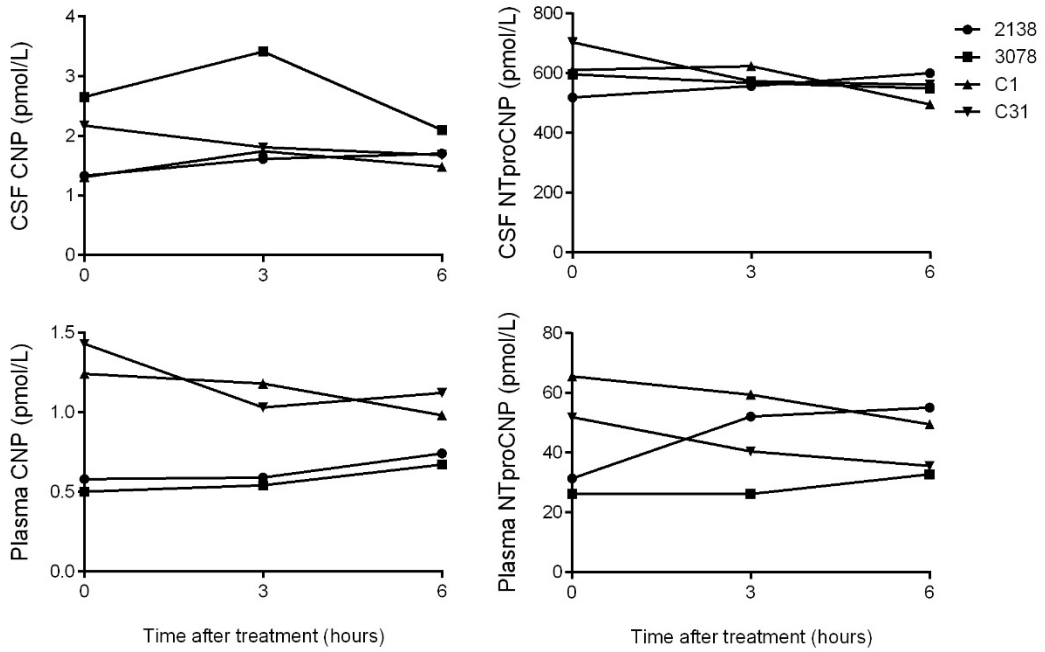

Figure 5.6 Effect of progesterone.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following exposure to progesterone for 3 h (intra-vaginal progesterone CIDR inserted at 0 h and removed after 3 h).

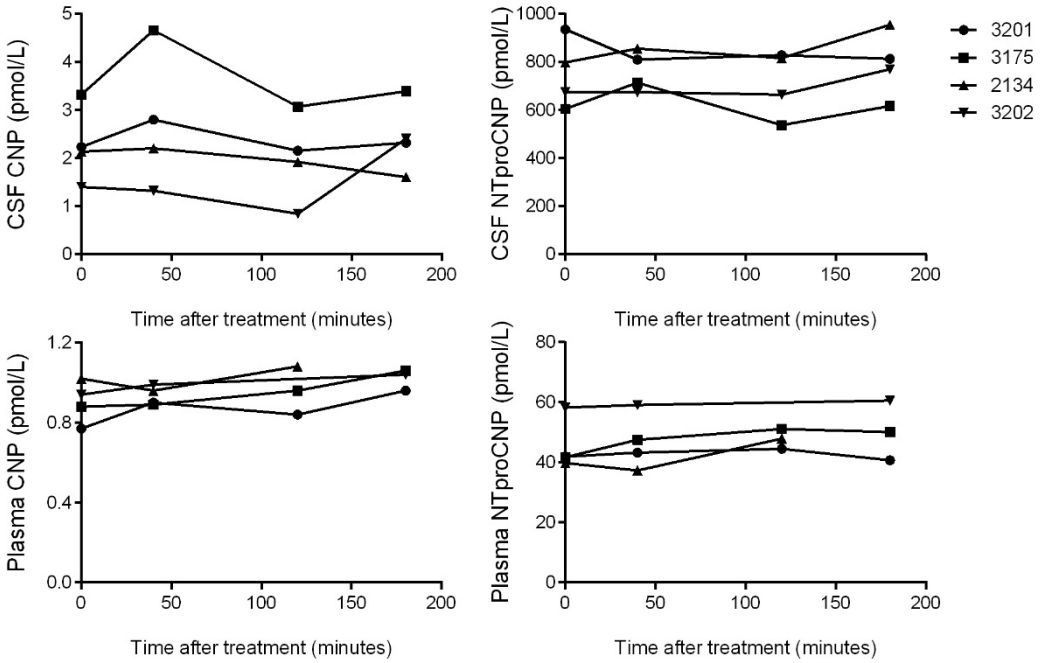

Figure 5.7 Effect of morphine.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following administration of a single bolus of morphine (0.84 mg/kg live weight)

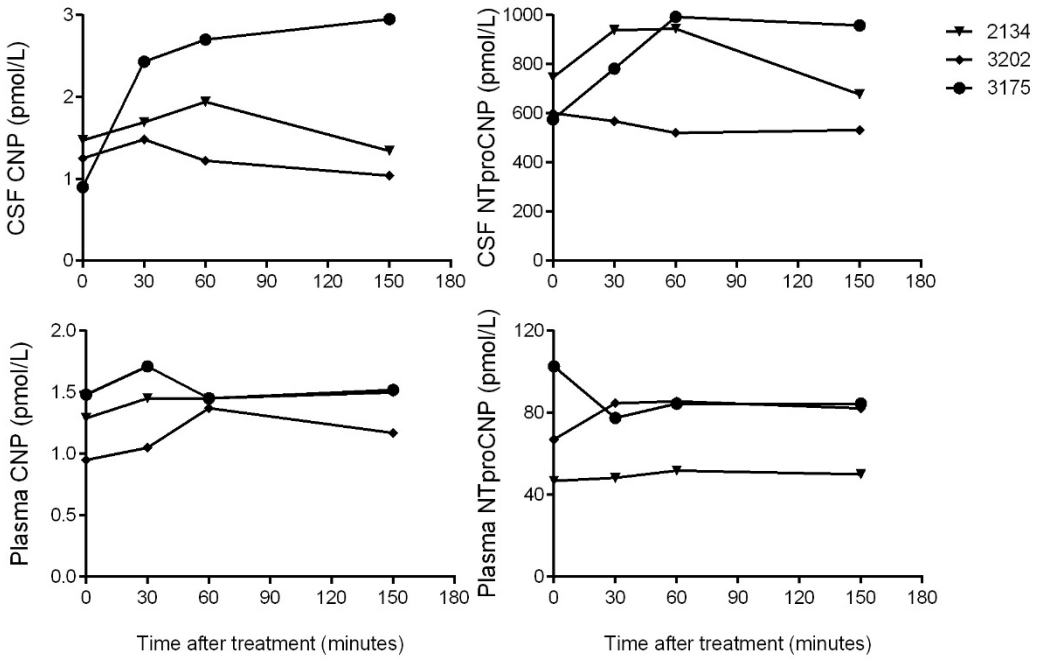

Figure 5.8 Effect of l-deprenyl.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following administration of a single bolus of l-deprenyl (10 mg/sheep, i.v.).

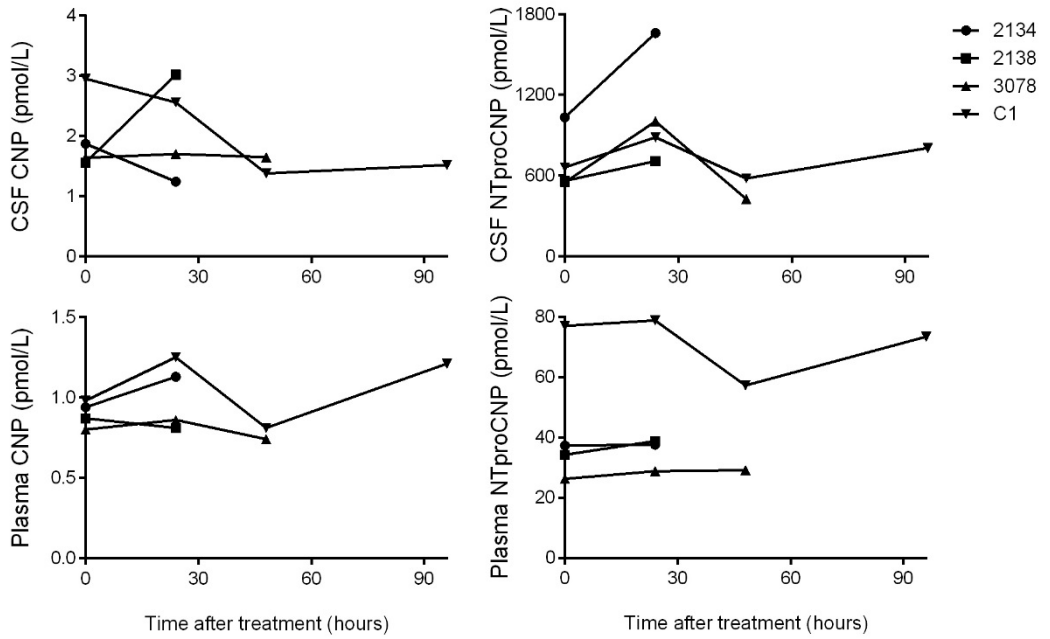

Figure 5.9 Effect of dexamethasone.

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 4) following administration of a single bolus of dexamethasone (0.25 mg/kg, i.m.).

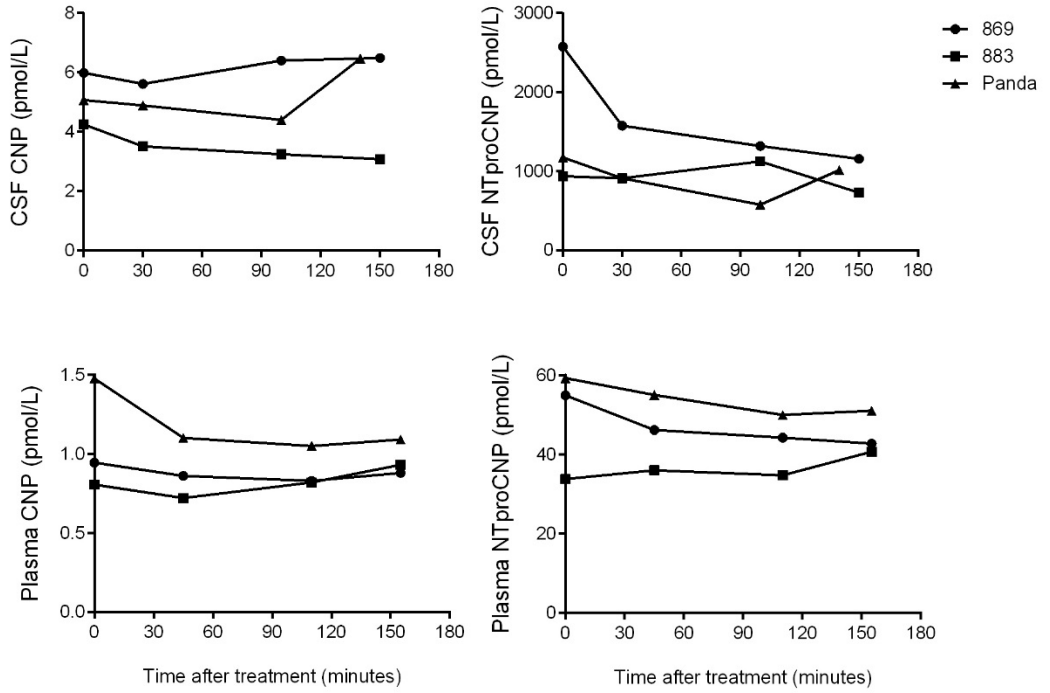

Figure 5.10 Effect of l-deprenyl (follow-up study).

CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 3) following a single bolus of l-deprenyl (10 mg/sheep, i.v.).

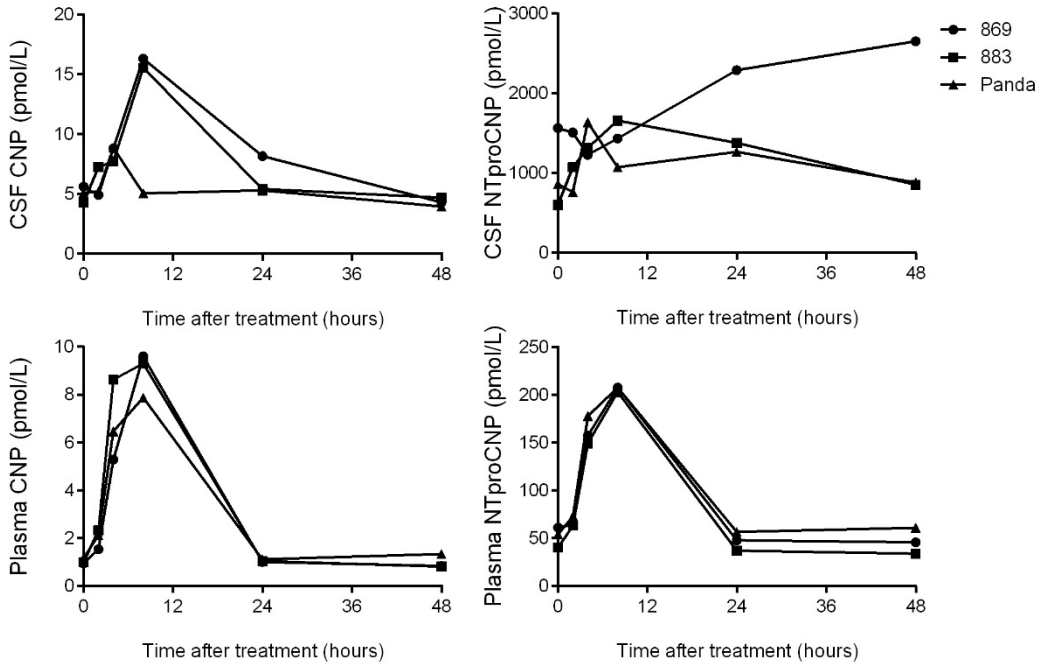

Figure 5.11 Effect of dexamethasone (follow-up study).
CNP (left) and NTproCNP (right) concentration in CSF (top) and plasma (bottom) of individual sheep (n = 3) following a single bolus of dexamethasone (0.25 mg/kg live weight, i.v.).

5.1.5 Discussion

This series of pilot studies confirms previous observations showing that CSF concentrations of the CNP peptides exceed those in plasma and are remarkably stable within individuals over a time period of several hours (Wilson *et al.* 2015). These results suggest that levels of CNP peptides in CSF remain unchanged even during significant alterations in brain activity that occur with anaesthesia. This finding, along with the fact that the structure of CNP is highly conserved across species (2.1.2), and that CNP concentrations are consistently higher in CSF (relative to circulating levels), supports the idea that the latter is necessary for some basic aspect of homeostasis in the brain that may be common to all mammalian species. Furthermore, it can be speculated that the integration of neuronal activity of cortical regions is not essential for the maintenance of the higher concentrations of CNP in CSF — as a reduction of the ability of the brain to integrate information from different cortical regions (via thalamic relay) is the common denominator of most anaethetics — despite their varying modes of action (Alkire *et al.* 2008).

The finding that high CNP levels in the CSF are maintained despite the depression of higher brain neuronal activity is supportive of existing literature which proposes CNP as an important signal in

the CNS and, suggests that CNP signalling may be necessary even during the unconscious state (reviewed by Prado *et al.* 2010). The finding that CSF concentrations of CNP peptides remain unchanged following administration of diazepam and ketamine is important for studies where CSF samples are collected from sedated or anaesthetised animals (Chapter 4 and Chapter 6), because it rules out anaesthesia as a potential confounding factor when determining concentrations of CNP and NTproCNP.

These studies achieved the objective of identifying a stimulus capable of acutely changing CSF concentrations of CNP peptides and identified dexamethasone as the reliable candidate for this task. Although plasma levels of CNP and NTproCNP were also increased, the elevation of NTproCNP concentration 24 h after dexamethasone — but not in plasma — suggests that the peptides in the two fluids are regulated independently and diminishes the possibility of a 'spillover effect' of peptides passing from the peripheral circulation to the CSF. Although this possibility deserves further consideration, data from a recent study in humans also indicate that CNP peptides in CSF and plasma are independently regulated (Schouten *et al.* 2011).

Although the objective was to screen candidates for their ability to provide an acute stimulus or to suppress levels of CNP peptides in the CNS (that could be used in future studies), controls, such as saline-treated animals, were not included in these studies. This means that some of the compounds used here can not be fully ruled out as potential modifiers of CNP levels in the CNS. Also, it was not always possible to ascertain whether an effective dose had been used in the pilot study. For instance, the elevation of rectal temperature following administration of LPS was minor (mean maximum of 39.7 °C) compared with expected values above 40 °C (Breen and Barrell, 2002). Likewise, other compounds, e.g. l-deprenyl, progesterone, etc. may have been effective at higher doses than were used here. In simplistic terms, these pilot studies were of the nature of a 'fishing expedition' and were conducted in this way using 'best guess' dosages to, rather hopefully, identify potential candidates for further study whilst minimising the effort and animal usage that would have been required for an exhaustive study in each case. Finding dexamethasone to be a suitable candidate, following this minimalistic approach, can be considered as serendipitous. Not only has it proven to be a reliable stimulator of CNP peptide levels in CSF, but it also points to involvement of an endogenous corticosteroid pathway in the regulatory physiology of the CNP peptides. Importantly, for the subsequent studies in this thesis, the pilot studies have achieved their objective in revealing a suitable candidate stimulus for

raising CNP peptide levels in the CSF and for alteration of gene expression in pituitary and central nervous tissues.

5.2 Investigation of the effect of seasonal changes in appetite and food intake on CNP peptide concentrations in red deer (*Cervus elaphus*) stag plasma and CSF

5.2.1 Introduction

Several studies suggest that CNP is involved in the regulation of energy balance and appetite. Following observations that *NPPC* and *NPRB* — the genes encoding the precursor and receptor of CNP, respectively — are detected throughout nuclei of the hypothalamus which are strongly linked with appetite regulation (Langub *et al.* 1995a, Langub *et al.* 1995b, Herman *et al.* 1996b), it was speculated that CNP might be involved in the regulation of energy balance and appetite.

The use of knockout mice in studies of CNP are complicated by the skeletal deformities that occur when the *NPPC* or *NPRB* gene is disrupted (Tamura *et al.* 2004), which compromises the animal's ability to eat. Consequently, Inuzuka *et al.* (2010) generated CNP-knockout mice with targeted chondrocyte-specific *NPPC* expression (CNP-Tg/*NPPC*$^{-/-}$), so that changes related to the absence of CNP could be observed, while minimising skeletal problems. These mice — with a rescued body length and lack of *NPPC* mRNA in the hypothalamus — were leaner and had a significantly reduced body weight at 20 weeks of age, when compared with CNP-Tg/*NPPC*$^{-/-}$ mice. Furthermore, they lost more body weight during 48 h of starvation, ate less after a refeed, had improved insulin sensitivity, and had significantly higher uncoupling protein-1 mRNA levels in brown adipose tissue, which is activated during thermogenesis (reviewed by Ricquier & Bouillaud 2000). Altogether, data from Inuzuka *et al.* (2010) support the hypothesis that CNP might increase body fat accumulation and reduce energy expenditure via a reduction in thermogenesis.

In contrast, Yamada-Goto *et al.* (2013) demonstrated that CNP suppresses food intake when administered centrally to mice prior to refeeding after a 48-h fasting period. This effect was blocked by an antagonist for melanocortin-3 and melanocortin-4 receptors, suggesting that CNP has a central anorexigenic action which acts partly through activation of the melanocortin system. This anorexigenic effect was observed only when CNP was administered centrally — and not peripherally. In the same study, CNP significantly suppressed neuropeptide Y- and ghrelin-induced food intake when CNP (both CNP-22 and CNP-53) was centrally co-administered with neuropeptide Y. CNP may exert its actions via direct or indirect stimulation of hypothalamic neurons, as indicated by the increase in the number of c-Fos-positive cells in multiple hypothalamic nuclei following central administration of CNP (Yamada-Goto *et al.* 2013).

Although there is evidence to suggest that CNP is implicated in the regulation of appetite, the nature of its involvement is unclear. As part of the same objective described in 5.1, studies were undertaken in a second animal model, the red deer stag. The appetite and live weight of red deer (*Cervus elaphus*), especially stags, vary markedly over different seasons of the year in response to changes in photoperiod. After summer, when the daily photoperiod begins to decrease, dramatic physiological changes occur in the stag in preparation for an active reproductive season (the 'rut') including marked testes growth, increase in circulating levels of testosterone and increased sperm production. During the rut (which occurs from late-March to late-April in NZ) stags become hyper-active and invest a large amount of energy into competition for access to hinds for mating and their appetite is greatly reduced — and consequently up to 30 % of their body weight is lost (Stevenson *et al.* 1992). When photoperiod begins to increase in late-winter/early-spring, the appetite and food intake of stags is greatly increased, and their body weight recovers until the beginning of the next rut season. In this study, it is hypothesised that red deer stags may have seasonal changes in CNP levels in their CSF that could be associated with their very pronounced appetite cycle. Using this physiologically unique setting, changes in central levels of CNP peptides were assessed *in vivo*, in relation to the dramatic changes in appetite and body weight.

5.2.2 Methods

Animal procedures

Six adult red deer stags were grazed on ryegrass/white clover pasture at the Lincoln University Research Farm for the duration of the trial, and were brought into yards on sampling days which occurred every 2 months from September 2013 to September 2014. Stags were transferred individually from a yard and secured in a standing position using a padded side loading mechanical deer crush (Keane Deer Yards, Rangiora, NZ) which was positioned over weighing scales during the sampling procedures. Once secured, live weight was recorded, and a 10 cm by 3 cm patch of hair was clipped from the neck. Body condition score was recorded at all sampling occasions except in November, and was assessed by the same person at all times. Two stags departed from the trial prior to the last 2 sampling occasions (one stag died of causes unrelated to the trial and one died unexpectedly following administration of the anaesthetic) and were replaced by 2 stags of similar live weight and age which were recruited into the group to maintain the sample size of 6.

Blood was collected via jugular venepuncture into 2 evacuated tubes containing 18 mg of potassium ethylenediamine tetra acetate (BD Vacutainer®, Becton Dickinson, Franklin Lakes, New Jersey, USA). Blood in EDTA tubes was placed on ice then centrifuged at 2500 rpm for 10 min at

4°C. The time from blood collection until centrifugation did not exceed 2 h. Plasma was stored in polycarbonate tubes at −20°C until assayed. Stags were individually anaesthetised with a single xylazine/ketamine combination bolus (Phoenix Xylaket 15/5, 150 mg/ml xylazine hydrochloride and 50 mg/ml ketamine hydrochloride, Phoenix Pharm Distributors Ltd, NZ) using 0.05 mL/kg live weight administered via the jugular vein. Hair was clipped from around the CSF sampling site (Plate 5.1) and the site was cleaned with povidone-iodine solution (Vetadine - Iodine Animal Wash, 1.6 % w/v available iodine, 19.7 mg/mL crude iodine, Bomac Laboratories Ltd, Auckland, NZ) and disinfected with 70 % isopropanol. Once the animal was anaesthetised, an assistant flexed the head fully downwards and a sterile hypodermic needle (22 G x 1½" (0.71 x 38 mm)) was inserted into the atlanto-occipital space (Plate 5.1) and 2 mL CSF was withdrawn. CSF samples were stored in polythene Eppendorf containers and placed directly on ice, prior to storage at -20 °C. Anaesthetic reversal agent was administered (2 mL yohimbine hydrochloride 10 mg/mL, Phoenix Pharm Distributors Ltd, NZ) by i.v. delivery whereupon the stag was released from the crush and monitored until fully conscious.

Plate 5.1 Collection of CSF from the atlanto-occipital space of a red deer stag.
Collection of CSF from the atlanto-occipital space of a red deer stag. The tissues penetrated, in order, are: the skin and subcutaneous tissue, nuchal ligament, dura mater and the arachnoid mater to reach the subarachnoid space.

Hormone assays

CSF and plasma samples were processed as described in 3.2 for measurement of CNP and NTproCNP concentration. Plasma testosterone concentration was measured using an in-house enzyme-linked immunosorbent assay (ELISA) at Canterbury Health Laboratories (Christchurch, NZ) as previously described (Elder & Lewis 1985). Briefly, 50 µL of each sample was manually

dispensed (in duplicate or quadruplet) into wells of a 96-well microtitre plate blocked with a conjugate to reduce non-specific antibody binding using an automated ELISA analyser (Behring ELISA Processor M, CSL Behring, King of Prussia, USA) and incubated overnight at 4°C with antiserum to testosterone (raised in rabbits). Plates were washed with phosphate-buffered saline solution containing a detergent and aspirated prior to the addition of a peroxidase-labelled goat-antirabbit IgG and incubated for a further 2 h. Plates were washed again and 100 µL of enzyme substrate was added. Colour development was allowed to occur in the dark for 15 minutes and the enzymatic reaction was terminated using 100 µL of 1.25 M H_2SO_4. The absorbance was read at 492 nm on the automatic analyser and results were interpolated from a standard curve. Assay precision was determined by analysing 6 sets of duplicates from 3 pools of plasma; intra-assay variation was 7.6, 9.8 and 12 % for samples with mean concentrations of 4.3, 13.8 and 22.7 nmol/L, respectively. Inter-assay variation did not exceed 12.5 % for the 3 pools (Elder & Lewis 1985).

Statistical analysis

Concentrations of CNP and NTproCNP in CSF and plasma, as well as live weight and plasma testosterone concentration were compared across time points using logged data (log_{10}) and a repeated-measures ANOVA in Genstat Version 16 (VSN International Ltd., Hemel Hempstead, UK) and least significant differences were used to determine differences between specific time points. The relationship between peptide levels in plasma, plasma testosterone concentration, live weight and body condition score was determined using GraphPad Prism version 6.01 for Windows (GraphPad Software Inc, La Jolla, California, USA, www.graphpad.com). Two ANOVA analyses were run using both possible data sets that resulted according to how the newly recruited stag data were arranged, and results did not differ between data sets.

5.2.3 Results

All data are presented in Figure 5.12 as mean ± s.e. As shown in Figure 5.12 A, mean live weight changed significantly through the year ($p < 0.001$) and increased from the first sampling occasion in December (beginning of summer) to January ($p < 0.05$) by approximately 50 kg. Mean live weight was significantly higher in January and March (192 ± 7 and 188 ± 6 kg, respectively) than during the following months, i.e. in May, July, and September ($p < 0.05$) when mean live weight decreased by approximately 30 kg (158 ± 3 kg in September). Body condition score followed the same pattern of change as live weight (Figure 5.12 A). Plasma testosterone concentration (Figure 5.12 B, closed circles) peaked in March (the beginning of autumn and the mating season),

whereby concentrations exceeded those in samples from all other time points by 5- to 30-fold ($p < 0.05$).

CSF concentrations of both peptides markedly exceeded those in the plasma (3- to 4- fold for CNP, 11- to 17-fold for NTproCNP) at all time points, except in March when the difference was smaller (2- and 7-fold for both peptides, respectively). Following a similar pattern to testosterone, plasma concentrations of CNP and NTproCNP varied throughout the year ($p < 0.001$), with a peak concentration occurring in March. This increase in concentration was more progressive ($p < 0.05$), i.e. appeared to commence earlier, than the rise in plasma testosterone concentration. In March, plasma CNP and NTproCNP concentration was approximately 2-fold higher than at other time points (Figure 5.12 D). Both CNP and NTproCNP concentrations were lower in plasma samples collected in May and July, than in samples from the previous sampling time points (November, January and March, $p < 0.05$).

Whereas plasma concentrations of CNP and NTproCNP changed significantly throughout the year, very little variation occurred in CSF (Figure 5.12 C). Mean concentrations of CNP in CSF ranged between 2.1 ± 0.4 and 3.7 ± 0.6 pmol/L and means were not significantly different between any time points considered here. Mean concentrations of NTproCNP in CSF were similar across different months in most cases — except there was a slight rise ($p < 0.05$) between September (437 ± 40 pmol/L) and the following March (557 ± 32 pmol/L).

Concentrations of CNP and NTproCNP were positively correlated in plasma ($r = 0.88$, $p < 0.0001$) but not in CSF (Figure 5.13). Plasma testosterone concentration was positively correlated with both plasma CNP ($r = 0.58$, $p < 0.001$) and plasma NTproCNP concentration ($r = 0.61$, $p < 0.0001$, Figure 5.14). Live weight was positively correlated with both CNP and NTproCNP concentration in plasma ($r = 0.70$, $p < 0.001$ and $r = 0.54$, $p < 0.05$, respectively, Figure 5.15) and with plasma testosterone concentration ($r = 0.4518$, $p < 0.05$, Figure 5.15). The lack of major change in CSF concentrations of CNP and NTproCNP throughout the year precluded any attempt to correlate these peptide levels with the other measurements.

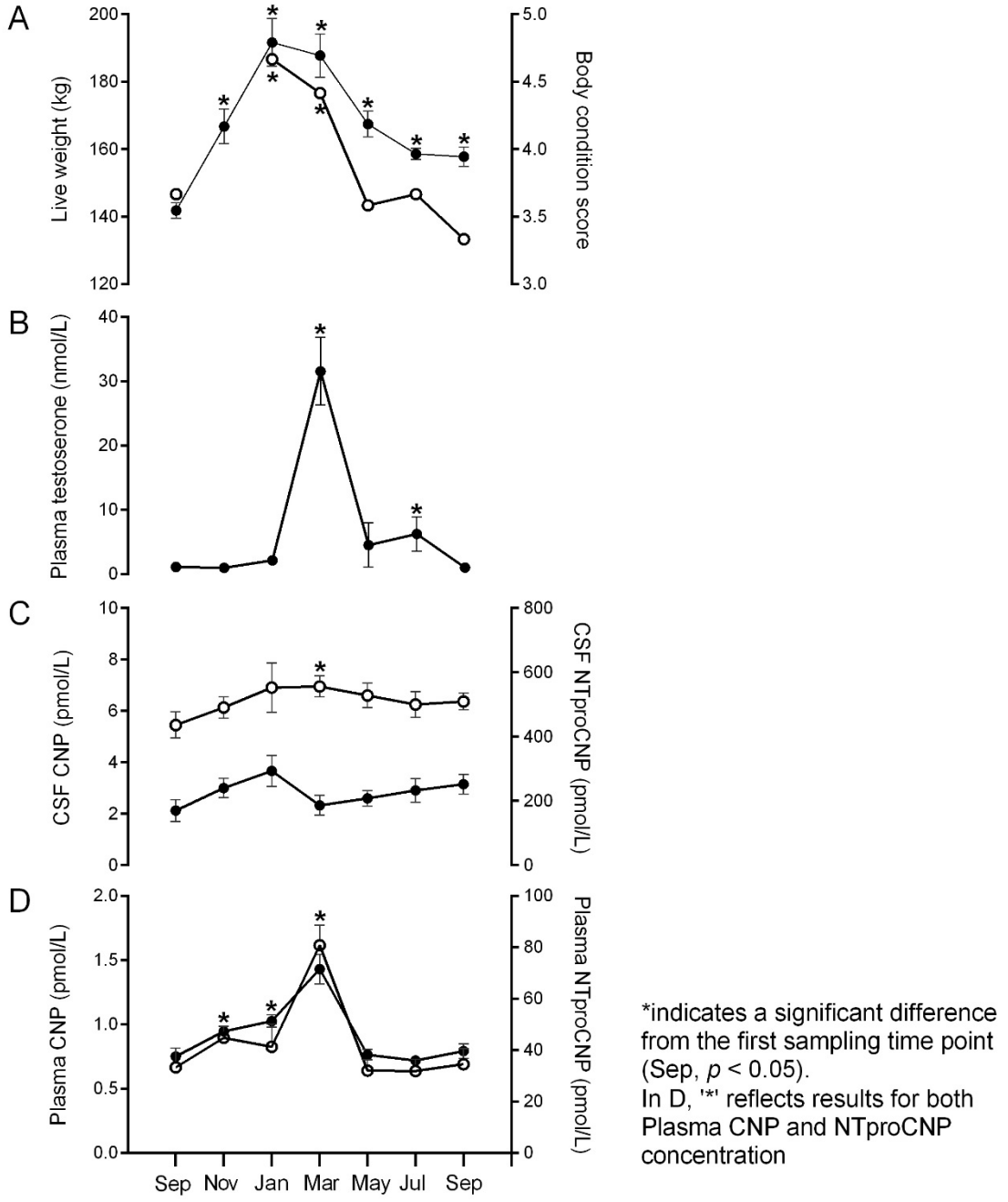

Figure 5.12. CNP and NTproCNP concentration in CSF and plasma of red deer stags, in relation to annual changes in live weight and plasma testosterone concentration.

Time trend of CNP (left axis) and NTproCNP (right axis) concentrations in CSF and plasma over a one year period in six red deer stags (pmol/L), in relation to seasonal indicators (testosterone level and live weight). All data is presented as mean ± s.e. A) Live weight (kg, closed circles) and body condition score (open circles). B) Plasma testosterone concentration (nmol/L). C) CSF concentrations of CNP (closed circles) and NTproCNP (open circles). D) Plasma concentrations of CNP (closed circles) and NTproCNP (open circles).

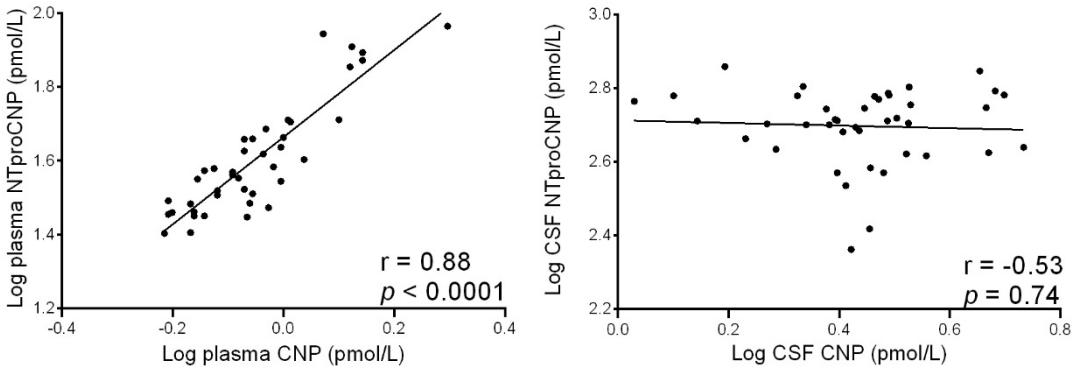

Figure 5.13 Relationship between CNP and NTproCNP in CSF and plasma in stags.

Relationship between CNP (log_{10}) and NTproCNP (log_{10}) concentration in plasma (left) and CSF (right) in samples collected from red deer stags (n = 6) every two months for a one year period.

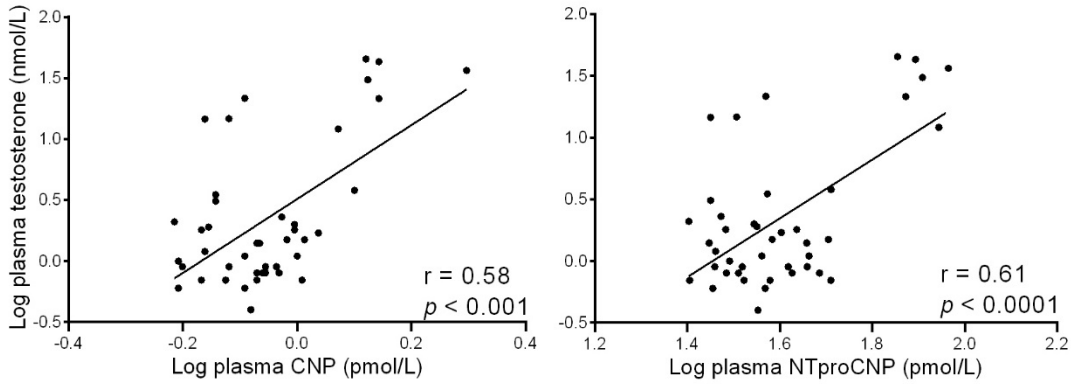

Figure 5.14. Relationship between testosterone and CNP peptides in stag plasma.

Relationship between testosterone (log_{10}) and CNP (log_{10}) concentration (left) and NTproCNP (log_{10}) concentration (right) in plasma of red deer stags (n = 6) collected every two months over a one year period.

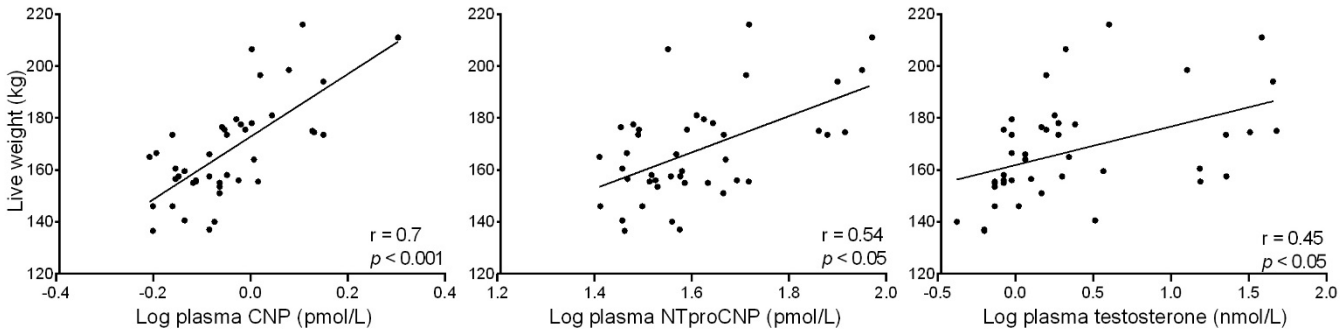

Figure 5.15. Relationship between live weight and (log_{10}) plasma concentrations of CNP, NTproCNP and plasma in red deer stags.

Relationship between live weight (kg) and plasma concentrations of A) CNP, B) NTproCNP (both pmol/L) and C) testosterone (nmol/L) in red deer stags over a one year period (n = 6).

5.2.4 Discussion

Despite the marked seasonal pattern of change in live weight that occurs in relation to the breeding season in red deer stags there were no related temporal changes in CSF concentrations of the CNP peptides. However, there was a marked increase in plasma concentrations of CNP and NTproCNP coinciding with the beginning of the rut which had a similar temporal pattern of change to that of the circulating levels of testosterone. To date, this is the second report of a relationship between the plasma concentrations of testosterone and the CNP peptides. Olney *et al.* (2007) reported an increase in plasma concentrations of NTproCNP that occurred in children receiving testosterone treatment for growth deficiency. As previously mentioned (5.1.2), a direct effect of testosterone on peripheral levels of CNP peptides was ruled out in another study in pre-pubertal lambs (Prickett *et al.* 2008).

Whether the temporal pattern in circulating concentrations of CNP peptides in stags (with peak concentrations occurring in autumn) reported here differs from those in red deer hinds is difficult to ascertain. A previous report of CNP peptides in non pregnant red deer hinds indicated a different temporal pattern, whereby highest CNP and NTproCNP values occurred in spring (McNeill *et al.* 2010). However, the variation reported by (McNeill *et al.* 2010) was not significant, and data from January to March were not available for these red deer hinds. Whereas there was a significant relationship between live weight and circulating concentrations of CNP peptides in red deer stags, McNeill *et al.* (2010) found no association between plasma CNP forms and live weight in red deer hinds or ewes.

The physiological significance (if any) of the coinciding peak concentrations of CNP, NTproCNP and testosterone in plasma at the start of the breeding season (autumn) reported here is unknown. However, there is a general trend for CNP peptide levels in plasma and CNP concentration in CSF to increase from winter to summer. This spring-related rise coincides with the increase in live weight and body condition score (recorded in the current study and the report by Gaspar-López *et al.* 2009), and increase in plasma leptin concentration (Gaspar-López *et al.* 2009). Also, S Voice (unpublished adult stag data from our laboratory, n = 8) showed a marked increase in plasma concentrations of CNP (0.55 to 0.9 pmol/L) and NTproCNP (12 to 22 pmol/L) for the same time period. Together, this fits a picture of a possible regulatory relationship with appetite — deserving of further study, but not substantiated by results from the current study.

CSF concentrations remained unchanged despite the increased levels in plasma, which is consistent with human studies reporting independent regulation between fluids (Schouten *et al.* 2011). As the stags were not cannulated for CSF collection, it was not possible to examine the effect of xylazine and ketamine on central concentrations of CNP and NTproCNP, however findings from sheep indicate that the effect of anaesthetics is not a confounding factor in these measurements (Chapter 4, and Figure 5.1 to Figure 5.4). Although CNP signalling in the brain may be altered during the rut, these findings indicate that appetite and live weight changes are not associated with changes in central concentrations of these peptides — and therefore for the purpose of these studies, were not considered to be worth pursuing as influencers of CNP secretion in central tissues.

Chapter 6. Dexamethasone increases production of C-type natriuretic peptide in the sheep brain

Statement

I was involved in all aspects of this chapter including study design, animal procedures, statistical analysis and writing of the manuscript, and all natriuretic peptide assays were carried out by me. Measurement of gene expression was carried out by me for 3 of the 7 tissue regions examined, and the rest by Bryony McNeill (Deakin University, Geelong, Australia). The writing of the manuscript which forms this thesis benefitted from contributions from all authors. This manuscript has been formatted for submission to the *Journal of Endocrinology* (currently under review), therefore the nomenclature has been selected accordingly and differs from other chapters. Here, NPR-B and NPR-C are referred to as NPR-2 and NPR-3, respectively. In the interest of formatting consistency in this thesis, short titles have been added to the existing figure legends.

Dexamethasone increases production of C-type natriuretic peptide in the sheep brain

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6.1 Abstract

Although C-type natriuretic peptide (CNP) has high abundance in brain tissues and cerebrospinal fluid (CSF), the source and possible factors regulating its secretion within the central nervous system (CNS) are unknown. Here we report the dynamic effects of a single IV bolus of dexamethasone or saline solution on plasma, CSF, CNS and pituitary tissue content of CNP products in adult sheep, along with changes in CNP gene expression in selected tissues. Both CNP and NTproCNP (the amino-terminal product of proCNP) in plasma and CSF showed dose responsive increases lasting 12-16 h after dexamethasone whereas other natriuretic peptides were unaffected. CNS tissue concentrations of CNP and NTproCNP were increased by dexamethasone in all of the 12 regions examined. Abundance was highest in limbic tissues, pons and medulla oblongata. Relative to controls, CNP gene expression (*NPPC*) was upregulated by dexamethasone in 5 of 7 brain tissues examined. Patterns of responses differed in pituitary tissue. Whereas the abundance of CNP in both lobes of the pituitary gland greatly exceeded that of brain

tissues, neither CNP nor NTproCNP concentration was affected by dexamethasone — despite an increase in *NPPC* expression. This is the first report of enhanced production and secretion of CNP in brain tissues in response to a corticosteroid. Activation of CNP secretion within CNS tissues by dexamethasone, not exhibited by other natriuretic peptides, suggests an important role for CNP in settings of acute stress. Differential findings in pituitary tissues likely relate to altered processing of proCNP storage and secretion.

6.2 Introduction

C-type natriuretic peptide (CNP), a paracrine growth factor which regulates cell proliferation and maturation, is widely expressed along with its receptor (*NPR2*) throughout the brain and spinal cord in mammals (Komatsu *et al.* 1991, Langub *et al.* 1995, Herman *et al.* 1996) including primates (Abdelalim & Tooyama 2011). In contrast to other natriuretic peptides, concentrations of products of CNP gene expression (CNP and amino-terminal proCNP, NTproCNP) in cerebrospinal fluid (CSF) greatly exceed those in the systemic circulation (Schouten *et al.* 2011, Wilson *et al.* 2015) and presumably reflect the high CNP abundance relative to other natriuretic peptides identified in brain tissues (Pemberton *et al.* 2002). Although the functional role of CNP in nervous tissues is unclear, *in vitro* evidence shows that CNP stimulates neural growth and connectivity (Zhao & Ma 2009) and neuroplasticity in hippocampal tissues (Decker *et al.* 2010). Changes in CNP gene expression in the perinatal and later periods of brain maturation (Müller *et al.* 2009) further suggest that CNP in brain tissues is subject to regulation but putative secretagogues have yet to be identified.

In the course of study of the regulation of CNP products in CSF in conscious sheep, we determined that dexamethasone — which suppresses plasma levels of CNP and NTproCNP when chronically administered to growing lambs (Prickett *et al.* 2009) or children (Prickett *et al.* 2012a) — conversely, abruptly increases concentrations of these peptides in CSF and plasma after an IV bolus injection. This novel observation has initiated a series of studies aimed to determine the temporal sequence and dose responsivity of CNP peptides in plasma and CSF to stimulation by dexamethasone and to identify the sites in the central nervous system (CNS) targeted by this glucocorticoid. Mindful of the well-recognised and profound effects of glucocorticoids on brain function (Wolkowitz *et al.* 2009), the focus of these studies has been the brain and nearby organs. Based on the initial observations of CNP responses in CSF, we hypothesised that i) the increases in CSF concentrations of CNP would be dose dependent and differ from the dynamic changes exhibited in plasma and ii) the increases in CSF concentration would be associated with corresponding increases in brain tissue abundance of CNP as well as increased CNP gene

expression. We further postulated that among the family of natriuretic peptides, the response would be specific to CNP products.

6.3 Methods

Animal procedures

All procedures involving animals were conducted at Lincoln University and carried out in accordance with the Animal Welfare Act 1999 (New Zealand) and were approved by the Lincoln University Animal Ethics Committee.

Responses of CNP peptides in plasma and CSF to graded doses of dexamethasone. (Study 1). We first determined the dose response of peripheral venous plasma and cisternal CSF concentrations of CNP peptides to dexamethasone in chronically cannulated conscious sheep (Study 1). Eight healthy yearling Coopworth ewes (average live weight 42 kg, 9–14 months old) were housed indoors for 1 week prior to study and fed concentrated lucerne pellets (SealesWinslow, Ashburton, New Zealand) and lucerne chaff at 0900 h every day at maintenance nutritional level, with water provided ad libitum. This feeding regime was continued for the duration of the study. One day before cannulation, sheep were fasted for 24 h and water withheld overnight. Initially CSF samples were collected from 2 of the sheep using cannulae that were placed into the cervical epidural space whilst the sheep were anaesthetised. Thereafter, with the need for improved cannula patency, all CSF samples from other sheep were collected from the cisterna magna via an indwelling cannula (Wilson & Barrell 2015). For samples collected from the cisterna magna, 0.5 mL of CSF — which occupied the dead space in the cannula — was withdrawn under aseptic conditions using a 3 mL disposable syringe and discarded. At each sampling time point, 1.0–1.2 mL of CSF was withdrawn and transferred immediately to a polycarbonate tube on ice, then stored at -20°C until assayed. Blood samples were obtained as described previously (Wilson *et al.* 2015) and were collected on the same occasions as the CSF samples. Dose response studies commenced at least 2 days after cannulation, and continued over a study period of 6 days. Dosing comprised a single IV bolus injection of dexamethasone sodium phosphate in aqueous solution (Dexa 0.2, PhoenixPharm Distributors Ltd, Auckland, New Zealand) at 0.025, 0.063, 0.125, and 0.25 mg dexamethasone/kg live weight, or saline solution (0.9 % w/v) that was delivered according to a balanced incomplete block design. This ensured that 4 different individuals were allocated to each dose of dexamethasone. The sampling was conducted immediately prior to administration of dexamethasone or saline solution, and at 4, 8, 12, and 16 h post administration for measurement of CNP and NTproCNP.

Responses of brain, pituitary and spinal cord levels of CNP peptides to dexamethasone. (Study 2).
In this study, 14 healthy Texel-Romney wethers (average live weight 77 kg, 2 years old) were sampled as described above except that CSF samples were obtained under light anaesthesia — as previously described (Wilson *et al.* 2015) — and blood samples were collected (Wilson *et al.* 2015) immediately prior to a single IV bolus of dexamethasone containing 0.25 mg/kg live weight (n = 7), or saline (control, n = 7), and again at 8 h post injection. At that point, sheep were individually euthanised by captive bolt and exsanguinated. The brain and pituitary gland were rapidly removed and approximately 0.5 g of selected tissues from 14 sites were excised and instantly placed in liquid nitrogen. Specific zones sampled were the anterior pituitary gland, posterior pituitary gland, olfactory bulb, septum, thalamus, hypothalamus, mammillary body, hippocampus, occipital cortex, pineal gland, cerebellum, pons, medulla oblongata, and cervical spinal cord. The frozen samples were stored on dry ice until they were transferred to a -80°C freezer within 2–3 h.

Measurement of peptide concentration and gene expression
Samples of frozen brain and pituitary tissue (mean 70 ± 10 mg) were finely diced on a chilled (dry ice) melamine chopping board, weighed and gently boiled for 5 minutes in 10 mL distilled water containing 10 µL 1 % Triton X-100. After boiling, the tissue suspension was cooled on ice and 610 µL of glacial acetic acid was added. The tissue suspension was homogenised (3 x 20 second bursts at 400 Hz) using an Ultra-Turrax homogeniser (IKA-Labortechnik, Staufen, Germany). The tissue homogenates were then centrifuged (3000 g, 4°C, 30 minutes), and processed thereafter in an identical manner to the CSF and plasma samples.

Hormone assays. Hormones levels in CSF, plasma, brain and pituitary tissue were measured by radioimmunoassay after extraction using solid phase cartridges (Sep Pak, Waters Corp., Milford, MA). All samples from an individual animal were processed in duplicate in a single assay. CNP and NTproCNP were assayed as previously described (Wilson *et al.* 2015) and tissue concentrations were calculated from wet weight of tissue homogenised, assay buffer reconstitution volume and radioimmunoassay result. The ratio of NTproCNP to CNP (NTproCNP:CNP) was calculated from molar concentrations of the respective peptides in each sample. Atrial natriuretic peptide (ANP) concentration was measured as previously reported (Yandle *et al.* 1986) except: 50 µL standard/sample was pre-incubated with 50 µL of primary rabbit antiserum diluted to 1:12500 for 24 h at 4 ^{o}C, to which 50 µL of iodinated ANP was added (2500 cpm/50 µL). Following a second

incubation period, bound and free-labelled antigen were separated by addition of 500 µL of solid phase secondary antibody (5 % v/v Donkey anti-Rabbit Sac-cell (IDS Ltd, UK) diluted in assay buffer containing 2 % polyethylene glycol. After 30 minutes incubation at room temperature, tubes were centrifuged for 10 minutes, and radioactivity of the pellet was counted following aspiration of the supernatant. B-type natriuretic peptide (BNP) concentration was measured as previously described (Pemberton *et al.* 1997) except: 50 µL standard/sample was pre-incubated for 24 h at 4 ^{o}C with 50 µL of primary rabbit antiserum, to which 50 µL of iodinated BNP was added (5000 cpm/50 µL). After a second incubation period, bound and free-labelled antigen were separated in a similar manner as the ANP protocol, except the assay was incubated for 30 minutes in an ice bath. The detection limit (pmol/L) for each assay was: 7.3 for ANP, 4.9 for BNP, 0.6 for CNP and 1.9 for NTproCNP. Intra- and inter-assay coefficients of variation respectively were 7.8 % (21-100 pmol/L) and 10.4 % (at 86 pmol/L) for ANP, 9.9 % (4-20 pmol/L) and 15 % (at 23.6 pmol/L) for BNP, 6.3 and 7.9 % at 9 pmol/L for CNP and 7.4 and 11.4 % at 64 pmol/L for NTproCNP.

Quantitative real-time PCR. Total RNA was extracted from approximately 40 mg of tissue using the ReliaPrep™ RNA Tissue Miniprep System (Promega, Madison, WI), according to the manufacturer's instructions. RNA purity was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). cDNA was synthesised from 1 µg of RNA template using an iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR was performed using iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA) and specific primers for ovine *NPPC* (Forward: GGT CAG AAG GGC GAC AAG A; Reverse: TGT ATT TGC GCG CGT TGG G), *NPR2* (Forward: TGC CCT CTA TGC CAA GAA GC; Reverse: GTA GAA AGG CCC ACT GCG AA) and *NPR3* (Forward: CAC CCA GGA GGT TAT TGG TGA; Reverse: AAG GAG AGC TGT TCG TGT GCT) on a Stratagene MX3000p thermal cycler (Agilent Technologies, Santa Clara, CA). Gene expression was normalised to cDNA concentration, quantified using a Quant-iT™ OliGreen® ssDNA assay kit (Thermo Fisher Scientific, Waltham, MA). To determine relative gene expression, mean Ct values were power transformed from their logarithmic format, and divided by sample cDNA concentration.

Statistical analyses

Changes in CNP and NTproCNP concentration in CSF, plasma and CNS tissue in response to saline or dexamethasone administration were analysed separately. In Study 1, a repeated measures ANOVA was used to compare area under the curve for the logged (log_{10} here and in all cases

thereafter) data using Genstat Version 16 (VSN International Ltd., Hemel Hempstead, UK). Data from one sheep were excluded when pregnancy became apparent at the time of sample collection. In Study 2, a repeated measures ANOVA was used to compare logged concentrations of the various peptides in CSF, plasma, and CNS tissues in response to dexamethasone or saline solution, as well as the ratio of CNP:NTproCNP concentration in CNS tissues which was calculated for each sheep. Least significant differences were used to identify differences in CNP and NTproCNP concentration between treatment groups in specific CNS tissues. The relation between peptide concentration in tissue (logged) and the percentage difference in peptide concentration after dexamethasone was determined using linear regression analysis in GraphPad Prism version 6.01 for Windows (GraphPad Software Inc, La Jolla, CA, www.graphpad.com). Gene expression levels of *NPPC*, *NPR2* and *NPR3* were compared separately using a repeated measures ANOVA in Genstat Version 16 (VSN International Ltd., Hemel Hempstead, UK) using logged data, and least significant differences were used to identify differences between saline- and dexamethasone-treated sheep for each tissue.

6.4 Results

All studies were accomplished as planned, and data collection was complete for Study 1. In Study 2, CSF samples were collected from 13 of 14 sheep at baseline, and from 10 of 14 sheep following treatment. No adverse events were noted in any animal during either study, nor were any signs of infection evident in cannulated animals.

Dose responses to dexamethasone (Study 1).

On the control day (saline injection), plasma and CSF concentration of both CNP and NTproCNP were stable during the 16 h period of sampling. As expected, mean concentrations of CNP and NTproCNP in CSF were higher than in plasma (3.32 ± 0.14 vs 0.94 ± 0.02 pmol/L; 817 ± 24 vs 47.7 ± 1.3 pmol/L respectively, $P < 0.001$ for both). There was no significant association between time matched plasma and CSF concentrations for either peptide. Serial changes in CSF and plasma levels evoked by a range of doses of dexamethasone are shown in Figure 6.1. Dose dependent increases in both CNP peptides were observed within 4 h in plasma and somewhat later in CSF, although the magnitude and duration of responses differed in the two circulations. Overall, relative responses of plasma CNP and NTproCNP concentration to the highest dose of dexamethasone (8-fold and 6-fold increase respectively) exceeded those in CSF (3.3-fold and 1.5-fold respectively). In CSF both the onset and the offset of responses were delayed relative to those observed in plasma. Further, time to peak CSF concentrations of NTproCNP (Cmax, 16 h)

was delayed compared with that of CNP (Cmax 8-12 h) and the NTproCNP concentration in CSF remained above pre injection levels 16 h after dexamethasone administration in 3 of the 4 studies. Whereas a significant increase above control levels of plasma CNP and NTproCNP concentration was generated by the two lowest doses of dexamethasone (0.025 and 0.063 mg/kg, $P < 0.05$), these doses failed to raise levels of these peptides in CSF.

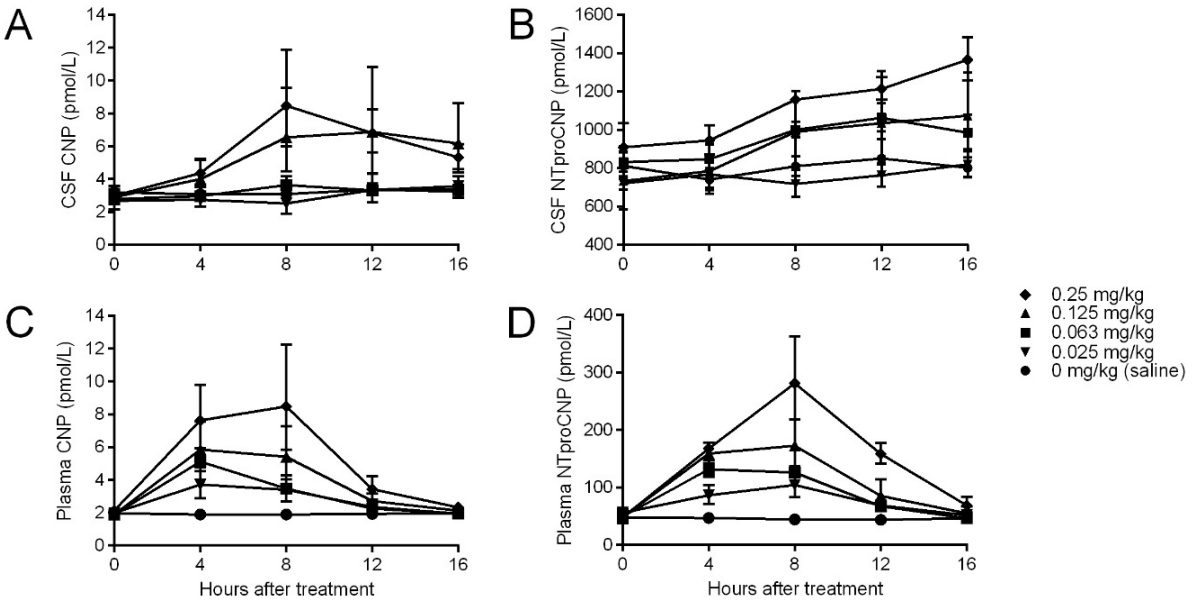

Figure 6.1 Effect of different doses of dexamethasone on CSF and plasma concentrations of CNP and NTproCNP.

Mean CNP (left) and NTproCNP (right) concentration in CSF (A and B) and plasma (C and D) of sheep following different doses of dexamethasone: 0 (saline), 0.025, 0.063, 0.125, 0.25 mg/kg live weight. Data are presented as geometric means. n = 8 (saline) and 4 per dexamethasone dose level.

CNS and pituitary tissue responses to dexamethasone (Study 2).

To ensure that responses in CSF and systemic concentrations of CNP products on the day of tissue collection were comparable to those observed in the dose response study, both peptides were measured in plasma (at 2 hourly intervals over 8 h) and CSF (pre injection and at 8 h after treatment). Concentrations of both peptides prior to injection were similar in saline- and dexamethasone-treated sheep and did not change significantly after saline injection in the 8 h period prior to tissue collection (Figure 6.2). Again, as in Study 1, significant increases of CNP and NTproCNP concentration in both plasma and CSF were recorded following IV dexamethasone (0.25 mg/kg).

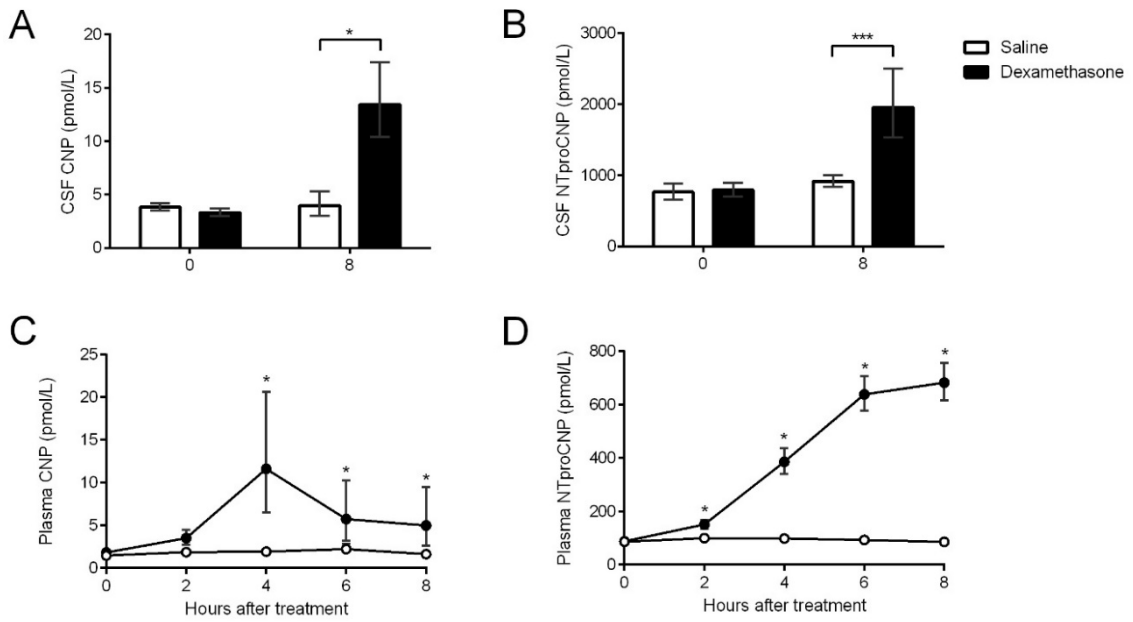

Figure 6.2 Confirmation of the effect of dexamethasone.

Mean CNP (left) and NTproCNP (right) concentration in CSF (A and B) and plasma (C and D) of sheep treated with saline solution (open bars/circles) or dexamethasone (closed bars/circles). Data are presented as geometric means. In CSF, n = 6 (saline) and 7 (dexamethasone) per group at 0 h, n = 5 per group at 8 h. In plasma, n = 7 per group. *significant difference between groups, $P < 0.05$.

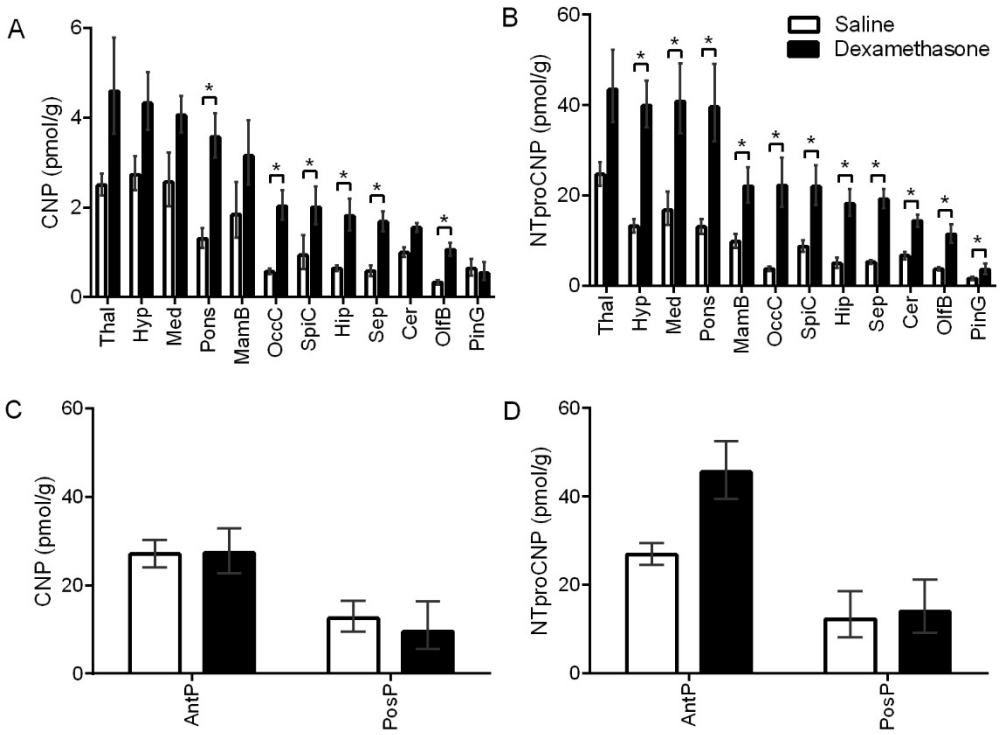

Figure 6.3 Tissue concentrations of CNP and NTproCNP.

Mean concentrations (wet weight basis) of CNP (left panels) and NTCNP (right panels) in brain (A and B) and pituitary gland (C and D) tissues in saline- (open bars) and dexamethasone-treated sheep (filled bars) 8 h following treatment. Data are presented as geometric means, n = 7 per group. *significant difference between groups, $P < 0.05$. Tissue regions were abbreviated to the following: AntP (anterior pituitary gland), PosP (posterior pituitary gland), Thal (thalamus), Hyp (hypothalamus), Med (medulla oblongata), Pons (Pons), MamB (mammillary body), OccC (occipital cortex), SpiC (spinal cord), Hip (hippocampus), Sep (septum), Cer (cerebellum), OlfB (olfactory bulb), PinG (pineal gland).

Across the wide range of neural tissues examined, both CNP and NTproCNP were detected in all tissue extracts. As shown in Figure 6.3 A, in control sheep, concentrations of CNP in the brain were highest in limbic tissues (thalamus, hypothalamus, mammillary body), medulla oblongata and pons. Low abundance was observed in the pineal gland, olfactory bulb and occipital cortex. In contrast, CNP concentrations were much higher in the anterior pituitary (28.2 ± 3.5 pmol/g) and posterior pituitary (15.7 ± 4.5 pmol/g) lobes (Figure 6.3 C). Excepting pituitary tissue, concentrations of NTproCNP (Figure 6.3 B and D) in tissues from saline injected (control) sheep were almost 10-fold higher (8.3 ± 0.7) than those of CNP. Dexamethasone increased concentrations of both peptides in most tissues examined (Figure 6.3 A and B). Compared with

controls, there were significant increments in CNP concentration following dexamethasone in 6 of the 14 selected tissues and those for NTproCNP were significant in 11 of these tissues. The relative abundance of both peptides across brain tissues from controls was largely preserved in tissues extracted from dexamethasone-treated animals. Again, responses in pituitary tissues differed. Neither CNP nor NTproCNP abundance in the posterior lobe was affected by dexamethasone (Figure 6.3 C and D). NTproCNP levels in the anterior pituitary lobe were increased after dexamethasone (Figure 6.3 D), however this was not significant — and in contrast to brain tissues — CNP concentration was unaffected. Considering all 14 tissues, the percentage difference (dexamethasone versus control) in CNP concentration was significantly greater in tissues with relatively low abundance ($r = 0.64$, $P < 0.05$, Supplemental Figure 6.1). A similar inverse relation was observed for NTproCNP ($r = 0.54$, $P < 0.05$).

Concentrations of the 2 peptides in control sheep were highly correlated in brain ($r = 0.68$, $P < 0.0001$) and pituitary gland ($r = 0.81$, $P < 0.05$), and even more so after dexamethasone: ($r = 0.80$ and 0.88 respectively, $P < 0.0001$ for both). However, as shown in Supplemental Figure 6.2, across all studies the concentration ratio of NTproCNP to CNP in both pituitary lobes was close to unity (1:1) — much lower than found in brain tissues (5:1 to 10:1).

Gene expression

NPPC, NPR2, and *NPR3* gene expression in selected tissues from saline- and dexamethasone-treated sheep is shown in Figure 6.4. *NPPC* expression levels were upregulated by dexamethasone ($P < 0.001$) in 5 of the 7 tissues examined: anterior pituitary, posterior pituitary, hypothalamus, hippocampus and pons. No significant change in *NPPC* expression was found in occipital cortex or olfactory bulb tissue. Except for the downregulation of *NPR2* in hypothalamic tissue following treatment with dexamethasone ($P < 0.05$), changes in *NPR2* and *NPR3* gene expression in the other tissues after dexamethasone were not significant (Figure 6.4). Despite markedly lower NTCNP:CNP ratios in the anterior and posterior pituitary gland, *NPR3* expression in these tissues did not differ from that in the other regions examined here.

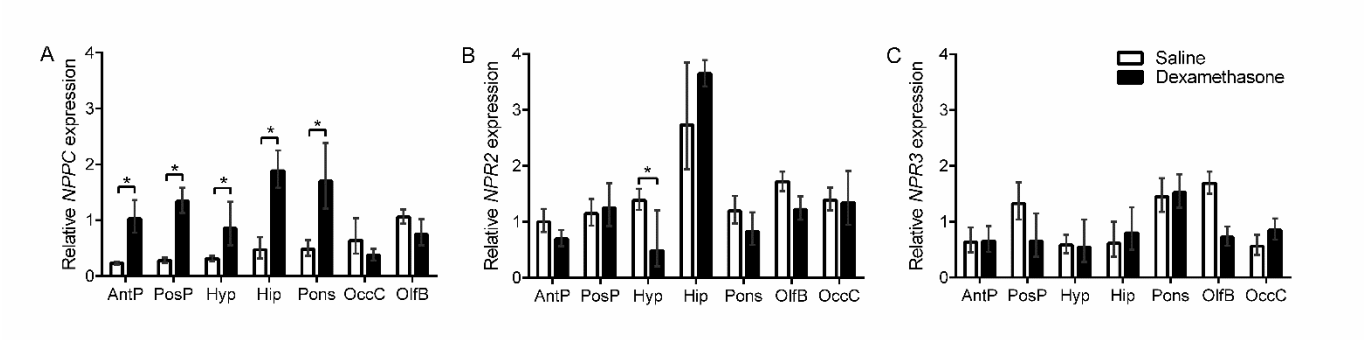

Figure 6.4 Effect of dexamethasone on gene expression of *NPPC*, *NPR2* and *NPR3.*

Relative gene expression of A) *NPPC* B) *NPR2* and C) *NPR3* in tissues from brain and pituitary gland of sheep obtained at 8 h following treatment with IV saline solution (open bars) or a single IV dose of dexamethasone (closed bars). Data are presented as geometric means, n = 7 per group. *significant difference between groups, $P < 0.05$.

Specificity of CNP responses.

Whether natriuretic peptide responses to dexamethasone are specific to CNP was addressed by measuring the concentration of ANP and BNP in samples obtained in Study 2. As shown in Figure 6.5, whereas small fluctuations in plasma ANP and BNP concentration were observed after saline or dexamethasone, the mean values for either hormone after dexamethasone (0.25 mg/kg live weight) did not differ from those following the saline treatment. Both ANP and BNP were undetectable in CSF, including samples obtained after dexamethasone. In brain tissue extracts, ANP was undetectable in 29 of 42 samples examined. In 3 tissues where ANP was detectable (olfactory bulb, thalamus and pons) concentrations after dexamethasone (mean 0.84 ± 0.43 pmol/L, n = 6) were low and did not differ from control sheep (mean 0.72 ± 0.30, n = 7). BNP was undetectable in 37 of 39 samples analysed.

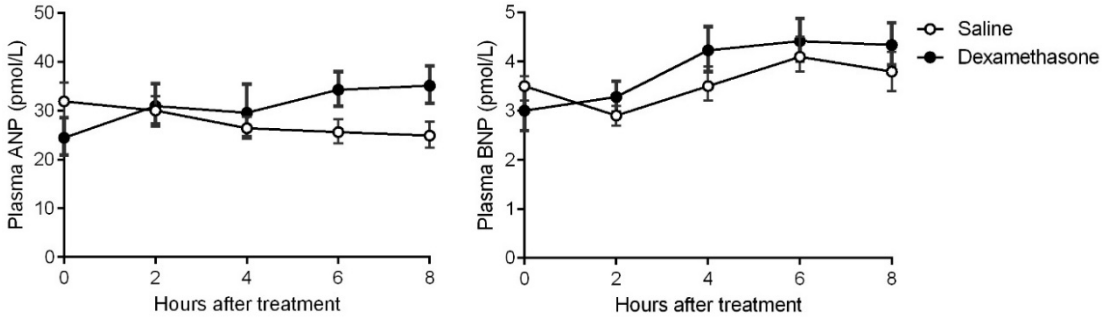

Figure 6.5 Specificity of CNP responses.

Geometric mean plasma ANP (left) and BNP (right) concentration in sheep treated with saline solution (open circles) or dexamethasone (filled circles). Data are presented as geometric means, n = 7 per group.

6.5 Discussion

CNP is the most abundant of the natriuretic peptides present in CNS tissues (Ueda *et al.* 1991, Kaneko *et al.* 1993). Whereas its role in the early development and maturation of cerebral neurons (Müller *et al.* 2009), and in axonal branching of sensory neurons entering the CNS (Zhao & Ma 2009) is well-defined, factors regulating CNP production within the brain and related tissues of adults *in vivo* have not been studied. Here we show that a single IV bolus injection of dexamethasone abruptly increases plasma and CSF concentrations of CNP peptides selectively and dose dependently. These changes are associated with marked increases in peptide concentrations in a wide range of CNS tissues, and upregulation of *NPPC* mRNA expression in 5 of the 7 cranial tissues examined here. Collectively these novel findings suggest that CNP may mediate some of the acute effects of stress on brain function, which now warrants further study. As part of physiological studies examining fluctuations of CNP peptides in CSF drawn from conscious adult sheep, we found that doses of dexamethasone — at levels that can reduce plasma concentrations of CNP peptides in lambs when administered for several days (Prickett *et al.* 2009) — actually raise concentrations of CNP in CSF during the first 12 h.

In more focussed studies we now show that in contrast to relatively unchanged levels that persist after administration of saline, a single IV bolus injection of dexamethasone induces a prompt increase in CNP and NTproCNP concentrations in both plasma and CSF. ANP and BNP levels were unaffected. As noted in humans (Schouten *et al.* 2011), in the absence of interventions (i.e. saline-treated animals here), concentrations of CNP and NTproCNP were much higher in CSF than in time matched samples of plasma. Lack of any significant correlation between levels in the two circulations suggests independent regulation and little, if any, exchange across the blood-brain barrier (BBB). After dexamethasone, onset of the response in CNP peptide levels occurred earlier in plasma (within 4 h), was dose dependent and of shorter duration than was observed in CSF. Presumably delayed entry of dexamethasone to the CNS (Balis *et al.* 1987), diffusion and bulk flow of CNP peptides from extra-cellular fluid to CSF (Leng & Ludwig 2008) and slower clearance of CNP peptides in CSF (particularly NTproCNP), are the basis of these temporal differences. Sustained elevations of NTproCNP in CSF 16 h after dexamethasone, when plasma levels had returned to baseline, suggests that any increase in permeability of the BBB by CNP (Bohara *et al.* 2014) is minimal in this experimental setting.

In order to examine likely sources of the response of CNP peptides in CSF, we measured their relative abundance in 12 selected tissues within the CNS 8 h after saline or dexamethasone (0.25 mg/kg live weight) – a dose which strongly stimulated plasma and CSF CNP peptides in these

same sheep when compared with the control study. In controls, the relative abundance of CNP peptides in CNS tissues was similar to that previously reported in adult sheep (Pemberton *et al.* 2002), rodents (Jankowski *et al.* 2004) and humans (Komatsu *et al.* 1991, Totsune *et al.* 1994). Highest concentrations were found in tissue sampled from thalamus, hypothalamus, mammillary body and medulla oblongata. Overall, concentrations of the two peptides were highly correlated in brain and pituitary gland of control sheep — with levels of NTproCNP approximately 5–10 fold those of CNP in brain tissue. Of the 12 CNS tissues examined, the concentration of both peptides was higher after dexamethasone when compared with controls — significantly so in 11 and 6 tissues for NTproCNP and CNP respectively. Tissues with lower abundance after saline exhibited proportionately higher levels after dexamethasone (Supplemental Figure 6.1). This trend was more obvious in the CNP response — possibly reflecting higher rates of secretion (loss to extracellular fluid and CSF) for this peptide, particularly in tissue zones adjacent to cerebral ventricles. Again, after dexamethasone significant associations were observed between CNP and NTproCNP but in 4 tissues (hypothalamus, pineal gland, occipital cortex and medulla oblongata) the ratio of NTproCNP to CNP was significantly increased compared with control values.

Presumably higher rates of CNP degradation and/or egress from the neuropil account for these glucocorticoid-induced differences. Viewed in relation to proportionate increases in CSF at 8 h after dexamethasone (0.25 mg/kg live weight) the increase in brain tissue CNP content (2-4 fold) is commensurate with that observed in CSF (3-4 fold) and lends credibility to the possibility that the changed levels in CSF are a consequence of enhanced secretion from tissues within the CNS. Proof that these changes involve increased synthesis of the peptides is provided by the evidence presented here for upregulation of *NPPC* in the hypothalamus, hippocampus, and pons. In contrast, *NPPC* expression in the occipital cortex and olfactory bulb was not increased by dexamethasone — despite significant increases in both CNP and NTproCNP concentration in these tissues. Presumably this discrepancy relates to differences in mRNA stability (rapid degradation compared with peptide loss) and/or co-production of inhibitory microRNAs.

Whether longer term treatment with dexamethasone could sustain the responses we observed here in CNS tissues is unknown but important to resolve – particularly since plasma levels of CNP peptides are suppressed by prolonged dosing with dexamethasone in growing lambs and children (Prickett *et al.* 2009, Prickett *et al.* 2012a). In the latter studies, contributions of NTproCNP to its plasma concentrations are likely to be sourced from growth plate proliferating chondrocytes (Prickett *et al.* 2005, Prickett *et al.* 2012b) — which are depleted by glucocorticoids (Siebler *et al.*

2002) — so it is not surprising that the temporal responses of CNS tissues to glucocorticoids are likely to vary from those in other body regions.

Although it is not part of the CNS, and is unprotected by the BBB, the pituitary gland in sheep and humans has an unusually high CNP content (Yandle *et al.* 1993, Pemberton *et al.* 2002, Thompson *et al.* 2009), so its potential to contribute to the amount of CNP in plasma or CSF was an important factor in selecting this organ for closer study. Findings in pituitary tissue differ from those in CNS tissues in several respects. First, in control sheep the concentration of CNP is much higher in both anterior and posterior lobes than in any of the regions of the CNS reported here. Second, in both lobes there are equimolar concentrations of CNP and NTproCNP, reducing the ratio of NTproCNP:CNP to unity (1:1) – much lower than that found in CNS tissues (5:1 to 10:1, Supplemental Figure 6.2). Previous work (Yandle *et al.* 1993) has also shown that the pituitary processing of proCNP is unique in that pituitary tissue contains predominantly CNP-53, contrasting with the presence of equal amounts of CNP-53 and the smaller peptide CNP-22 in hypothalamic extracts. These findings suggest that under physiological conditions different functions are subserved in the pituitary gland — with less degradation and reduced processing being consistent with accumulation and storage. Thirdly, although *NPPC* expression in both lobes is significantly upregulated after dexamethasone, abundance of peptides was not changed.

Together these unexpected findings raise the possibility that both peptides are actively secreted from the pituitary gland into the systemic circulation in response to dexamethasone, and thus could make significant contributions to the responses recorded in plasma after IV administered dexamethasone. If so, a disproportionate amount of CNP-53 would be expected to appear in the systemic circulation. More recent studies in our laboratory confirm this and show that the profile of high molecular weight immunoreactive CNP forms in plasma closely reflect the profile found in anterior pituitary tissue extracts (manuscript in preparation, Chapter 7). Others have shown that CNP in anterior pituitary tissue appears to be exclusively associated with gonadotroph cells (Thompson *et al.* 2009, McArdle *et al.* 1994). Reports that acute stress or glucocorticoid administration stimulate gonadotrophin secretion in some settings (Maeda & Tsukamura 2006) may be relevant in this context. Although we have not found evidence of a CNP arterio-venous concentration gradient across the pituitary gland in samples of plasma drawn from the inferior petrosal vein in human subjects with Cushing's disease (unpublished), further study of the acute effect of glucocorticoids on pituitary secretion of CNP peptides is warranted.

Our study was not designed to address either the origins or actions of CNP at the cellular level but these questions become highly relevant in light of the present findings — and of the manifold effects of glucocorticoids on brain function (Wolkowitz *et al.* 2009). Notably, CNP responses in CSF were observed after a dexamethasone dose of 0.125 mg/kg live weight — which corresponds to the therapeutic range for adult humans. The wide array of glucocorticoid-responsive CNS tissues identified here suggests that commonalities — such as capillary networks (Vigne & Frelin 1992) or glial tissues (Parpura & Zorec 2010) — all of which are recognised sites of CNP production — are likely to be involved. One possible action of dexamethasone, increasing the CNP mRNA response to shear stress in brain capillaries (Zhang *et al.* 1999), might account for the present results. In cultured murine cerebral cortex neurons, activation of voltage-sensitive calcium channels during potassium-induced depolarisation strongly upregulates *NPPC* expression over a 6 h period (Kim *et al.* 2010). Since glucocorticoids specifically enhance L-type calcium channel amplitude in a variety of neurons sourced from brain tissues (Joëls & Karst 2012), the glucocorticoid-induced increases in CNP we observed may have resulted from such membrane-level events (Wolkowitz *et al.* 2009).

Responses from astrocytes or microglia — ubiquitous in CNS tissues and an important source of natriuretic peptides, including CNP (Deschepper 1998) — may also have contributed to these findings. Of note, dexamethasone elicits a dose dependent CNP response from cells of monocyte/macrophage lineage (Kubo *et al.* 2001) which, within the BBB, constitute the microglia. To our knowledge no glucocorticoid response element has been identified in the CNP gene in any species. Interestingly, glucocorticoids act directly and specifically to increase ANP gene transcription in rodent cardiomyocytes (Gardner *et al.* 1988) and other tissues (Gardner *et al.* 1986) yet we found no evidence that ANP or BNP is affected by dexamethasone in either the systemic circulation or CNS tissues. Concerning possible actions of CNP in glial tissues, there is a strong body of evidence that cGMP, a downstream mediator of CNP activity and more responsive to CNP than either ANP or BNP (Deschepper & Picard 1994), regulates several crucial intercellular actions including Na^+/H^+ exchangers, neurotransmitter re-uptake, gap junctions, cell pH and brain cell water content (Kim *et al.* 2010). In this context, it is important to note that dexamethasone mitigates cerebral glioma tumour oedema – a cell type highly responsive to CNP (Eguchi *et al.* 1992). Conceivably, this well-described pharmacological action of dexamethasone is mediated at least in part by CNP which could have therapeutic implications now that CNP agonists are available for use in humans.

Declaration of interest

E.A.E. is a consultant for BioMarin Pharmaceutical. The authors have no conflicts of interest to disclose.

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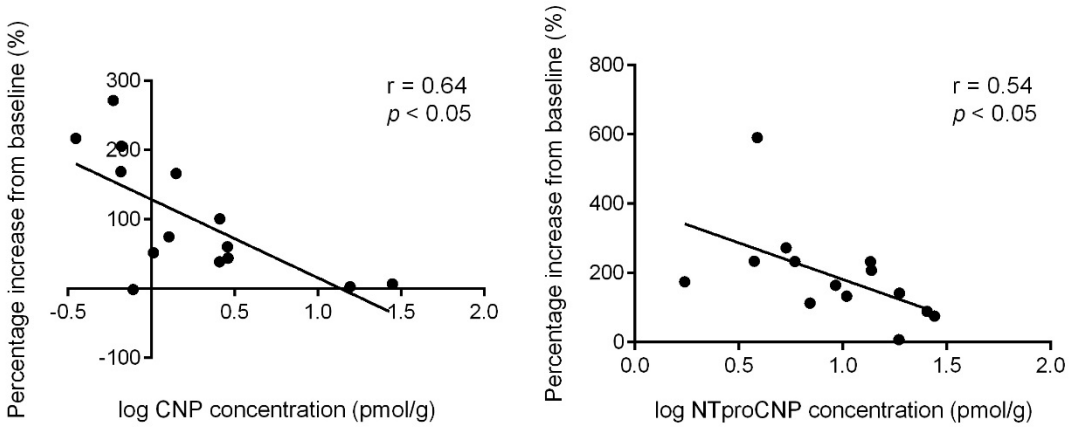
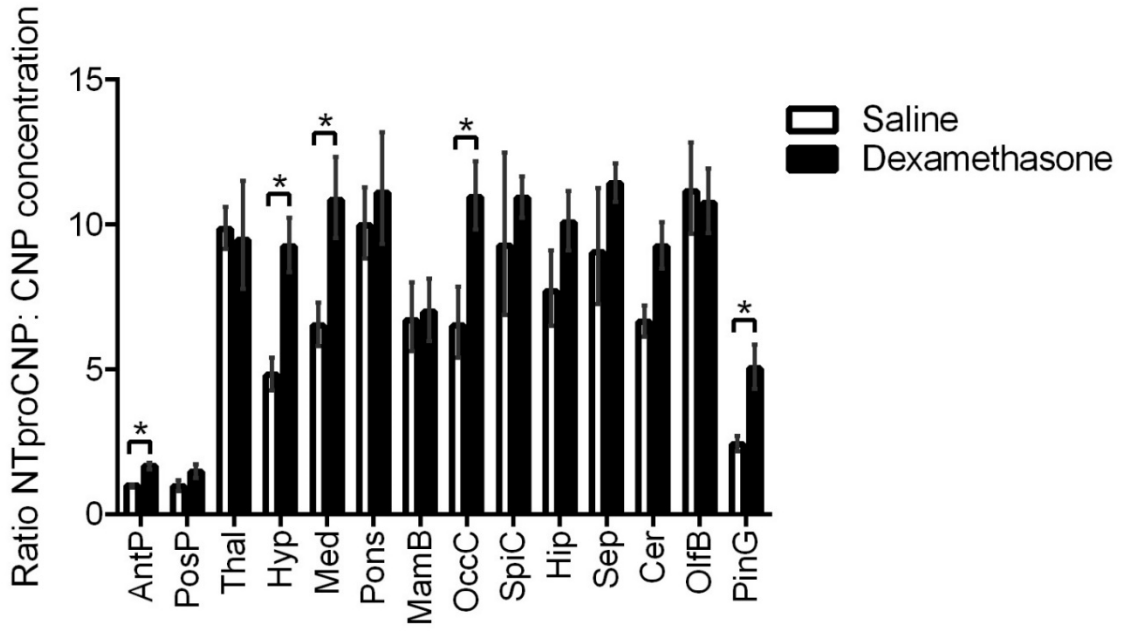


Supplemental Figure 6.1 Relationship of the percentage increase in CNP peptide concentration following dexamethasone and levels in control sheep.

Relationship of the percentage increase in mean $\log_{10}$ CNP (left) and NTproCNP (right) concentration following dexamethasone (n = 7) above that of saline-treated sheep (n = 7) for 14 cranial tissues.



Supplemental Figure 6.2 Ratio of CNP:NTproCNP concentration in tissues.

Mean ratio of NTproCNP:CNP in 14 cranial tissues from saline- (open bars) and dexamethasone-treated (filled bars) sheep obtained at 8 h following treatment. Data are presented as geometric means, n = 7 per group. *significant difference between groups, $P < 0.05$. Tissue regions were abbreviated to the following: AntP (anterior pituitary gland), PosP (posterior pituitary gland), Thal (thalamus), Hyp (hypothalamus), Med (medulla oblongata), Pons (Pons), MamB (mammillary body), OccC (occipital cortex), SpiC (spinal cord), Hip (hippocampus), Sep (septum), Cer (cerebellum), OlfB (olfactory bulb), PinG (pineal gland).

Additional data

The following data were collected for studies in this chapter, however were excluded from the submitted manuscript.

Confirmation of correct pituitary gland dissection

Prior to commencing tissue collection for measurement of CNP peptide concentration and gene expression analysis, dissections were practiced using brains from sheep killed at a local abattoir (Canterbury Fresh Lamb, Christchurch). In order to confirm that the pituitary gland was accurately dissected, tissues were sent to a pathology laboratory (Gribbles Veterinary Pathology, Christchurch) for a routine haematoxylin and eosin staining, and viewed under a bright field microscope (Nikon Eclipse 50i microscope, Tokyo, Japan). Images were captured using microscope imaging software (NIS Elements Imaging Software, Basic Research, Tokyo, Japan, Plate 6.1). Correct dissection was confirmed by the higher density of nuclei (stained blue) in the anterior pituitary gland (left), compared with the posterior pituitary gland (right).

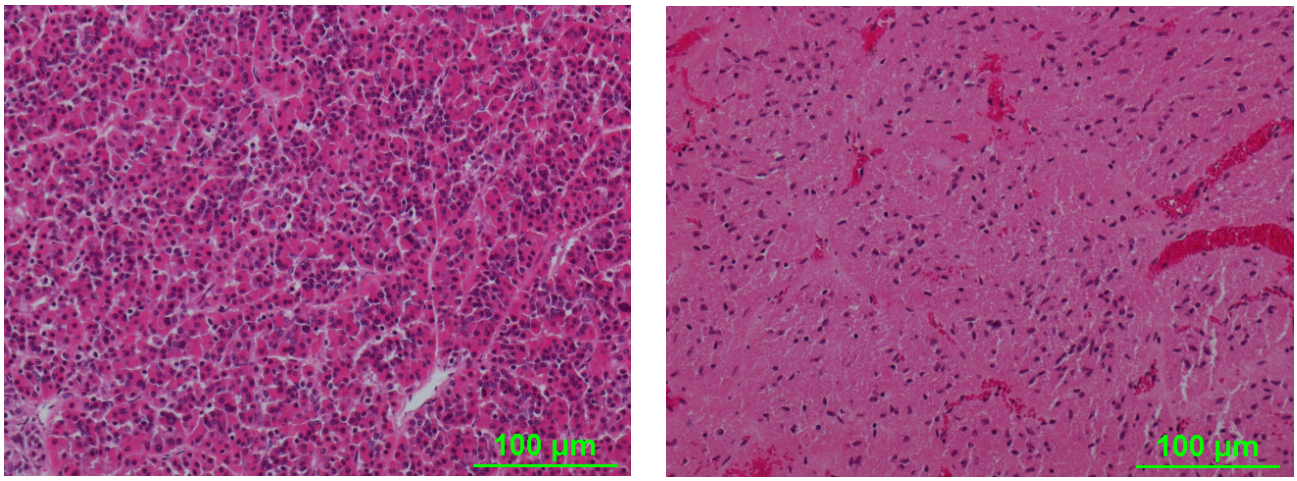


Plate 6.1 Ovine anterior (left) and posterior (right) pituitary gland stained with haematoxylin and eosin.

Comparison of gene expression levels of NPPC, NPR2 and NPR3 across tissues

To compare gene expression levels of *NPPC*, *NPR2* and *NPR3* within groups, gene expression was calculated relative to the lowest Ct (threshold cycle) value of all samples analysed, and compared using a repeated-measures ANOVA. Significant differences between tissues were identified using least significant differences. The main finding was that despite markedly different concentration ratios of NTproCNP:CNP in the brain (5:1 to 10:1) compared with the anterior and posterior pituitary glands (1:1), the expression level of *NPR3* (*NPRC)* was similar between tissues within the control group (Figure 6.6 C). This suggests that differences in clearance rates of CNP may be a result of higher enzymatic degradation occurring in the brain — such as insulin-degrading enzyme or neprilysin — compared with in the pituitary gland. It is relevant to note that neprilysin expression was not detected in the posterior pituitary gland of the rat, and was found in low levels in the anterior pituitary gland (Facchinetti *et al.* 2003). In contrast, neprilysin expression levels were highly variable throughout the brain (Facchinetti *et al.* 2003) — which may account for some of the variation in NTproCNP:CNP concentration ratios measured in the current study. Additionally, there may be some differences in clearance receptor activity that are not reflected by mRNA levels; Ardaillou *et al.* (1996) reported that NPR3 protein (NPR-C) is increased by dexamethasone without affecting mRNA levels.

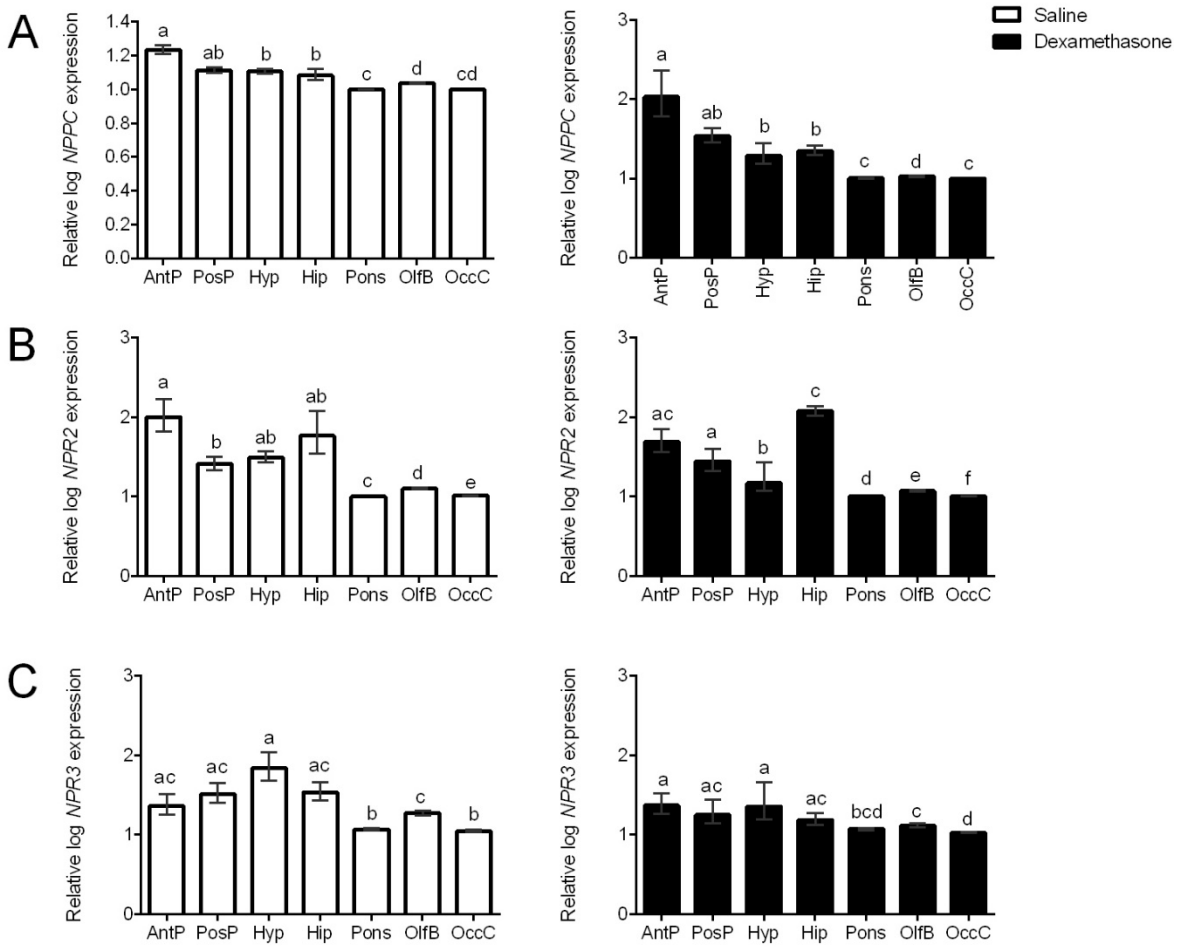


Figure 6.6 Comparison of gene expression levels in brain and pituitary gland tissue within groups of saline- and dexamethasone-treated sheep.

Relative gene expression of A) *NPPC*, B) *NPR2*, and C) *NPR3* are presented as geometric means (n = 7 per group). Gene expression levels do not differ between tissues given the same letter. Tissues with different letters have significantly different levels of gene expression for that respective gene ($p < 0.05$).

Effect of dexamethasone on glucose concentration

In order to confirm that dexamethasone was being administered at an effective dose concentration capable of producing effects that were typical of glucocorticoids, glucose concentration was measured in plasma from sheep given saline solution or dexamethasone (0.025, 0.063, 0.125, 0.25 mg/kg live weight, Figure 6.7), as described in 6.3. Glucose concentration was also measured in CSF of sheep given dexamethasone (0.25 mg/kg live weight, n = 3) and saline solution (n = 1, Figure 6.8). Plasma and CSF glucose concentration were measured using the hexokinase method in an automated analyser (Enzymatic Assay Glucose Hexokinase, Abbott c8000 analyser, Abbott reagents) by Canterbury Health Laboratories, Christchurch, NZ. Two-tailed unpaired Student's *t*-tests were used to compare glucose concentrations in plasma using GraphPad Prism version 6.01 for Windows (GraphPad Software Inc, La Jolla, California, USA, www.graphpad.com).

Mean plasma glucose concentration did not differ between groups in Study 1 prior to treatment, ranging from 3.5 to 4.9 mmol/L in individual sheep at baseline, and did not differ between 0 and 8 h in control sheep (mean change 0.00 ± 0.11 mmol/L). All doses of dexamethasone used here elevated glucose levels in plasma sampled at 8 h after administration ($p < 0.001$) where mean concentrations had increased to between 5.98 ± 0.11 mmol/L (lowest dose) and 7.28 ± 0.77 mmol/L (highest dose). This response was sustained for a period of 24 h following the highest dose of dexamethasone (0.25 mg/ live weight, n = 2) in contrast to control levels which remained consistently lower (mean change -0.23 ± 0.17, n = 3).

In control sheep, glucose concentration in CSF was approximately 65 % of concomitant plasma levels (n = 1) and levels were similar on each of the 3 days they were measured. Following the highest dose of dexamethasone (0.25 mg/kg live weight), CSF glucose concentration was increased by 1.5-fold to 2-fold in 2 sheep, and was unchanged in 1 sheep (Figure 6.8).

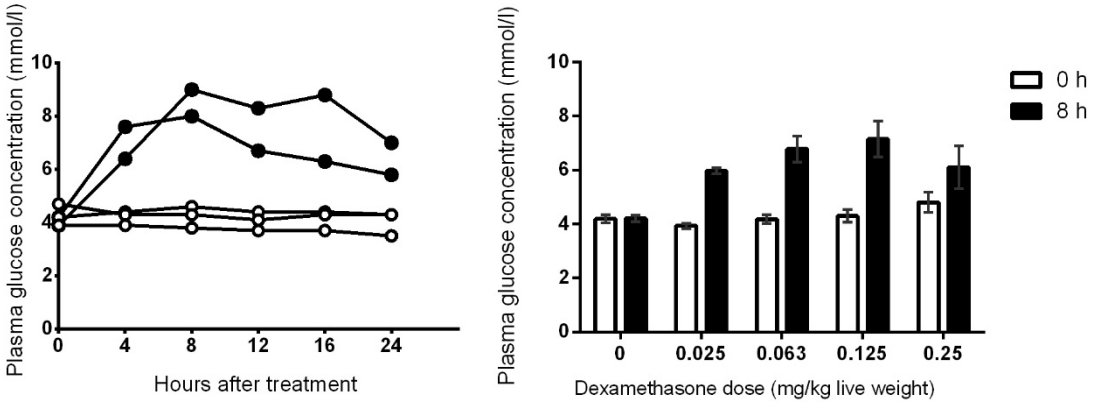


Figure 6.7 Plasma glucose concentration in sheep administered with dexamethasone or saline. Left: Glucose concentration in plasma of sheep administered with saline solution (open circles, n = 3) or dexamethasone (closed circles, n = 2) 24 h after treatment. Right: Mean glucose concentration (± s.e.) in plasma from sheep immediately before (open bars) and 8 h after (closed bars) i.v. injection with saline solution or dexamethasone (n = 4 per group).

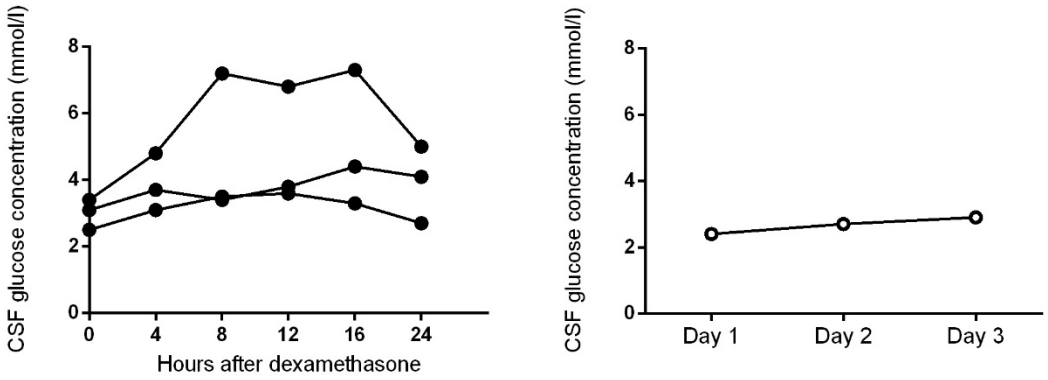


Figure 6.8 CSF glucose concentration in sheep administered with dexamethasone or saline Left: 24 h profile of glucose concentration in CSF of sheep (n = 3) given a single i.v. injection of dexamethasone (0.25 mg/kg). Right: CSF glucose concentration in one sheep following administration with saline solution.

Effect of dexamethasone on the transfer of small metabolites from plasma to CSF

Concentrations of glucose and β-hydroxybutyrate were analysed in 6 matched pairs of CSF and plasma, collected immediately before and 8 h after a single i.v. bolus of saline or dexamethasone (0.25 mg/kg live weight). Samples were analysed by a commercial laboratory (Gribbles Veterinary Pathology, Christchurch, NZ) as previously described (Chapter 6) and were a mixture of samples from across the several studies described in this chapter. The purpose of this was to assess the effect of dexamethasone on the movement of small molecules from plasma to CSF. However, CSF concentrations of β-hydroxybutyrate were below the assay detection limit (possibly due to sample degradation as some samples were several years old) so it was not possible to assess this for β-hydroxybutyrate. Two-tailed unpaired Student's *t*-tests were used to compare glucose concentrations in CSF and plasma, and β-hydroxybutyrate concentration in plasma of saline- and dexamethasone-treated sheep using GraphPad Prism version 6.01 for Windows (GraphPad Software Inc, La Jolla, California, USA, www.graphpad.com).

B-hydroxybutyrate concentration in plasma did not differ between groups prior to treatment (saline: 0.26 ± 0.05 mmol/L, dexamethasone: 0.26 ± 0.03 mmol/L). 8 h after treatment, concentrations of β-hydroxybutyrate increased in both groups, however this was not significant (saline: 0.34 ± 0.01 mmol/L, dexamethasone: 0.43 ± 0.07 mmol/L).

In time-matched samples, baseline concentrations of glucose did not differ between groups in plasma (4.4 ± 0.1, 4.4 ± 0.2 mmol/L) or CSF (3.0 ± 0.2, 3.0 ± 0.2 mmol/L) in saline- or dexamethasone-treated sheep, respectively. Plasma and CSF concentrations of glucose in control sheep were unchanged after 8 h (4.4 ± 0.03 and 3.1 ± 0.2 mmol/L, respectively). In contrast, dexamethasone increased glucose concentrations in both plasma (8.1 ± 0.9 mmol/L) and CSF (5.35 ± 0.7 mmol/L) at 8 h ($p < 0.05$ for both). Despite increases in glucose concentration in both fluids, the concentration ratio of glucose in plasma:CSF was unchanged following dexamethasone treatment (0.7 ± 0.06, 0.7 ± 0.03, 0 and 8 h, respectively), and was 0.7 ± 0.05 at both time points in control sheep.

Without any indication from CSF measures of β-hydroxybutyrate, it is difficult to ascertain whether dexamethasone or increased concentrations of CNP has any effect on the blood-brain barrier in this setting. However, the finding that there was no change in the concentration ratio of glucose in CSF:plasma suggests that the rate of transfer of glucose from the blood to CSF was unaltered by dexamethasone or raised levels of CNP, and was consistent with findings reported in

Chapter 4 — whereby the CSF:plasma ratio of both glucose and β-hydroxybutyrate was unaltered by the high concentrations of CNP that occur in plasma in pregnant sheep. Whereas Bohara *et al.* (2014) reported that supra-physiological doses of CNP reduced the presence and gene expression of blood-brain barrier proteins (zonula occludins-1), and increased the permeability of the BBB to sodium fluorescein in mice, an opposite effect on the BBB has been reported for dexamethasone (Hedley-Whyte & Hsu 1985). Therefore, in this case, is possible that any effect on the BBB by either dexamethasone or CNP could be countered by the other.

Chapter 7. Molecular forms of C-type natriuretic peptide in cerebrospinal fluid and plasma reflect differential processing in brain and pituitary tissues

Statement

This chapter contains a manuscript which has been formatted for submission to *Peptides*. Submission of this chapter is pending acceptance of the manuscript in Chapter 6. I was involved in the study design, animal procedures, statistical analysis and writing of the manuscript, and carried out all aspects of the laboratory work. The manuscript which forms this chapter benefitted significantly from contributions from all authors.

Molecular forms of C-type natriuretic peptide in cerebrospinal fluid and plasma reflect differential processing in brain and pituitary tissues

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7.1 Abstract

C-type natriuretic peptide (CNP) is a paracrine growth factor present in CSF at concentrations that far exceed its circulating levels in blood. It is widely distributed in the brain and both major lobes of the pituitary gland. Following our recent finding that an intravenous bolus of dexamethasone (0.25 mg/kg) in sheep acutely raises concentrations of CNP peptides throughout the brain as well as in CSF and peripheral plasma, we have characterised the molecular forms of CNP in cranial tissues following administration of dexamethasone or saline solution using size-exclusion high performance chromatography (SE-HPLC) and radioimmunoassay for CNP and NTproCNP. Three immunoreactive-CNP components were identified as proCNP (1-103), CNP-22 and CNP-53, and the presence and ratio of these different fragments differed between tissues. Similar profiles were obtained in CSF extracts from saline- and dexamethasone-treated sheep, whereby the predominant fragment was CNP-53, with CNP-22 present as a minor peak in 6 of 6 extracts. Profiles were similar for hypothalamic and posterior pituitary gland extracts, except an earlier eluting product (proCNP 1-103) was consistently present, and more abundant in profiles from dexamethasone-treated sheep — consistent with increased synthesis in these tissues. In contrast, SE-HPLC profiles of anterior pituitary gland and plasma extracts were devoid of CNP-22 and

contained mainly the CNP-53 and proCNP (1-103) fragments. This is the first report of the presence of proCNP (1-103) in plasma. The similarity in SE-HPLC profiles of plasma extracts and those from the anterior pituitary gland supports existing evidence for release of CNP from the anterior pituitary gland into the circulation.

7.2 Introduction

C-type natriuretic peptide (CNP) is a paracrine growth factor widely expressed in tissues of the central nervous system (CNS) where the peptide exhibits actions promoting neurogenesis[1], learning[2] and axonal connectivity[3]. Consonant with the prominence of CNP in CNS tissues, concentrations of CNP peptides in cerebrospinal fluid (CSF) greatly exceed those in plasma of humans[4] and sheep[5]. Concentrations are especially enriched in pituitary tissues[6,7], yet CNP concentrations in the systemic circulation are low and close to detection limits in adult mammals[8]. Although CNP in pituitary tissue appears to be co-located with gonadotrophic cells[9], its regulation, function and metabolism in this tissue are unknown.

CNP is synthesized as a 103 amino residue peptide (proCNP 1-103, proCNP) which is highly conserved in mammals[10]. The penultimate 22 amino acids form a ring structure at the carboxy terminal. ProCNP is cleaved intracellularly by furin[11] yielding a 53-amino acid residue peptide (proCNP 51-103, CNP-53) and an inactive amino-terminal fragment (proCNP 1-50, NTproCNP) which are likely to be secreted in equimolar proportions. CNP-53 is further cleaved – presumably extracellularly and at unknown sites – to CNP-22 (proCNP 82-103) which retains the ring structure and full biological activity. Both bioactive forms (CNP-53 and CNP-22) are subject to rapid degradation at source[12] whereas the inactive fragment NTproCNP remains intact for longer periods and is readily detectable in plasma prior to clearance by renal filtration and excretion[13].

Study of CNP in pathophysiology has employed immunoassays using antisera directed to proCNP 51-103 (therefore detecting both CNP-53 and CNP-22), or alternatively assays detecting the amino-terminal inactive fragment (NTproCNP) using antisera directed to proCNP 1-50. Depending on the objective, results from these assays add to our knowledge of CNP's regulation and metabolism but cannot specify the precise molecular form(s) – necessary for delineating pathways connecting production, secretion and metabolism. When analyses enabling identity of specific forms have been undertaken – for example using size-exclusion high performance liquid chromatography (SE-HPLC) to separate products by molecular size – the predominant form in most tissues expressing CNP has been CNP-53[8,14,15]. Notably, processing of proCNP appears to

differ in hypothalamic tissues (where both CNP-53 and CNP-22 residue forms are detected) and pituitary – in which CNP-22 is low or undetectable[15]. In plasma, when CNP immunoreactivity has been detected it has been attributed to CNP-22 based on gel permeation chromatography[8] although the low concentrations limit reliability and accuracy of analyses using SE-HPLC. To our knowledge, higher molecular weight forms such as proCNP have not been identified in plasma in any species, and no study has examined tissue and circulating molecular forms concurrently in the course of changes in CNP production.

An opportunity to characterize CNP molecular forms in tissues and associated fluids was recently provided by the availability of samples collected during the course of a study of the CNP response in brain and related tissue to dexamethasone (paper in submission). Compared with a saline injection, concentrations of CNP in brain tissues increased 2-4 fold and were commensurate with increases (3-4 fold) in CSF 8 h after a single injection of intravenous dexamethasone (0.25 mg/kg). In light of evidence of differential processing in hypothalamic and pituitary tissues[15], we hypothesized that molecular forms would differ in hypothalamic tissue and CSF when compared with forms in the pituitary gland and plasma. Accordingly we have used SE-HPLC and radioimmunoassay (RIA) to examine relative abundance of proCNP, CNP-53 and CNP-22, together with amino-terminal proCNP forms, in samples of tissue and associated fluids collected from controls and dexamethasone-treated sheep.

7.3 Methods

All procedures involving animals were carried out in accordance with the Animal Welfare Act 1999 (New Zealand) and were approved by the Lincoln University Animal Ethics Committee.

Animal procedures

Appropriate brain and pituitary gland samples suitable for HPLC-RIA analysis were obtained from 13 of the 14 healthy Texel-Romney wethers sampled in our recent study (Chapter 6). Details of animal housing and sample collection have been described previously (paper in submission). Briefly, CSF and plasma samples were collected prior to and 8 h after administration with intravenous (i.v.) saline (controls) or dexamethasone (0.25 mg/kg live weight), and sheep were sacrificed 8 h after the injection.

In the current study, after grazing on pasture at Lincoln University Research Farm, sheep were brought indoors for one week prior to the study and fed concentrated lucerne pellets

(SealesWinslow, Ashburton, New Zealand) and lucerne chaff at 0900 h every day at the maintenance nutritional level, with water provided *ad libitum*. At least 2 days before the study, the cisterna magna was catheterised under anaesthesia as previously described[16]. Sample collection began at least 2 days after the cannulation procedures. Groups of sheep (Supplementary Table 7.1) received a single dose of intravenous dexamethasone (Dexa 0.2, PhoenixPharm Distributors Ltd, Auckland, New Zealand, 2mg/mL, 0.25 mg/kg live weight) or saline solution (0.9 % w/v).

CSF and plasma sample collection

For study of the molecular forms of CNP using SE-HPLC-RIA, at least 10 mL of CSF and 40 mL of plasma were required for each analysis. This requirement was met on the control day (saline injection) by withdrawal of 7 (2 mL) CSF samples and jugular blood (5-6 mL) at intervals of not less than 20 minutes. For the sheep receiving dexamethasone, CSF and blood samples for SE-HPLC were collected at known peak response times of CNP and NTproCNP concentration, which were approximately 7-9 h and 4-6 h post treatment for CSF and plasma, respectively. Additional CSF samples were collected for measurement of CNP and NTproCNP immunoreactivity. Concentrations are listed in Supplementary Table 7.1. These samples were drawn immediately prior to injection of saline solution or dexamethasone and again at 8 h post injection. All plasma and CSF samples were rapidly placed on ice, centrifuged within 10 min and stored at -20 °C until assayed.

Tissue collection and extraction

After euthanasia by captive bolt stunning and exsanguination, brain and pituitary gland were rapidly removed. Approximately 0.5 g of tissue was excised from anterior pituitary gland, posterior pituitary gland and hypothalamus and rapidly frozen in liquid nitrogen. Later, tissues were treated as previously described (paper in submission), homogenised, centrifuged and then processed identically to CSF and plasma samples (Wilson *et al.* 2015). Tissue concentrations of CNP and NTproCNP immunoreactivity are listed in Supplementary Table 7.1.

Size-exclusion HPLC-RIA

CSF, plasma, and tissue homogenates from each sheep were extracted using three Sep-Pak C18 cartridges (Waters Corp., Milford, Massachusetts, USA) as previously described[17]. Tissue homogenate was extracted over a single cartridge, while CSF and plasma samples were extracted across several (3-5) cartridges. 100 µl of 20 % CH_3CN in 0.1 % trifluoroacetic acid (TFA) was added

to each extract, and extracts were vortex mixed then centrifuged at 780 g for one minute and combined, after which the total volume was spun at 3600 g for five minutes. 10 µl of detergent (0.1 % Triton™ X-100, Sigma-Aldrich, St Louis, Missouri, USA) was added to each collecting well on the HPLC block, which was then placed ready for collection.

Prior to loading, 20 % CH_3CN in 0.1 % TFA was run through the column. 50 µl of sample was injected onto the column with a Hamilton syringe, and fractions were collected at a rate of 0.5 mL/minute. Fractions from CSF and plasma were assayed for measurement of CNP and NTproCNP concentrations, and fractions from hypothalamic and pituitary extracts were assayed for CNP concentration only. Collected fractions were dried under an air stream and assayed as previously described for measurement of CNP and/or NTproCNP[5] using commercial CNP-22 antiserum (catalogue number G-012-03, Phoenix Pharmaceuticals, Belmont, California, USA) and rabbit antiserum J39 (raised locally against human proCNP (1-15), respectively. CNP-22 and CNP-53 exhibit equal cross reactivity with the antiserum provided by Phoenix Pharmaceuticals. The detection limit for each assay was 0.6 and 1.9 pmol/L for CNP and NTproCNP, respectively.

Chemicals and reagents

Purified standards of CNP-53 for SE-HPLC were obtained from Peninsula Labs (San Carlos, California, USA) and CNP-22 was obtained from BaChem (Bubendorf, Switzerland). NTproCNP standard was synthesised by Mimotopes Pty Ltd, Clayton, Victoria, Australia (proCNP 1-19, Lot #93001) and was reported to be > 95 % purity according to the manufacturers. The column was calibrated with four molecular weight markers: bovine serum albumin (BSA, 66.5 kDa), cytochrome C (12 kDa), aprotinin (6.5 kDa) and tyrosine (181 Da) (Sigma Aldrich, St Louis, Missouri, USA).

Statistical analysis

To calculate relative abundance, peptide concentrations in fractions corresponding to respective peaks were summed (i.e. area under the curve — AUC) and expressed as a percentage of the total CNP immunoreactants in the sample. Two-tailed unpaired Student's *t*-tests were used to detect significant differences in the respective proportions between groups in various tissues, using GraphPad Prism version 6.01 for Windows (GraphPad Software Inc, La Jolla, California, USA, www.graphpad.com).

7.4 Results

Elution profiles of CNP peptides and molecular weight standards following SE-HPLC are shown in Supplemental Figure 7.1. CNP-53 and CNP-22 eluted in fractions 27-29 and 34-36, respectively. Higher molecular weight forms consistent with proCNP eluted in fractions 24-25.

Hypothalamic and posterior pituitary tissue

In hypothalamic tissue, remarkably similar HPLC profiles were observed in 6 individual sheep (Figure 7.1). The major molecular form (i.e. largest peak) was CNP-53. Preceding this peak was a smaller peak, consistent with proCNP, and this peak appeared to be higher (although not statistically significant) in the 3 dexamethasone-treated sheep (Figure 7.1). Also, in all 6 cases, there was a clearly defined, late eluting, smaller peak consistent with CNP-22 (Figure 7.1). In posterior pituitary lobe tissues (Supplemental Figure 7.2) remarkably similar proportions of molecular forms consistent with proCNP and CNP-53 were observed in saline- and dexamethasone-treated sheep. Late eluting material (CNP-22) was more variable than observed in the hypothalamic extracts but was clearly identifiable in 6 of the 8 cases.

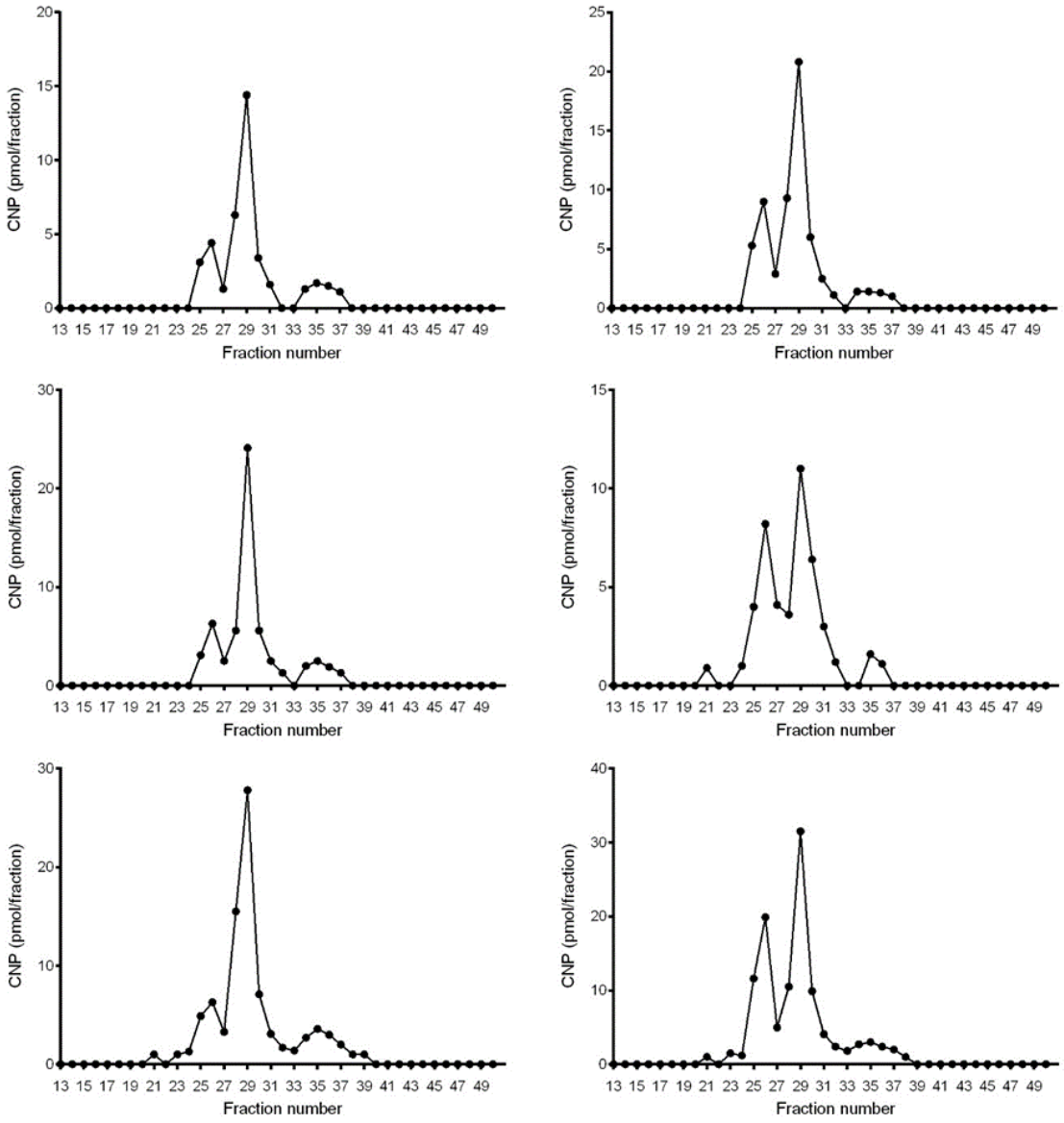

Figure 7.1 Size-exclusion HPLC profiles of CNP-immunoreactive fractions from hypothalamic tissue in saline-treated (left) and dexamethasone-treated (right) sheep.

Anterior pituitary tissue

In all 6 HPLC profiles for anterior pituitary gland tissue, the major molecular form was CNP-53 (Figure 7.2). Smaller peaks consistent with proCNP were also identified. In contrast, any products eluting in fractions 34-36 (CNP-22) were either very low in presence or undetectable. As noted in hypothalamic and posterior pituitary tissue (above), proCNP was consistently present and was increased following dexamethasone ($p < 0.05$).

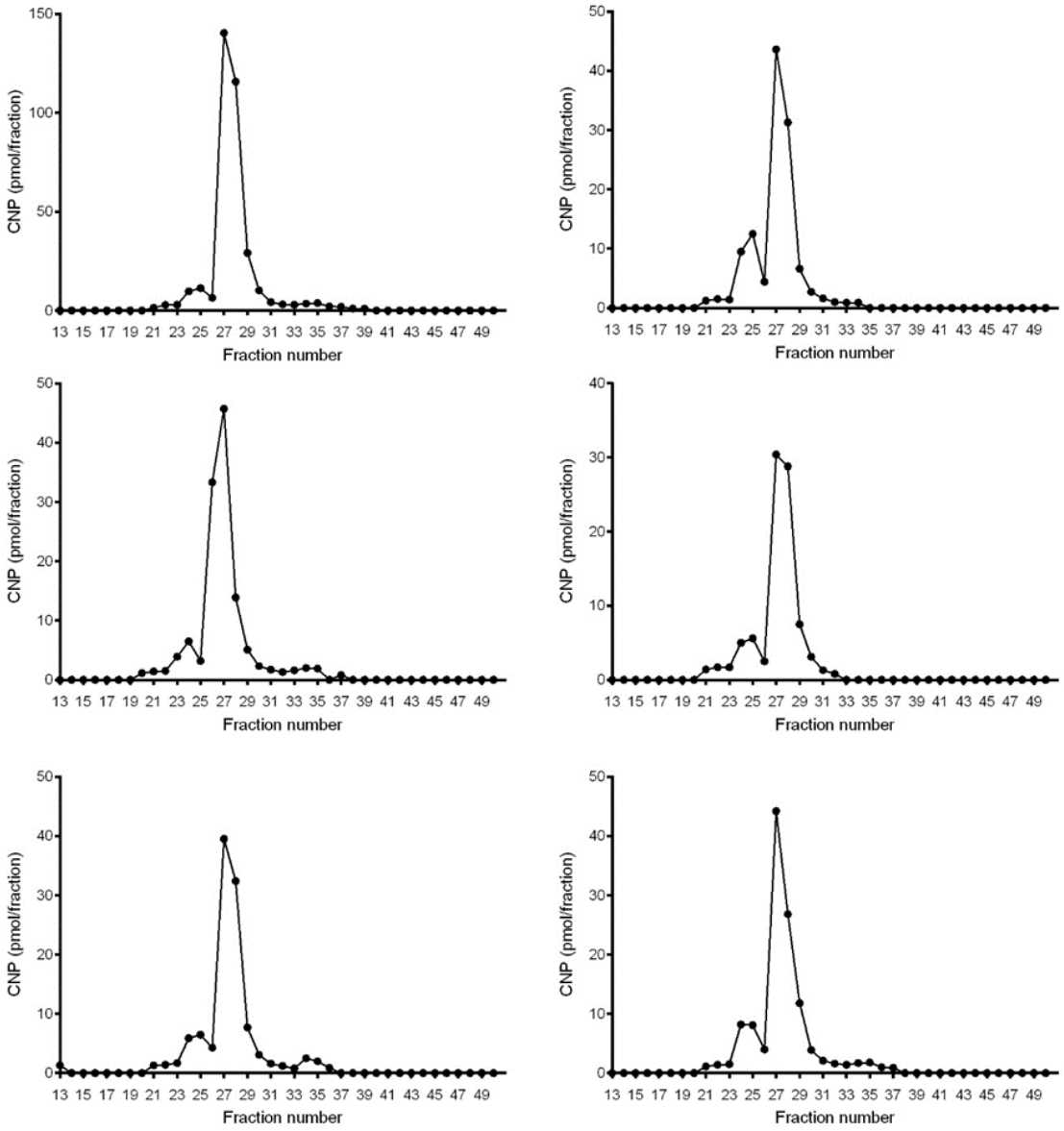

Figure 7.2 Size-exclusion HPLC profiles of CNP-immunoreactive fractions from anterior pituitary tissue extracts in saline-treated (left) and dexamethasone-treated (right) sheep.

CSF

CNP-53 was the dominant molecular form observed in HPLC profiles for CSF (Figure 7.3) and constituted a broad based peak, suggesting the presence of slightly smaller immunoreactive fragments of the molecule. A small peak, presumably CNP-22, eluted in fractions 34-36 all 6 cases. In contrast to hypothalamic and posterior pituitary tissues, peaks consistent with proCNP in the CSF profiles were variable, very small and not affected by dexamethasone. Immunoreactive NTproCNP profiles (Supplemental Figure 7.3) contained a major and clearly defined peak consistent with the 5 kDa peptide NTproCNP in all 8 cases, together with a smaller, later eluting product of lower molecular weight. There was no evidence of proCNP in any of the NTproCNP profiles for CSF.

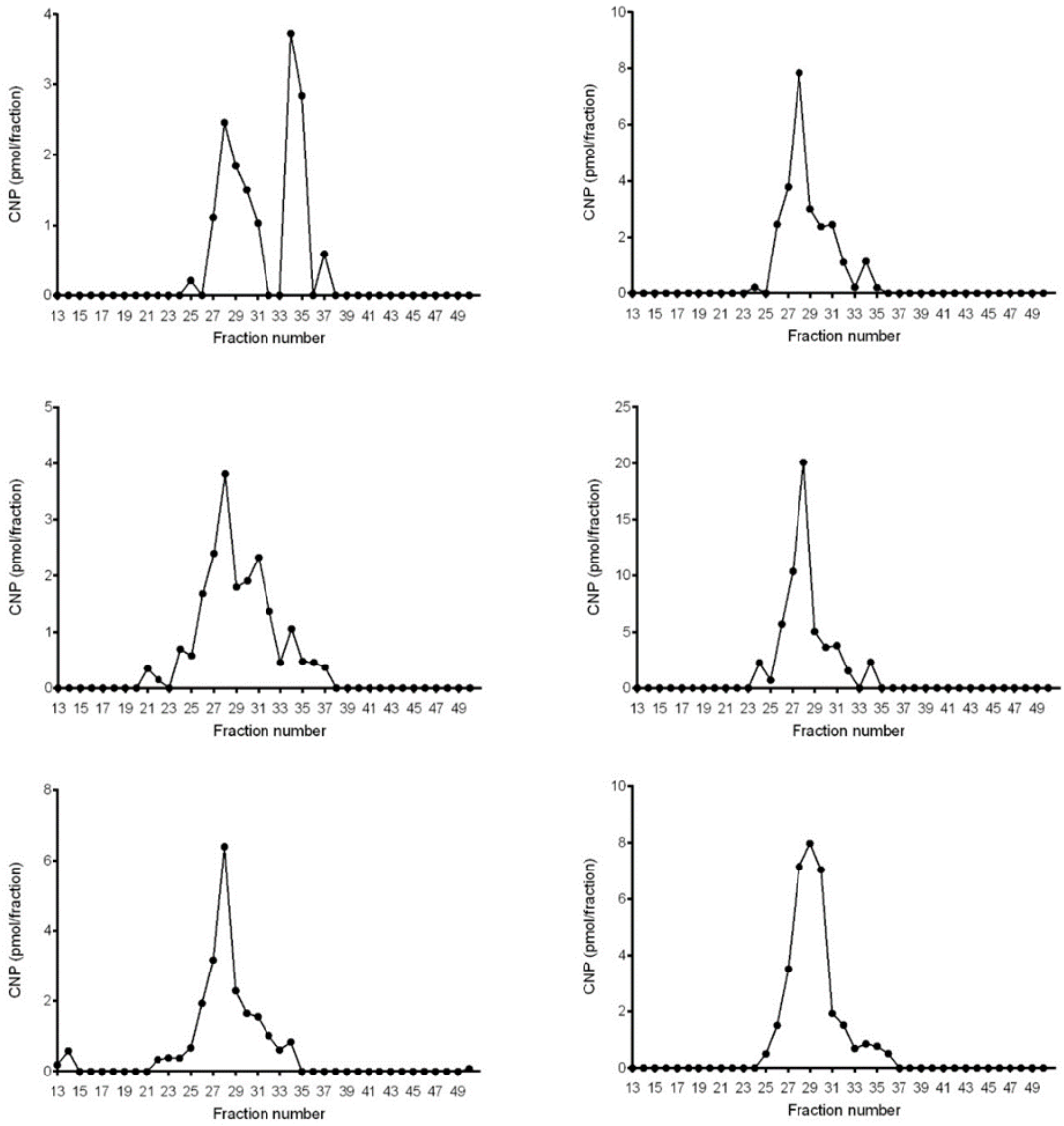

Figure 7.3 Size-exclusion HPLC profiles of CNP-immunoreactive fractions from CSF extracts in saline-treated (left) and dexamethasone-treated (right) sheep.

Plasma

As in the case of CSF, a broad immunoreactive peak consistent with CNP-53 was evident in HPLC profiles from plasma – preceded by a large amount of higher molecular weight peptide in fractions 24-25 in all 6 sheep, consistent with the presence of proCNP in this fluid (Figure 7.4). In 3 of the 6 cases (including both dexamethasone-treated sheep), the magnitude of the proCNP peak was similar to that of CNP-53. With the possible exception of profiles obtained after treatment with dexamethasone, CNP-22 was not identified in plasma. Immunoreactive NTproCNP HPLC profiles obtained from plasma are shown in Figure 7.5. The predominant peak eluting in fractions 26-31 was consistent with the 5kDa peptide NTproCNP 1-50. Two other smaller peaks were present in all 6 cases — one eluting early (fractions 23-25 consistent with proCNP) and the other being an unidentified late eluting form (fractions 33-35).

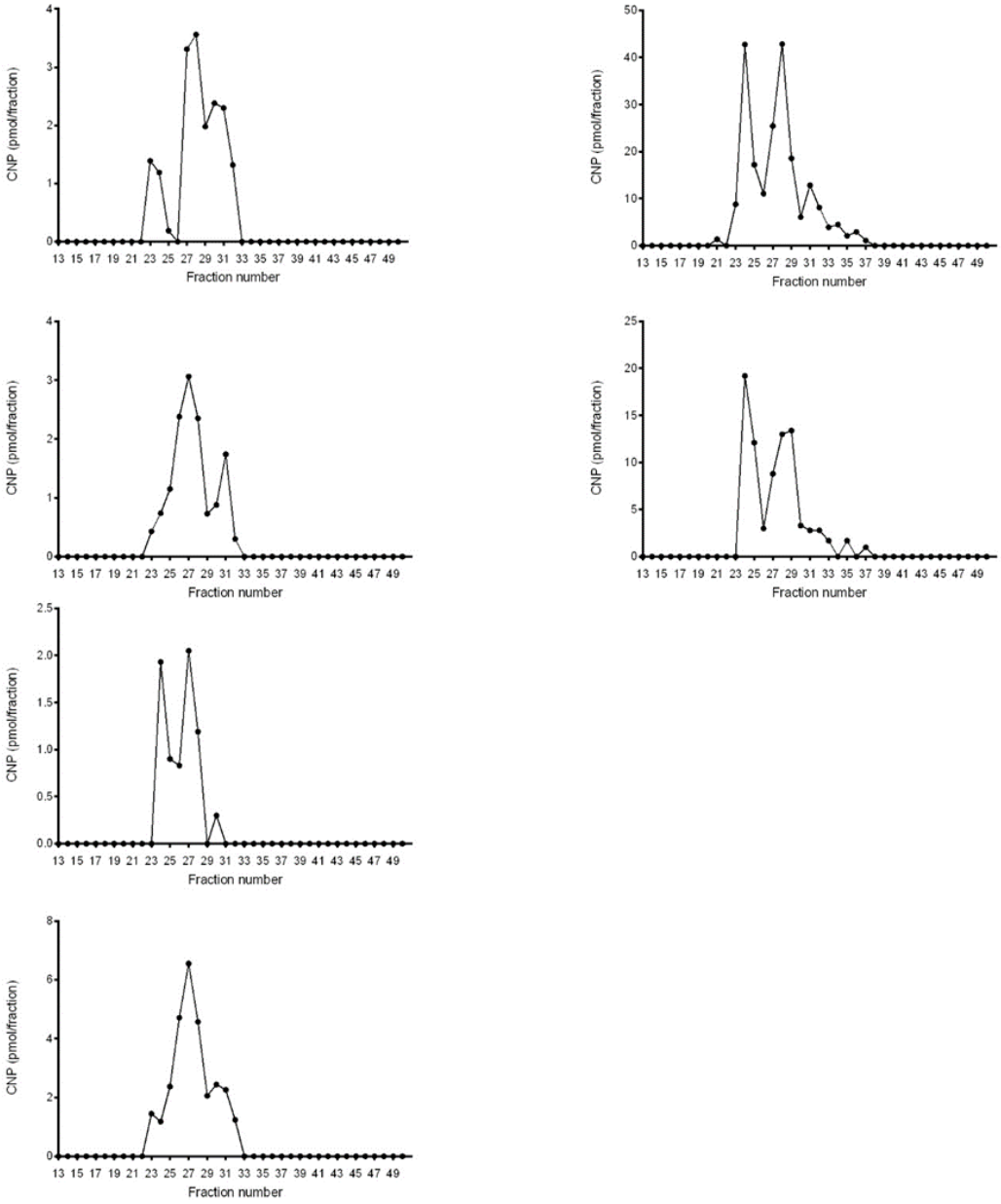

Figure 7.4 Size-exclusion HPLC profiles of CNP-immunoreactive fractions from plasma extracts in saline-treated (left) and dexamethasone-treated (right) sheep.

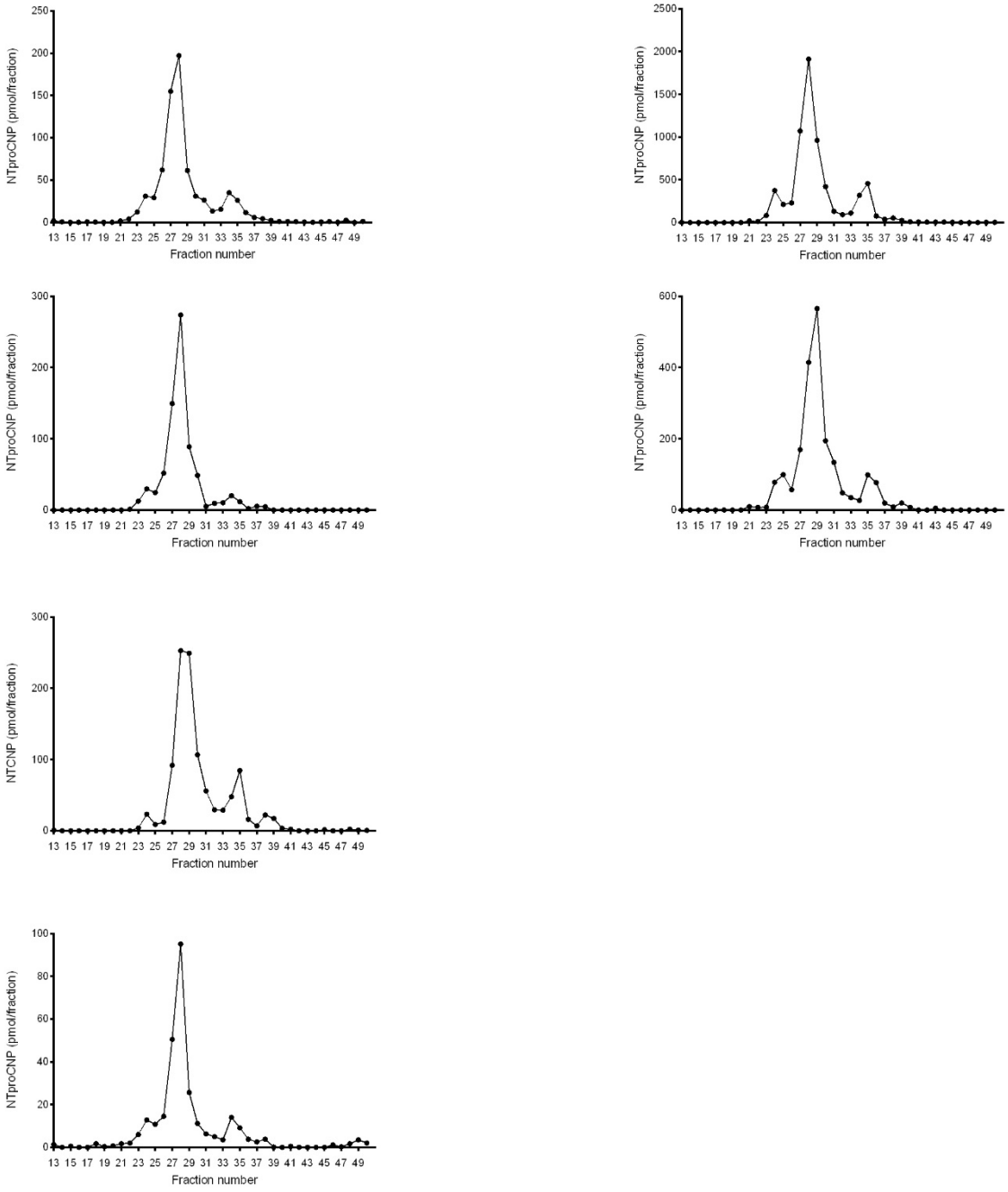

Figure 7.5 Size-exclusion HPLC profiles of NTproCNP-immunoreactive fractions from plasma extracts in saline-treated (left) and dexamethasone-treated (right) sheep.

Relative percentage abundance of CNP forms

Table 7.1 lists the relative contributions of each of the 3 molecular forms to CNP immunoreactivity in tissue and fluids analysed in saline- and dexamethasone-treated animals. CNP-53 is the most abundant molecular form identified in all extracts. Notably CNP-22 is markedly lower in anterior pituitary gland and plasma, but higher in hypothalamus, posterior pituitary gland and CSF. ProCNP is proportionately increased after dexamethasone in hypothalamus, anterior and posterior pituitary tissues and possibly in plasma, whereas in CSF the proportion of proCNP is low and falls even lower after dexamethasone.

Table 7.1 Percentage of CNP immunoreactivity attributed to different fragments in plasma, CSF, and tissue samples from sheep subject to size-exclusion HPLC, following dexamethasone or saline.

Astericks (*) indicate a significant difference ($p < 0.05$) in the proportion of the fragment in the respective tissue, between saline- and dexamethasone-treated sheep. Data are presented as mean ± s.e.

		ProCNP (1-103)	CNP-53	CNP-22	n
CSF	Control	6 ± 2.1	72 ± 9.3	22 ± 11.4	3
	Dex	2 ± 0.8	92 ± 0.8	6 ± 0.8	3
Plasma	Control	20 ± 6.6	80 ± 6.6	0 ± 0.0	4
	Dex	37 ± 4.5	57 ± 3.0	6 ± 1.4	2
Hypothalamus	Control	17 ± 3.7	68 ± 2.3	15 ± 1.5	3
	Dex	31 ± 4.6	61 ± 4.9	8 ± 1.9*	3
Anterior	Control	11 ± 2.0	84 ± 2.3	5 ± 0.3	3
pituitary	Dex	19 ± 1.7*	79 ± 1.9	2 ± 1.7	3
Posterior	Control	12 ± 0.7	71 ± 7.9	17 ± 8.5	4
pituitary	Dex	16 ± 3.5	69 ± 3.8	15 ± 6.8	4

7.5 Discussion

Despite the increasing recognition of CNP's role in CNS pathophysiology[18], there are only limited reports of the precise molecular forms of CNP – either in selected tissues or in circulating fluids such as plasma and CSF. When molecular forms are reported, commonly only a single analysis is shown and to our knowledge no study has characterised the CNP forms in multiple tissues in the course of interventions that stimulate CNP production. Mindful of the differential processing of proCNP in brain and pituitary tissue[15], we have taken advantage of a physiological setting where CNP secretion has been markedly stimulated over a short time frame (8 h), and show that the 3 molecular forms in tissues reproducibly carry their signature profiles into their respective circulations within and outside the blood-brain barrier. As postulated, proportions of these 3 molecular forms were similar in hypothalamus, posterior pituitary gland and CSF. Quite unexpectedly, profiles in anterior pituitary tissue closely simulated the profile in plasma and were distinguished by the virtual absence of CNP-22 and the presence of clearly defined peaks of the high molecular weight peptide, proCNP.

Using SE-HPLC immunoassay techniques, most reports identify CNP-53 as the major component in tissue extracts[6,8,14] with smaller but variable amounts of CNP-22. ProCNP has also been identified as a small component in some studies where a large amount of tissue has been extracted[19,20]. In sheep, we have previously identified small peaks of proCNP using SE-HPLC-RIA of CNP in whole pituitary extracts (unpublished) and in placenta[21] both of which tissues have high CNP abundance. In contrast, neither rat nor fetal lamb tissue extracts contain detectable levels of proCNP when measured by SE-HPLC-RIA of NTproCNP[22,23]. These discrepancies are likely to be due to weight of tissue extracted, CNP enrichment and the relative sensitivity of the immunoassay employed.

In the current study, although CNP-53 was the major molecular form, clearly defined peaks of proCNP were found in all studies — excepting in extracts of CSF. Notably proCNP — but not other forms — was proportionately increased by dexamethasone, compared with control (saline). These observations are consistent with a direct and prompt effect of dexamethasone upregulating *NPPC* expression in both hypothalamic and posterior pituitary gland tissues (paper in submission), and with delayed processing to smaller forms — although rapid degradation of lower molecular weight forms cannot be excluded. As previously reported in sheep hypothalamus[15], CNP-22 was clearly defined as the late eluting peak in the present study. However the concentration ratio of CNP-22 to CNP-53 (1:5) differed from that reported previously[15] (1:1). CNP-22 was also identified in posterior pituitary tissue. It constituted a prominent peak in several of the profiles but was undetectable in 2 out of the 8 studies. Such variation could be related to

uneven distribution of CNP-22 along the length of the hypothalamo-hypophyseal stalk, which might also account for the lower relative abundance of CNP-22 in the hypothalamus if the plane of excision of this tissue was not uniform. In extracts of CSF, although CNP immunoreactivity was much lower than found in hypothalamic fractions, CSF SE-HPLC profiles were similar except for the very low content of proCNP which was also undetectable in assays using NTproCNP. Presumably proCNP, even when enriched by dexamethasone, is processed prior to exocytosis so little enters the extracellular space from either hypothalamic or posterior pituitary lobe tissue.

This is the first report of CNP molecular forms in ovine CSF, profiles of which strongly resemble those reported in humans[4]. CNP-53 was the major component — contributing some 70-90 % of the total immunoreactivity although the broad and irregular late eluting material observed in some of the profiles is likely to represent smaller degraded immunoreactive products. CNP-22 was identified in all 6 CSF profiles — and in one was a major peak — and was more conspicuous than seen in the single study of human CSF[4]. Profiles derived from assays of NTproCNP are almost identical in sheep and human in showing a predominant peak (fractions 26-32) consistent with the 5 kDa form (NTproCNP), and a much smaller but consistent, unidentified, peak eluting in fractions 33-35 which has been observed previously in sheep plasma[24].

Molecular forms of CNP in the anterior pituitary gland, and possible links with those in plasma were of special interest not least for the unusually high abundance of immunoreactive CNP in the anterior lobe[6,7] but also the apparent lack of processing of CNP-53 to CNP-22[15]. We have recently confirmed the unusually high content of CNP in ovine anterior lobe tissue (paper in submission) which is some 15-fold greater than was found in hypothalamic tissue. Similar enrichment (10-fold) is reported in rat anterior pituitary tissues[6]. Here we confirm that CNP-53 is the predominant form. Smaller peaks of proCNP were also present in SE-HPLC profiles from the anterior pituitary gland, increasing after dexamethasone in all 3 cases – which aligns with the marked increase in *NPPC* expression in this tissue after dexamethasone (paper in submission). CNP-22 content of the anterior lobe profiles – in contrast to clearly defined peaks in profiles from hypothalamic tissue – was very low or absent but trended towards even lower relative abundance after dexamethasone. A similar trend was also noted for hypothalamic tissue. As previously reported (Supplemental Figure 6.2) the ratio of NTproCNP:CNP is much higher in the hypothalamus (5:1) compared with both the anterior and posterior pituitary glands (1:1) — consistent with higher degradation rates of CNP in the former tissue. Since we find no difference in *NPRC* expression in the two tissues (Figure 6.6), hydrolysis by neprilysin, prominently expressed in brain tissues[25] may

account for these observations. However to our knowledge, neprilysin, which cleaves CNP-22 at multiple sites, does not affect generation of CNP-22 from CNP-53 or proCNP[12]. Clearly, further work is required to clarify the mechanism regulating CNP-22 production and its role in pathophysiology which would logically be pursued in hypothalamic tissues.

Quite unexpectedly, profiles using SE-HPLC-RIA CNP assays of plasma show a virtual absence of CNP-22, yet major peaks of a high molecular weight form — consistent with the presence of proCNP — in all 6 samples. Smaller but clearly detectable peaks were visible in the same fractions using RIA NTproCNP assays. This co-location rules out the presence of proCNP 1-81 (a putative cleavage by-product of CNP-22 formation) since the antiserum in the CNP assay is solely directed to the C-terminal ring structure of proCNP. Differences in sensitivity of the two assays (lower for NTproCNP) presumably account for the differences in peak size of proCNP, and also may explain in part the absence of detectable proCNP in CSF using SE-HPLC-RIA for NTproCNP. Prominent peaks consistent with CNP-53 were evident in all 6 studies of plasma. Previous reports of CNP immunoreactive forms directed to the C-terminal ring in human plasma find only CNP-22[8,26]. Low concentrations of bioactive CNP immunoreactivity in plasma in most species limits the ability to characterise molecular forms but when plasma concentrations are high, as in pregnant sheep[27], both CNP-53 and CNP-22 were identified. On the other hand, as found in the present study, antisera directed to the N terminus of proCNP consistently detect a 5 kDa peptide which is likely to be proCNP 1-50 or closely related peptide. The smaller unidentified peak eluting in fractions 33-35 — also found previously in sheep plasma[27] and consistently found here in CSF — likely represents a degraded product of proCNP of molecular weight similar to CNP-22 but containing residues 1-15 of the prohormone. Taken together, our findings of proCNP and CNP-53 — but not CNP-22 — in ovine plasma strongly suggest that the very high abundance of both of these high molecular weight forms in the anterior pituitary gland is the likely source. Consistent with this is the relative increase in proCNP after dexamethasone in both anterior pituitary and plasma — linkages that are not observed in hypothalamic or CSF profiles.

In further support of active secretion from anterior pituitary stores are our previous findings; where plasma concentrations of both CNP and NTproCNP increase markedly after dexamethasone, yet content of immunoreactive CNP in the anterior pituitary gland was unaffected — even when *NPPC* was clearly upregulated by dexamethasone (paper in submission). The absence of CNP-22 in plasma — a further signature of pituitary origin — suggests that processing from larger forms to CNP-22 does not occur in the systemic circulation at least in adult

sheep. Although rapid degradation of CNP-22 in blood cannot be excluded, this is unlikely to explain its absence as clearly detectable peaks of CNP-22 are observed in CSF, whereby CNP concentrations are typically only 3-fold higher than in plasma. It will be instructive to further define molecular forms in plasma and other tissues in other mammals in the light of these findings which have the potential to illuminate links between CNP production and sites of CNP-22 generation in tissues and its concentration in the systemic circulation.

7.6 References

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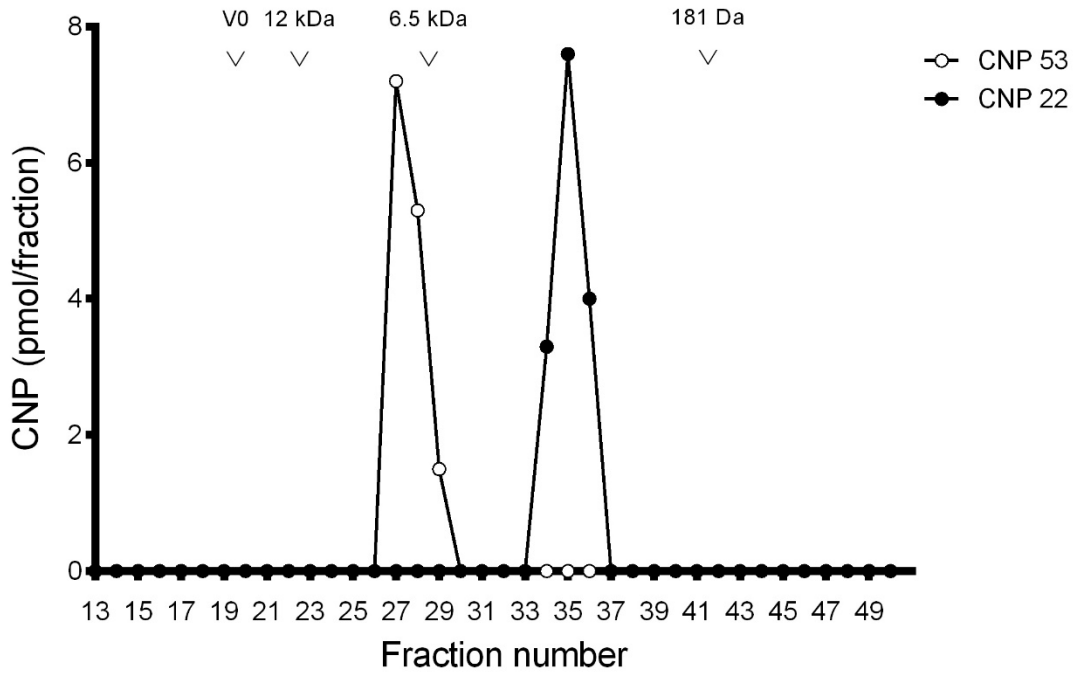
Supplementary material

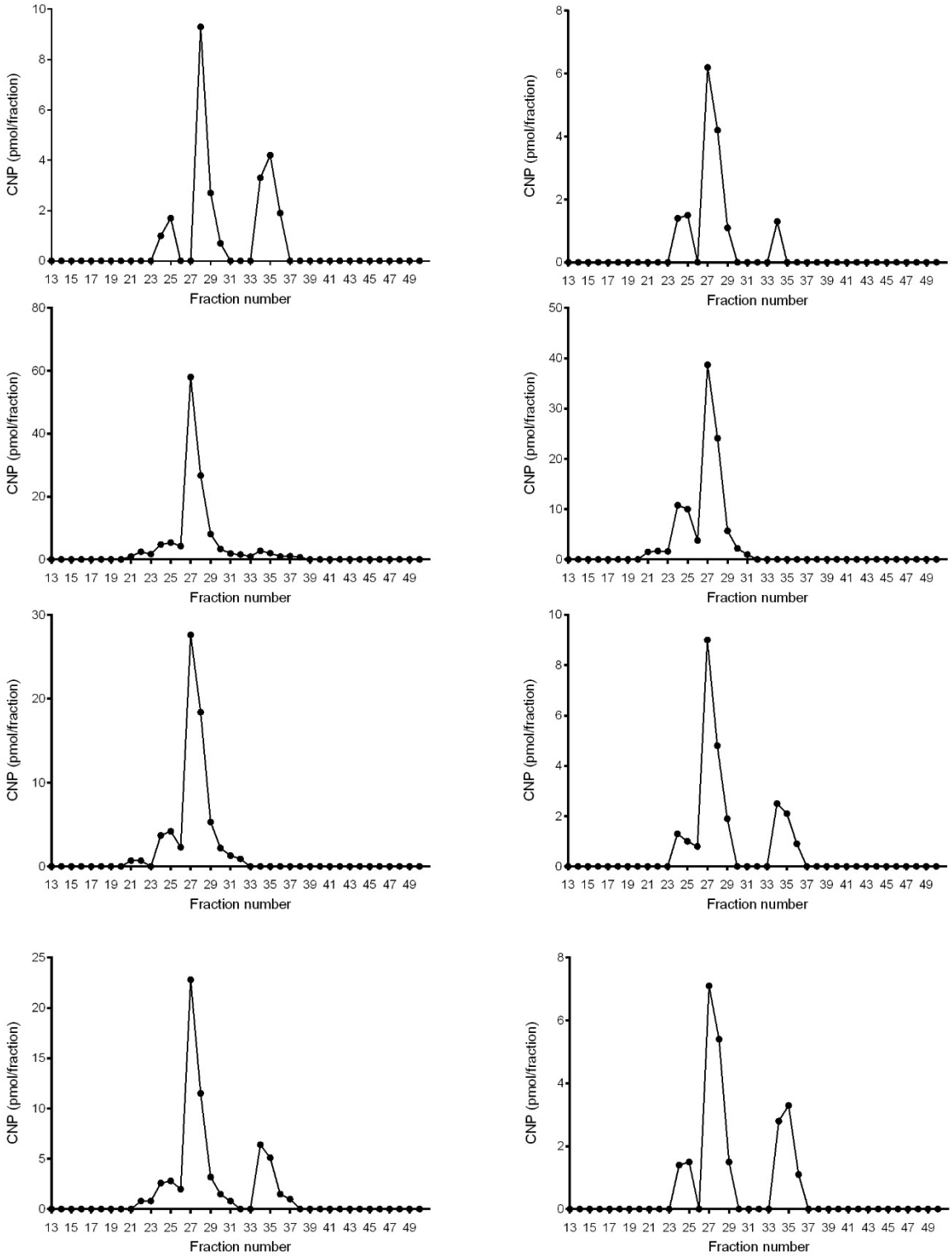
Supplementary Table 7.1 Mean (± s.e.) concentration of CNP and NTproCNP in tissues subject to size-exclusion HPLC from saline- and dexamethasone-treated sheep.

Analyte	Treatment	**Hypothalamus** (pmol/g, n = 3/group)	**Posterior pituitary** (pmol/g, n = 4/group)	**Anterior pituitary** (pmol/g, n = 4/group)	**CSF** (pmol/L, n = 4/group, 0 and 8 h , respectively)
CNP	Saline	3.24 ± 0.3	21.49 ± 6.3	21.28 ± 0.7	2.23 ± 0.05, 2.93 ± 0.6
	Dexa	5.91 ± 1.0	19.43 ± 2.9	30.97 ± 4.0	3.13 ± 0.6, 11.36 ± 3.6
NTproCNP	Saline	2.55 ± 0.5	27.13 ± 7.8	22.15 ± 1.5	849.23 ± 68, 976.08 ± 50
	Dexa	50.11 ± 13.2	26.62 ± 5.1	54.11 ± 8.8	888.7 ± 38, 1398 ± 33.8

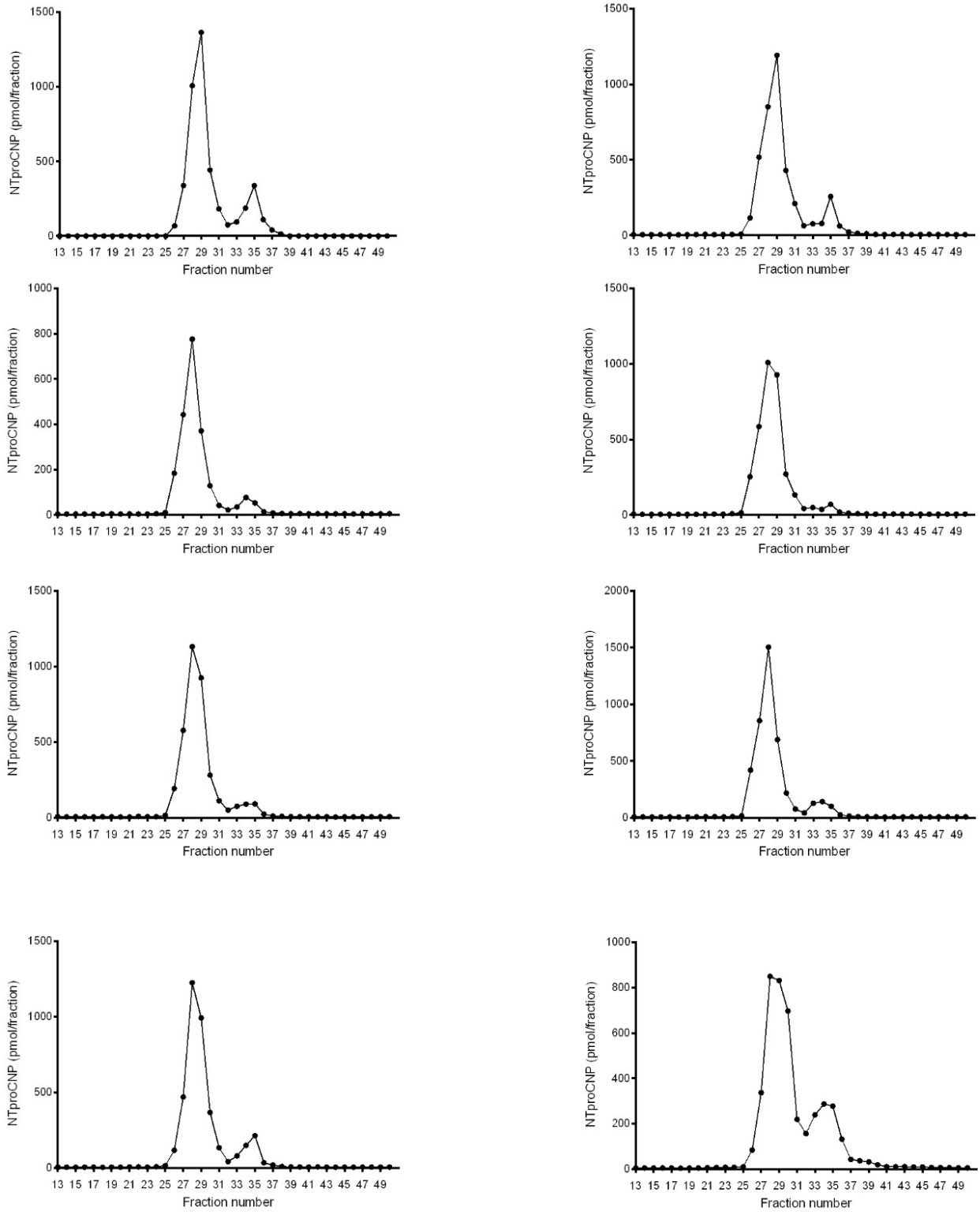


Supplemental Figure 7.1 Size-exclusion HPLC profiles of ovine CNP-53 (fractions 27-29) and CNP-22 (fractions 34-36) standards.

Molecular weight markers are indicated by arrow heads; bovine serum albumin: V_0, cytochrome C: 12 kDa, aprotinin: 6.5 kDa, tyrosine: 181 Da



Supplemental Figure 7.2 Size-exclusion HPLC profiles of CNP-immunoreactive fractions from posterior pituitary gland extracts in saline-treated (left) and dexamethasone-treated (right) sheep.



Supplemental Figure 7.3 Size-exclusion HPLC profiles of NTproCNP-immunoreactive fractions from CSF extracts in saline-treated (left) and dexamethasone-treated (right) sheep.

Chapter 8. Final discussion and future directions

8.1 Major contributions of this thesis

The findings of this thesis contribute significantly towards elucidating the abundance of CNP in the CNS, by relating changes in CSF concentrations of CNP peptides to their distribution, form and regulation in related tissues. *In vivo* studies such as those described in this thesis are necessary as they are an essential part of establishing fundamental aspects of physiology — particularly as CNP may be an invaluable therapeutic and/or diagnostic tool in certain settings of central pathophysiological disorders (Mahinrad *et al.* 2016). While numerous studies have mapped out sites of *NPPC* and *NPRB* expression in the CNS (Minamino *et al.* 1991, Yeung *et al.* 1996b, Moriyama *et al.* 2006), relatively little has been reported on sites and abundance of CNP peptides themselves. Advancing our knowledge about the regulation of CNP synthesis *in vivo* and identifying central sources of CNP in the brain is part of the crucial groundwork necessary to allow for the development of practical applications in future.

The demonstration that markedly elevated concentrations of CNP and NTproCNP in the circulation — as occurs during ruminant pregnancy — do not result in increased levels in the CSF, is supportive of the hypothesis that central sources of CNP peptides exist (Chapter 4). This observation is in agreement with the single report by Schouten *et al.* (2011) describing concurrent levels of CNP peptides in human CSF and plasma, where concentrations were independent in the respective fluids. Prior to this study, there were no known physiological states or compounds capable of acutely altering concentrations of CNP peptides in CSF or nervous tissue. A method for cannulation of the cisterna magna was developed to facilitate repeated collection of CSF samples from conscious sheep (Chapter 3), following which a series of pilot studies were conducted in order to identify a compound or physiological state capable of altering CSF concentrations of CNP peptides (5.1.2). The consequences of the finding that CSF concentrations of CNP peptides concentration were not altered during or after anaesthesia were two-fold; firstly, it added to the growing body of evidence that CNP peptide concentrations in CSF are remarkably stable and presumably necessary for some aspect of brain homeostasis. Secondly, it established that anaesthesia was unlikely to be a confounding factor in studies where anaesthesia was necessary for obtaining single samples of CSF.

Upon finding that a single intravenous dose of dexamethasone resulted in elevated concentrations of CNP peptides in CSF (and plasma), the change in concentration of CNP peptides

in response to different doses of dexamethasone was characterised in the respective fluids (Chapter 6) which indicated a dose-response relationship with dexamethasone and a differential response between CSF and plasma. Hypothesising that the increase in CSF concentration of CNP peptides was a result of increased peptide synthesis and release into extracellular fluid/CSF from one or more regions of brain tissue, CNP and NTproCNP concentration was measured in a wide selection of tissues sampled from brains of dexamethasone- and saline-treated sheep (Chapter 6). This revealed a widespread response to dexamethasone in central tissues. The demonstration that gene expression levels of *NPPC* increased in response to stimulation with dexamethasone suggests that increased peptide concentration is a result of increased synthesis of CNP peptides. Of the natriuretic peptide family, this effect was shown to be specific to CNP, as no response was shown for ANP or BNP in plasma, CSF and brain tissue. The measurement of the gene expression levels of *NPRB* and *NPRC*, which encode for the CNP receptor and clearance receptor, respectively, provided an insight into CNP signalling and clearance pathways in response to dexamethasone. Characterisation of different molecular size forms of CNP through size-exclusion HPLC revealed different profiles and proportions of the respective forms in CSF, plasma, brain tissue and anterior and posterior pituitary glands (Chapter 6).

These studies establish multiple sources for CNP in the CNS. The identification of dexamethasone as a secretagogue for CNP in the CNS implicates CNP as a potentially important mediator of glucocorticoid-mediated pathways. Together, these findings highlight a difference in the processing of proCNP among brain and anterior and posterior pituitary glands and portray the pituitary gland as a major peripheral source. The novel contributions of this thesis are outlined in Table 8.1.

Table 8.1 Summary of novel findings presented in this thesis.

The demonstration that both CSF and plasma concentrations of CNP and NTproCNP remained stable throughout anaesthesia
The finding that markedly elevated plasma concentrations of CNP and NTproCNP throughout gestation in sheep do not lead to increased concentrations in CSF
Demonstration of independent regulation of peripheral and central levels of CNP and NTproCNP in pregnant sheep and red deer stags
First report of a stimulus (dexamethasone) capable of acutely increasing CSF concentrations of CNP and NTproCNP
The finding that concentrations of CNP and NTproCNP are increased in multiple brain tissues following dexamethasone administration
The demonstration that the stimulating effect of dexamethasone on peptide synthesis is specific to CNP, as ANP and BNP concentrations were unchanged in plasma, CSF and brain tissue
Demonstration of increased *NPPC* expression in brain tissues following dexamethasone, suggesting that increased peptide concentrations reflect increased synthesis
The finding that the ratio of NTproCNP: CNP in the anterior and posterior pituitary gland (1:1) is markedly different from the ratio in brain tissues (5:1 to 10:1)
Evidence from differing HPLC profiles suggesting that CNP processing differs between the anterior and posterior pituitary gland
Identification of large irCNP fragments in plasma, consistent with proCNP (1-103)
The finding that *NPRC* gene expression levels are similar between brain and both lobes of the pituitary gland— despite large differences in the NTproCNP:CNP ratio between the brain and pituitary gland

8.2 Elucidating the role of CNP in the central nervous system: future studies

The identification of dexamethasone as a stimulant of CNP synthesis is a crucial step towards elucidating the abundance of CNP peptides in the CNS. Now that a secretagogue for CNP peptides in brain tissue has been identified, dexamethasone can be used to facilitate studies designed to answer existing questions, as well as ones that arose from this study.

8.2.1 Identification of sources of CNP in brain at the cellular level

In order to determine the central role/s of CNP *in vivo*, the next key step will be to identify specific cell types which synthesise CNP following dexamethasone stimulation. A comparison of brain tissue from both dexamethasone- and saline-treated sheep using double-labelling immunohistochemistry techniques will be necessary to identify cells which show an increased presence of CNP and/or NTproCNP, whilst simultaneously confirming the cell type through the use of specific markers for neurons (e.g. neurotrace), subtypes of glial cells (e.g. glial fibrillary acidic protein, GFAP) and cerebral capillary endothelial cells (e.g. vascular endothelial growth factor, VEGF).

There are many possible candidates for the central source of CNP peptides, because *NPPC* expression and the CNP peptide have been measured in both neuronal and glial cells — as previously discussed. Had there been only one region in the CNS identified as being responsive to one of the well-characterised secretagogue candidates with known specific actions — such as l-deprenyl — it may have been easier to narrow down the precise (presumably neural) pathways and mechanisms by which CNP secretion was stimulated. Given that glucocorticoids have many diverse effects on brain function, there is a large array of possible mechanisms that lead to the increase of CNP peptide concentration in brain tissue and CSF following dexamethasone administration to sheep. It has been estimated that glucocorticoids regulate between 10 and 100 genes per cell (Hayashi *et al.* 2004). However, the finding that stimulation with dexamethasone increased the concentration of CNP and NTproCNP in multiple tissues sampled throughout the brain suggests that responding cells may be those that are common across the respective stimulated regions, e.g. pericytes, astrocytes, microglia or brain capillary endothelial cells.

Originally it was thought that the only function of glial cells was to provide structural support for neurons. However, it is becoming increasingly clear that glial cells are involved in the regulation of a multitude of functions including fluid, ion, pH and transmitter homeostasis plus development and the processing of synaptic information (reviewed by Sofroniew & Vinters 2010). Some of these actions, including the regulation of cerebral blood flow, water balance and neuroinflammation are common to both NPs (Prado *et al.* 2010) and dexamethasone (Leenders *et al.* 1985, Tajima *et al.* 1990, Sorrells *et al.* 2009). The substantial presence of CNP in astrocytic endfeet of rat Müller cells — which are essentially retinal astrocytes which wrap around ganglion cells and their fibres and blood vessels (Newman & Reichenbach 1996) — suggests a role for CNP in glia-ganglion cell communication, and/or the regulation of blood flow in vessels/intraocular pressure (Cao *et al.* 2004). Also, CNP (but not ANP) is capable of stimulating cGMP accumulation in cultures of rat astrocyte cells (Kobayashi *et al.* 1993).

Dexamethasone is known to alter cerebral blood flow in some settings, although the mechanism is unclear (Leenders *et al.* 1985). It is possible that CNP is secreted by astrocytes following stimulation by dexamethasone and acts to alter blood flow in cerebral vessels. *NPRB* transcripts are expressed in the human retina, providing evidence for a local NP system (Rollín *et al.* 2004), which is supported by Fernández-Durango *et al.* (1999) who demonstrated that CNP potently stimulated guanylate cyclase activity in ciliary process membranes, and decreased intraocular pressure in rabbits. It is plausible that CNP has a similar effect on glial cells that exist outside of

the retina. Of note, cell water content of glial cells is reduced following exposure to ANP by approximately 30 % (Latzkovits *et al.* 1993).

It is also relevant to note that monocytes and macrophages are capable of secreting natriuretic peptides (Naruko *et al.* 1996). Whether microglia — which originate from monocyte precursors and constitute the brain immune cells (Ginhoux *et al.* 2013) — are sources of CNP in this setting is worth investigating. Although glucocorticoids are known for their anti-inflammatory properties, recent studies have shown that glucocorticoids can mediate pro-inflammatory effects in certain settings — particularly in the CNS (reviewed by Sorrells *et al.* 2009). This mostly occurs during chronic stress (Sorrells *et al.* 2009), however in the absence of injury, acute stress can activate inflammatory mediators in the CNS, for example increases in prostaglandin E_2 in the rat cerebral cortex occur following psychological stress induced by immobilisation (Madrigal *et al.* 2003). Microglia can be 'primed' by glucocorticoids, such that the cells can undergo changes and become 'sensitised' where although they do not immediately produce an inflammatory response, they produce exaggerated levels of inflammatory mediators when they are further stimulated (Frank *et al.* 2010). Using a murine restraint model of psychological stress and corticosterone administration, it has been shown that both glucocorticoids and stress are capable of activating microglia via the glucocorticoid receptor (GR), and these are blocked by GR antagonists (Nair & Bonneau 2006). It is relevant to note here that dexamethasone is a specific agonist of the GR, and its affinity for the GR is 6- to 10-fold greater than that of cortisol (Arriza *et al.* 1988, Spencer *et al.* 1990). In contrast, the mineralocorticoid receptor (MR) has a high affinity for cortisol (Funder 1997), and none for dexamethasone (Rebuffat *et al.* 2004). Frank *et al.* (2010) suggested that the GR may be the predominant mediator of corticosterone-induced sensitisation of pro-inflammatory processes. Whether CNP contributes to the priming of microglia is now a key question.

Identifying which cell type(s) in the CNS are stimulated to produce CNP following stimulation with dexamethasone may provide an insight into the actions of CNP downstream. As this is a key line of future investigation, some preliminary efforts were directed towards double-staining fluorescent immunohistochemistry, whereby sheep brain tissues were labelled using antibodies for CNP and NTproCNP, neurons (neurotrace) and glial cells (GFAP). This staining was inconclusive due to strong background staining and could not be pursued further because of time and resource constraints. Strong immunohistochemistal staining of tissues with DAB (3,3'-Diaminobenzidine) showed some promise as some individual cells could be identified, however

the staining of even thin sections of wax-embedded tissue (3-5 µm) was too strong to discriminate between most cells, so this was also inconclusive.

Furthermore, pinpointing the mechanism by which dexamethasone administration leads to increased concentrations of CNP peptides in brain tissues and in the CSF will be revealing. Although some rapid effects of glucocorticoids have been attributed to non-classical signalling via receptors located on the cell membrane of neuronal cells in specific regions (reviewed by Tasker *et al.* 2005), the upregulation of *NPPC* in brain tissue following dexamethasone implicates classical glucocorticoid receptor signalling in the central regulation of CNP synthesis — at least partially. Currently, it is unknown whether CNP synthesis is increased by direct upregulation of *NPPC* by dexamethasone, or whether this occurs indirectly, i.e. as a response to altered changes in fluid dynamics such as CSF pressure, volume, or shear stress. To remove the latter influences, dexamethasone-induced CNP secretion should be measured *in vitro* in cultures of various cell types. This would also allow studies of steroid specificity, modulators and the effect of alterations in related receptor levels.

8.2.2 The effect of changes in physical fluid dynamics

Considering that dexamethasone can alter physical factors of fluids in certain settings — i.e. intracranial pressure and cerebral blood flow (Behrens *et al.* 1998, Rohde *et al.* 2015), and is routinely used to decrease intracranial pressure in patients with brain tumours (Rangel-Castillo *et al.* 2008 — it would be useful to determine the effect of changes in these properties on CSF concentrations of CNP peptides, and vice versa, via central administration of CNP. *In vitro* studies reveal opposing effects of CNP and dexamethasone on the expression of water channel aquaporin-4 (AQP4), which supports the possibility that CNP may act to counterbalance some actions of dexamethasone; AQP4 expression is upregulated by CNP in cultured astrocytes (Miyajima *et al.* 2004) and downregulated by dexamethasone in the perihematomal area of rats with intracerebral haemorrhage (Gu *et al.* 2007).

Although such studies would be valuable, they would be expensive due to the large amount of peptide needed to substantially alter CSF concentrations in sheep, and the technology required — such as functional magnetic resonance imaging. For these studies, it would be advantageous to identify a compound capable of suppressing secretion of CNP in brain tissue, and/or inhibiting its action, so that the effect of a decrease in CNP peptide concentration or activity on these physical aspects could also be studied.

When investigating the influence of CNP on cerebral blood flow, it will be necessary to bear in mind previous ovine studies which support a role for CNP in fetal-maternal signalling and effects on blood vessel delivery of nutrients to the fetus. In parallel to the observation that central and peripheral concentrations of CNP peptides are independently regulated, it has been shown that fetal and maternal plasma concentrations of CNP peptides are also independently regulated in sheep (Prickett *et al.* 2007). Trophoblast binucleate cells, which are unique to ruminants and facilitate the transport of fetal products into the maternal circulation, have been identified as an important site of CNP production (McNeill *et al.* 2011). This and other evidence implicates CNP as an important fetal-maternal signal that serves to favour fetal growth. Maternal circulating concentrations of CNP peptides are increased following nutrient restriction in early (Madhavan *et al.* 2015) and late gestation (Prickett *et al.* 2007), and are positively correlated with fetal number, which can also be seen as a form of caloric restriction (McNeill *et al.* 2009). Whether there are parallel roles for CNP in meeting the metabolic demands of neural tissue and the fetus by affecting blood supply in the brain and placenta, respectively, remains to be seen.

8.2.3 Sources and function of CNP in the pituitary gland

The novel finding that the concentration ratio of NTproCNP:CNP differs markedly in both the anterior and posterior pituitary gland (both 1:1) compared with other brain regions (5:1 to 10:1) is of significance for several reasons. Firstly, it highlights the importance of measuring NTproCNP in studies involving CNP. Prior to this finding, it was widely accepted that the highest concentrations of CNP in any tissue existed in the pituitary gland, following reports that concentrations of CNP in the anterior and posterior pituitary gland of rats exceeded those in the brain by approximately 13- and 3-fold, respectively (Komatsu *et al.* 1991). However, the data provided in Chapter 6 indicate that although CNP concentration is higher in the anterior and posterior pituitary gland than in most brain tissues sampled, this difference is minimal when NTproCNP concentration — which is a better indicator of CNP secretion due the short half-life of CNP (Hunt *et al.* 1994, Prickett *et al.* 2001) — is compared. The difference in concentration ratio of NTproCNP:CNP between the brain tissues sampled and pituitary gland tissue indicates that relatively very little clearance of CNP occurs in the pituitary gland of healthy normal individuals. This results in an apparent abundance of the peptide in the pituitary — relative to brain tissue — which may lead to the incorrect assumption that this is a result of pituitary gland having a markedly higher level of secretion than the brain.

Given that CNP concentrations in the peripheral circulation are reported to be close to assay detection limits (Hama *et al.* 1994) in most species, it was assumed that the relatively high abundance of CNP in the pituitary gland solely reflected a paracrine/autocrine role for the peptide. Other studies have since provided evidence to support this including; a) CNP potently stimulates cGMP accumulation in pituitary cell lines, including somatotrophs, gonadotrophs (reviewed by Fowkes & McArdle 2000), b) *NPRB* is expressed in normal human adult and fetal pituitary cells, and the identification of NPR-B protein and related mRNA expression in human pituitary adenomas regardless of the cellular origin of the tumour (Thompson *et al.* 2012), c) the visualisation of NPR-B receptors in the anterior and posterior pituitary gland as well as in the *pars intermedia* (Konrad *et al.* 1992), d) CNP inhibits LH secretion in rats when centrally administered (Huang *et al.* 1993). However, there are several findings described in Chapter 6 and Chapter 7 which support the possibility of an endocrine role for CNP — in addition to these paracrine actions. Firstly, the finding that expression of *NPPC* is increased in both the anterior and posterior pituitary glands following stimulation with dexamethasone — despite no significant change in peptide content — suggests that CNP is synthesised in the pituitary gland and released into the circulation. Furthermore, the high degree of similarity between HPLC profiles of CNP immunoreactive forms in the anterior pituitary gland and in plasma — both containing a high proportion of proCNP (1-103), CNP-53, and minimal CNP-22 — provide further support for the anterior pituitary gland as a major source of "CNP" in the circulation; including after stimulation with dexamethasone. It is apparent that little degradation of CNP occurs in the pituitary gland, as indicated by the 1:1 NTproCNP:CNP concentration ratio, which is indicative of accumulation and storage for future release into the circulation — possibly stimulated at times of high stress. Taken together, these points indicate that it is likely that targets of CNP are both local and distant.

Addressing the question of whether the release of CNP from other peripheral sources (for example muscle, liver or adipose tissues) is increased in response to dexamethasone — and to what degree — may provide insight into the significance of the contribution of the pituitary gland to the peripheral circulation. More specifically, obtaining samples of blood from the inferior petrosal sinus for measurement of CNP peptide concentration would also be valuable in determining the pituitary gland contributions to circulating levels of CNP under normal and dexamethasone-stimulated conditions. It is unknown why both the anterior and posterior pituitary glands — two tissues of very different origin, histology and function — possess high concentrations of CNP peptides. However given the ubiquitous nature of CNP, roles may differ between the endocrine aspect of the pituitary gland, and the glial-cell containing posterior

pituitary gland (Hatton 1988). Characterising the type of CNP-positive cells in both the anterior and posterior pituitary glands of sheep is a key step necessary for identifying the physiological relevance of CNP in the pituitary gland. McArdle *et al.* (1994) identified the majority of CNP-positive cells in the rat pituitary to be gonadotropes and found no CNP-positive cells in the posterior pituitary gland — or in heart tissue. Although McArdle *et al.* (1994) and other earlier reports (Komatsu *et al.* 1991, Minamino *et al.* 1993) failed to detect CNP in heart tissue, or attributed its cross reactivity with ANP (Minamino *et al.* 1991), later studies have in fact identified CNP transcripts in rat atria and ventricles (reviewed by Nishikimi *et al.* 2006). In light of the recent findings of this thesis, closer examination of CNP peptides using immunohistochemical staining and *in situ* hybridisation in both anterior and posterior pituitary glands is warranted. NTproCNP antibodies should also be considered in these studies.

8.3 Limitations

Although the studies that comprise the research chapters of this thesis contributed significant novel findings regarding CNP in the CNS, there are limitations — some of which were inherent to the methods used here.

Whilst the series of pilot studies described in 5.1.2 were successful in identifying a secretagogue for CNP in the CNS, they were by no means an exhaustive study designed to exclude the compounds previously described and the aim was to identify a potential candidate that could be examined more closely subsequent studies. Therefore, in the interest of time, cost and reduction of animal manipulations, study sizes were small (n = 4-6), and in some cases there were no secondary measures that could confirm the efficacy of the compound in question, e.g. l-deprenyl. In 5.2, ovine NTproCNP standards had to be used for the study of CNP in red deer stags, because the amino acid sequence of deer NTproCNP has not been characterised. However, a 5 kDa NTproCNP-immunoreactive product has been identified in deer plasma, which eluted where NTproCNP was expected to (Prickett *et al.* 2003). Overall, the separate temporal pattern and marked difference in CNP peptide concentrations between CSF and plasma that were recorded from the deer stags have provided relevant information to these studies.

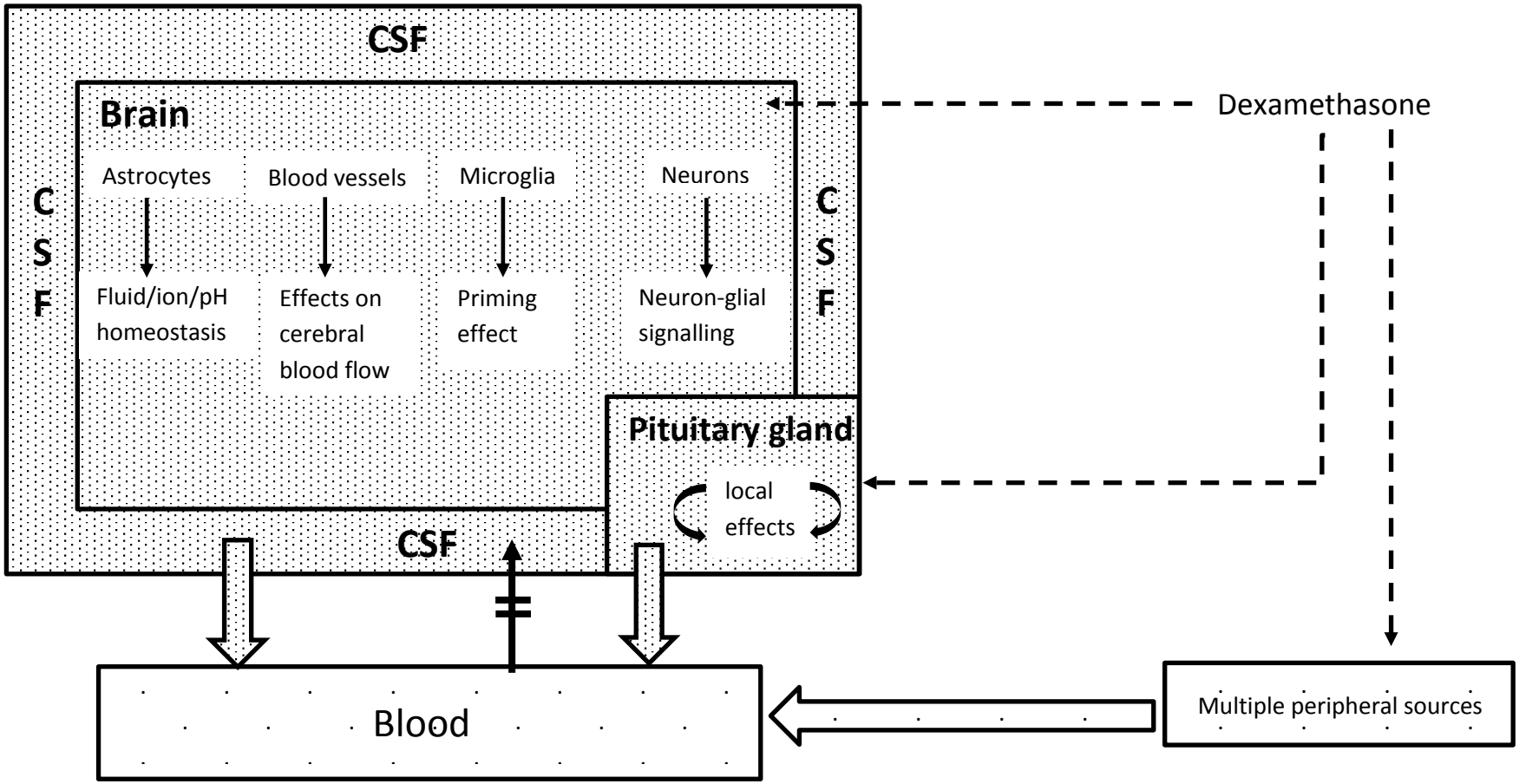
Profiles of CNP- and NTproCNP-immunoreactive fractions eluted by SE-HPLC suffered from poor resolution between peaks, in particular in CNP profiles of plasma and CSF, presumably where more peptide degradation had occurred — compared with profiles from tissue extracts. This is presumably due to the actions of C- and N-terminally directed exopeptidases in extracellular fluids. However, despite these limitations and the inherent time-consuming and laborious nature

of size-exclusion HPLC-RIA, multiple profiles were obtained (n = 2-4) and the results were highly reproducible — as demonstrated by one sample which was subject to two SE-HPLC runs and two separate CNP assays carried out in different weeks (see Appendix).

It is relevant to note that the current understanding of the processing and release of proCNP can now be challenged, following the surprising finding that the large molecular weight form — presumably proCNP (1-103) is present in ovine plasma (Chapter 7). Very few previous reports have exposed plasma extracts to analysis by size-exclusion HPLC-RIA, largely due to the low concentration of CNP in plasma and consequently, the large amount of plasma required to generate HPLC data. The use of gel-permeation chromatography was used to identify the presence of CNP-22 in human plasma (Stingo *et al.* 1992), which contrasts to the virtual absence of CNP-22 in ovine plasma extracts reported in this thesis. Small amounts of CNP-22 were detected in plasma extracts containing higher concentrations of CNP (from dexamethasone-treated sheep). This latter finding may reflect release from the anterior pituitary and/or minimal conversion from CNP-53 to CNP-22, or some level of sample degradation following collection. Determining how CNP-22 is generated may shed light here in the future.

The studies described in Chapter 6 which examined the effect of dexamethasone administration to sheep on CNP peptide concentration in a selection of brain and pituitary gland tissues were not designed to identify either the mechanism behind dexamethasone-stimulated secretion or the regulation of specific CNS functions by CNP. Also, it is necessary to be cautious when extrapolating studies involving glucocorticoids, given that the down-stream effects vary widely depending on the dose, duration and target cell. For example, whereas repeat administration of dexamethasone reduces circulating concentrations of CNP peptides for several days in growing lambs (Prickett *et al.* 2009), these studies reveal that CNP concentration is acutely increased following a single dose. The effect of sustained dexamethasone administration on central levels of CNP remains unknown and will be necessary to resolve, in order to determine the relevance of CNP in settings of chronic stress and sustained exposure to high levels of glucocorticoids. In this context it will also be important to study the effects of cortisol and other glucocorticoids on CNP peptides in the CNS — although it is possible that other glucocorticoids stimulate CNP synthesis, this cannot be assumed. As circulating concentrations of cortisol exceed those of aldosterone by 100-fold (free) to 1000-fold (bound), and the affinity of corticosteroids for the MR is 10-fold that of the GR (ter Heegde *et al.* 2015), most MR are occupied by glucocorticoids under non-stressful conditions — and it is during stress that GRs are activated by cortisol, when MR are saturated

(Gomez-Sanchez & Gomez-Sanchez 2014). As dexamethasone only binds to the GR, and has no affinity for the MR, it is likely that the action of dexamethasone on CNP synthesis reflects stressful conditions. Therefore, it may be worthwhile to consider basal levels of cortisol when studying dexamethasone actions on CNP, as high stress levels may lead to a higher GR occupancy, and less availability for binding with dexamethasone.



8.4 Conceptual model

Conceptual model displaying the possible contribution of CNP from central and peripheral tissues to the blood, transfer of CNP between CSF and the circulation, and its potential mechanisms in the brain.

Key: contribution no contribution synthesis is upregulated possible mechanisms as supported by the literature

CNP is represented by '.'

8.5 Implications

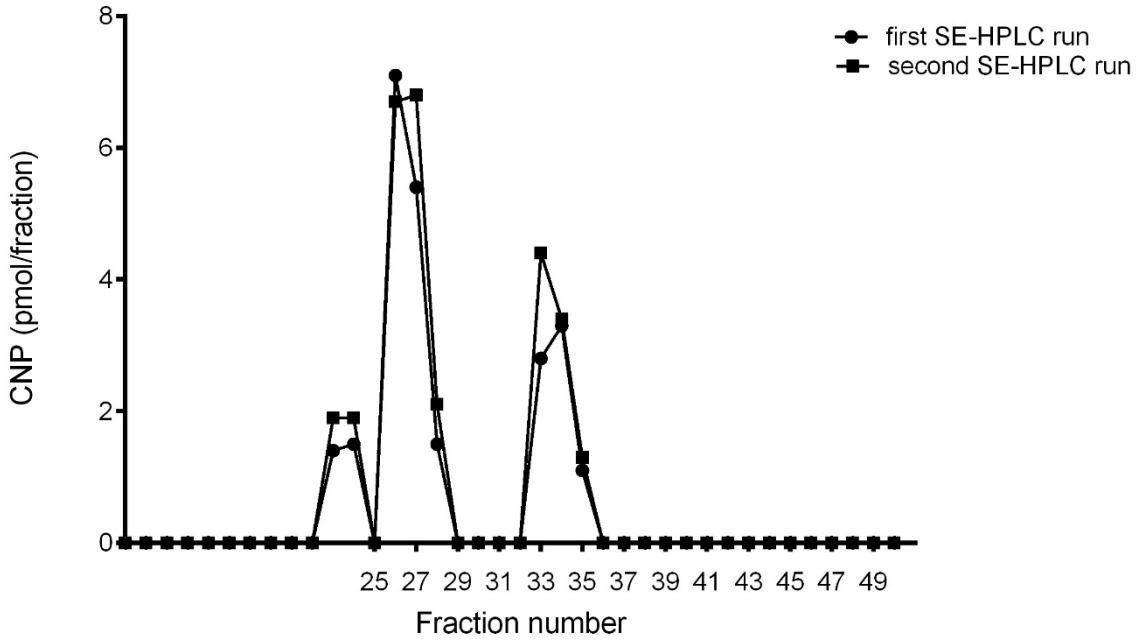
The discovery of dexamethasone as a potent secretagogue in the CNS paves the way for many other future studies which should be aimed at identifying functions in the CNS regulated by CNP under normal physiological conditions, as well as under times of high stress and in neuropathological diseases. Furthermore, more questions have been raised that are particularly important to resolve given the high amount of glucocorticoid use, the known detrimental effects of chronic glucocorticoid exposure on brain function (including learning and memory; Conrad 2010) which may be implicated with CNP (Telegdy *et al.* 1999), and the untapped potential of CNP as a diagnostic and/or therapeutic target for cognitive impairment pathophysiological disorders of the CNS. Dexamethasone can be used as a 'tool' to answer these new fundamental questions which include: Which cell type(s) secretes CNP in response to dexamethasone stimulation? Which signalling pathways lead to dexamethasone-induced CNP secretion in the brain? Can the dexamethasone-induced increase in CNP concentration be blocked? What are the downstream effects of such widespread increases in CNP secretion in the brain? The ultimate purpose for answering these questions is to determine whether there are neuropathological states that this is relevant to, and to establish how these new findings can be applied in clinical settings.

These studies established CNP as a peptide derived from central sources, whereby concentrations are independent of those in the peripheral circulation and are remarkably stable in the face of changes in neuronal activity, consistent with a role for CNP in maintaining some essential, constitutive, aspect of normal brain health. The involvement of CNP in different forms of neurodegeneration such as in Alzheimer's disease, Parkinson's disease and multiple sclerosis deserves consideration, given that CNP has vasoactive effects on cerebral arterioles (Mori *et al.* 1997), neuroprotective properties *in vitro* (Ma *et al.* 2010) and potentially *in vivo* (Espiner *et al.* 2014), and the realisation that cerebrovascular dysfunction may precede cognitive decline and onset of neurodegenerative changes (Bell & Zlokovic 2009). It has been recognised that normal brain functioning requires communication between cells of the neurovascular unit — which involves all of the major cellular components of the brain including neurons, astrocytes, brain endothelium, pericytes, vascular smooth muscle cells, microglia and perivascular macrophages (Bell & Zlokovic 2009). Therefore, understanding how CNP sends signals within and between these cells will likely help us to understand mechanisms behind the onset and progression of neurodegeneration.

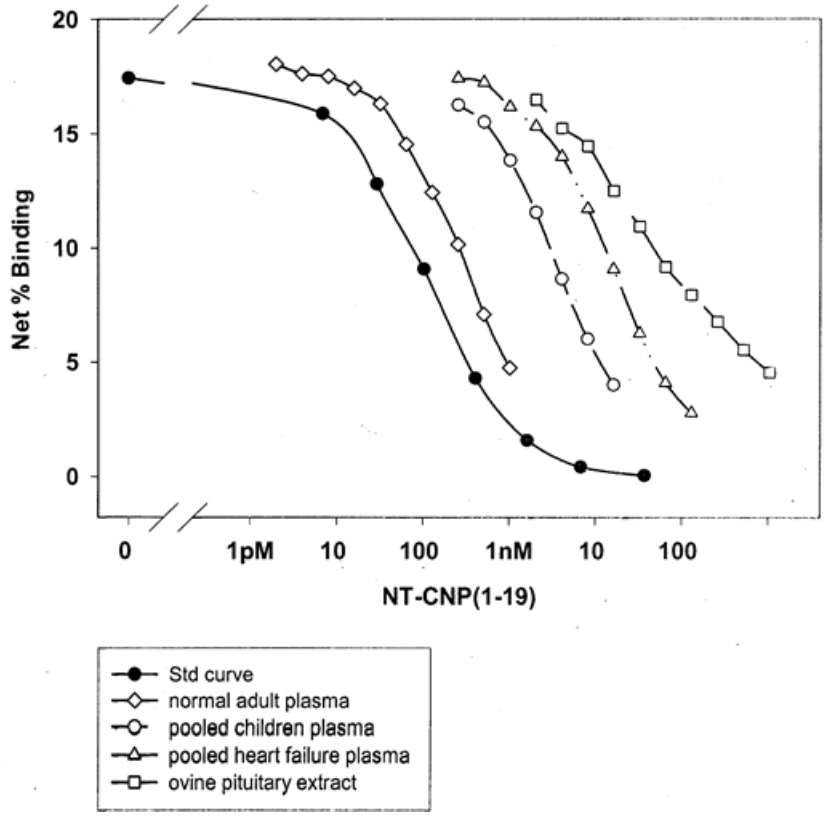
The possibility that changes in intracellular concentrations of Ca^{2+} in astrocytes constitutes an extraneuronal signalling system in the CNS has been a focus of research over recent years (reviewed by Bazargani & Attwell 2016). It is now recognised that Ca^{2+} transients exist with varying spatial and temporal patterns that differ between the cell soma and cell processes, but how these transients are communicated from a cell process across to the endfeet is unknown, as is the mechanism by which these transients are decoded and translated into functional effects (Bazargani & Attwell 2016). Investigating a role for CNP in this (and other) signalling system/s is certainly justified, particularly as cGMP is able to induce branching and elongation of astrocyte processes by redistributing GFAP filaments and depolymerising actin (Borán & García 2007).

Findings arising from this thesis open up many avenues of study that have the potential to resolve not only questions surrounding the presence of CNP in the CNS, but the mechanism of action of many other functions in the CNS. The identification of dexamethasone as a secretagogue for CNP highlights the complex nature of the action of glucocorticoids, whereby effects depend on the dose, concentration and cellular target. Although there are many possible mechanisms for this glucocorticoid-induced increase in CNP secretion, many are testable and identifying the cell type(s) where CNP secretion is increased following dexamethasone stimulation is the most urgent next step that will certainly influence the direction of further investigation.

Appendix



Size-exclusion HPLC profiles of CNP-immunoreactive fractions from one posterior pituitary extract of one dexamethasone-treated sheep. The extract was subject to two size-exclusion HPLC runs and two separate CNP assays carried out in different weeks.



A standard curve (human NTproCNP) and dilution curves of normal adult human plasma, pooled children (aged 5-18) plasma, pooled plasma from patients with heart failure and one sheep pituitary extract. Due to the slight divergence from parallelism of samples from sheep, (compared with human samples), ovine standards were introduced for the work produced in this thesis.

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